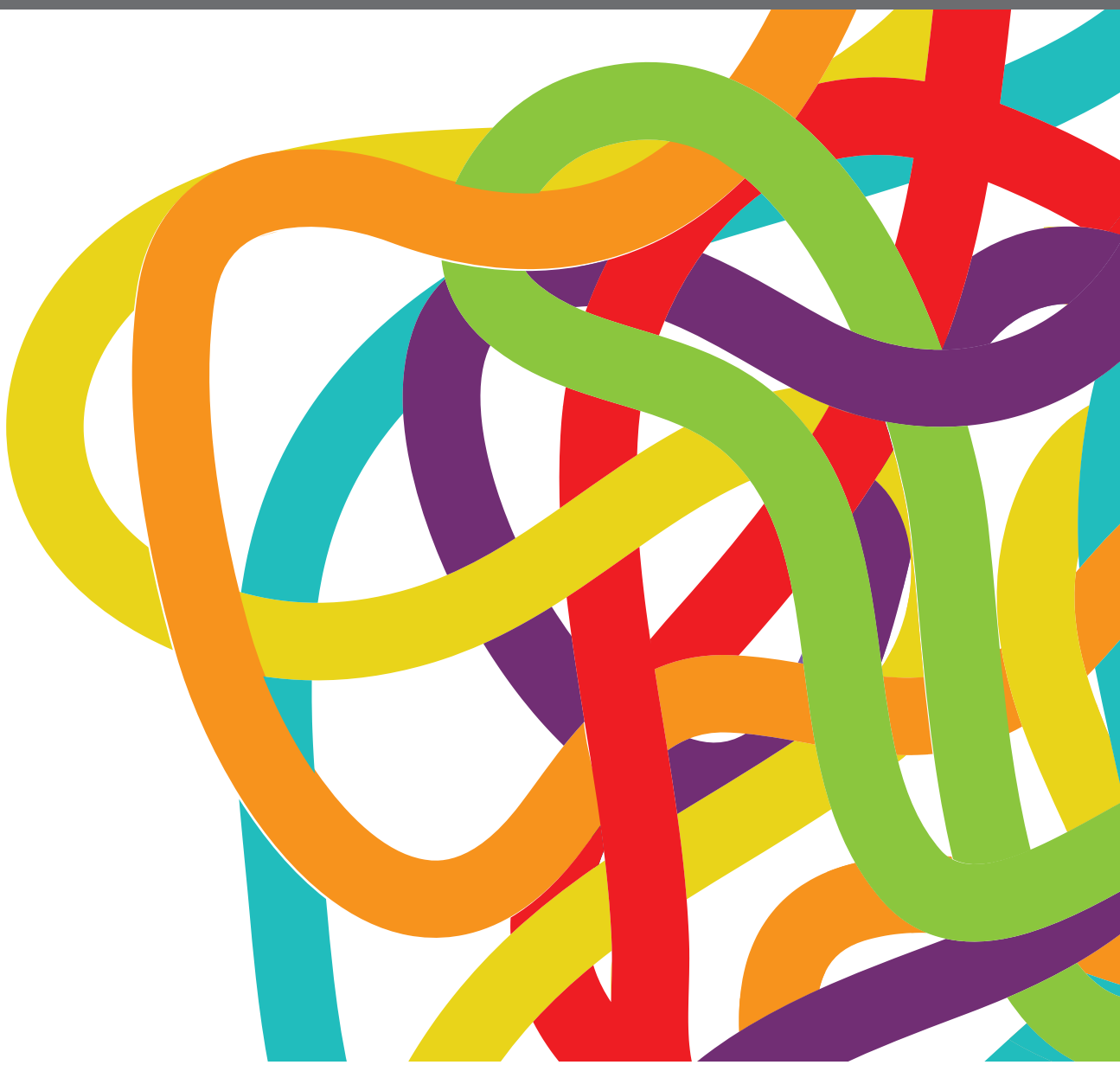
ENDOMETRIAL CANCER: FROM BIOLOGICAL TO CLINICAL APPROACHES

EDITED BY: Massimo Nabissi, Frederic Amant and Paola Gehrig
PUBLISHED IN: Frontiers in Oncology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88963-049-3
DOI 10.3389/978-2-88963-049-3

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

ENDOMETRIAL CANCER: FROM BIOLOGICAL TO CLINICAL APPROACHES

Topic Editors:
Massimo Nabissi, University of Camerino, Camerino, Italy
Frederic Amant, KU Leuven, Belgium
Paola Gehrig, University of North Carolina at Chapel Hill, United States

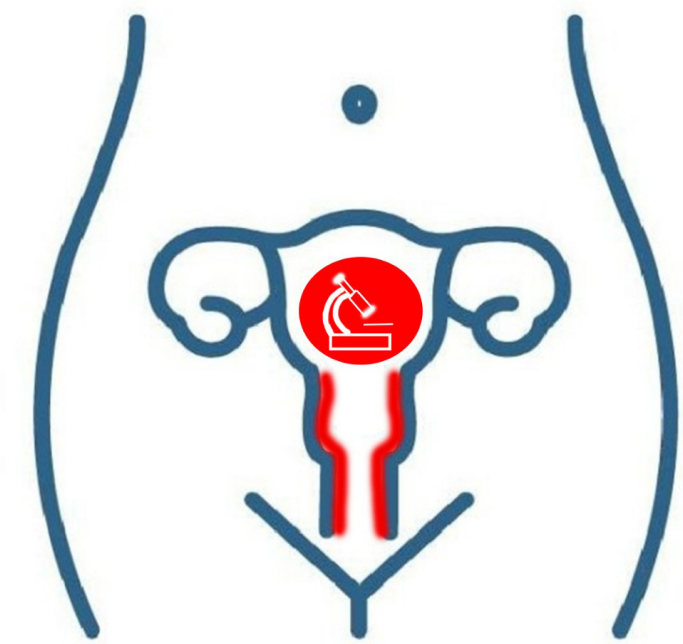


Image: Endometrial cancer, New therapeutical options need more research, by Eng. Giacomo Nabissi.

In developed countries, cancer of the endometrium (EC) is the most common cancer of the female reproductive organs, affecting mainly postmenopausal women. The highest incidence of EC is in Northern America and Europe, but its incidence is growing around the world. Several factors influence the risk of developing EC, including: impaired hormone levels, obesity, physical activity, family history, and having been diagnosed with breast or ovarian cancer in the past. For EC, a standard screening test does not exist. It is often diagnosed at stage I/II due to frequent vaginal bleeding, while invasive EC (stage III/IV) is mainly diagnosed in its advanced stage.

Despite the different clinical improvements when EC is progressed to the advanced/metastatic stage, the patients still have poor prognoses and unsatisfactory outcomes with conventional chemotherapy. The need for precision drugs is underscored by

the limited number of options these patients have. So, the introduction of new techniques and the discovery of new biological characteristics, such as identification of transcriptome, proteome and metabolomics profiles, and new potential diagnostic/prognostic biomarkers are fundamental to improve our knowledges on EC.

The scope of this Research Topic is to welcome articles and reviews from clinicians and scientists around the world in order to give an updated vision of the recent clinical insights, technical advances, and cellular and molecular targets related to endometrial cancer.

Dedicated to Mr. Dario Conti, which supported endometrial cancer research.

Citation: Nabissi, M., Amant, F., Gehrig, P., eds. (2019). Endometrial Cancer: From Biological to Clinical Approaches. Lausanne: Frontiers Media.
doi: 10.3389/978-2-88963-049-3

Table of Contents

frontiers in Oncology

ORIGINAL RESEARCH
published: 24 April 2019
doi: 10.3389/fonc.2019.00256

Endometrial Cytology as a Method to Improve the Accuracy of Diagnosis of Endometrial Cancer: Case Report and Meta-Analysis

Qing Wang[1,2†], *Qi Wang*[1,2†], *Lanbo Zhao*[3], *Lu Han*[1,2], *Chao Sun*[1,2], *Sijia Ma*[1,2], *Huilian Hou*[4], *Qing Song*[1] *and Qiling Li*[1,2*]

[1] *Center for Single-Cell Biology, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China,* [2] *Department of Obstetrics and Gynecology, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China,* [3] *Guipei 77, Health Science Center, Xi'an Jiaotong University, Xi'an, China,* [4] *Department of Pathology, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China*

More and more researchers have reported that dilatation and curettage (D&C) or Pipelle had low accuracy, high misdiagnosis, and insufficient rate. Endometrial cytology is often compared with histology and seems to be an efficient method for the diagnosis of endometrial disorders, especially endometrial cancer. We report a case of misdiagnosed endometrial cancer by D&C, but with a positive cytopathological finding. Following that, a meta-analysis including 4,179 patients of endometrial diseases with cyto-histopathological results was performed to assess the value of the endometrial cytological method in endometrial cancer diagnosis. The pooled sensitivity and specificity of the cytological method in detecting endometrial atypical hyperplasia or cancer was 0.91[95% confidence interval (CI) 0.74–0.97] and 0.96 (95% CI 0.90–0.99), respectively. The pooled positive likelihood ratio and negative likelihood ratio was 25.4 (95% CI 8.1–80.1) and 0.10 (95% CI 0.00–0.30), respectively. The diagnostic odds ratio which was usually used to evaluate the diagnostic test performance reached 260 (95% CI 36–1905). So we recommend that D&C and Pipelle are still practical procedures to evaluate the endometrium, cytological examinations should be utilized as an additional endometrial assessment method.

Keywords: cytology, histology, endometrial cancer, diagnosis, atypical hyperplasia

OPEN ACCESS

Edited by:
Massimo Nabissi,
University of Camerino, Italy

Reviewed by:
Johanna Pijnenborg,
Radboud University Nijmegen Medical Centre, Netherlands
Gökhan Açmaz,
Erciyes University, Turkey

***Correspondence:**
Qiling Li
liqiling@mail.xjtu.edu.cn

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *15 September 2018*
Accepted: *21 March 2019*
Published: *24 April 2019*

Citation:
Wang Q, Wang Q, Zhao L, Han L, Sun C, Ma S, Hou H, Song Q and Li Q (2019) Endometrial Cytology as a Method to Improve the Accuracy of Diagnosis of Endometrial Cancer: Case Report and Meta-Analysis. Front. Oncol. 9:256. doi: 10.3389/fonc.2019.00256

INTRODUCTION

Endometrial cancer is becoming the primary reason of female deaths of genital track cancer in developed countries (1). Dilatation and curettage (D&C), as the traditional gold standard procedure for diagnosing endometrial cancer, is painful, expensive, requires general anesthesia and has a high rate of misdiagnosis (2). It has been reported that less than half of the uterine cavity is curetted in 60% of cases (3), and over 40% of women with complex atypical hyperplasia as a preoperative diagnosis have a final confirmation of endometrial cancer during hysterectomy (4, 5). Endometrial cytology is recently reported as a useful diagnostic method with high sensitivity and specificity in detecting endometrial malignancies (6–9), but no meta-analysis, which is considered more credible, has yet been performed to evaluate the diagnostic accuracy of endometrial cytology for endometrial carcinoma compared with histological diagnosis.

Here, we report a case of misdiagnosed endometrial cancer by D&C, but with a positive cytopathological finding. The patient has provided her written informed consent for the publication of this manuscript and any identifying images or data. After searching on PubMed, we believe it is the first case report of a misdiagnosis of endometrial cancer detected by cytopathology. Following this, a random-effects meta-analysis including 4,179 patients with both cytopathological and histopathological results was performed to assess the value of the endometrial cytology method in the diagnosis of endometrial atypical hyperplasia or cancer.

CASE REPORT

A 60-year-old post-menopause female, from Baoji City of the Shaanxi province in China, went to a local hospital complaining of abnormal uterine bleeding for 2 months. No high risk factor for endometrial cancer was observed, such as genetic factors, obesity, diabetes, a history of tamoxifen use and so on. Curettage was performed with a histopathological diagnosis of complex hyperplasia endometrium. No medicine or therapeutic curettage was effective for her with a continued bleeding. Her type B ultrasound in Shaanxi Provincial People's hospital showed a 0.8 cm-thick endometrium. Then, she turned to the First Affiliated Hospital of Xi'an Jiaotong University for further treatment. After written informed consent, she volunteered to get cytological endometrial samplings by Li Brush (Xi'an Meijiajia Bio-Technologies Co. Ltd., China, 20152660054) for cytological examination before D&C. Her histopathological report revealed that papillary epithelial hyperplasia was found, and cancer was a concern according to the structure of tissue but could not be diagnosis due to insufficient tissue (**Figure 1A**). Meanwhile, the cytopathological report revealed that some malignant cells were found (**Figure 1B**). Her serum markers showed high serum carbohydrate antigen 19-9 (CA19-9, 42.08 U/ml) and squamous cell carcinoma antigen (SCC, 6.10 ng/ml). A diagnostic laparoscopic hystero-salpingo-oophorectomy was performed and the patient was converted to a laparotomy when intraoperative frozen section examination revealed an endometrial serous carcinoma with ovarian metastasis. Omentum resection, pelvic lymphadenectomy and para-aortic nodes dissection were performed. She was finally diagnosed with stage IIIc endometrial serous carcinoma.

MATERIALS AND METHODS

Source of Material

We searched the PubMed and Embase databases with the heading terms and keywords as "cytology" and "endometrial" from Jan 1, 1995 to June 1, 2018. Then, the results were manually selected for studies to include and repeatedly checked by a second investigator. We searched the full-text articles about the comparison of cytological results and the histological results in endometrial samples.

Standard of Inclusion and Exclusion

All candidate studies were evaluated and extracted by two independent investigators. Inclusion criteria: (1) patients were diagnosed by histopathological and cytological examination; (2) the histopathological results were paired with cytological results; (3) sufficient information was provided to conduct a statistical analysis; (4) endometrial cells were sampled by endometrial brushes; (5) studies were limited to human trials and published in English. Exclusion criteria include: (1) news, abstracts, case reports, letters, commentaries, and reviews studies; (2) other kinds of endometrial cells sampler like endometrial aspiration cytology; (3) different cytopathology report formats with others, that made it hard to re-group and analyze; and (4) studies with different positive result definition or duplicate data.

Data Extraction

We set atypical hyperplasia and endometrial carcinoma as the positive results and the others as the negative results, including normal endometrium, non-atypical hyperplasia, endometrial polyp, simple endometrial hyperplasia, complex endometrial hyperplasia and so on.

Two investigators separately extracted the following information from each research: the name of the first author, year of publication, cytological sampling method, cytological specimen preparation, histological sampling method, number of patients enrolled, and true positive (TP), false negative (FN), false positive (FP), and true negative (TN) results. Any discrepancies between the two investigators were discussed by all the authors.

Quality Assessment

A quality assessment of eligible studies was evaluated using the quality assessment of studies of diagnostic accuracy included in systematic reviews-2 (QUADAS-2). There were 13 questions (each of which was scored as yes, no, or unclear): (1) Was a consecutive or random sample of patients enrolled? (2) Was a case–control design avoided? (3) Did the study avoid inappropriate exclusions? (4) Were the cytopathological diagnoses interpreted without knowledge of the results of the gold standard (histopathological diagnosis)? (5) whether the blind method was used for pathologists? (6) Was the histopathological diagnosis likely to correctly classify the target condition? (7) Was there an appropriate interval between the cytological sampling and histological sampling? (8) Did all patients receive the histopathological diagnosis? (9) Were all patients included in the analysis? (10) Whether the diagnostic test steps were detailed? (11) Were there concerns that the included patients and setting do not match the review question? (12) Were there concerns that the target condition as defined by the gold standard does not match the question? (13) Were there concerns that the index test, its conduct, or its interpretation differ from the review question?

Statistical Analysis

The publication bias was checked by a Deeks funnel plot, and $P < 0.05$ was considered a significant publication bias. Statistical heterogeneity was detected by a Q test and an inconsistency index (I^2), with significant heterogeneity set at $P \leq 0.05$ and $I^2 > 50\%$.

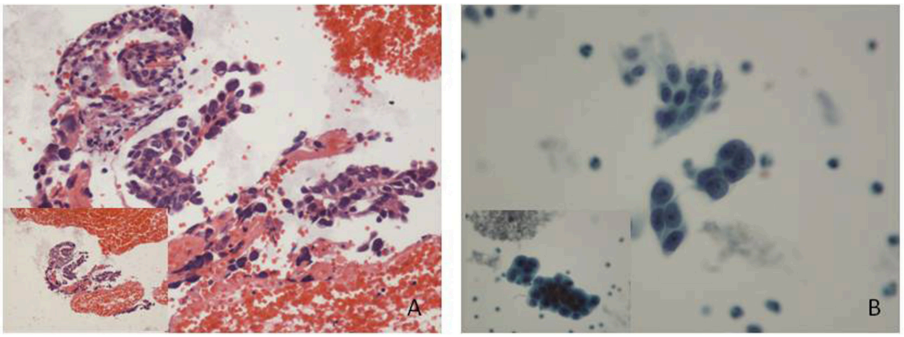


FIGURE 1 | Histological and cytological images. **(A)** Some papillary arranged epithelial dysplasia cells could be found in plenty of blood cells, with no tissue structure. (Hematoxylin-eosin staining; original magnification x10). **(B)** Endometrial carcinoma cells: cell clumps with irregular protrusions were rich in dimensional sense. Variable sizes, different shapes and hyperchromatic nuclei showed a loss of polarity within the epithelial sheet with irregularly clumped chromatin (Papanicolaou stain; original magnification x 20).

If there was no significance in heterogeneity ($P > 0.05$), a fixed effects model was chosen. If it was the opposite ($P < 0.05$), a random effects model was chosen.

According to TP, FN, FP and TN results, we calculated sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (PLR) (>10 suggested strong concordance), negative likelihood ratio (NLR) (<0.1 suggested strong concordance), diagnostic odds ratio (DOR). PLR was calculated as: positive likelihood ratio = sensitivity/(1–specificity). NLR was calculated as: negative likelihood ratio = (1–sensitivity)/specificity. DOR was estimated by the Mantel-Haenszel formula. All statistical analyses, including 95% Confidence Interval (CI) were performed using STATA software (version 12.1, StataCorp LP) with the Midas module.

RESULTS

Search Results

After searching on PubMed and Embase, 9 of 4,182 studies were included in meta-analysis. **Figure 2** showed a flow diagram of the selection process. All data in researches were screened rigorously by our team.

Basic Characteristics of Studies

Our analysis included 9 eligible studies, which were shown in **Table 1**. In total, 2 studies were from Italy, 2 from the USA, 1 from China, 1 from Japan, 1 from England, 1 from Indonesia, and 1 from Greece. A total of 4,179 patients were included. Different endometrial brushes were used in these 9 studies, including the Tao brush (2), Endoflower (2), Endogyn (1), Cytobrush (1), and Uterobrush (1), and 1 study used six different devices. In all, 8 studies prepared the cytology specimens with a liquid-based cytology, and 1 study used the conventional way. In sum, 2 studies compared the cytological results to the D&C results, 3 studies compared the cytological results to the hysterectomy results, 2 studies compared the cytological results to the hysteroscopy and biopsy results, 1 study compared the cytological results to the biopsy or D&C results and 1 study compared the cytological results to the biopsy, D&C or hysterectomy results. Additionally, 5 studies research the pre/post-menopausal patients, 2 studies researched peri/post-menopausal patients, 1 study researched post-menopausal patients, and in 1 study, the menopause situation was unknown.

Study Quality

We assessed the quality of eligible studies by QUADAS-2 and found that the quality of all the studies was good (**Table 2**).

Diagnostic Accuracy

The pooled sensitivity and specificity of the cytological method in detecting endometrial atypical hyperplasia or cancer was 0.91 (95% CI 0.74–0.97) and 0.96 (95% CI 0.90–0.99), respectively (**Figure 3**). The pooled PLR and NLR were 25.4 (95% CI 8.1–80.1) and 0.10 (95% CI 0.00–0.30), respectively. The DOR which used to evaluate the diagnostic test performance,reached 260 (95% CI 36–1905).

Heterogeneity and Sensitive Analysis

I^2 values of pooled sensitivity and specificity were 96.53 (95% CI, 95.23–97.83) and 98.29 (95% CI, 97.78–98.80), which indicated a statistically significant heterogeneity. A sensitivity analysis was performed to assess the influence of each study, in which each individual study was removed each time. No significant change or reversal of result was found (**Table 3**). The I^2-value showed that none of the single study affected the heterogeneity of this meta-analysis.

Subgroup Analysis

Subgroup 1: the corresponding values of the subgroup with sample size <300 were 0.89 (95% CI: 0.55, 0.98) for sensitivity and 0.93 (95% CI: 0.81, 0.98) for specificity. While in subgroup sample size ≥300, the sensitivity was 0.93 (95% CI: 0.71, 0.99) and specificity was 0.98 (95% CI: 0.91, 1.00). Subgroup 2: the corresponding values of the studies of European countries showed the sensitivity of 0.96 (95% CI: 0.86, 0.99) and specificity of 0.99 (95% CI: 0.94, 1.00) and in other countries were 0.84(95% CI: 0.50, 0.96) and 0.92 (95% CI: 0.79, 0.97), respectively (**Table 3**).

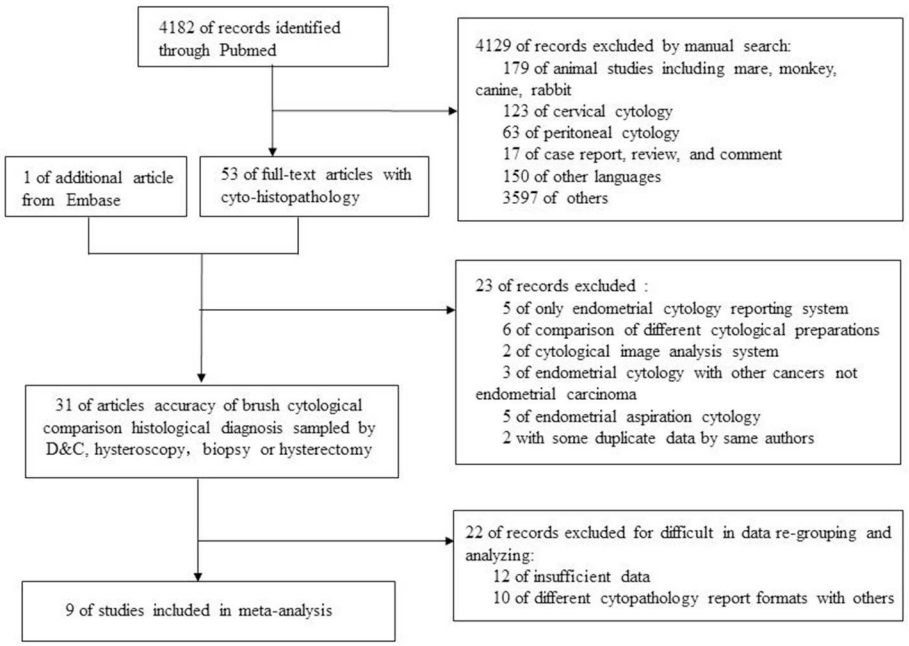


FIGURE 2 | Study selection process.

Publication Bias Analysis

Deeks funnel plot asymmetry test was conducted to evaluate publication bias in this study (**Figure 4**), which showed statistically nonsignificant publication bias ($P = 0.60$).

Clinical Utility

Given the PLR and NLR, the cytological detection method of endometrial atypical hyperplasia or cancer was located in the left upper quadrant (**Figure 5A**), indicating that the cytological detection method could serve as a test to confirm and exclude endometrial atypical hyperplasia or cancer. Fagan's plot indicated a dramatic improvement in posttest probability. When the pretest probability of endometrial atypical hyperplasia or cancer was set to 20%, using the cytological method as a source to detect the above diseases could significantly raise the posttest probability of a positive result to 86% and lower the posttest probability of a negative result to 2% (**Figure 5B**).

DISCUSSION

It is currently estimated, that 60 million cervical cytology examinations are performed every year in the United States (19). Cytopathological screening, histopathological diagnosis and even the human papillomavirus vaccine are used to prevent and to make early diagnosis of cervical cancer, which helps the early detection and lowers the mortality of cervical cancer. In the absence of such effective screening programs and prevention methods, endometrial malignant diseases are becoming the most prevalent cancer of the female genital tract in developed countries, accounting for nearly 50% of all new diagnoses of gynecological cancer (20, 21). Nearly 75–80% of all endometrial cancer patients diagnosed at early stage (22, 23). But researchers are still paying attention to the early diagnosis of endometrial malignant diseases, especially its precancerous lesion.

Histological (D&C and Pipelle) and cytological diagnosis are two classes of endometrial sampling modalities. Both D&C and the most golden standard of evaluating the endometrium, hysteroscopic-guided uterine biopsy, are painful, expensive, and requires dilatation and anesthesia (24–26). Insufficient samples carry negative ramifications and increase the difficulty for the pathologist (27). An insufficient rate was reported as 6.4% (810/12,745) of curettage and 6.5% (310/4,777) of endometrial biopsy, and multiple factors contributed to such variation, including patient age, parity, endometrial thickness, sampling device, and provider technique. When stratified by age, the insufficient rate was 2.7% in the group of patients under 40 years old (3,454 cases), 5.8% in the group of 40 to 59 years old (11,838 cases), and 14.6% in the group of 60 years and older (2,230 cases) (28, 29). Sakhdari et al. (27) also showed that 15% (226/1,768) of the samples of women age 60 and older were reported as insufficient, and Barut et al. reported the insufficient rate was likely associated with menopause, with 6.5% (26/401) in premenopausal and 49.2% (120/244) in postmenopausal women (25). However, 75% of endometrial cancers occurred in women older than 55 years of age, with a median age of 62 (30). A meta-analysis evaluated the diagnostic rate of D&C and hysteroscopy in postmenopausal women. It pointed out that D&C had a high rate of non-diagnostic samples 31% (range 7–76%) and a high failure rate of 11% (range 1–53%), which lead to a missing diagnosis rate of 7% (range 0–18%) (31).

Pipelle, as another widely used endometrial biopsy apparatus, is safe, cost-effective, and easily preformed (24). A meta-analysis

TABLE 1 | Study characteristics of the nine included studies on the diagnostic accuracy of endometrial cytological sampling.

Study	Year/ country	Cytological sampling and preparation	Histologic sampling	Menopausal status	Sample size	TP	FP	TN	FN	PPV %	NPV %
Maksem et al. (10)	1997 USA	Tao brush/ LBC	Hysterectomy	Pre/post	100	18	1	81	0	94.7	100.0
Garcia et al. (11)	2003 England	Uterobrush/ LBC	Biopsy/D&C/ hysterectomy	Pre/post	60	7	2	49	2	77.8	96.1
Papaefthimiou et al. (12)	2005 Greece	Endogyn/ LBC	Hysterectomy	Peri/post	491	191	5	292	3	97.4	99.0
Andrijono et al. (13)	2005 Indonesia	Cytobrush/ LBC	D&C	Peri/post	45	5	3	24	13	62.5	64.9
Buccoliero et al. (14)	2007 Italy	Endoflower/ LBC	Hysteroscopy and biopsy	Pre/post	531	29	0	501	1	100.0	99.8
Kipp et al. (15)	2008 USA	Tao Brush/LBC	Hysterectomy	Pre/post	137	83	17	33	4	83.0	89.2
Yanoh et al. (16)	2012 Japan	Uterobrush/ endocyte/ endosearch/ softcyto/tube /cottonswab/NA	Biopsy/D&C	NA	1045	328	25	605	87	92.9	87.4
Remondi et al. (17)	2013 Italy	Endoflower/ LBC	Hysteroscopy and biopsy	Post	98	11	4	82	1	73.3	98.8
Yang et al. (18)	2017 China	SAP-1 sampler/ LBC	D&C	Pre/post	1672	154	167	1286	65	48.0	95.2

LBC, Liquid - based cytology; NA, not available; D&C, dilatation and curettage.

TABLE 2 | Risk of bias and concerns of applicability by study using a modified Quadas-2 tool.

	Risk of bias				Applicability concerns		
	Patient selection	Index test	Reference standard	Flow and timing	Patient selection	Index test	Reference standard
Maksem et al. (10)	Unclear	Low	Low	Low	Low	Low	Low
Garcia et al. (11)	Low	Low	Low	Unclear	Low	Low	Low
Papaefthimiou et al. (12)	Low	Low	Low	Low	Low	Low	Low
Andrijono et al. (13)	Unclear	Low	Low	Low	Low	Low	Low
Buccoliero et al. (14)	Unclear	Low	Low	Unclear	Low	Low	Low
Kipp et al. (15)	Unclear	Low	Low	Unclear	Low	Low	Low
Yanoh et al. (16)	Low	Low	Low	Unclear	Low	Low	Low
Remondi et al. (17)	Low	Low	Low	Unclear	Low	Low	Low
Yang et al. (18)	Low	Low	Low	Unclear	Low	Low	Low

of 39 studies, including 7,914 patients, revealed the concordance rate between Pipelle and D&C/hysteroscopy/hysterectomy in endometrial cancer detection of postmenopausal and premenopausal women was 99.6% and 91%, respectively (32). However, the Pipelle is random point sampling and said to sample 4.2% of the uterine cavity (33), and 25–36% women using Pipelle were found to have insufficient tissue for pathologic assessment (34).

Both D&C and Pipelle have their limitations in detecting endometrial cancer. Hysteroscopic guided biopsy showed a high diagnostic accuracy for endometrial cancer diagnosis (estimated sensitivity of 82.6% and specificity of 99.7%), data from a meta-analysis over 9,000 patients (35), but it could not be performed on asymptomatic women or used as a screening method. Are histological procedures (curettage or biopsy) enough to be the only methods in the diagnosis of endometrial diseases?

Endometrial cytology examination may be an inevitable method for endometrial cancer screening and a combined diagnostic procedure. It might have been hampered by the frequent presence of excess blood, mucus and overlapping cells and varied endometrium cell morphology with different sex

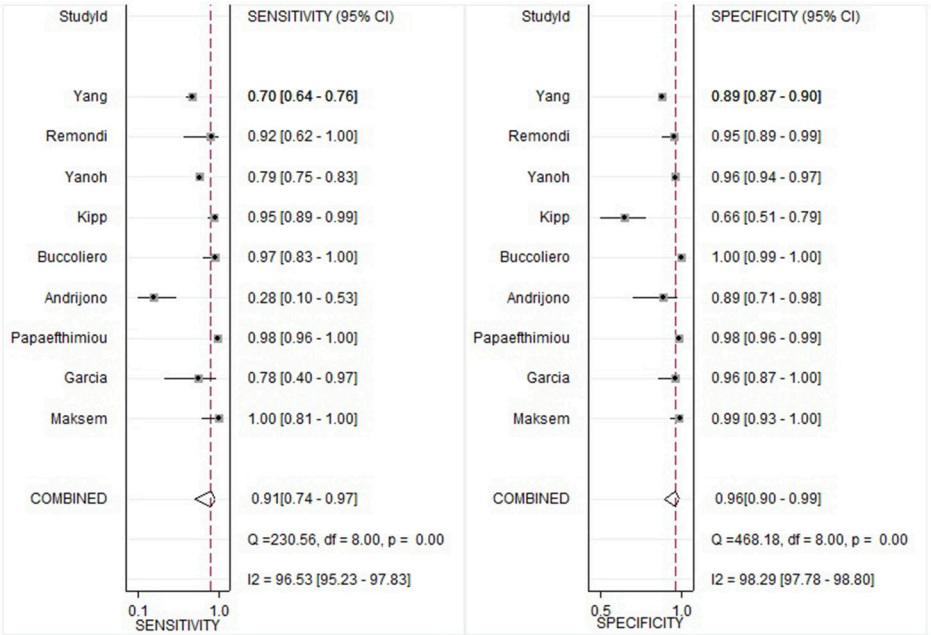


FIGURE 3 | Forest plots of sensitivity and specificity.

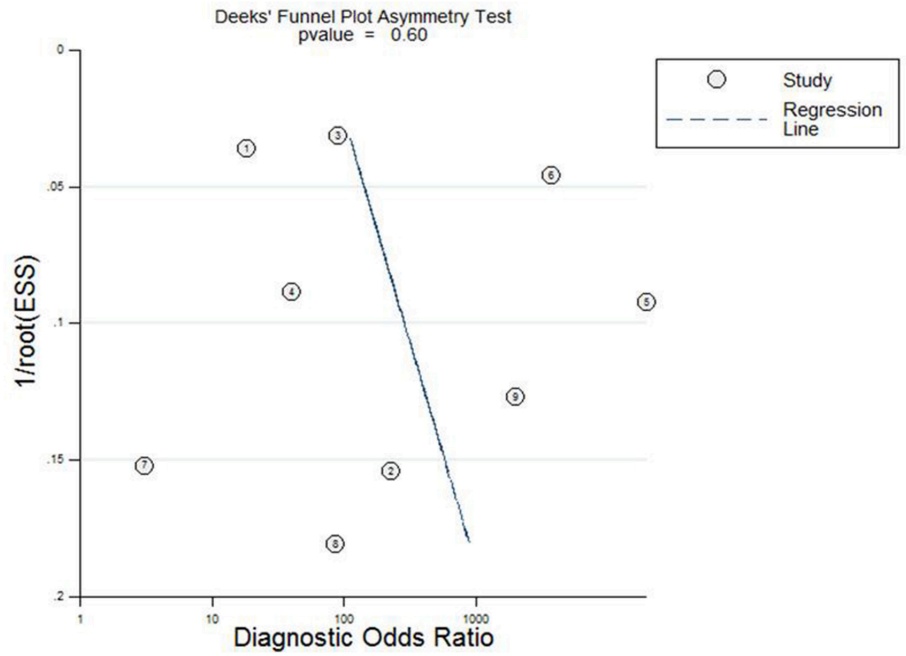


FIGURE 4 | Deeks regression line showed no significant publication bias of studies.

hormone levels. However, liquid-based preparation techniques improve the diagnostic accuracy of endometrial cytology (6, 36), and more and more scholars have made efforts on the endometrial cytology reporting system (37–39). With the establishment and maturation of universal standards for the reporting system, endometrial cytology will truly play an important role in the diagnosis of endometrial diseases and endometrial cancer screening. Kondo et al. tested some different methods in 114 consecutive symptomatic women, and they reported that the sensitivity of detecting malignancy increased

TABLE 3 | Sub-analysis and sensitivity analysis on the diagnostic accuracy of endometrial cytological sampling.

Variables	Study number	SEN (95%CI)	I^2	SPE (95%CI)	I^2	PLR (95%CI)	NLR (95%CI)	DOR (95%CI)
SUBGROUP ANALYSES								
Sample size								
<300	5	0.89 (0.55, 0.98)	96.38	0.93 (0.81, 0.98)	92.26	13.5 (4.2, 43.6)	0.11 (0.02, 0.65)	119 (10, 1359)
≥300	4	0.93 (0.71, 0.99)	99.04	0.98 (0.91, 1.00)	99.62	50.5 (9.0, 284.1)	0.07 (0.01, 0.03)	727 (28, 18689)
Country								
Europe	4	0.96 (0.86, 0.99)	76.62	0.99 (0.94, 1.00)	80.56	73.6 (15.4, 351.9)	0.04 (0.01, 0.15)	1769 (160, 19516)
Other	5	0.84 (0.50, 0.96)	93.05	0.92 (0.79, 0.97)	96.41	10.2 (3.6, 29.3)	0.18 (0.04, 0.72)	58 (7, 477)
SENSITIVITY ANALYSES								
Maksem et al. (10)		0.88 (0.70, 0.96)	96.03	0.96 (0.87, 0.99)	98.06	21.4 (6.4, 72.0)	0.12 (0.04, 0.36)	172 (24, 1247)
Garcia et al. (11)		0.92 (0.73, 0.98)	97.20	0.96 (0.88, 0.99)	98.61	26.1 (7.1, 95.1)	0.09 (0.02, 0.31)	299 (31, 2864)
Papaefthimiou et al. (12)		0.87 (0.68, 0.96)	95.30	0.96 (087, 0.99)	98.04	22.7 (6.1, 83.9)	0.13 (0.04, 0.38)	174 (22, 1381)
Andrijono et al. (13)		0.93 (0.83, 0.97)	97.19	0.97 (0.90, 0.99)	98.92	30.8 (8.8, 107.7)	0.07 (0.03, 0.19)	435 (65, 2930)
Buccoliero et al. (14)		0.89 (0.69, 0.97)	95.05	0.94 (0.88, 0.97)	97.15	15.7 (6.8, 36.7)	0.12 (0,04, 0.37)	135 (23, 782)
Kipp et al. (15)		0.90 (0.70, 0.97)	96.89	0.97 (0.93, 0.99)	98.41	31.6 (10.8, 92.7)	0.10 (0.03, 0.35)	318 (33, 3031)
Yanoh et al. (16)		0.92 (0.74, 0.98)	97.21	0.97 (0.88, 0.99)	98.46	26.6 (7.1, 99.8)	0.08 (0.02, 0.31)	320 (32, 3158)
Remondi et al. (17)		0.90 (0.71, 0.97)	97.08	0.97 (0.88, 0.99)	98.58	26.6 (7.1, 100.2)	0.10 (0.03, 0.34)	267 (28, 2543)
Yang et al. (18)		0.92 (0.76, 0.98)	96.04	0.97 (0.90, 0.99)	96.69	31.2 (8.7, 111.4)	0.08 (0.02, 0.28)	389 (45, 3397)
Total		0.91 (0.74, 0.97)	96.53	0.96 (0.90, 0.99)	98.29	25.4 (8.1, 80.1)	0.10 (0.03, 0.30)	260 (36, 1905)

SEN, sensitivity; SPE, specificity; PLR, positive likelihood ratio; NLR, negative likelihood ratio; DOR, diagnostic odds ratio; CI, confidence interval.

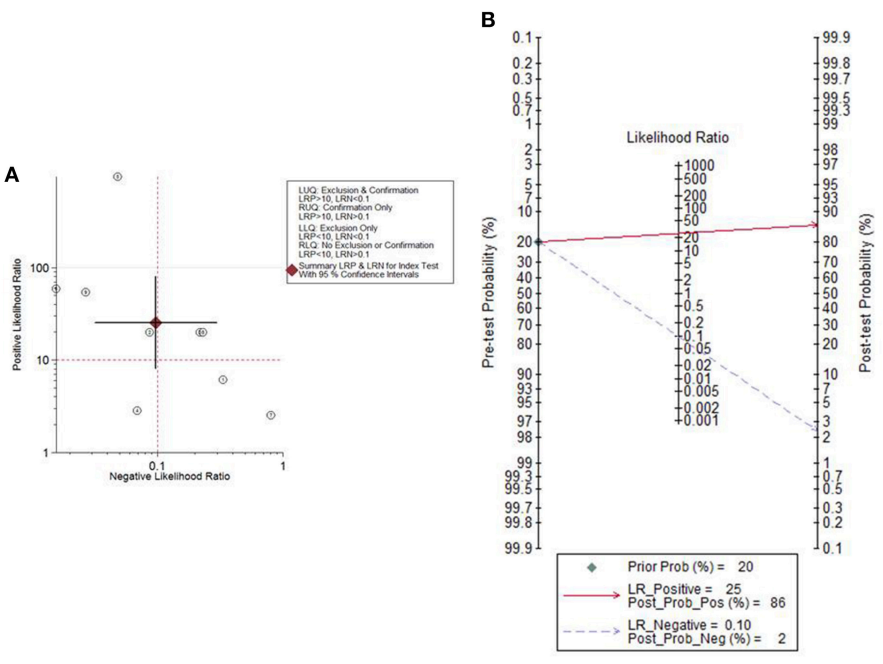


FIGURE 5 | The likelihood ratio matrix and Fagan's plot. **(A)** The likelihood ratio matrix of the cytological method for the detection of endometrial atypical hyperplasia or cancer. **(B)** Fagan's plot presented the clinical utility of the cytological method for the detection of endometrial atypical hyperplasia or cancer.

from 92% to 98% when endometrial cytology was combined with suction curettage (40).

Our meta-analysis showed that endometrial cytology had a high diagnostic accuracy and could serve as a test to confirm or exclude endometrial atypical hyperplasia or cancer. The pooled sensitivity and specificity of the cytological method in detecting endometrial atypical hyperplasia or cancer was 0.91 (95% CI 0.74–0.97) and 0.96 (95% CI 0.90–0.99), respectively. Its diagnostic odds ratio reached 260 (95% CI 36–1905). The pooled positive likelihood ratio and negative likelihood ratio was 25.4 and 0.10, respectively. Therefore, we can conclude that the test results of endometrial cytology are

very accurate in diagnosing endometrial atypical hyperplasia or cancer.

Therefore, we recommend that D&C and Pipelle are still practical procedures to evaluate the endometrium, cytological examinations should be utilized as an additional endometrial assessment method, especially for women at high-risk for endometrial cancer.

Additionally, endometrial cytology is inexpensive, tolerated well and can be performed without anesthesia in an outpatient clinic. It is now the most common test for an initial evaluation of endometrial cancer in Japan (7) and has been encouraged as the first level screening method for women at high risk for endometrial cancer (37). Japanese epidemiological data revealed that the overall death rate of endometrial cancer decreased from 20.0 per 100,000 in 1950 to 8.0 per 100,000 in 1999, and this was thought to be a consequence of cytological screening (41).

Many researchers reported a high risk of endometrial cancer with positive cervical cytology (42, 43). Abnormal cervical cytology was associated with high-grade endometrial cancer, worse 5-year median recurrence-free survival and worse disease-specific survival (44). Positive cervical cytology should also be considered as a high risk of endometrial cancer, and endometrial cytology may benefit this kind of patients even with no clinical symptom.

An important strength of this meta-analysis is that we performed a thorough search for articles on the diagnostic accuracy in women with endometrial atypical hyperplasia or cancer using endometrial cytology. This article has several limitations. First, the risk of missing potentially relevant articles is a concern. Otherwise, the relatively small number of studies and variability in methods did not allow for more standard statistical analyses. Higher sensitivity and specificity could be found in subgroup of studies with sample size $\geq$ 300 and studies in European countries. However, after the subgroup analyses and sensitivity analysis, no factor showed associated with high heterogeneity. Patient age, menopause or not, different kinds of clinical symptoms, varies of cytological samplers and histological sampling methods might contribute to the high heterogeneity, and further study should approve it with enough data. What's more, the studies that are included in the meta-analysis are performed in symptomatic women. More data are needed before endometrial cytology being an effective screening tool for asymptomatic women with high-risks of endometrial cancer.

CONCLUSION

In conclusion, endometrial cytology is an efficient diagnostic method and could be applied in the diagnosis of endometrial disorders. The diagnostic accuracy of endometrial carcinoma will surely be improved by the combination of cyto-histopathological procedures and vaginal ultrasonography. Moreover, cytological examination, as a proper outpatient procedure, should be advised for endometrial screening, especial for those with high-risks of endometrial cancer.

AUTHOR CONTRIBUTIONS

QingW and QiW drafted the manuscript. QingW, LZ, and CS collected the case. QiW, LH, and SM performed the meta analysis. HH performed the pathological figure. QS helped to revise the manuscript. QL conceptualized the study.

FUNDING

This study was partly supported by the Shaanxi Provincial Collaborative Technology Innovation Plan (2017XT-026, 2018XT-002), Key Research and Development Project of Shaanxi Province Science and Technology Department (2017ZDXM-SF-068) and Medical Research Project of Xi'an Social Development Guidance Plan (2017117SF/YX011-3).

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* (2015) 65:5–29. doi: 10.3322/caac.21254
2. Telner DE, Jakubovicz D. Approach to diagnosis and management of abnormal uterine bleeding. *Can Fam Physician.* (2007) 53:58–64. doi: 10.1093/bmb/ldm014
3. Clark TJ, Gupta JK. Endometrial sampling of gynaecological pathology. *Obstetr Gynaecol.* (2002) 4:169–74. doi: 10.1576/toag.2002.4.3.169
4. Trimble CL, Kauderer J, Zaino R, Silverberg S, Lim PC, Burke JJ, et al. Concurrent endometrial carcinoma in women with a biopsy diagnosis of atypical endometrial hyperplasia. *Cancer.* (2006) 106:812–819. doi: 10.1002/cncr.21650
5. Suh BE, Hung YY, Armstrong MA. Complex atypical endometrial hyperplasia: the risk of unrecognized adenocarcinoma and value of preoperative dilation and curettage. *Obstetr Gynecol.* (2009) 114:523–9. doi: 10.1097/AOG.0b013e3181b190d5
6. Norimatsu Y, Kouda H, Kobayashi TK, Shimizu K, Yanoh K, Tsukayama C, et al. Utility of liquid-based cytology in endometrial pathology: diagnosis of endometrial carcinoma. *Cytopathology.* (2010) 20:395–402. doi: 10.1111/j.1365-2303.2008.00589.x
7. Fujiwara H, Takahashi Y, Takano M, Miyamoto M, Nakamura K, Kaneta Y, et al. Evaluation of endometrial cytology: cytohistological correlations in 1,441 cancer patients. *Oncology.* (2015) 88:86–94. doi: 10.1159/000368162
8. Fambrini M, Sorbi F, Sisti G, Cioni R, Turrini I, Taddei G, et al. Endometrial carcinoma in high-risk populations: is it time to consider a screening policy? *Cytopathology.* (2014) 25:71–7. doi: 10.1111/cyt.12131
9. Yanaki F, Hirai Y, Hanada A, Ishitani K, Matsui H. Liquid-based endometrial cytology using surepath^{TM} is not inferior to suction endometrial tissue biopsy in clinical performance for detecting endometrial cancer including atypical endometrial hyperplasia. *Acta Cytol.* (2017) 61:133—9. doi: 10.1159/000455890
10. Maksem MDJ, Sager DOF, Bender MDR. Endometrial collection and interpretation using the Tao brush and the CytoRich fixative system: a feasibility study. *Diagnost Cytopathol.* (1997) 17:339–46. doi: 10.1002/(SICI)1097-0339(199711)17:5<339::AID-DC6>3.0.CO;2-5
11. Garcia FAR, Barker B, Davis JD, Shelton T, Harrigill K, Schalk N, et al. Thin-layer cytology and histopathology in the evaluation of abnormal uterine bleeding. *J Reprod Med.* (2003) 48:882–8. doi: 10.1023/B:JARG.0000006710.64788.99
12. Papaefthimiou M, Symiakaki H, Mentzelopoulou P, Giahnaki AE, Voulgaris Z, Diakomanolis E, et al. The role of liquid-based cytology

associated with curettage in the investigation of endometrial lesions from postmenopausal women. *Cytopathology.* (2005) 16:32–9. doi: 10.1111/j.1365-2303.2004.00224.x
13. Andrijono A, Prayitno GD, Hamdani C. Diagnostic test of endometrial cytobrush in cases of perimenopausal and postmenopausal hemorrhage. *Med J Indonesia.* (2005) 14:87–91. doi: 10.13181/mji.v14i2.181
14. Buccoliero AM, Gheri CF, Castiglione F, Garbini F, Barbetti A, Fambrini M, et al. Liquid-based endometrial cytology: cyto-histological correlation in a population of 917 women. *Cytopathology.* (2007) 18:241–9. doi: 10.1111/j.1365-2303.2007.00463.x
15. Kipp BR, Medeiros F, Campion MB, Distad TJ, Peterson LM, Keeney GL, et al. Direct uterine sampling with the Tao brush sampler using a liquid-based preparation method for the detection of endometrial cancer and atypical hyperplasia: a feasibility study. *Cancer.* (2008) 114:228–35. doi: 10.1002/cncr.23636
16. Yanoh K, Hirai Y, Sakamoto A, Aoki D, Moriya T, Hiura M, et al. New terminology for intrauterine endometrial samples: a group study by the japanese society of clinical cytology. *Acta Cytol.* (2012) 56:113–4. doi: 10.1159/000336258
17. Remondi C, Sesti FF, Bonanno E, Pietropolli A, Piccione E. Diagnostic accuracy of liquid-based endometrial cytology in the evaluation of endometrial pathology in postmenopausal women. *Cytopathology.* (2013) 24:365–71. doi: 10.1111/cyt.12013
18. Yang X, Ma K, Chen R, Zhao J, Wu C, Zhang N, et al. Liquid-based endometrial cytology associated with curettage in the investigation of endometrial carcinoma in a population of 1987 women. *Arch Gynecol Obstetr.* (2017) 296:1–7. doi: 10.1007/s00404-017-4400-2
19. Cohen CJ, Gusberg SB. Screening for endometrial cancer. *Clin Obstetr Gynecol.* (2008) 114:219–21. doi: 10.1002/cncr.23705
20. Sorosky JI. Endometrial cancer. *Obstetr Gynecol.* (2012) 120:383–397. doi: 10.1097/AOG.0b013e3182605bf1
21. Bakkum-Gamez JN, Gonzalez-Bosquet J, Laack NN, Mariani A, Dowdy SC. Current issues in the management of endometrial cancer. *Mayo Clinic Proceed.* (2008) 83:97–112. doi: 10.4065/83.1.97
22. Bijen CBM, Bock GHD, Vermeulen KM, Arts HJG, Brugge HGT, Sijde RVD, et al. Laparoscopic hysterectomy is preferred over laparotomy in early endometrial cancer patients, however not cost effective in the very obese. *Eur J Cancer.* (2011) 47:2158–65. doi: 10.1016/j.ejca.2011.04.035
23. Seebacher V, Schmid M, Polterauer S, Hefler-Frischmuth K, Leipold H, Concin N, et al. The presence of postmenopausal bleeding as prognostic parameter in patients with endometrial cancer: a retrospective multi-center study. *BMC Cancer.* (2009) 9:460. doi: 10.1186/1471-2407-9-460
24. Abdelazim IA, Aboelezz A, Abdulkareem AF. Pipelle endometrial sampling versus conventional dilatation & curettage in patients with abnormal uterine bleeding. *J Turkish German Gynecol Assoc.* (2013) 14:1–5. doi: 10.5152/jtgga.2013.01
25. Barut A, Barut F, Arikan I, Harma M, Harma MI, Ozmen BU. Comparison of the histopathological diagnoses of preoperative dilatation and curettage and hysterectomy specimens. *J Obstetr Gynaecol Res.* (2012) 38:16–22. doi: 10.1111/j.1447-0756.2011.01633.x
26. Su H, Huang L, Huang KG, Yen CF, Han CM, Lee CL. Accuracy of hysteroscopic biopsy, compared to dilation and curettage, as a predictor of final pathology in patients with endometrial cancer. *Taiwanese J Obstetr Gynecol.* (2015) 54:757–60. doi: 10.1016/j.tjog.2015.10.013
27. Sakhdari A, Moghaddam PA, Liu Y. Endometrial samples from postmenopausal women: a proposal for adequacy criteria. *Int J Gynecol Pathol.* (2016) 35:525–30. doi: 10.1097/PGP.0000000000000279
28. Williams A, Brechin S, Porter A, Warner P, Critchley H. Factors affecting adequacy of Pipelle and Tao Brush endometrial sampling. *BJOG.* (2008) 115:1028–36. doi: 10.1111/j.1471-0528.2008.01773.x
29. Kandil D, Yang X, Stockl T, Liu Y. Clinical outcomes of patients with insufficient sample from endometrial biopsy or curettage. *Int J Gynecol Pathol.* (2014) 33:500–6. doi: 10.1097/PGP.0000000000000085
30. Howlader N, Noone AM, Krapcho M, Garshell J, Miller D, Altekruse SF, et al. *SEER Cancer Statistics Review*, 1975-2012. National Cancer Institute (2013).
31. Van HN, Prins MM, Bongers MY, Opmeer BC, Sahota DS, Mol BW, et al. The accuracy of endometrial sampling in women with postmenopausal bleeding: a systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol.* (2016) 197:147–55. doi: 10.1016/j.ejogrb.2015.12.008
32. Dijkhuizen FP, Mol BW, Brölmann HA, Heintz AP. The accuracy of endometrial sampling in the diagnosis of patients with endometrial carcinoma and hyperplasia: a meta-analysis. *Cancer.* (2000) 89:1765–72. doi: 10.1002/1097-0142(20001015)89:8<1765::AID-CNCR17>3.0.CO;2-F
33. Demirkiran F, Yavuz E, Erenel H, Bese T, Arvas M, Sanioglu C. Which is the best technique for endometrial sampling? Aspiration (pipelle) versus dilatation and curettage (D&C). *Arch Gynecol Obstetr.* (2012) 286:1277–82. doi: 10.1007/s00404-012-2438-8
34. Tanriverdi HA, Barut A, Gun BD, Kaya E. Is pipelle biopsy really adequate for diagnosing endometrial disease. *Medical Sci Monitor.* (2004) 10:CR271–4. doi: 10.1051/medsci/2004206-7710
35. Gkrozou F, Dimakopoulos G, Vrekoussis T, Lavasidis L, Koutlas A, Navrozoglou I, et al. Hysteroscopy in women with abnormal uterine bleeding: a meta-analysis on four major endometrial pathologies. *Arch Gynecol Obstetr.* (2015) 291:1347. doi: 10.1007/s00404-014-3585-x
36. Norimatsu Y, Kouda H, Kobayashi TK, Moriya T, Yanoh K, Tsukayama C, et al. Utility of thin-layer preparations in the endometrial cytology: evaluation of benign endometrial lesions. *Ann Diagnost Pathol.* (2008) 12:103–11. doi: 10.1016/j.anndiagpath.2007.05.005
37. Fulciniti F, Yanoh K, Karakitsos P, Watanabe J, Di LA, Margari N, et al. The Yokohama system for reporting directly sampled endometrial cytology: the quest to develop a standardized terminology. *Diagnost Cytopathol.* (2018) 46:400–12. doi: 10.1002/dc.23916
38. Shinagawa A, Kurokawa T, Yamamoto M, Onuma T, Tsuyoshi H, Chino Y, et al. Evaluation of the benefit and use of the new terminology in endometrial cytology reporting system. *Diagnost Cytopathol.* (2018) 46:314–9. doi: 10.1002/dc.23901
39. Margari N, Pouliakis A, Aninos D, Meristoudis C, Stamataki M, Panayiotides I, et al. Internal quality control in an academic cytopathology laboratory for the introduction of a new reporting system for endometrial cytology. *Diagnost Cytopathol.* (2017) 45:883–8. doi: 10.1002/dc.23787
40. Kondo E, Tabata T, Koduka Y, Nishiura K, Tanida K, Okugawa T, et al. What is the best method of detecting endometrial cancer in outpatients?-Endometrial sampling, suction curettage, endometrial cytology. *Cytopathology.* (2008) 19:28–33. doi: 10.1111/j.1365-2303.2007.00509.x
41. Nagase E, Yasuda M, Kajiwara H, Osamura RY, Yoshitake T, Hirasawa T, et al. Uterine body cancer mass screening at Tokai University Hospital. *Tokai J Exp Clin Med.* (2004) 29:43–8.
42. Tate K, Yoshida H, Ishikawa M, Uehara T, Ikeda S, Hiraoka N, et al. Prognostic factors for patients with early-stage uterine serous carcinoma without adjuvant therapy. *J Gynecol Oncol.* (2018) 29:e34. doi: 10.3802/jgo.2018.29.e34
43. van Doom HC, Opmeer BC, Kooi GS, Ewing Graham PC, Kruitwagen RF, Mol BW. Value of cervical cytology in diagnosing endometrial carcinoma in women with postmenopausal bleeding. *Acta Cytol.* (2009) 53:277–82. doi: 10.1159/000325308
44. Lcm A, Jma P, Dwl J, Hjmm M, Smj VK, Mja E, et al. Contribution of cervical cytology in the diagnostic work-up of patients with endometrial cancer. *Cytopathology.* (2018) 29:63–70. doi: 10.1111/cyt.12511

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 19 February 2019
doi: 10.3389/fonc.2019.00067

An Efficacious Endometrial Sampler for Screening Endometrial Cancer

*Lu Han[1,2], Jiang Du[3], Lanbo Zhao[4], Chao Sun[1,2], Qi Wang[1,2], Xiaoqian Tuo[1,2], Huilian Hou[5], Yu Liu[5], Qing Wang[1,2], Qurat Ulain[1,2], Shulan Lv[2], Guanjun Zhang[5], Qing Song[1,6] and Qiling Li[1,2]**

[1] Center for Single-Cell Biology, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China, [2] Department of Obstetrics and Gynecology, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China, [3] Department of Gynecology and Obstetrics, Shaanxi Provincial People's Hospital, Xi'an, China, [4] Guipei 77, Health Science Center, Xi'an Jiaotong University, Xi'an, China, [5] Department of Pathology, First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China, [6] Cardiovascular Research Institute, Morehouse School of Medicine, Atlanta, GA, United States

Recently, the research on early detection of precancerous change and endometrial carcinoma has been focusing on minimally invasive procedures for screening. On this basis, we aim to verify the feasibility of endometrial samplers for screening endometrial cancer using Li Brush. We recruited patients undergoing hysterectomy for different diseases from the Inpatient Department of the Department of Obstetrics and Gynecology. Before surgery, endometrial cells were collected by Li Brush. The cytopathologic diagnosis from Li Brush and the histopathologic diagnosis from hysterectomy in the same patient were compared to calculate sensitivity (Se), specificity (Sp), false-negative rate (FNR), false-positive rate (FPR), positive predictive value (PV+) %, and negative predictive value (PV-). The research enrolled 293 women into this self-controlled trial. According to the hypothesis test of paired four lattices, we obtained the following indicators: Se 92.73, Sp 98.15, FNR 7.27, FPR 1.85, PV+92.73, and PV−98.15%. The endometrial sampler Li Brush is an efficacious instrument for screening endometrial cancer.

Keywords: endometrial cancer, endometrial sampler, cytology, histopathology, screening

OPEN ACCESS

Edited by:
Massimo Nabissi,
University of Camerino, Italy

Reviewed by:
Margaret Liang,
University of Alabama at Birmingham, United States
Saori Furuta,
University of Toledo, United States

***Correspondence:**
Qiling Li
Liqiling@mail.xjtu.edu.cn

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *09 September 2018*
Accepted: *24 January 2019*
Published: *19 February 2019*

Citation:
Han L, Du J, Zhao L, Sun C, Wang Q, Tuo X, Hou H, Liu Y, Wang Q, Ulain Q, Lv S, Zhang G, Song Q and Li Q (2019) An Efficacious Endometrial Sampler for Screening Endometrial Cancer. Front. Oncol. 9:67. doi: 10.3389/fonc.2019.00067

INTRODUCTION

The morbidity and mortality of endometrial carcinoma is on the rise around the world in recent years. It has been the most common gynecologic malignancy in some developed countries such as Japan and US and ranked second in many developing countries (1, 2). The cancer-related costs are increasing significantly, constituting a challenge for social economics and female health. Efforts focusing on primary and secondary prevention remain central to the global charge to reduce the incidence of cancer and avoid one-third to one-half of cancer deaths (2, 3). With developing morbidity of endometrial cancer around the world, early detection and diagnosis would undoubtedly become the most important part. For endometrial carcinoma, the 5-year survival rate gradually decreases with the development of the stages. Eighty percent of the patients diagnosed with endometrial cancer are in stage I, with a 5-year survival rate of >95% (4). Endometrial atypical hyperplasia is considered to be the precancerous lesions of endometrial cancer. Thirty percent of atypical hyperplasia will develop into cancer a long time in the future; thus, we have the opportunity to screen for endometrial cancer within this long time period (5). The aim of screening is to detect endometrial atypical hyperplasia and the early stages of endometrial cancer. The ability to save lives would mean great social significance and economic benefits.

Endometrial carcinoma is a type of epitheliogenic malignant tumor that originates from the endometrium. As one of three major malignant tumors of the female reproductive system, the average onset age of endometrial carcinomas is 63 years, and > 90% occur in women above 50 years of age, and ∼4% occur in women younger than 40 years of age (4). Risk factors for endometrial cancer include early menarche (6), late menopause, nulliparity, Lynch syndrome (7), diabetes (8, 9), obesity (10), hypertension, estrogen, and tamoxifen treatment after menopause (11, 12), a family history of endometrial cancer or breast cancer (13), and polycystic ovary syndrome (14).

Because of the rising morbidity, the window period, and an explicit screening population, endometrial cancer screening is feasible. Until now, histopathology with dilatation and curettage (D&C) with or without hysteroscopy and surgery has been the gold standard for the diagnosis of endometrial carcinoma and precancerous lesions (15). However, the injury and discomfort caused by D&C have influenced its widespread use for screening. In recent years, more and more non-invasive endometrial devices have been invented and proposed for screening endometrial cancer, such as Pipelle, which was found to have an 86% sensitivity in one study (16); Tao Brush, which was found to have a 95.5% sensitivity (16); and SAP-1, which was found to have a 73% sensitivity, 95.8% specificity, 75% positive predictive value, and 95.3% negative predictive value (17). However, no available, specific, and effective screening method could be applied popularly for women until now. In this study, we compared the cytopathologic diagnosis of the Li Brush (**Figure 1A**, Xi'an Meijiajia Medical Co. 20152660054) with the histopathologic diagnosis of hysterectomy to evaluate the feasibility of the endometrial samplers for screening endometrial cancer.

MATERIALS AND METHODS

Patients and Study Procedures

From January 2015 to July 2016, we recruited patients undergoing hysterectomy because of different diseases from the Inpatient Department (IPD) of the Department of Obstetrics and Gynecology. Patients in the IPD were excluded if they had already been diagnosed with pregnancy, acute inflammation of the genital system besides atrophic vaginitis, endogenous cervical carcinoma, dysfunction of blood coagulation, and other hematologic diseases that might influence coagulation function. Women with a body temperature >37.5°C in two subsequent measurements in 1 day were also ruled out.

According to the following procedures, we collected endometrium specimens using the Li Brush and obtained the cytopathologic diagnosis from the Department of Pathology before surgery. First, the patients were placed in the lithotomy position, and the conventional perineal and vaginal disinfection were performed after emptying the bladder. Second, the uterine cervix was exposed by vaginal speculum and the uterine depth was detected with uterine probe. After the brush head was hidden in the drivepipe, the sampler was put into the fundus of uterus (**Figure 1B**). Then, the drivepipe was drawn out ∼5 mm to show the brush, and the handle was rotated 5–10 complete circles to gather cells of the uterine corpus (**Figure 1C**). Third, the drivepipe was advanced 3 mm and the handle was again rotated to gather cells from the uterine fundus (**Figure 1D**). Finally, the brush was removed from uterine cavity after protecting the brush head under the casing (**Figure 1E**). When sampling was complete, the brush head was placed into the preservation solution and shaken several times to release the cells into the solution.

The cell specimens were prepared for testing with a liquid-based cytologic test, and the tissues were embedded in paraffin and cut into cross-sections. Both cell and tissue specimens were stained using hematoxylin and eosin. After concealing identity information, all the samples were sent to the Department of Pathology of First Affiliated Hospital of Xi'an Jiaotong University and randomly diagnosed by two independent professors. The diagnoses of histology and cytology were independently conducted. According to the method of sample size calculation in the diagnosis experiment (18) and using the sensitivity of SAP-1 brush (4, 17) to predict the sensitivity of Li Brush, a minimum of 113 cases were required for the study. The self-control method was used in this study. The histopathologic diagnosis from the hysterectomy was defined as the standard. The outcomes were obtained by comparing the cytopathologic and histopathologic results of the same individuals.

The study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University(XJTU1AHCR2014-007). All the patients involved in the research were sufficiently informed of the content of the study and provided written informed consent.

Data Collection

For all eligible patients, the following information was collected: age, age at menarche, last menstrual period or menopausal age, childbearing history, endometrial thickness, tumor history, smoking history, with or without hormone replacement, and history of other diseases such as hypertension and diabetes. The data were used to determine sampling satisfaction, cytopathologic diagnosis, and histopathologic diagnosis.

Definition of Outcomes

According to the International Society of Gynecological Pathologists, the histopathologic diagnoses included the following: proliferative endometrium, secretory endometrium, atrophic endometrium, mixed endometrium, and simple hyperplasia including cystic glandular hyperplasia, complex hyperplasia defined as adenomatous hyperplasia without atypia, endometrial atypical hyperplasia, and endometrial carcinoma. The cytopathologic diagnoses were classified into seven categories, as follows: proliferative endometrial cells, secretory endometrial cells, atrophic endometrial cells, mixed endometrial cells, endometrial hyperplasia cells, endometrial atypical cells, and endometrial cancer cells. Positive results were defined as endometrial carcinoma, endometrial cancer cells, endometrial atypical hyperplasia, and endometrial atypical cells. Other categories were defined as negative results. When both cellular and histionic diagnostic results were positive, it was judged as true positive; if both were negative, it was

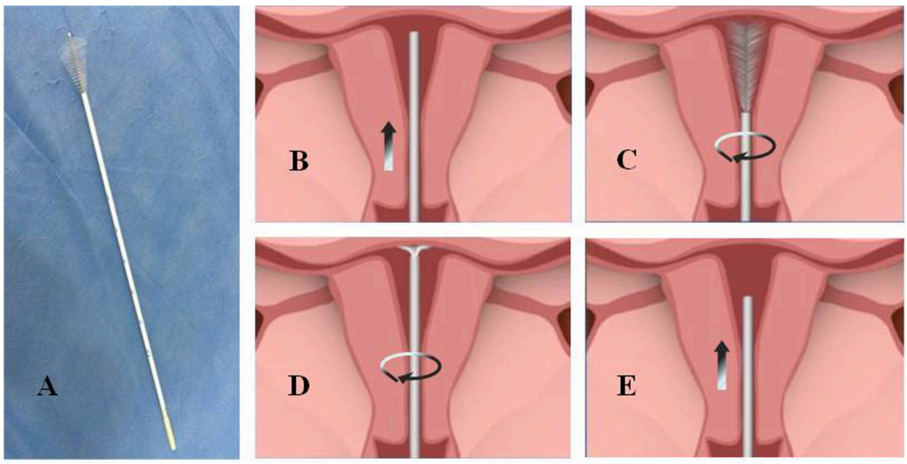


FIGURE 1 | The physical map and sampling procedure of endometrial samples using Li Brush**. (A)** a photo of Li Brush; **(B)** protect the brush head by placing it into the drivepipe and put sampler into the fundus of the uterus; **(C)** withdraw the drivepipe approximately 5 mm to show the brush and rotate the handle in 5 to 10 complete circles to gather cells of the uterine corpus; **(D)** advance the drivepipe 3 mm and rotate the handle again to gather cells of the uterine fundus; **(E)** cover the head with the casing and withdraw the brush from the uterine cavity.

judged as true negative. If the cytopathologic result was positive and the histopathologic result was negative, it was judged as false positive; if the cytopathologic result was negative and the histopathologic result was positive, it was judged as false negative. Consistent outcome was when the cytopathologic and histopathologic diagnoses were both positive or both negative; otherwise, the outcomes were considered inconsistent. The sampling satisfaction was reflected in a sufficient number of cells and the correct location.

Statistical Analysis

Using the hypothesis test of paired four lattices, the following indicators were calculated: sensitivity (Se), false-negative rate (FNR), specificity (Sp), false-positive rate (FPR), positive predictive value (PV+), and negative predictive value (PV-). The differences of endometrial histopathology and cytopathology using Li Brush in the diagnosis of endometriosis were evaluated by the calculation of P-value. If $P < 0.05$, the difference was statistically significant. In contrast, if $P > 0.05$, the difference was not statistically significant.

RESULTS

Patients

We aimed to collect a total of 420 patients from the IPD of the Department of Obstetrics and Gynecology. We ruled out 112 patients because of insufficient or incomplete information. The remaining 308 women completed the study (**Table 1**). Satisfactory endometrial cells were not obtained in 37 patients (**Figure 2**).

Pathologic Images

Using pathological slide and microscopic camera technology, the following histrionic images were obtained: proliferative endometrium (**Figure 3A**, left), secretory endometrium (**Figure 3B**, left), atrophic endometrium (**Figure 3C**, left), mixed endometrium (**Figure 3D**, left), endometrial atypical hyperplasia (**Figure 3E**, left), and endometrial carcinoma (**Figure 3F**, left). The corresponding cytopathologic images were included: proliferative endometrial cells (**Figure 3A**, right), secretory endometrial cells (**Figure 3B**, right), atrophic endometrial cells (**Figure 3C**, right), mixed endometrial cells (**Figure 3D**, right), endometrial atypical cells (**Figure 3E**, right), and endometrial cancer cells (**Figure 3F**, right).

Data Calculation

According to the hypothesis test of paired four lattices, there were 51 true-positive, 212 true-negative, 4 false-positive, and 4 false-negative cases. The following indices were obtained: Se 92.73, Sp 98.15, FNR 7.27, FPR 1.85, PV+ 92.73, and PV- 98.15%. The data showed that there were no significant difference between cytopathologic results from the Li Brush and histopathologic results from hysterectomy ($\chi^2 = 0.125 < \chi^2_{0.05}$, $\alpha = 0.05$, $P > 0.05$).

Furthermore, we compared the histopathologic results obtained from hysterectomy and the cytopathologic results of our samplers accurately to evaluate the feasibility of using Li Brush in the diagnosis of endometrial types. Through the statistics, there were 228 cases consistent and 34 cases inconsistent. In addition, 9 cases were diagnosed as endometrial simple hyperplasia with local polyps by hysterectomy and endometrial hyperplasia cells by our samplers. The overall degree of satisfaction and sensitivity of sampling were 87.02 and 87.63%, respectively (**Table 2**). The sensitivities of different types of endometrium were 85.88% for proliferative endometrium, 72.73% for secretory endometrium, 88.24% for atrophic endometrium, 83.33% for mixed endometrium, 94.64% for simple hyperplasia, 100% for complex hyperplasia, 80.00% for endometrial atypical hyperplasia, and 87.02% for endometrial carcinoma. Meanwhile,

TABLE 1 | Patient characteristics.

Characteristics	*n*
SOURCE	
IPD[a]	308
AGE	
<40 years old	32
≥40 years old	276
MENSTRUAL STATUS	
Premenopausal	200
Postmenopausal	83
AUB[b]	6
ENDOMETRIAL THICKNESS[c]	
<5 mm	24
≥5 mm	211
Intrauterine heterogeneity echo	5
Unclear display	3
OTHER DISEASE	
Ovarian cancer	3
Hypertension	7
Diabetes	4
Hormone replacement therapy	2

Some information of the patients is missing.
[a]IPD, Inpatient Department.
[b]AUB, Abnormal uterus bleeding.
[c]Some patients were not examined by ultrasound, whose endometrial thickness is missing.

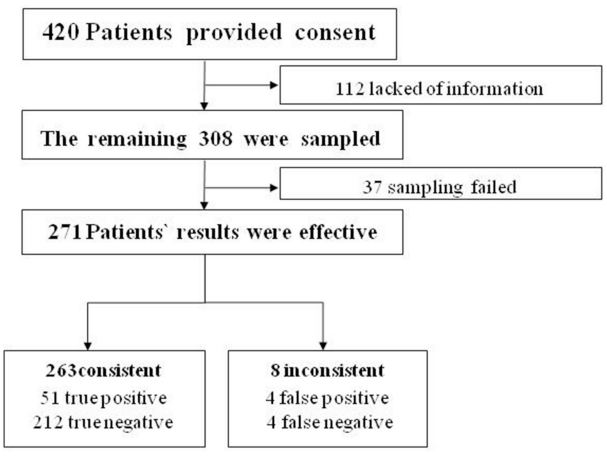


FIGURE 2 | Diagram of study participants.

the results of cells and tissues were separately analyzed and compiled into a bar chart by composition ratios (**Figure 4**).

DISCUSSION

Endometrial curetting or D&C have long been the standard diagnosis or treatment for evaluating suspicious endometrial lesions, especially in mainland China. Only patients who display symptoms that are geared to the indication of D&C, such as abnormal uterine bleeding, would refer themselves to medical help for diagnosis and treatment, which restricts the early diagnosis and treatment of endometrial carcinomas. Because only approximately 60% of curetting procedures can evaluate less than half of the uterine cavity, even when performed by the most experienced physician, the rate of false-negative results is high (19). Moreover, the pain and suffering caused by the procedure were not widely accepted patients. Despite its diagnostic value for patients who display symptoms, this method shows its deficiency as a screening procedure for endometrial lesions (20).

Recently, research on the early detection of endometrial carcinoma has been focusing on minimally invasive histopathologic and cytopathologic procedures (16, 21–24). Along with the improvement and widespread use of liquid-based preparation (LBP) of endometrial cell samples, direct cellular sampling has more commonly used as the primary screening procedure for endometrial lesions (25). Among the sampling techniques, endometrial brush cytology is minimally invasive, more economical, and more convenient compared with traditional diagnostic and curettage techniques. Therefore, it has already become a partly accepted method for the detection of endometrial lesions (26). The endometrial cytology by direct intrauterine sampling has a relatively high specificity and sensitivity for the diagnosis of endometrial cancer reported by some researchers (27). Related samplers have been studied, including histology samplers such as Pipelle (28), and cytology samplers, such as Tao brush (19) and SAP-1 sampler (4, 17), as well as Uterobrush (26). However, up to this point, we still required a more convenient, economical, and non-invasive tool to screen for endometrial cancer.

On that basis, we have invented a new endometrial sampler—Li Brush. Our sampler was awarded a utility model patent certificate from the State Patent Office (number: ZL.2014 2 0720056.8). The brush is made up of four parts: head,tube core, drivepipe, and hand shank (**Figure 1A**). The head is T shaped, which is close to the physiologic form of the uterine cavity. The fusiform brush allows easy access to the uterine cavity, fundus, and horn of the uterus. The elastic drivepipe works with the handle to protect the head from contamination of cervical cells. In addition, our samplers have other advantages, such as low cross-infection, good flexibility, less damage, low cost, and higher acceptability. In this study, we compared the diagnosis of cytology by the Li Brush with the diagnosis of histopathology by hysterectomy. A total of 271 cases were analyzed, with a sensitivity of 92.73% and a specificity of 98.15%. The proportions of the endometrial types were similar between histology and cytology. These findings showed that Li Brush will be able to play a role in the screening for endometrial cancer.

The two common causes of sampling failure using Li Brush were the inaccurate location of sampling and insufficient number of endometrial cells. The lower sampling satisfaction of atypical hyperplasia and complex hyperplasia are because of the limited sample size. The lower sampling satisfaction of atrophic endometrium is attributed to the atrophic cervix and the adhesion of cervix tube in post-menopausal women. To improve the sampling satisfaction, we considered whether using cervical

TABLE 2 | The comparison of diagnosis between cytopathology and histopathology.

Endometrial types	Cytopathology and histopathology			Total	Se(%)	Sampling satisfaction(%)
	Consistent	Inconsistent	Unsatisfied sampling			
Proliferative endometrium	73	12	10	95	85.88	89.47
Secretory endometrium	24	9	5	38	72.73	86.84
Atrophic endometrium	15	2	4	21	88.24	80.95
Mixed endometrium	10	2	1	13	83.33	92.31
Simple hyperplasia	53	3	9	65	94.64	86.15
Complex hyperplasia	4	0	1	5	100.00	80.00
Atypical hyperplasia	4	1	1	6	80.00	83.33
Endometrial carcinoma	45	5[a]	6	56	90.00	89.29
Total	228	34	37	299[b]	87.02	87.63

Se, Sensitivity.

[a] *There were 4 cases which the histopathological results were endometrial cancer but the cytopathologic results were proliferative endometrial or endometrial hyperplasia cells. One of this 5 cases which the histopathological result of was endometrial cancer but the cytopathologic result was endometrial atypical cells, so this case was considered to be true positive but inconsistent.*

[b] *There were 9 of all 308 cases diagnosed endometrial simple hyperplasia with local polyp.*

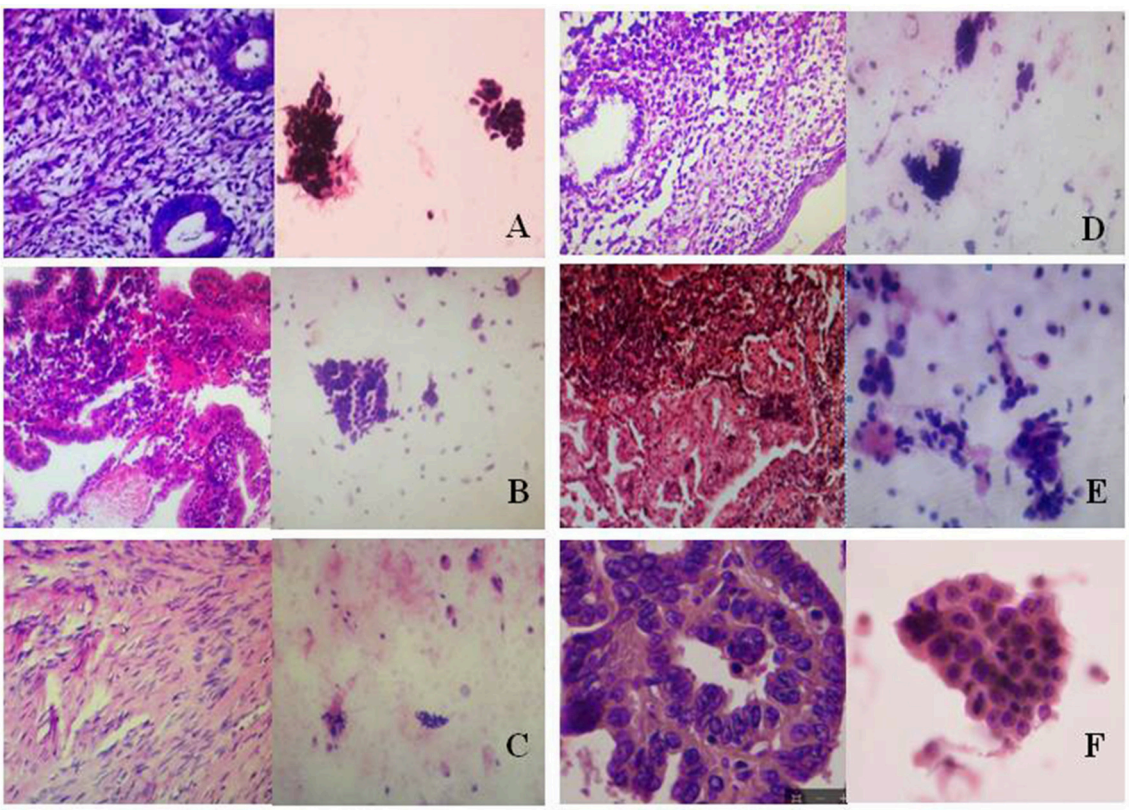


FIGURE 3 | Histopathologic and cytopathologic images**. (A)** proliferative endometrium (Left: HE × 400) and proliferative endometrial cells (Right: HE × 100); **(B)** secretory endometrium (Left: HE × 10) and secretory endometrial cells (Right: HE × 10); **(C)** atrophic endometrium (Left: HE × 10) and atrophic endometrial cells (Right: HE × 10); **(D)** mixed endometrium (Left: HE × 10) and mixed endometrial cells (Right: HE × 10); **(E)**: endometrial atypical hyperplasia (Left: HE × 10) and endometrial atypical cells (Right: HE × 200); **(F)** endometrial carcinoma (Left: HE × 400) and endometrial cancer cells (Right: HE × 400).

clamps when using the brush could make it easier for the brush to smoothly enter the uterine cavity. We can also mechanically expand the cervical canal for patients with cervical adhesion or narrowing if necessary.

For endometrial polyps, Li Brush showed a high false-negative rate. According to histology, endometrial polyps are classified into four categories: non-functional glandular endometrial polyp, functional glandular endometrial polyp, adenomatoid polyp,

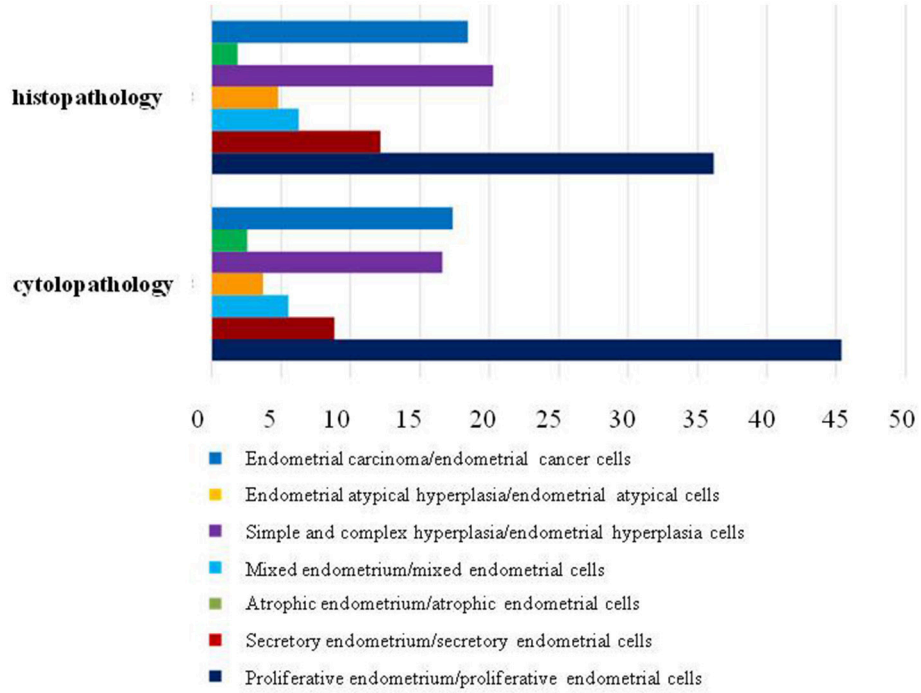


FIGURE 4 | The proportions of histopathologic and cytologic diagnosis.

and polyp with malignant transformation (5). Polyps consist of proliferating glands, blood vessels, and stroma because of the hyperplasia due to the high sensitivity of endometrium to estrogen (17). When brushing, we only tend to sample the superficial cells or glands of polyps, which makes the sample look like endometrial hyperplasia, and ignores its real structure. Reagan and Ng et al. Study pointed out that when sampled cells were out of the endometrial cycle, only a quarter of them were from polyp (5). Thus, histology often shows more sensitive results than cytology for the diagnosis of polyps.

Our study found that Li Brush will be able to be a reliable approach for screening endometrial cancer and may provide great benefits for the social economy and women's health. However, there are still some shortcomings to the technique, such as the false-negative rate for the diagnosis of endometrial polyps is high and the sampling satisfaction rates are not low enough. In the future, after obtaining more data, we hope to use the brush to diagnose the detailed pathologic types of endometrium.

AUTHOR CONTRIBUTIONS

LH, JD, and CS performed the clinic experiments. LZ, SL, and QL designed the endometrial sampler. HH and GZ performed the pathologic diagnosis. YL performed the pathological sections. HH also performed the pathological sections. QiW, QinW, and XT helped to perform the statistical analysis. LH and QL wrote the manuscript. QU revised the English grammar. QS helped to designed the experiments. QL conceived the study. All authors gave feedback and approved the final version of the manuscript.

FUNDING

This study was supported by the Key Research and Development Project of Shaanxi Provincial Science and Technology Department (2017ZDXM-SF-068), Shaanxi Provincial Collaborative Technology Innovation Plan (2017XT-026, 2018XT-002), and Medical Research Project of Xi'an Social Development Guidance Plan (2017117SF/YX011-3).

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin.* (2015) 65:87–108. doi: 10.3322/caac.21262
2. Batista TP, Cavalcanti CL, Tejo AA, Bezerra AL. Accuracy of preoperative endometrial sampling diagnosis for predicting the final pathology grading in uterine endometrioid carcinoma. *Eur J Surg Oncol.* (2016) 42:1367–71. doi: 10.1016/j.ejso.2016.03.009
3. del Carmen MG, Rice LW, Schmeler KM. Global health perspective on gynecologic oncology. *Gynecol Oncol.* (2015) 137:329–34. doi: 10.1016/j.ygyno.2015.03.009
4. Colombo N, Creutzberg C, Amant F, Bosse T, Gonzalez-Martin A, Ledermann J, et al. ESMO-ESGO-ESTRO consensus conference on endometrial cancer:

diagnosis, treatment and follow-up. *Radiother Oncol*. (2015) 117:559–81. doi: 10.1016/j.radonc.2015.11.013
5. Shu Y, Aikle F. *Cytopathology of the Endometrium: Correlation With Histopathology*. Beijing: People's Medical Publishing House (2008).
6. Braun MM, Overbeek-Wager EA, Grumbo RJ. Diagnosis and management of endometrial cancer. *Am Fam Phys*. (2016) 93:468–74.
7. Mas-Moya J, Dudley B, Brand RE, Thull D, Bahary N, Nikiforova MN, et al. Clinicopathological comparison of colorectal and endometrial carcinomas in patients with Lynch-like syndrome versus patients with Lynch syndrome. *Hum Pathol*. (2015) 46:1616–25. doi: 10.1016/j.humpath.2015.06.022
8. Friberg E, Mantzoros CS, Wolk A. Diabetes and risk of endometrial cancer: a population-based prospective cohort study. *Cancer Epidemiol Biomarkers Prev*. (2007) 16:276–80. doi: 10.1158/1055-9965.EPI-06-0751
9. Friberg E, Orsini N, Mantzoros CS, Wolk A. Diabetes mellitus and risk of endometrial cancer: a meta-analysis. *Diabetologia* (2007) 50:1365–74. doi: 10.1007/s00125-007-0681-5
10. Dallal CM, Brinton LA, Bauer DC, Buist DSM, Cauley JA, Hue TF, et al. Obesity-related hormones and endometrial cancer among postmenopausal women: a nested case-control study within the B similar to FIT cohort. *Endocr Related Cancer* (2013) 20:151–60. doi: 10.1530/Erc-12-0229
11. Ngo C, Brugier C, Plancher C, de la Rochefordiere A, Alran S, Feron JG, et al. Clinico-pathology and prognosis of endometrial cancer in patients previously treated for breast cancer, with or without tamoxifen: a comparative study in 363 patients. *Eur J Surg Oncol*. (2014) 40:1237–44. doi: 10.1016/j.ejso.2014.05.007
12. Dallal CM, Lacey JVJr, Pfeiffer RM, Bauer DC, Falk RT, Buist DS, et al. Estrogen metabolism and risk of postmenopausal endometrial and ovarian cancer: the B approximately FIT Cohort. *Horm Cancer* (2016) 7:49–64. doi: 10.1007/s12672-015-0237-y
13. Win AK, Reece JC, Ryan S. Family history and risk of endometrial cancer: a systematic review and meta-analysis. *Obstet Gynecol*. (2015) 125:89–98. doi: 10.1097/AOG.0000000000000563
14. Gottschau M, Kjaer SK, Jensen A, Munk C, Mellemkjaer L. Risk of cancer among women with polycystic ovary syndrome: a danish cohort study. *Gynecol Oncol*. (2015) 136:99–103. doi: 10.1016/j.ygyno.2014.11.012
15. Practice Bulletin, No 149. endometrial cancer. *Obstet Gynecol*. (2015) 125**:**1006–26. doi: 10.1097/01.AOG.0000462977.61229.de
16. Del Priore G, Williams R, Harbatkin CB, Wan LS, Mittal K, Yang GC. Endometrial brush biopsy for the diagnosis of endometrial cancer. *J Reprod Med*. (2001) 46:439–43. doi: 10.1097/00006254-200109000-00014
17. Wen J, Chen R, Zhao J, Dong Y, Yang X, Liao QP. Combining endometrium sampling device and SurePath preparation to screen for endometrial carcinoma: a validation study. *Chin Med J*. (2015) 128:648–53. doi: 10.4103/0366-6999.151664
18. Malhotra RK, Indrayan A. A simple nomogram for sample size for estimating sensitivity and specificity of medical tests. *Indian J Ophthalmol*. (2010) 58:519–22. doi: 10.4103/0301-4738.71699
19. Kipp BR, Medeiros F, Campion MB, Distad TJ, Peterson LM, Keeney GL, et al. Direct uterine sampling with the Tao brush sampler using a liquid-based preparation method for the detection of endometrial cancer and atypical hyperplasia: a feasibility study. *Cancer* (2008) 114:228–35. doi: 10.1002/cncr.23636
20. Munro MG, Southern California Permanente Medical Group's Abnormal Uterine Bleeding Working G. Investigation of women with postmenopausal uterine bleeding: clinical practice recommendations. *Perm J*. (2014) 18:55–70. doi: 10.7812/TPP/13-072
21. Zorlu CG, Cobanoglu O, Isik AZ, Kutluay L, Kuscu E. Accuracy of pipelle endometrial sampling in endometrial carcinoma. *Gynecol Obstet Invest*. (1994) 38:272–5.
22. Guido RS, Kanbour-Shakir A, Rulin MC, Christopherson WA. Pipelle endometrial sampling. sensitivity in the detection of endometrial cancer *J Reprod Med*. (1995) 40:553–5.
23. Machado F, Moreno J, Carazo M, Leon J, Fiol G, Serna R. Accuracy of endometrial biopsy with the Cornier pipelle for diagnosis of endometrial cancer and atypical hyperplasia. *Eur J Gynaecol Oncol*. (2003) 24:279–81.
24. Abdelazim IA, Aboelezz A, Abdulkareem AF. Pipelle endometrial sampling versus conventional dilatation & curettage in patients with abnormal uterine bleeding. *J Turk Ger Gynecol Assoc*. (2013) 14:1–5. doi: 10.5152/jtgga.2013.01
25. Norimatsu Y, Yanoh K, Kobayashi TK. The role of liquid-based preparation in the evaluation of endometrial cytology. *Acta Cytol*. (2013) 57:423–35. doi: 10.1159/000353148
26. Iavazzo C, Vorgias G, Mastorakos G, Stefanatou G, Panoussi A, Alexiadou A, et al. Uterobrush method in the detection of endometrial pathology. *Anticancer Res* (2011) 31:3469–74.
27. Fujiwara H, Takahashi Y, Takano M, Miyamoto M, Nakamura K, Kaneta Y, et al. Evaluation of endometrial cytology: cytohistological correlations in 1,441 cancer patients. *Oncology* (2015) 88:86–94. doi: 10.1159/000368162
28. Matsuo K, Yabuno A, Hom MS, Shida M, Kakuda M, Adachi S, et al. Significance of abnormal peritoneal cytology on survival of women with stage I-II endometrioid endometrial cancer. *Gynecol Oncol*. (2018). doi: 10.1016/j.ygyno.2018.02.012

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 12 April 2019
doi: 10.3389/fonc.2019.00265

A Novel Approach to Preoperative Risk Stratification in Endometrial Cancer: The Added Value of Immunohistochemical Markers

*Vit Weinberger[1], Marketa Bednarikova[2], Jitka Hausnerova[3], Petra Ovesna[4], Petra Vinklerova[1], Lubos Minar[1], Michal Felsinger[1], Eva Jandakova[3], Marta Cihalova[3] and Michal Zikan[5]**

[1] Department of Gynecology and Obstetrics, University Hospital in Brno and Masaryk University, Brno, Czechia, [2] Department of Internal Medicine – Hematology and Oncology, University Hospital in Brno and Masaryk University, Brno, Czechia, [3] Department of Pathology, University Hospital in Brno and Masaryk University, Brno, Czechia, [4] Faculty of Medicine, Institute of Biostatistics and Analyses, Masaryk University, Brno, Czechia, [5] Institute of Biostatistics and Analyses, Faculty of Medicine, Masaryk University, Brno, Czechia

Background: The current model used to preoperatively stratify endometrial cancer (EC) patients into low- and high-risk groups is based on histotype, grade, and imaging method and is not optimal. Our study aims to prove whether a new model incorporating immunohistochemical markers, L1CAM, ER, PR, p53, obtained from preoperative biopsy could help refine stratification and thus the choice of adequate surgical extent and appropriate adjuvant treatment.

Materials and Methods: The following data were prospectively collected from patients operated for EC from January 2016 through August 2018: age, pre- and post-operative histology, grade, lymphovascular space invasion, L1CAM, ER, PR, p53, imaging parameters obtained from ultrasound, CT chest/abdomen, final FIGO stage, and current decision model (based on histology, grade, imaging method).

Results: In total, 132 patients were enrolled. The current model revealed 48% sensitivity and 89% specificity for high-risk group determination. In myometrial invasion >50%, lower levels of ER ($p = 0.024$), PR (0.048), and higher levels of L1CAM ($p = 0.001$) were observed; in cervical involvement a higher expression of L1CAM ($p = 0.001$), lower PR ($p = 0.014$); in tumors with positive LVSI, higher L1CAM ($p = 0.014$); in cases with positive LN, lower expression of ER/PR ($p < 0.001$), higher L1CAM ($p = 0.002$) and frequent mutation of p53 ($p = 0.008$).

Cut-offs for determination of high-risk tumors were established: ER <78% ($p = 0.001$), PR <88% ($p = 0.008$), and L1CAM ≥4% ($p < 0.001$). The positive predictive values (PPV) for ER, PR, and L1CAM were 87% (60.8–96.5%), 63% (52.1–72.8%), 83% (70.5–90.8%); the negative predictive values (NPV) for each marker were as follows: 59% (54.5–63.4%), 65% (55.6–74.0%), and 77% (67.3–84.2%). Mutation of p53 revealed PPV 94% (67.4–99.1%) and NPV 61% (56.1–66.3%). When immunohistochemical markers were included into the current diagnostic model, sensitivity improved (48.4 vs. 75.8%, $p < 0.001$). PPV was similar for both methods, while NPV (i.e., the probability of extremely low risk in negative test cases) was improved (66 vs. 78.9%, $p < 0.001$).

OPEN ACCESS

Edited by:
Massimo Nabissi, University of Camerino, Italy

Reviewed by:
Kruti P. Maniar, Northwestern University, United States
Tonya J. Webb, University of Maryland, Baltimore, United States

***Correspondence:**
Michal Zikan
michal.zikan@lf1.cuni.cz

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *07 December 2018*
Accepted: *22 March 2019*
Published: *12 April 2019*

Citation:
Weinberger V, Bednarikova M, Hausnerova J, Ovesna P, Vinklerova P, Minar L, Felsinger M, Jandakova E, Cihalova M and Zikan M (2019) A Novel Approach to Preoperative Risk Stratification in Endometrial Cancer: The Added Value of Immunohistochemical Markers. Front. Oncol. 9:265. doi: 10.3389/fonc.2019.00265

Conclusion: We proved superiority of new proposed model using immunohistochemical markers over standard clinical practice and that new proposed model increases accuracy of prognosis prediction. We propose wider implementation and validation of the proposed model.

Keywords: endometrial cancer, ER, imaging method, L1CAM, PR, preoperative biopsy, p53, risk stratification

INTRODUCTION

Endometrial carcinoma (EC) is one of the most common female cancers. It predominantly has a favorable prognosis, due to the early onset of signs and symptoms such as postmenopausal bleeding or spotting, which lead to early-stage diagnosis in most patients and five-year overall survival rates of up to 85% (1). However, 20% of those EC patients who are estimated to be at low risk of recurrence will nevertheless recur while up to 50% of those designated "high-risk" will not (2, 3). It is clear the prognostic markers currently used (FIGO stage, tumor subtype, and histological grade) are far from optimal in terms of preoperative stratification of patients into low- or high-risk groups regarding surgical planning and adjuvant treatment.

One of the currently used prognostic markers is FIGO stage. This is obligatory and determined by transvaginal ultrasound of the pelvis (US). Computed tomography (CT) of the chest and abdomen is an imaging method of choice and is routinely used to exclude retroperitoneal lymphadenopathy and metastases in parenchymal organs. The other prognostic markers, histotype, and grade of tumor differentiation, are assessed from a biopsy obtained either by dilatation and curettage of the uterus or by hysteroscopy. Based on the established FIGO stage, histotype, and tumor grade, patients are divided into two groups regarding the recurrence risk. Low-risk patients are treated with surgery alone, consisting of hysterectomy and bilateral salpingo-oophorectomy, while high-risk patients undergo more aggressive surgical treatment, including pelvic (PLN) and/or paraaortic lymphadenectomy (PALN) with or without adjuvant radiotherapy or chemotherapy. An aggressive therapeutic approach is associated with significantly higher side effects, such as increased blood loss, risk of thrombosis, infection, lymphoceles, lymphatic ascites, and lymphedema (4, 5).

The discovery of new histotype-specific and prognostic biomarkers for better stratification into high- or low-risk EC seems to be urgently needed in order to avoid over- or undertreatment of EC patients. The results of studies on potential new biomarkers assessed immunohistochemically (IHC), related to EC patient prognosis, were recently published. L1 cell adhesion molecule (L1CAM) overexpression and the loss of estrogen receptors (ER) and/or progesterone receptors (PR) are associated with poor prognosis and a high risk of relapse and death (6–9). Mutations of the tumor protein p53 are associated with L1CAM expression, but not universally (10). However, to our best knowledge, neither the significance of L1CAM, ER, and PR expression nor knowledge of their relevant cut-offs together with determination of p53 mutation status in preoperative biopsies for pretreatment stratification into low- or high-risk have been established yet. No IHC biomarkers from preoperative biopsy are currently routinely used in the decision-making process for EC management.

The purpose of this study was to evaluate the clinical usefulness and added value of preoperatively assessed IHC biomarkers L1CAM, ER, PR, and p53 in differentiation between low- and high-risk EC patients through comparison of the current clinical practice model with a proposed model that includes immunohistochemical markers. The secondary objective of our study was to evaluate the correlation of IHC biomarkers with specific clinical (according to preoperative ultrasound and CT chest/abdomen) and pathological parameters.

PATIENTS AND METHODS

Patients

Patients undergoing surgical treatment for histologically proven or suspicious EC in the oncogynecological center of University Hospital Brno, Czech Republic, from January 2016 to August 2018 were consecutively included. The study was approved by the Institutional Ethical Board as was a version of written informed consent regarding tissue and clinical data use for scientific purposes obtained from each eligible patient.

Preoperative Imaging

All patients underwent a clinical examination, preoperative ultrasound staging examination, and CT of the chest/abdomen according to the local guidelines (11, 12). Each patient underwent both a transabdominal and a transvaginal US scan within 14 days before a board discussion led by one of the two oncogynecologists experienced in the field of US diagnostics in gynecologic oncology. Each US examination was immediately described in a written report; these reports were used for study analysis. Descriptions and examination reports were based on the standards applied by our center (13). During US staging examination of the uterine cavity, myometrium and cervix and pelvic lymph nodes were carefully assessed in every patient to describe the local extent of the tumor (14, 15).

Each patient underwent a CT scan of the chest, abdomen, and pelvis within 14 days before board discussion and admission to the operating theater. CT was performed with oral and intravenous contrast in order to exclude bowel wall implants, parenchymatous metastasis, and pathological lymphadenopathy. When lymph nodes measured >1 cm in the shorter axis or morphological changes as a rounded shape or necrosis were observed, tumor involvement was marked as suspicious.

Risk Stratification and Clinical Management

The extent of the surgery was determined by the multidisciplinary board after dividing patients into the low- or high-risk group based on clinical staging and the preoperative histopathological examination and determination of the histotype and grading. The low-risk group was defined as endometrioid or mucinous carcinoma TNM stage cT1a or cT1b, grade 1 and/or endometrioid or mucinous carcinoma TNM stage cT1a, grade 2, all without clinical or imaging evidence of lymphadenopathy (cN0) or distant metastases (cM0). Patients were defined as high-risk unless these low-risk criteria were met. Type A radical hysterectomy with bilateral salpingo-ophorectomy was performed in all patients (16). Systematic pelvic and paraaortic lymphadenectomy was performed in the high-risk group only; in high-grade serous uterine cancer cases, total omentectomy and appendectomy were added to the staging procedure. The definitive histopathological examination was provided by one of three pathologists with experience in gynecological malignancies and contained data about stage, histotype, and grade, lymphovascular space involvement (LVSI), and measures of the IHC expression of markers L1CAM, ER, PR, and p53. Based on final histopathological findings, the patients were once again stratified into low- or high-risk groups based on the same preoperative criteria (i.e., irrespective of known IHC status of ER, PR, L1CAM, and p53) and, thereafter, decisions regarding adjuvant treatment and follow-up were made by the multidisciplinary board.

Clinical Data

Age, results of US and CT scan with respect to depth of myometrial invasion, cervical involvement, lymphadenopathy, parenchymal organ involvement, and pathological data from biopsies (histotype, grading, IHC status of L1CAM, ER, PR, p53) were recorded.

Tissue and Immunohistochemistry Analysis

All hematoxylin and eosin-stained slides were read by one of three experienced gynecological histopathologist to confirm histological subtype, grade, and (definitive excision specimen) stage and the presence or absence of LVSI. The evaluator was blinded to patient characteristics. All specimens were assessed according to the WHO Classification of Tumors of Female Reproductive Organs, 2014 (17). No additional later review of the slides was performed for the purpose of this study because it would not copy our real clinical practice. Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded (FFPE) tissue sections. Immunohistochemistry for ER (clone SP1, product no. RBK 018-05, Zytomed, dilution 1:300), PR (clone 16, product no. NCL-L-PGR-312, Novocastra, dilution 1:80), L1CAM/CD171 (clone 14.10, product no. 826701, BioLegend, dilution 1:100), and p53 (clone DO-7, product no. M7001, DAKO, dilution 1:300) were performed using an automatic immunostainer (BenchMark Ultra, Ventana Medical Systems, Tucson, AZ, USA) according to the manufacturer's instructions. For ER, PR, and p53, only nuclear staining was scored as positive. Positivity of L1CAM was defined as distinct membrane staining. For ER, PR, and L1CAM, the percentage of positive tumor cells was assessed. p53 was classified into wild type or mutant (excessive = strong diffuse overexpression in more than 90% of tumor cells or completely negative) phenotypes. Representative microphotographs of the expression of estrogen receptor (ER), progesterone receptor (PR), L1CAM and p53 in serous (high risk) and grade 1 endometrioid (low risk) carcinoma are shown in **Figures 1A–H**.

Statistical Analysis

Categorical data were summarized using absolute and relative frequencies and compared by Fisher's exact test. Continuous variables were summarized as median with 10 and 90th percentile and tested by the Mann-Whitney *U*-test.

A model for the best classification of final risk was built using the CHAID growing method with crossover validation. Misclassification cost for wrongly determining high-risk patients as low-risk was set twice higher because of the preference for the correct high-risk group EC patient determination.

The success of risk-group classification was evaluated using four standard measures: (i) *sensitivity* is the ability of the test to correctly identify those with an occurrence of the assessed marker (true positive rate), whereas (ii) *specificity* is the ability of the test to correctly identify those without an occurrence of the assessed marker (true negative rate), (iii) *positive predictive value* (PPV) is the probability that the marker is present when the test is positive, whereas (iv) *negative predictive value* (NPV) is the probability that the marker is not present when the test is negative. All these statistics were accompanied by 95% confidence intervals (CI).

The comparison of sensitivities and specificities of the two binary diagnostic tests in a paired study design was performed using McNemar's test with continuity correction. Differences in (positive and negative) predictive values of two binary diagnostic tests were tested using a generalized score statistic proposed by Leisenring, Alonzo, and Pepe (18). All tests were performed as two-sided at the significance level 0.05. Analyses were done in IBM SPSS Statistics and R.

RESULTS

Clinical and Histopathological Characteristics

From January 2016 to August 2018, 132 patients underwent surgical treatment for EC in the oncogynecological center of University Hospital Brno, Czech Republic, and have been consecutively enrolled in the study. The median age was 66 years. According to ultrasound and CT staging, before operation 95 patients (72%) were evaluated as FIGO stage IA, while 25 (19%) were stage IB, 5 (4%) stage II, 3 (2%) stage III, and 4 (3%) were at an unknown stage. Preoperative biopsy was available for all 132 patients; 102 patients had endometrioid cancer, of whom 50 (49%) had endometrioid or mucinous carcinoma grade 1 (EG1), 45 (44%) grade 2 (EG2), 6 (6%) grade 3 (EG3), and one had a non-diagnostic grade. Seventeen (13%) patients were diagnosed with non-endometrioid carcinoma (NEC). Furthermore, there were

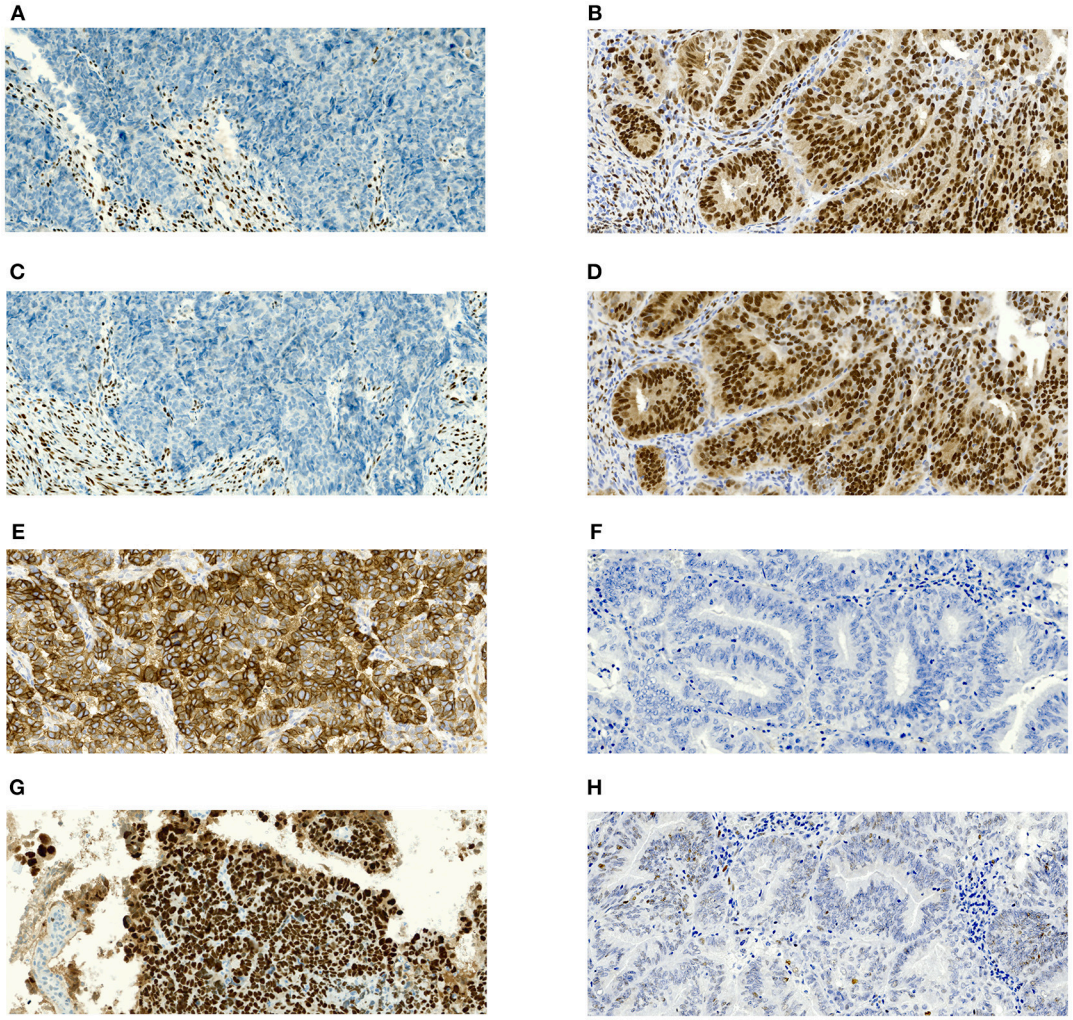


FIGURE 1 | Microphotographs showing representative examples of immunohistochemical expression of estrogen receptors (ER), progesterone receptor (PR), L1CAM and p53 in tissue specimens of endometrial carcinomas. Magnification 100x. **(A)** Complete negativity of ER expression in serous carcinoma with 0% cells positive; **(B)** Complete negativity of PR expression in serous carcinoma with 0% cells positive; **(C)** Strong diffuse membranous positivity of L1CAM expression in serous carcinoma with 100% cells positive; **(D)** p53 nuclear overexpression (mutant pattern) in serous carcinoma; **(E)** Nuclear positivity of ER expression in grade 1 endometrioid carcinoma with almost 100% cells positive; **(F)** nuclear positivity of PR expression in grade 1 endometrioid carcinoma with almost 100% cells positive; **(G)** complete negativity of L1CAM expression in grade 1 endometrioid carcinoma with 0% cells positive; **(H)** p53 wildtype immunohistochemical pattern in grade 1 endometrioid carcinoma.

eight cases (6%) of endometrial intraepithelial neoplasia (EIN) in preoperative biopsy (**Table 1**). Based on both histological and clinical findings, 94 (71%) patients were preoperatively classified as low-risk and 38 (29%) as high-risk by current model and, consequently, the recommendation for the extent of surgery was issued. In the high-risk group, PLN and PALN were performed for 26 (20%) patients, apart from hysterectomy and bilateral salpingo-oophorectomy. After the surgical procedure and definitive histopathological examination, 82 (62%) patients were at FIGO stage IA, 17 (13%) at FIGO stage IB, 19 (14%) FIGO stage II, 12 (9%) FIGO stage III and two (2%) with FIGO stage IV. Regarding endometrioid or mucinous carcinoma grade, 30 (28%) were at EG1, 69 (65 %) at EG2, 8 (7 %) EG3, 20 (15%) NEC, and 2 (2%) EIN. In contrast to the preoperative risk determination, the final post-operative stratification in risk groups was as follows: 70 (53%) low-risk patients and 62 (47%) high-risk patients (**Table 1**).

Immunohistochemical Characteristics

The expression of markers ER, PR, L1CAM, and p53 status were immunohistochemically evaluated from the specimen obtained both by diagnostic procedure and definitive surgery. The correlation of IHC markers between preoperative examination and definitive histopathological findings was statistically significant for all of the evaluated markers ($p < 0.005$). In the preoperative specimen, the expression was evaluable in 98 patients for ER and PR, 97 for L1CAM, and 98 for p53 mutational status; results are listed in **Table 2**.

TABLE 1 | Patients' clinical and histopathological characteristics.

Age at diagnosis	**66 (50–78)**		***p*-value**
Histology	**Preoperative (biopsy or imaging)**	**Final specimen**	
Endometrioid (incl. mucinous)	102 (77%)	107 (81%)	0.216
Non-endometrioid	17 (13%)	20 (15%)	
Serous	6 (35%)	2 (10%)	
Clear cell	4 (24%)	2 (10%)	
Carcinosarcoma	1 (6%)	2 (10%)	
Undifferentiated carcinoma	2 (11%)	3 (15%)	
Mixed carcinoma	4 (24%)	11 (55%)	
EIN	8 (6%)	2 (2%)	
Non-diagnostic	5 (4%)	3 (2%)	
GRADE (ONLY ENDOMETRIOID)			
G1	50 (49%)	30 (28%)	0.006
G2	45 (44%)	69 (65%)	
G3	6 (6%)	8 (7%)	
Non-diagnostic	1 (1%)		
MYOMETRIAL INVASION			
<50%	96 (73%)	93 (70%)	0.557
≥50%	33 (25%)	39 (30%)	
Unknown	3 (2%)		
CERVICAL INVASION			
Yes	9 (7%)	24 (18%)	0.015
No	120 (91%)	108 (82%)	
Unknown	3 (2%)		
LYMPHADENOPATHY			
Yes	3 (2%)	9 (7%)	0.070
No	129 (98%)	123 (93%)	
TUMOR BOARD DECISION			
Low-risk EC	94 (71%)	70 (53%)	<0.001
High-risk EC	38 (29%)	62 (47%)	

values denote median (10-90th percentile) or n (%); p-values of chi-square or McNemar's test; G, grade; EC, endometrial cancer.

Correlation of IHC Markers With Disease Extent (FIGO Staging)

The correlation was assessed between IHC markers in preoperative tissue samples and the final histopathological findings (e.g., myometrial invasion, cervical, and lymph node involvement). Moreover, the correlation with LVSI was evaluated because LVSI is one of the important markers for adjuvant treatment strategy decisions. There were statistically significant lower levels of ER ($p = 0.024$) and PR (0.048) and higher levels of L1CAM ($p = 0.001$) in tumors with myometrial invasion >50%. In tumors with cervical involvement, a significantly higher expression of L1CAM was observed ($p = 0.001$), while differences among levels of ER ($p = 0.236$) and PR ($p = 0.108$) did not reach statistical significance. PR were significantly lower ($p = 0.014$) and L1CAM higher ($p = 0.014$) in tumors with positive LVSI. In patients with positive LN, levels of ER and PR were lower ($p = 0.001$ and $p < 0.001$, respectively); on the other hand, levels of L1CAM were higher ($p = 0.002$) and the mutation of p53 more frequent ($p = 0.008$), see **Table 3**.

The Precision of EC Risk Stratification Based on Markers Currently Used in Clinical Praxis

Concerning depth of myometrial invasion, we classified all the patients in whom invasion reached ≥50% as high-risk ($n = 36$) and the patients with invasion <50% as low-risk ($n = 96$). Our approach to preoperative risk stratification of EC patients revealed a sensitivity of 48% and specificity of 89% in terms of high-risk group determination. Taking all current standard prognostic markers together, it can be concluded that, whereas low-risk EC patients are preoperatively classified with relatively high accuracy (62/70, 82%), the determination of high risk is far from optimal, since more than half of EC patients with actual high-risk disease were established as low-risk. See **Table 4**.

The Accuracy of EC Risk Stratification by Using IHC Markers

IHC markers were assessed in a preoperative tumor sample, and optimal cut-offs for continuous markers (obtained preoperatively) were designed using ROC analyses. At a cut-off for ER <78% ($p = 0.001$), PR <88% ($p = 0.008$), and L1CAM ≥4% ($p < 0.001$), high-risk tumors were determined with a sensitivity of 28% for ER (15.6–42.6%), 62% for PR (46.4–75.5%), and 72% for L1CAM (57.4–84.4%). Specificity was 96% (86.5–99.5%), 68% (52.1–79.2%), and 86% (73.3–94.2%), respectively. The PPV was 87% for ER (60.8–96.5%), 63% for PR (52.1–72.8%), and 83% for L1CAM (70.5–90.8%); the NPV for each marker were as follows: 59% (54.5–63.4%), 65% (55.6–74.0%), and 77% (67.3–84.2%), respectively. The sensitivity of p53 mutated status ($p < 0.001$) for high-risk detection was low (34%), but the specificity was high (98.0%, CI 88.7–99.9%), which represents PPV 94% (67.4–99.1%) and NPV 61% (56.1–66.3%), respectively. If p53 mutated, there was a high probability the patient fit into the high-risk group (15 out of 16 patients in our series). As far as accuracy of high-risk determination in the largest number of patients was concerned, the marker L1CAM seemed to be the most robust: 34 from 41 patients who had L1CAM values ≥4% were classified as high-risk (**Table 5**, **Figure 2**).

The Added Value of IHC Markers for Improvement of EC Risk Stratification

To evaluate whether IHC markers would contribute to more accurate stratification of EC patients into risk groups, a model consisting of parameters obtained by imaging methods (myometrial invasion, cervical involvement, lymph node involvement), histology (endometrioid or mucinous vs. non-endometrioid), grade and IHC markers (ER, PR, L1CAM, p53) was introduced. According to risk stratification, the following parameters have been shown as statistically significant and crucial for the model: L1CAM, PR, and myometrial invasion.

TABLE 2 | Immunohistochemical biomarkers in preoperative biopsies.

IHC markers	Overall values ($n = 98$)	EG1 ($n = 36$)	EG2 ($n = 37$)	EG3 ($n = 9$)	EIN ($n = 3$)	NEC ($n = 13$)
ER (%), $n = 98$	86 (27)	95 (15)	96 (8)	71 (34)	100 (0)	44 (42)
	99 (40–100)	100 (90–100)	99 (85–100)	80 (0–100)	100 (100–100)	30 (0–100)
PR (%), $n = 98$	73 (34)	88 (21)	79 (28)	48 (39)	87 (23)	28 (33)
	90 (5–100)	99 (60–100)	95 (30–100)	70 (0–95)	100 (60–100)	20 (0–85)
L1CAM (%), $n = 97$	16 (29)	2 (4)	7 (13)	33 (35)	0 (1)	72 (33)
	3 (0–70)	1 (0–8)	3 (0–15)	30 (0–100)	0 (0–1)	85 (15–100)
p53, $n = 98$						
Mut	16 (16.3%)	1 (2.8%)	2 (5.4%)	2 (22.2%)	0 (0%)	11 (84.6%)
Wt	75 (76.5%)	35 (97.2%)	32 (86.5%)	4 (44.4%)	2 (66.7%)	2 (15.4%)
Non-specific	7 (7.1%)	0 (0%)	3 (8.1%)	3 (33.3%)	1 (33.3%)	0 (0%)

Values denote mean (SD) and median (10–90th percentile) or n (%); means and SD are shown only for exploratory purpose since data are not normally distributed; IHC, immunohistochemical markers; ER, estrogen receptors; PR, progesterone receptors; L1CAM, L1cell adhesion molecule; mut, mutated; wt, wild type; EG1, endometrioid or mucinous cancer, grade 1; EG2, endometrioid or mucinous cancer, grade 2; EG3, endometrioid or mucinous cancer, grade 3; EIN, endometrioid intraepithelial neoplasia; NEC, non-endometrioid cancer.

TABLE 3 | Correlation of IHC markers from preoperative biopsy with staging after surgery.

	ER (%) ($N = 98$)	PR (%) ($N = 98$)	L1CAM (%) ($N = 97$)	p53 mut ($N = 16$)		P53 wt ($N = 75$)
Myometrial invasion	$p = 0.024$	$p = 0.048$	$p = 0.001$		$p = 0.382$	
<50% ($N = 66$)	88 (26) 100 (60–100)	77 (31) 95 (20–100)	14 (29) 1 (0–70)	9 (14.8%)		52 (85.2%)
≥50% ($N = 32$)	83 (30) 98 (40–100)	65 (38) 82.5 (0–100)	20 (29) 5 (1–70)	7 (23.3%)		23 (76.7%)
Cervical involvement	$p = 0.236$	$p = 0.108$	$p = 0.001$		$p = 0.070$	
Yes ($N = 19$)	82 (31) 95 (0–100)	60 (39) 70 (0–100)	32 (38) 8 (1–95)	6 (35.3%)		11 (64.7%)
No ($N = 79$)	88 (26) 99 (40–100)	76 (32) 90 (15–100)	12 (26) 2 (0–50)	10 (13.5%)		64 (86.5%)
LN (lymph node) metastases	$p = 0.001$	$p < 0.001$	$p = 0.002$		$p = 0.008$	
Yes ($N = 6$)	43 (47) 35 (0–95)	11 (20) 0.5 (0–50)	60 (36) 70 (4–100)	4 (66.7%)		2 (33.3%)
No ($N = 92$)	89 (23) 99 (70–100)	77 (31) 92.5 (20–100)	13 (27) 2 (0–50)	12 (14.1%)		73 (85.9%)
LVSI	$p = 0.111$	$p = 0.014$	$p = 0.014$		$p = 0.260$	
Yes ($N = 14$)	74 (41) 95 (0–100)	48 (43) 45 (0–100)	23 (32) 6 (2–70)	4 (22.2%)		10 (55.6%)
No ($N = 84$)	89 (24) 99 (60–100)	77 (31) 93 (20–100)	15 (29) 2 (0–70)	12 (13.0%)		65 (70.7%)

Values denote mean (SD) and median (10-90th percentile) or n (%), p-value of Mann-Whitney U-test or Fisher's exact test; means and SD are shown only for exploratory purpose since data are not normally distributed; LVSI, lymphovascular space involvemen; mut, mutated; wt, wild type.

The procedure of classification is shown in **Figure 3**. The overall EC risk stratification success was 78% for this model. Successful group inclusion was observed for 80% of the low-risk patients (56/70), and for 76% of high-risk patients (47/62 patients).

New Model in Comparison to Current Practice

Immunohistochemical markers included in the current diagnostic practice would significantly improve sensitivity (48.4 vs. 75.8%, $p < 0.001$) associated with a slightly, statistically non-significant decrease in specificity (to 80%, $p = 0.238$). Positive predictive values were similar for both methods, while negative predictive value (i.e., the probability of extremely low risk in negative test cases) was significantly improved (66 vs. 78.9%, $p < 0.001$) (**Table 6**, **Figure 4**).

DISCUSSION

The determination of an appropriate surgery and its adequate extent is a crucial part of treatment in newly diagnosed EC patients and significantly differs between the high- and low-risk groups. Existing models determining patient risk are based on the

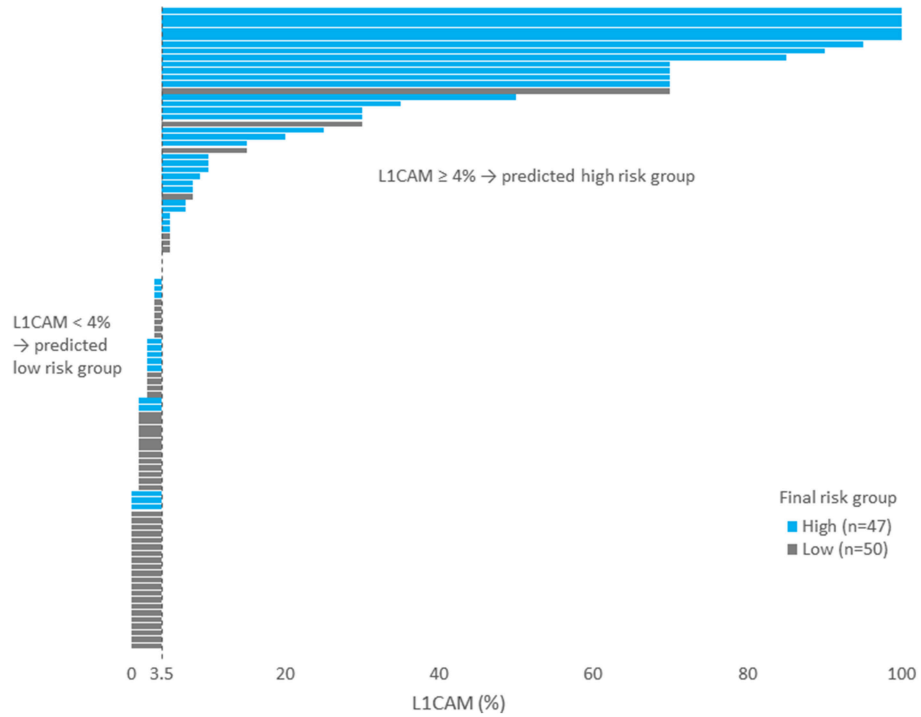


FIGURE 2 | Each patient is represented by one bar. Patients on the left part of the figure (i.e., with L1CAM<4) was predicted as low risk. Blue bars represent wrongly predicted patients here. On the contrary, gray bars on the right side of the figure represent patients wrongly predicted as high risk.

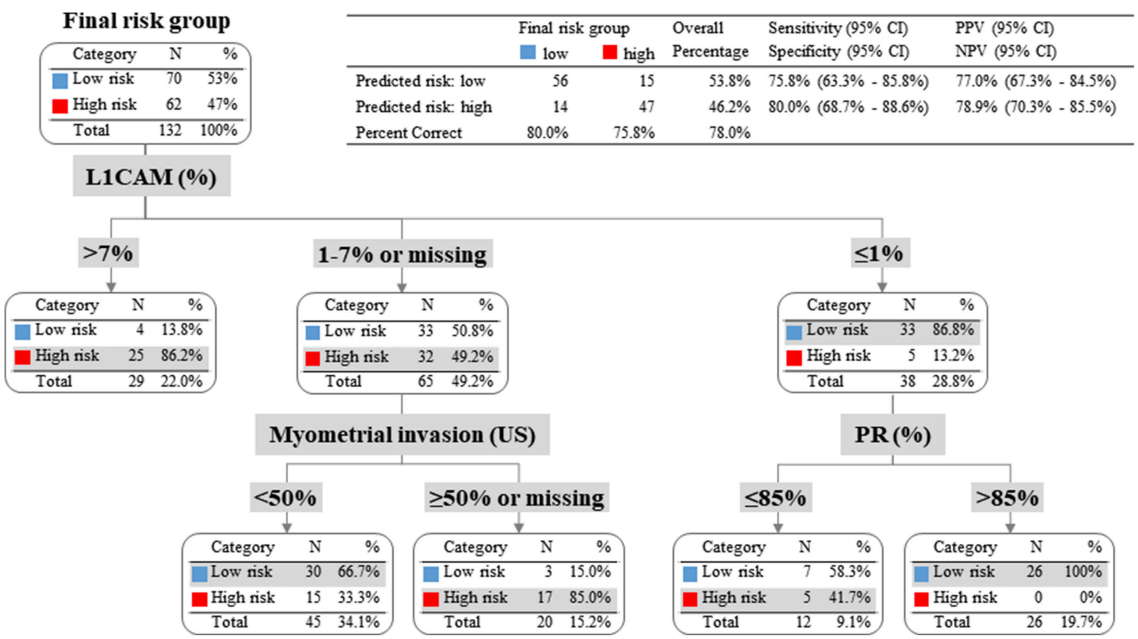


FIGURE 3 | Patients with L1CAM positivity >4% were identified as high risk; in fact 86% of them were high-risk indeed. Patients with L1CAM value from 1 to 4% were further divided according to myometrial invasion. In case of myometrial invasion <50% they were stratified as low-risk, on the contrary in case of myometrial invasion >50% or unknown they were classified as high-risk. Patients with L1CAM <1% were further disaggregated by PR value. In case of PR >85% they were identified as low-risk (with success rate of 100%), on the other hand in case of PR ≤85% they were stratified as high-risk according to clinical preference for more precise high-risk group determination even with the expectation of higher false positivity.

TABLE 4 | Accuracy of low-/high-risk group classification according to current practice.

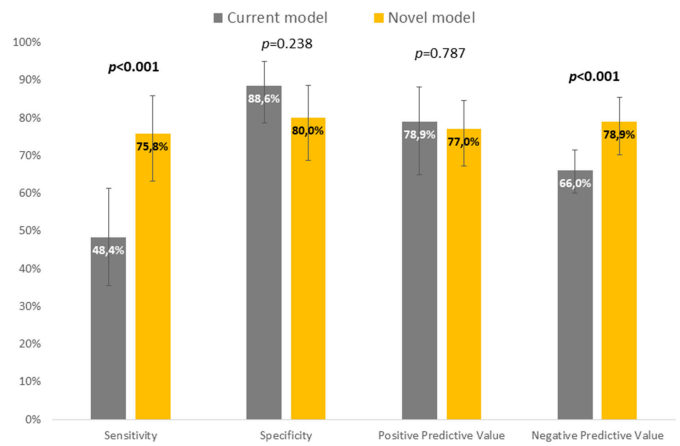
	Final risk				
	Low-risk	**High-risk**	**Total *N***	**Sensitivity (95% CI) Specificity (95% CI)**	**PPV (95% CI) NPV (95% CI)**
Current model: low-risk	62	32	94	48.4% (35.5–61.4%)	78.9% (65.0–88.3%)
Current model: high-risk	8	**30**	38	88.6% (78.7–94.9%)	66.0% (60.0–71.4%)
Total *N*	70	62	132		

CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value. Bold values are significant.

TABLE 5 | Correlation of IHC with final EC risk stratification.

	Final risk					
	Low-risk	**High-risk**	**Total *N***	***p*-value**	**Sensitivity (95% CI) Specificity (95% CI)**	**PPV (95% CI) NPV (95% CI)**
ER <78 (high-risk)	2	**13**	15	0.001	27.7% (15.6–42.6%)	86.7% (60.8–96.5%)
ER 78+ (low-risk)	49	34	83		96.1% (86.5–99.5%)	59.0% (54.5–63.4%)
Total *N*	51	47	98			
PR <88 (high-risk)	17	**29**	46	0.008	61.7% (46.4–75.5%)	63.0% (52.1–72.8%)
PR 88+ (low-risk)	34	18	52		66.7% (52.1–79.2%)	65.4% (55.6–74.0%)
Total *N*	51	47	98			
L1CAM <4 (low-risk)	43	13	56	<0.001	72.3% (57.4–84.4%)	82.9% (70.5–90.8%)
L1CAM 4+ (high-risk)	7	**34**	41		86.0% (73.3–94.2%)	76.8% (67.3–84.2%)
Total *N*	50	47	97			
p53 mut (high-risk)	1	**15**	16	<0.001	34.1% (20.5–49.9%)	93.8% (67.4–99.1%)
P53 wt (low-risk)	46	29	75		97.9% (88.7–99.9%)	61.3% (56.1–66.3%)
Total *N*	47	44	91			

p-value of Fisher's exact test; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; mut, mutated; wt, wild type. Bold values are significant.



Current model=histotype, grade, imaging method; Novel model=immunohistochemical biomarkers, imaging methods

FIGURE 4 | Comparison of current and novel model for prediction of risk tumor type.

synthesis of information obtained from the result of preoperative biopsy (histotype, grading) and imaging methods. Based on the results, patients are included in a risk group before the surgery and so only hysterectomy and salpingo-oophorectomy are indicated, or the procedure is extended by pelvic and para-aortic lymphadenectomy. Proper preoperative inclusion of a

TABLE 6 | Comparison of standard and new diagnostic approaches.

		Final risk: Low			Final risk: High		
		Current model: low-risk	**Current model: high-risk**	**Total**	**Current model: low risk**	**Current model: high risk**	**Total**
Novel model (IMG+IHC)	Low	50 (71.4%)	**6 (8.6%)**	56 (80%)	15 (24.2%)	**0 (0%)**	15 (24,2%)
	High	**12 (17.1%)**	2 (2.9%)	14 (20%)	**17 (27.4%)**	30 (48.4%)	47 (75,8%)
Total		62 (88.6%)	8 (11.4%)	70 (100%)	32 (51.6%)	30 (48.4%)	62 (100%)

IMG, imaging method; IHC, immunohistochemical markers; current model, histology, grading, imaging method. Bold values are significant.

patient in the risk group is crucial for her treatment and overall survival and is a clinically crucial question.

Other approaches to assess the biological behavior of endometrial cancers are under development. Several research groups have defined immunohistochemical and/or mutation profiles to allow distinguishing endometrial cancer subtypes. The Cancer Genome Atlas (TCGA) project provided the most comprehensive molecular study on endometrial cancer so far. They identified four group with distinct molecular changes that correlate with progression free survival – *POLE* (Polymerase Epsilon subunit) ultramutated, MSI (microsatellite instability) hypermutated, copy-number low, and copy-number high (19). This approach allows objective categorization of endometrial cancers, however, methodologically remains costly, complex and unsuitable for wider clinical application.

Others introduced a concept of sentinel lymph node detection in endometrial cancer patients. The large prospective study led by Rossi (20) showed very high sensitivity (97.2%) and low false negativity rate (3%) for sentinel lymph node (SLN) detection. SLN detection concept is based on low risk of paraaortic lymph nodes involvement in patients with negative pelvic lymph nodes (21). However, controversy regarding sentinel lymph node detection in high-risk disease and management of low volume nodal disease on ultrastaging still remains.

In many centers, frozen section of uterus is still standard-of-care in terms to confirm or to more specify type and grade of the tumor. Accuracy of frozen section histopathological evaluation is, however, comparable to imaging methods and interobserver agreement regarding both the categories, type and grade, is poor (22–26).

The current development of risk prediction model is mainly focused on combination of imaging and molecular predictors, as our study does. In 2014, Van Holsbeke et al. published a study that externally validated two mathematical models of preoperative risk group prediction in a particular patient (27). The models were based on histology, grading, and the preoperative sonographic evaluation of tumor invasion into the myometrium and cervix. Both models achieved sensitivity of 78–83% and specificity of 68–72% in the detection of high-risk EC patients. In our study, the current clinical model reliably determined low-risk patients (correctly in 83% of cases), while only 8 (11%) patients in the study were false positives included in the high-risk group. The current model preoperatively stratified patients to high-risk with sensitivity of only 48% (35.5–61.4%) and specificity 89% (78.7–94.9%), NPV 66% (60.0–71.4%), and PPV 79% (65.0–88.3%) (**Table 4**).

A number of ultrasound studies have been published for the assessment of individual staging parameters to determine the depth of tumor invasion into the myometrium, with ultrasound sensitivity from 61 to 93% and specificity from 71 to 92%, when performed by an expert sonography specialist (28–32). The sensitivity reported in the evaluation of tumor invasion in cervical stroma was lower, from 25 to 93%, and specificity from 85 to 99% (30, 33, 34). In a study utilizing expert sonography in a specialized center, Fruhauf et al. reported a PPV of 67.6% and NPV of 83.3% for the detection of deep myometrial invasion and PPV of 60.0% and NPV of 88.1% in the detection of tumor affection of the uterine cervix. According to a recent meta-analysis of 18 studies, CT sensitivity is 47% and specificity 93%; as for ultrasound, sensitivity is 55% and specificity up to 85% for the detection of malignant lymphadenopathy (35). In our group of 132 female patients, the invasion of the tumor to half the thickness of the myometrium was determined correctly in 90%; a false-negative result in the high-risk group of patients was reported in 39 cases. CT did not detect pathological lymphadenopathy in six cases out of nine. A total of nine patients were classified as false positives on the basis of US and CT as validated by the definitive histology.

Studies showing the discrepancy between histology obtained from preoperative curettage or hysteroscopy and definitive histological findings have been published (36–38). On the other hand, there are studies showing good concordance between histology, grade, and immunohistochemical staining in curettage and hysterectomy samples (39, 40). We confirmed that the preoperatively determined histological type, grade, and immunohistochemical biomarkers L1CAM, ER, PR, p53 correlated with the final preparation.

In our study, 70 (70/132) patients were classified as low-risk and 62 (62/132) as high-risk. According to the pre-operative staging, PLN and PALN were performed in 26 patients (20%) in our cohort. However, if the definitive risk were known, staging lymphadenectomy would be performed in all 62 patients in the high-risk group (47%). Due to an inappropriate staging surgery, patients underwent repeated surgery or adjuvant radiotherapy, which may have been avoided if a complete surgical staging with negative histological findings of the presence of the tumor in the lymph nodes had been performed. On the contrary, there are onco-gynecological centers which report extensive PLN + PALN in EC patients, thereby increasing post-operative morbidity without an oncology safety increase (41). We focused on currently promising prognostic IHC markers ER, PR, L1CAM, and p53 mutation to determine whether these markers can help

to refine the preoperative stratification of patients into high- and low-risk categories to assist the gynecological oncology surgeon selecting the adequate surgical extent.

A study published by van der Putten et al. revealed that L1CAM expression in curettage specimens is associated with features of aggressive endometrial cancer disease and poor survival of EC patients (42). van der Putten et al. (42) stated that L1CAM, ER, PR were associated with advanced stage, high-grade, non-endometrioid histology, lymphovascular space invasion (LVSI), and reduced disease-free survival (42). Trovik et al. (43) reported that combined ER/PR loss is a significant predictor of nodal affection and overall poor prognosis of patients. ER and PR are prospectively investigated in an ongoing study where the decision of whether to perform or not to perform lymphadenectomy is based on the pre-operational condition of hormone receptors (44). Prospective studies PIPENDO and PORTEC 4 are currently underway. The first study examines the use of molecular risk markers to identify high-risk patients requiring extensive surgery and/or adjuvant therapy (8). PORTEC 4 uses molecular risk factors for the stratification and indication of adjuvant radiotherapy (45).

In our study, ER, PR, L1CAM and p53 values from preoperative histology were related to definitive histology and grading. In endometroid carcinoma, there was a greater percentage of ER, PR receptors and no or ultimately low percentages of L1CAM mutations. The opposite ratio was seen in the occurrence of markers in non-endometroid ECs; p53 was mutated dominantly in endometrial grade 3 and non-endometroid carcinoma. The correlation of IHC markers with the extent of disease shows a decrease in ER and PR expression in higher stages of the disease. Furthermore, an increase in L1CAM expression can be observed when compared with early stages. Similarly, the p53 mutation was more common. Our results are in line with the published data in larger patient cohorts (43, 46). The correlation of markers with the presence of distant metastases could not be assessed as there were only two patients with distant metastases at the time of diagnosis in our study group.

To our best and honest knowledge, this is the first study to evaluate the added value of L1CAM, ER, PR and p53 markers in low- and high-risk EC preoperative diagnostics. This is the first study attempting to determine the cut-off of the individual markers for this classification.

We focused on the correlation of IHC markers with the determination of high-risk EC and the assessment of optimal cut-offs for continuous markers using ROC analyses. At the cut-off for ER <78% ($p = 0.001$), PR <88% ($p = 0.008$), and L1CAM ≥4% ($p < 0.001$), high-risk tumors were determined with a sensitivity of 28, 62, and 72%, respectively, and with respective specificity of 96, 72, and 86%. The PPV for ER, PR, and L1CAM were 87, 63, 83; the NPV for each marker were as follows: 59%, 65%, and 77%. The sensitivity of p53 mutated status ($p < 0.001$) for high-risk detection was low (34%), but the specificity was high (98%), which represents PPV 94% and NPV 61%, respectively.

A cut-off of 10% for positive L1CAM staining has been reported (6, 40, 47). van Gool et al. reported that when using a cut-off of 10% for positive staining, tumors in the study were classified as L1CAM-positive, with no significant association between L1CAM positivity and the rate of distant metastasis ($p = 0.195$). However, increasing the threshold for L1CAM positivity to 50% resulted in a reduction of the frequency of L1CAM-positive tumors and a significant association with the rate of distant metastasis ($p = 0.018$) (10). Estrogen receptors and PR are considered lost when expression is seen in <10% of the tumor cells. This cut-off used with breast cancer management in the prediction of hormone resistance was also evaluated in EC for prognosis prediction and published (42, 43, 48). In our cohort, we determined optimal cut-offs to distinguish low- and high-risk EC for ER <78% ($p = 0.001$), PR <88% ($p = 0.008$), and L1CAM ≥4% ($p < 0.001$). These cut-offs were established in a prospectively assessed cohort of consecutively included patients. In contrast to the cut-offs we defined, the published values are determined in retrospective cohorts of women with recurrence during follow-up, or with an adverse course of their disease with metastatic spread in parenchymatous organs and retroperitoneal lymph nodes. The incidence of L1CAM positivity and loss of ER, PR in these patients in retrospective cohorts may be significantly higher as it is an already pre-selected group of patients. The explanation may be based on the fact that patients who do not exceed the published cut-off values (10% for L1CAM, ER, and PR or 50% for L1CAM) but exceed the cut-offs set for the high-risk group in our study cannot be traced back in the retrospective studies, as they had not been radically treated with combined surgical and ± adjuvant therapy (radiotherapy, chemotherapy) and there was no recurrence during the long follow-up. Our results were confronted with 10 and 50% cut-offs for L1CAM; this setting led to good differentiation (high specificity) but at a very low sensitivity and good specificity.

To evaluate whether IHC markers would contribute to more accurate stratification of EC risk stratification, a new model was established. Parameters were obtained by imaging methods (myometrial invasion, cervical involvement, lymph node involvement), histology (endometrioid or mucinous vs. non-endometrioid), grade and IHC markers (ER, PR, L1CAM, p53). As deciding for risk stratification, the following parameters have been shown as crucial: L1CAM, PR, and myometrial invasion. The procedure of classification is shown in **Figure 3**. EC risk stratification's overall success was 78% for this model. Successful inclusion into a low-risk group was observed in 80% (56/70 patients), while for high-risk it was 76% (47/62 patients). We provided the comparison of current procedure represented by a model based on histotype, grading, and imaging methods with a new model consisting of imaging examination along with markers (IMG+IHC) (**Table 6**). IHC markers included in the current diagnostic would significantly improve sensitivity (48.4 vs. 75.8%, $p < 0.001$) associated with a slightly, statistically non-significant decrease in specificity to 80% ($p = 0.238$). Positive predictive value was similar for both methods, while negative predictive value (i.e., the probability of being true negative if the test is negative) was significantly improved (66 vs. 78.9%, $p < 0.001$), (**Figure 4**). Using our model, a significantly higher proportion of patients would be properly determined as high-risk.

When comparing the accuracy of the parameters used in the old model with the definite histology, we find discrepancies, especially in the grade and cervical invasion category (**Table 1**). At the present level of knowledge, no significant improvement in preoperative diagnostic accuracy can be expected by, for example, using imaging methods. Therefore, we are introducing a new model using molecular markers that are not dependent on imaging or other methods of clinical examination.

The strength of our study is that it is a cohort from a real clinical practice with prospective data collection and complete knowledge of preoperative and postoperative data. This is the first study to deal with the real implementation of new IHC markers in the pre-operational decision model. Our study design represents daily routine practice.

We acknowledge the study also has weaknesses. This is a relatively small cohort of EC patients, where all stages are not adequately represented; only two female patients in the FIGO IV stage were present. Considering the excellent correlation between preoperative and postoperative histology and grading, the weakness of the model is in its imaging method, which in the case of ultrasound is dependent on the expert skills of a particular sonographer or imaging specialist.

CONCLUSION

We have demonstrated in our cohort that incorporating IHC markers into preoperative practice in endometrial cancer patients increases prognosis prediction accuracy and allows for the development of a new model for more accurate patient clinical management. Should we prefer a higher specificity model, then the most accurate classification is based on L1CAM values, myometrial invasion, and the condition of PR receptors. However, for wider implementation and the validation of proposed model, additional study is needed. Ideally, a prospective randomized trial would evaluate the role of IHC markers L1CAM, ER, PR, and p53 in a preoperative setting together with imaging method and histology/grade. To further improve the new model, it would be interesting to focus on general weaknesses in the accuracy of preoperative imaging methods and in the quality of preoperatively obtained samples with a full IHC examination on a routine daily basis. Incorporating IHC markers seems to be the best way to treat EC patients more accurately.

ETHICS STATEMENT

The text of informed consent, information for patients and the study protocol was approved by Institutional Ethical Board of University Hospital Brno.

AUTHOR CONTRIBUTIONS

VW and MZ: idea of research project, overseeing, coordination of analyses; MB, PV, LM, and MF: collection and preparation of clinical data; JH, EJ, and MC: immunohistochemistry and interpretation of pathological data; PO: statistics; VW, MB, JH, PO, PV, and MZ: writing manuscript. All authors: final reading and approval of manuscript.

FUNDING

This work was supported by the Ministry of Health, Czech Republic—conceptual development of a research organization (FNBr 65269705) and grant project No. 17-32030A.

REFERENCES

1. Kurman RJ, International Agency for Research on Cancer, World Health Organization (eds.). *WHO Classification of Tumours of Female Reproductive Organs*. Lyon: International Agency for Research on Cancer (2014).
2. Salvesen HB, Haldorsen IS, Trovik J. Markers for individualised therapy in endometrial carcinoma. *Lancet Oncol.* (2012) 13:e353–61. doi: 10.1016/S1470-2045(12)70213-9
3. Dedes KJ, Wetterskog D, Ashworth A, Kaye SB, Reis-Filho JS. Emerging therapeutic targets in endometrial cancer. *Nat Rev Clin Oncol.* (2011) 8:261–71. doi: 10.1038/nrclinonc.2010.216
4. Abu-Rustum NR, Barakat RR. Observations on the role of circumflex iliac node resection and the etiology of lower extremity lymphedema following pelvic lymphadenectomy for gynecologic malignancy. *Gynecol Oncol.* (2007) 106:4–5. doi: 10.1016/j.ygyno.2007.03.026
5. Weinberger V, Cibula D, Zikan M. Lymphocele: prevalence and management in gynecological malignancies. *Expert Rev Anticancer Ther.* (2014) 14:307–17. doi: 10.1586/14737140.2014.866043
6. Zeimet AG, Reimer D, Huszar M, Winterhoff B, Puistola U, Azim SA, et al. L1CAM in early-stage type I endometrial cancer: results of a large multicenter evaluation. *J Natl Cancer Inst.* (2013) 105:1142–50. doi: 10.1093/jnci/djt144
7. van der Putten LJ, Visser NC, van de Vijver K, Santacana M, Bronsert P, Bulten J, et al. L1CAM expression in endometrial carcinomas: an ENITEC collaboration study. *Br J Cancer.* (2016) 115:716–24. doi: 10.1038/bjc.2016.235
8. Visser NCM, Bulten J, van der Wurff AAM, Boss EA, Bronkhorst CM, Feijen HWH, et al. PIpelle Prospective ENDOmetrial carcinoma (PIPENDO) study, pre-operative recognition of high risk endometrial carcinoma: a multicentre prospective cohort study. *BMC Cancer.* (2015) 15:487. doi: 10.1186/s12885-015-1487-3
9. Hua T, Liu S, Xin X, Jin Z, Liu Q, Chi S, et al. Prognostic significance of L1 cell adhesion molecule in cancer patients: A systematic review and meta-analysis. *Oncotarget.* (2016) 7:85196–207. doi: 10.18632/oncotarget.13236
10. Van Gool IC, Stelloo E, Nout RA, Nijman HW, Edmondson RJ, Church DN, et al. Prognostic significance of L1CAM expression and its association with mutant p53 expression in high-risk endometrial cancer. *Mod Pathol.* (2016) 29:174–81. doi: 10.1038/modpathol.2015.147
11. Lin MY, Dobrotwir A, McNally O, Abu-Rustum NR, Narayan K. Role of imaging in the routine management of endometrial cancer. *Int J Gynaecol Obstet.* (2018) 143 (Suppl. 2):109–17. doi: 10.1002/ijgo.12618
12. Frühauf F, Dvorák M, Haaková L, Hašlík L, Herboltová P, Chaloupková B, et al. [Ultrasound staging of endometrial cancer - recommended methodology of examination]. *Ceska Gynekol.* (2014) 79:466–76.
13. Fischerova D. Ultrasound scanning of the pelvis and abdomen for staging of gynecological tumors: a review. *Ultrasound Obstet Gynecol.* (2011) 38:246–66. doi: 10.1002/uog.10054

14. Fischerova D, Frühauf F, Zikan M, Pinkavova I, Kocián R, Dundr P, et al. Factors affecting sonographic preoperative local staging of endometrial cancer. *Ultrasound Obstet Gynecol.* (2014) 43:575–85. doi: 10.1002/uog.13248
15. Eriksson LSE, Lindqvist PG, Flöter Rådestad A, Dueholm M, Fischerova D, Franchi D, et al. Transvaginal ultrasound assessment of myometrial and cervical stromal invasion in women with endometrial cancer: interobserver reproducibility among ultrasound experts and gynecologists. *Ultrasound Obstet Gynecol.* (2015) 45:476–82. doi: 10.1002/uog.14645
16. Querleu D, Morrow CP. Classification of radical hysterectomy. *Lancet Oncol.* (2008) 9:297–303. doi: 10.1016/S1470-2045(08)70074-3
17. FIGO Committee on Gynecologic Oncology. FIGO staging for carcinoma of the vulva, cervix, and corpus uteri. *Int J Gynaecol Obstet.* (2014) 125:97–8. doi: 10.1016/j.ijgo.2014.02.003
18. Leisenring W, Alonzo T, Pepe MS. Comparisons of predictive values of binary medical diagnostic tests for paired designs. *Biometrics.* (2000) 56:345–51. doi: 10.1111/j.0006-341X.2000.00345.x
19. Cancer Genome Atlas Research N, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, et al. Integrated genomic characterization of endometrial carcinoma. *Nature*. (2013) 497:67–73. doi: 10.1038/nature12113
20. Rossi EC, Kowalski LD, Scalici J, Cantrell L, Schuler K, Hanna RK, et al. A comparison of sentinel lymph node biopsy to lymphadenectomy for endometrial cancer staging (FIRES trial): A multicentre, prospective, cohort study. *Lancet Oncol.* (2017) 18:384–92. doi: 10.1016/S1470-2045(17)30068-2
21. Abu-Rustum NR, Gomez JD, Alektiar KM, Soslow RA, Hensley ML, Leitao MM Jr., et al. The incidence of isolated paraaortic nodal metastasis in surgically staged endometrial cancer patients with negative pelvic lymph nodes. *Gynecol Oncol.* (2009) 115:236–8. doi: 10.1016/j.ygyno.2009.07.016
22. Gilks CB, Oliva E, Soslow RA. Poor interobserver reproducibility in the diagnosis of high-grade endometrial carcinoma. *Am J Surg Pathol.* (2013) 37:874–81. doi: 10.1097/PAS.0b013e31827f576a
23. Clarke BA, Gilks CB. Endometrial carcinoma: controversies in histopathological assessment of grade and tumour cell type. *J Clin Pathol.* (2010) 63:410–5. doi: 10.1136/jcp.2009.071225
24. Hussein YR, Broaddus R, Weigelt B, Levine DA, Soslow RA. The genomic heterogeneity of FIGO grade 3 endometrioid carcinoma impacts diagnostic accuracy and reproducibility. *Int J Gynecol Pathol.* (2016) 35:16–24. doi: 10.1097/PGP.0000000000000212
25. Han G, Sidhu D, Duggan MA, Arseneau J, Cesari M, Clement PB, et al. Reproducibility of histological cell type in high-grade endometrial carcinoma. *Mod Pathol.* (2013) 26:1594–604. doi: 10.1038/modpathol.2013.102
26. Alkushi A, Kobel M, Kalloger SE, Gilks CB. High-grade endometrial carcinoma: serous and grade 3 endometrioid carcinomas have different immunophenotypes and outcomes. *Int J Gynecol Pathol.* (2010) 29:343–50. doi: 10.1097/PGP.0b013e3181cd6552
27. Van Holsbeke C, Ameye L, Testa AC, Mascilini F, Lindqvist P, Fischerova D, et al. Development and external validation of new ultrasound-based mathematical models for preoperative prediction of high-risk endometrial cancer. *Ultrasound Obstet Gynecol.* (2014) 43:586–95. doi: 10.1002/uog.13216
28. Antonsen SL, Jensen LN, Loft A, Berthelsen AK, Costa J, Tabor A, et al. MRI, PET/CT and ultrasound in the preoperative staging of endometrial cancer - a multicenter prospective comparative study. *Gynecol Oncol.* (2013) 128:300–8. doi: 10.1016/j.ygyno.2012.11.025
29. Savelli L, Ceccarini M, Ludovisi M, Fruscella E, De Iaco PA, Salizzoni E, et al. Preoperative local staging of endometrial cancer: transvaginal sonography vs. magnetic resonance imaging. *Ultrasound Obstet Gynecol.* (2008) 31:560–6. doi: 10.1002/uog.5295
30. Akbayir O, Corbacioglu A, Numanoglu C, Guleroglu FY, Ulker V, Akyol A, et al. Preoperative assessment of myometrial and cervical invasion in endometrial carcinoma by transvaginal ultrasound. *Gynecol Oncol.* (2011) 122:600–3. doi: 10.1016/j.ygyno.2011.05.041
31. Sawicki W, Spiewankiewicz B, Stelmachów J, Cendrowski K. The value of ultrasonography in preoperative assessment of selected prognostic factors in endometrial cancer. *Eur J Gynaecol Oncol.* (2003) 24:293–8. doi: 10.1002/uog.14905
32. Alcazar JL, Pineda L, Martinez-Astorquiza Corral T, Orozco R, Utrilla-Layna J, Juez L, et al. Transvaginal/transrectal ultrasound for assessing myometrial invasion in endometrial cancer: a comparison of six different approaches. *J Gynecol Oncol.* (2015) 26:201–7. doi: 10.3802/jgo.2015.26.3.201
33. Mascilini F, Testa AC, Van Holsbeke C, Ameye L, Timmerman D, Epstein E. Evaluating myometrial and cervical invasion in women with endometrial cancer: comparing subjective assessment with objective measurement techniques. *Ultrasound Obstet Gynecol.* (2013) 42:353–8. doi: 10.1002/uog.12499
34. Frühauf F, Zikan M, Semeradova I, Dundr P, Nemejcova K, Dusek L, et al. The diagnostic accuracy of ultrasound in assessment of myometrial invasion in endometrial cancer: subjective assessment versus objective techniques. *Biomed Res Int.* (2017) 2017:1318203. doi: 10.1155/2017/1318203
35. Gong Y, Wang Q, Dong L, Jia Y, Hua C, Mi F, et al. Different imaging techniques for the detection of pelvic lymph nodes metastasis from gynecological malignancies: a systematic review and meta-analysis. *Oncotarget.* (2016) 8:14107–25. doi: 10.18632/oncotarget.12959
36. Lampe B, Kürzl R, Hantschmann P. Reliability of tumor typing of endometrial carcinoma in prehysterectomy curettage. *Int J Gynecol Pathol.* (1995) 14:2–6.
37. Frumovitz M, Singh DK, Meyer L, Smith DH, Wertheim I, Resnik E, et al. Predictors of final histology in patients with endometrial cancer. *Gynecol Oncol.* (2004) 95:463–8. doi: 10.1016/j.ygyno.2004.07.016
38. Leitao MM, Kehoe S, Barakat RR, Alektiar K, Gattoc LP, Rabbitt C, et al. Accuracy of preoperative endometrial sampling diagnosis of FIGO grade 1 endometrial adenocarcinoma. *Gynecol Oncol.* (2008) 111:244–8. doi: 10.1016/j.ygyno.2008.07.033
39. Stelloo E, Nout RA, Naves LCLM, Ter Haar NT, Creutzberg CL, Smit VTHBM, et al. High concordance of molecular tumor alterations between pre-operative curettage and hysterectomy specimens in patients with endometrial carcinoma. *Gynecol Oncol.* (2014) 133:197–204. doi: 10.1016/j.ygyno.2014.02.012
40. Bosse T, Nout RA, Stelloo E, Dreef E, Nijman HW, Jürgenliemk-Schulz IM, et al. L1 cell adhesion molecule is a strong predictor for distant recurrence and overall survival in early stage endometrial cancer: pooled PORTEC trial results. *Eur J Cancer.* (2014) 50:2602–10. doi: 10.1016/j.ejca.2014.07.014
41. Amant F, Mirza MR, Koskas M, Creutzberg CL. Cancer of the corpus uteri. *Int J Gynecol Obstetr.* (2018) 143:37–50. doi: 10.1002/ijgo.12612
42. van der Putten LJM, Visser NCM, van de Vijver K, Santacana M, Bronsert P, Bulten J, et al. Added value of estrogen receptor, progesterone receptor, and L1 cell adhesion molecule expression to histology-based endometrial carcinoma recurrence prediction models: an ENITEC collaboration study. *Int J Gynecol Cancer.* (2018) 28:514–23. doi: 10.1097/IGC.0000000000001187
43. Trovik J, Wik E, Werner HMJ, Krakstad C, Helland H, Vandenput I, et al. Hormone receptor loss in endometrial carcinoma curettage predicts lymph node metastasis and poor outcome in prospective multicentre trial. *Eur J Cancer.* (2013) 49:3431–41. doi: 10.1016/j.ejca.2013.06.016
44. *Biomarker Guided Treatment in Gynaecological Cancer - Full Text View - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/show/NCT02543710 (accessed March 28, 2017).
45. PORTEC-4a. *Molecular Profile-based Versus Standard Adjuvant Radiotherapy in Endometrial Cancer - Full Text View - ClinicalTrials.gov*. Available online at: https://clinicaltrials.gov/ct2/show/NCT03469674 (accessed March 19, 2018).
46. Tangen IL, Kopperud RK, Visser NC, Staff AC, Tingulstad S, Marcickiewicz J, et al. Expression of L1CAM in curettage or high L1CAM level in preoperative blood samples predicts lymph node metastases and poor outcome in

endometrial cancer patients. *Br J Cancer.* (2017) 117:840–7. doi: 10.1038/bjc.2017.235

47. Geels YP, Pijnenborg JMA, Gordon BBM, Fogel M, Altevogt P, Masadah R, et al. L1CAM expression is related to non-endometrioid histology, and prognostic for poor outcome in endometrioid endometrial carcinoma. *Pathol Oncol Res.* (2016) 22:863–8. doi: 10.1007/s12253-016-0047-8
48. Jongen V, Briët J, de Jong R, ten Hoor K, Boezen M, van der Zee A, et al. Expression of estrogen receptor-alpha and -beta and progesterone receptor-A and -B in a large cohort of patients with endometrioid endometrial cancer. *Gynecol Oncol.* (2009) 112:537–42. doi: 10.1016/j.ygyno.2008.10.032

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 29 May 2019
doi: 10.3389/fonc.2019.00448

Integrated Bioinformatic Analysis of a Competing Endogenous RNA Network Reveals a Prognostic Signature in Endometrial Cancer

Leilei Xia[1†], Ye Wang[2,3†], Qi Meng[1†], Xiaoling Su[4], Jizi Shen[1], Jing Wang[1], Haiwei He[1], Biwei Wen[1], Caihong Zhang[1] and Mingjuan Xu[1]*

[1] Department of Obstetrics and Gynecology, Changhai Hospital, Second Military Medical University, Shanghai, China, [2] Department of Cell Biology, Center for Stem Cell and Medicine, Second Military Medical University, Shanghai, China, [3] Department of Urology, Changhai Hospital, Second Military Medical University, Shanghai, China, [4] Department of Obstetrics and Gynecology, No. 455 Hospital, Second Military Medical University, Shanghai, China

OPEN ACCESS

Edited by:
Massimo Nabissi,
University of Camerino, Italy

Reviewed by:
Changqing Su,
Eastern Hepatobiliary Surgery Hospital, China
Xiaofan Lu,
China Pharmaceutical University, China
Qin Yang,
Shanghai Jiaotong University, China

***Correspondence:**
Mingjuan Xu
13636373419@163.com

[†] These authors have contributed equally to this work

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: 21 September 2018
Accepted: 13 May 2019
Published: 29 May 2019

Citation:
Xia L, Wang Y, Meng Q, Su X, Shen J, Wang J, He H, Wen B, Zhang C and Xu M (2019) Integrated Bioinformatic Analysis of a Competing Endogenous RNA Network Reveals a Prognostic Signature in Endometrial Cancer. Front. Oncol. 9:448. doi: 10.3389/fonc.2019.00448

In endometrial carcinoma, the clinical outcome directly correlates with the TNM stage, but the lack of sufficient information prevents accurate prediction. The molecular mechanism underlying the competing endogenous RNA (ceRNA) hypothesis has not been investigated in endometrial cancer. Multi-bioinformatic analyses, including differentially expressed gene analysis, ceRNA network construction, Cox regression analysis, function enrichment analysis, and protein-protein network analysis, were performed on the sequence data acquired from The Cancer Genome Atlas (TCGA) data bank. A ceRNA network comprising 366 mRNAs, 27 microRNAs (miRNAs), and 66 long non-coding RNAs (lncRNAs) was established. Survival analysis performed with the univariate Cox regression analysis revealed nine lncRNAs with prognostic power in endometrial carcinoma. In multivariate Cox regression analysis, a signature comprising LINC00491, LINC00483, ADARB2-AS1, and C8orf49 showed remarkable prognostic power. Risk score and neoplasm status, but not TNM stage, were independent prognostic factors of endometrial carcinoma. A ceRNA network comprising differentially expressed mRNAs, miRNAs, and lncRNAs may reveal the molecular events involved in the progression of endometrial carcinoma. In addition, the signature with prognostic value may discriminate patients with increased risk for poor outcome, which may allow physicians to take accurate decisions.

Keywords: endometrial cancer, ceRNA network, prognostic factor, lncRNA, TCGA

INTRODUCTION

Endometrial cancer was shown to cause ∼11,350 deaths in the United States this year, and its incidence has increased mainly owing to the rise in obesity, a known risk factor. Clinical outcomes directly correlate with the clinical stages, and the 5-year overall survival rate has sharply decreased from 95% in patients with stage I cancer to 16% in those with stage IV cancer (1, 2). In clinical follow-up, aside from imageological examination such as B-mode ultrasound and magnetic resonance imaging (MRI), serum tumor markers, including CA125, CA199, and CEA, may serve as the indicators for the predictive outcome in patients (3, 4). Recently, L Salmena et al. proposed

that mRNAs, long non-coding RNAs (lncRNAs), and pseudogenes may regulate the expression of each other by targeting microRNAs (miRNAs) (5). lncRNAs were the biggest and most diverse class of non-coding RNAs in the human genome (6). Plenty lncRNAs play a part in pathogenesis of cancer such as unmanageable proliferation, or metastasis (7, 8), and can serve as oncogenes or antioncogenes, or by interreacting with famous oncogenes or antioncogenes such as MYC or p53, on both a transcriptional or post-transcriptional level (9, 10). Although the molecular events involved in the progression of endometrial carcinoma have been well studied, the complicated interaction between mRNAs, miRNAs, and lncRNAs that exerts crucial influence on the progression and prognosis of endometrial cancer is yet unclear.

The Cancer Genome Atlas (TCGA), a public integrated database, provides multiplatform genomic data along with the clinical information of matched patients. This database has driven the development of genomics to characterize the molecular landscape of cancers (11). Using TCGA, we analyzed differentially expressed genes, including mRNA, miRNA, and lncRNA, and constructed a lncRNA-miRNA-mRNA competing endogenous (ceRNA) network in endometrial cancer. Furthermore, we used Cox regression analysis to identify a signature based on LINC00491, LINC00483, ADARB2-AS1, and C8orf49. Of note, this signature may serve as an independent prognostic factor in endometrial cancer. This study demonstrates that these lncRNAs would allow identification of patients with endometrial cancer that are at higher risk for poor clinical outcome.

METHODS AND MATERIALS

Data Source

All the foundation data of TCGA-UCEC project, including genetic data, transcriptome profiling, and clinical information, were acquired from the Genomic Data Commons of the National Cancer Institute (http://portal.gdc.cancer.gov). Among 587 endometrial cancer profiles, 35 were obtained from para-carcinoma tissues, while others were endometrial cancer tissues. These data were available with no restrictions for research, and this study was performed under the guidelines of TCGA. GENCODE v.27 was used to annotate RNAs in the original transcriptome profiling, and total of 19676 mRNAs, 14447 lncRNAs, 1881 miRNAs were annotated. In clinical information, overall survival data were calculated from the date of diagnosis to the date of death or last follow-up.

Differentially Expressed mRNAs, miRNAs, and lncRNAs

The genomic data and transcriptome data from TCGA were downloaded and subjected to normalization with the calcNormFactors function with method of trimmed mean of M-values (TMM) in edgeR package. In addition, to avoid low abundance impact on the next procedure, RNAs with an average value of <1 were excluded. The differentially expressed mRNAs, miRNAs, and lncRNAs were analyzed with the exactTest function using the edgeR package. RNAs with a cutoff false discovery rate (FDR) adjusted $p < 0.01$ and $|\log FC| \geq 2$ were considered statistically different between cancer and normal groups (12). Heatmap was plotted using pheatmap R package.

Construction of the ceRNA Network

According to the hypothesis of ceRNA, it is vital to match the differentially expressed mRNAs, miRNAs, and lncRNAs; thus, the network could highlight a new molecular mechanism involved in the development of endometrial cancer. Pairs of miRNA-lncRNA were establish using the miRcode database (13). Pairs of miRNA-mRNA were built using the basic data supplied by TargetScan (14), and the mRNA predicted by the database was characterized as the target mRNA and used in the subsequent step. Pearson correlation was calculated between lncRNAs and mRNAs mediated by miRNAs (15), only the pairs with coefficient >0.4 were considered may involved in ceRNA network (16). Then, to quantify the regulatory effect of lncRNA/mRNA over mRNA/lncRNA via a specific miRNA, Sumazin et al. proposed the use of conditional mutual information (17), which was calculated in JAMI software implemented in Java (18). In this analysis, pairs with a value of $p < 0.05$ was considered statistically significant.

The clusterProfiler R package created by Guang et al. (19) was used to perform functional enrichment analyses, including Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. Terms with a value of $p < 0.05$ was considered statistically significant.

Identification of a Prognostic Signature Based on the ceRNA Network

Prognostic data were created on the matrix of lncRNAs involved in the ceRNA network and matched follow-up data. Patients were classified according to the median expression of lncRNAs into high or low expression groups. Univariate Cox regression analysis was used to identify the lncRNA with prognostic value. In addition, lncRNA with a $p < 0.05$ was used in the multivariate Cox regression analysis. As the number of lncRNA was high, it is important to create a signature comprising a limited number of variables and the best Akaike information criterion (AIC). These steps in the multivariate Cox regression analysis used the function of coxph in survival R package. After the identification of the best signature that predicted the outcome of the patients with endometrial cancer, the risk score was calculated as the summation of the product of each gene and its coefficient. In addition, patients were classified into high and low risk groups with the cutoff of the median risk score. Log-rank test was used to compare the survival distribution of these two groups, as estimated by the Kaplan-Meier analysis. In addition, a receiver operating characteristic (ROC) analysis was used to estimate the predictive power of this signature using 3 years as the predicted time.

The relativity between risk score and clinical factors, including age at diagnosis, TNM stage, and neoplasm tumor status, was analyzed using the chi-square test. Both univariate and multivariate Cox regression analyses were employed to discriminate between prognostic factors in endometrial carcinoma.

Protein-Protein Interaction Network Construction

mRNAs involved in ceRNA network based on lncRNA from the signature were subjected to protein-protein interaction network analysis using the STRING website (20).

Statistical Analysis

Kaplan-Meier curve was conducted by SPSS 21.0 using log-rank test (21), while other statistical tests were executed by R 3.5.1 using the corresponding R package mentioned above, hazard ratios was used in Cox model (22).

RESULTS

Differentially Expressed mRNAs, miRNAs, and lncRNAs

By employing differential gene expression analysis between cancer tissues and normal adjunct tissues, as per the cutoff FDR adjusted $p < 0.01$and $|\log FC| \geq 2$, 2,609 differentially expressed mRNAs (1,648 overexpressed and 961 down-regulated), 189 differentially expressed miRNAs (140 overexpressed and 49 down-regulated), and 1,121 differentially expressed lncRNAs (798 overexpressed and 323 down-regulated) were identified (**Supplement Figure 1**).

Construction of the ceRNA Network

We constructed the ceRNA network comprising mRNAs, miRNAs, and lncRNAs. The pairs of lncRNA-miRNA were matched using miRcode, 95 lncRNAs and 27 miRNAs formed 530 potential lncRNA-miRNA pairs. The miRNA-mRNA pairs were matched based on TargetScan. As a result, 1126 pairs of lncRNAs-mRNAs have the Pearson correlation >0.4. Then, in conditional mutual information calculated in JAMI software, 1605 pairs of lncRNA-miRNA-mRNA have the $p < 0.05$ which means that in these pairs, miRNAs are mediating that interaction. Thus, the ceRNA network was completely constructed and constituted of mRNAs, miRNAs, and lncRNAs (**Supplement Table 1**).

It is well known that hub nodes play critical roles in biological networks. Therefore, we calculated all node degrees of the lncRNA involved in ceRNA network. According to the previously study by Han et al., in which they defined a hub as a node degree exceeding 5, we found that 15 lncRNAs could be chosen as hub nodes, and the results are shown in **Supplement Figure 2**.

To discover the biological terms associated with these dysregulated genes, GO and KEGG function enrichment analyses were separately performed on dysregulated mRNAs. Terms with a value of $p < 0.05$ were considered as statistically significant.

In GO analysis, the overexpressed mRNAs were mainly enriched in epidermis development, intermediate filament, and transcription factor activity, RNA polymerase II proximal

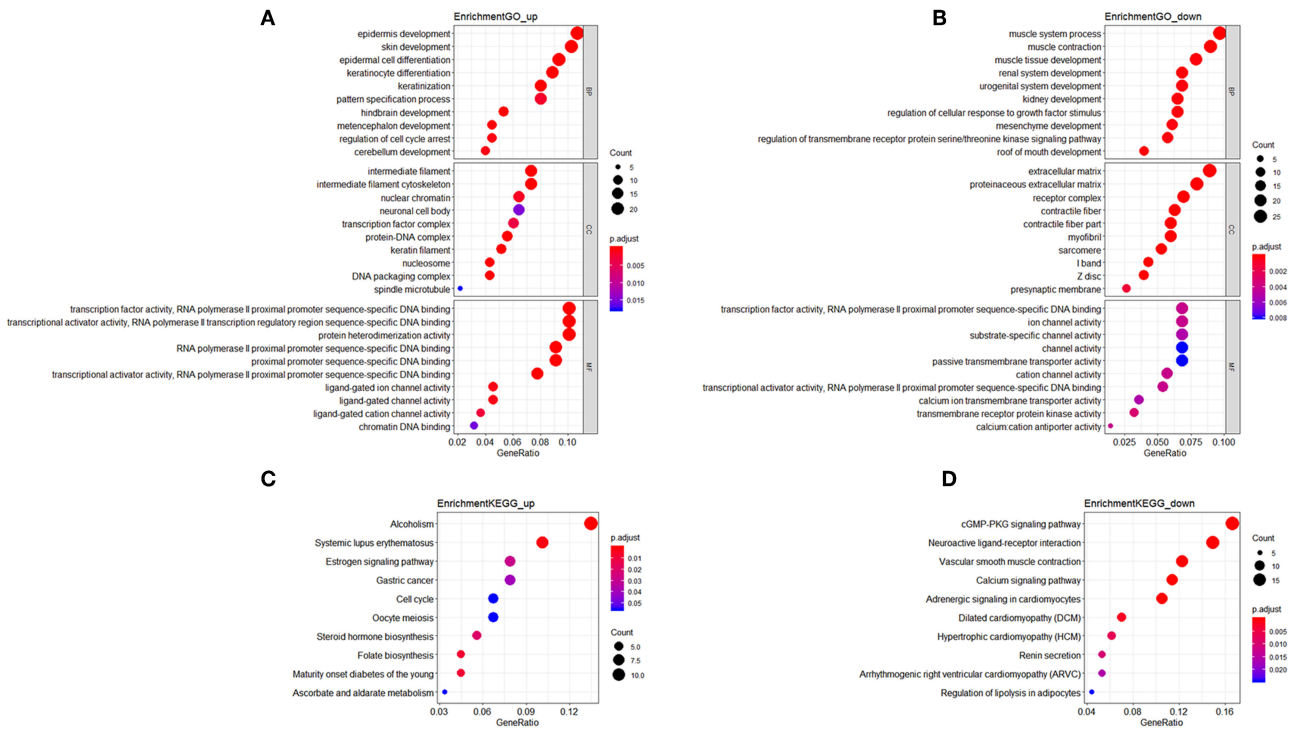


FIGURE 1 | Functional enrichment analysis of mRNAs in the ceRNA network. **(A)** Gene Ontology enrichment analysis of upregulated mRNAs. **(B)** Gene Ontology enrichment analysis of downregulated mRNAs. **(C)** KEGG pathway analysis of upregulated mRNAs. **(D)** KEGG pathway analysis of downregulated mRNAs. Horizontal axis represents gene count. Vertical axis represents enrichment analysis terms. Color of each plot represents the *p* value while the size represents the gene number in this term.

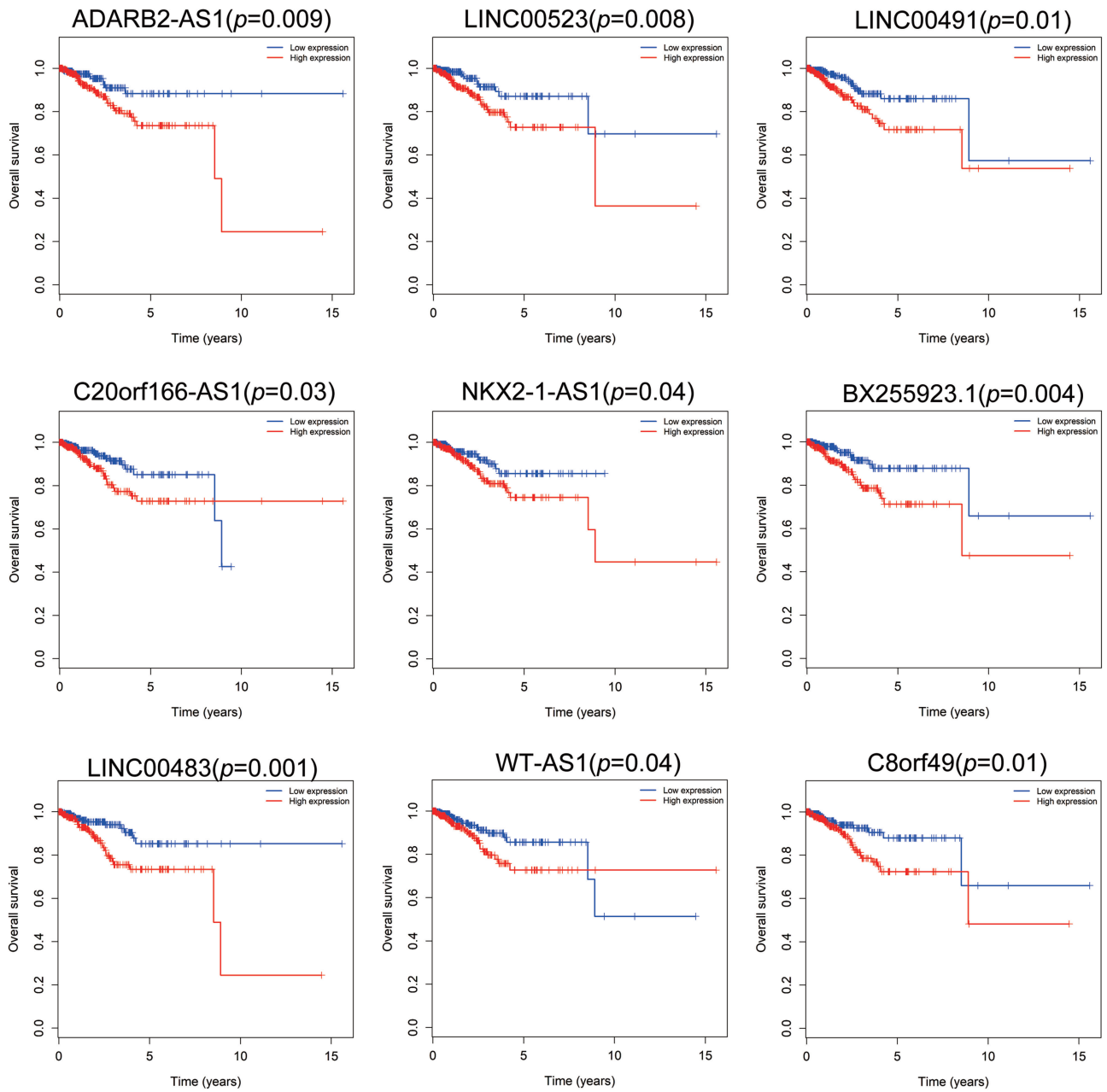


FIGURE 2 | Survival analysis of nine lncRNAs. Red plot indicates overexpression, while the blue plot represents low expression. Univariate Cox regression analysis was used to identify the lncRNA with prognostic value.

promoter sequence-specific DNA biding. The down-regulated mRNAs were significantly enriched in functions such as muscle system process, extracellular matrix, and transcription factor activity, RNA polymerase II proximal promoter sequence-specific DNA binding. In KEGG analysis, the overexpressed mRNAs were mainly enriched in Maturity onset diabetes of the young, and Alcoholism. The down-regulated mRNAs were mainly enriched in cGMP-PKG signaling pathway and Vascular smooth muscle contraction (**Figure 1**).

Construction of Prognostic Signature Based on the ceRNA Network

Patients with incomplete clinical information (age, race, TNM stage, tumor grade, histological type, type of neoplasm, and follow-up information) were excluded from the following procedure. Univariate Cox regression analysis was applied to lncRNAs involved in the ceRNA network. Survival status and overall survival time analyses revealed nine lncRNAs with prognostic values in endometrial carcinoma (**Figure 2**). These

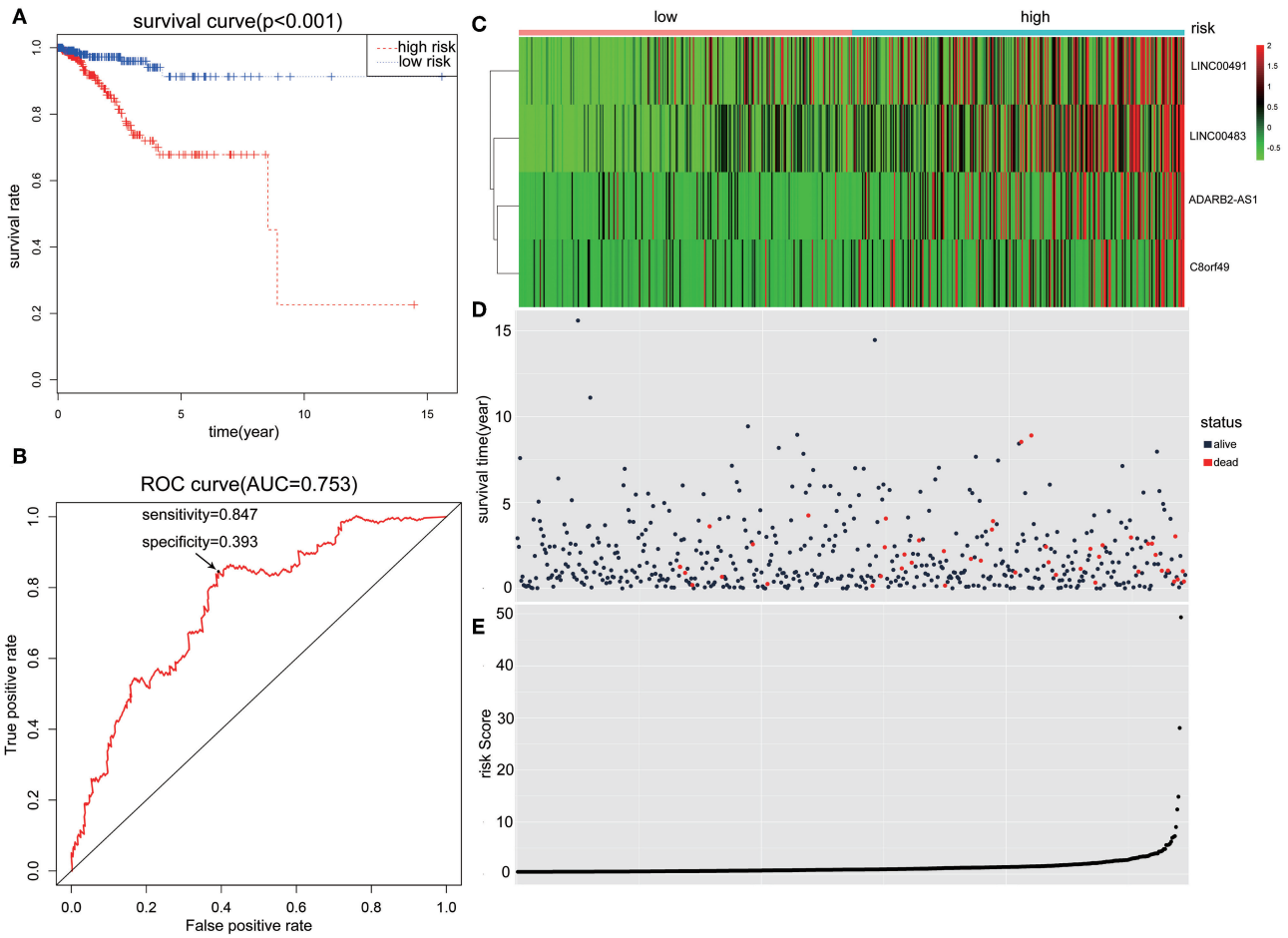


FIGURE 3 | The predictive value of the risk score calculated by LINC00491, LINC00483, ADARB2-AS1, and C8orf49. **(A)** Kaplan-Meier survival analysis of the risk score for overall survival. Log-rank test was used to compare the survival distribution of these two groups. **(B)** ROC for the prediction of the 3-years survival based on risk score. Area under the curve is 0.753, the sensitivity and specificity are 0.847 and 0.393 respectively. **(C)** Heatmap of LINC00491, LINC00483, ADARB2-AS1, and C8orf49 in high-risk and low-risk groups. The color of each block represents the relative expression of lncRNA in this patient. **(D)** Survival status and survival time of each individual. Color of each plot represents the survival status of each patient. **(E)** Risk score of each individual.

nine lncRNAs were subjected to multivariate Cox regression analysis. In this step, a function of step was applied to identify the best signature that predict the outcome of patients with endometrial carcinoma. A risk score formula based on LINC00491, LINC00483, ADARB2-AS1, and C8orf49 had the lowest AIC and was selected as the best signature. The risk assessment score for the prediction of overall survival was calculated as follows: Risk score $= \exp_{\mathrm{LINC00491}} \times 0.13335 + \exp_{\mathrm{LINC00483}} \times 0.32495 + \exp_{\mathrm{ADARB2-AS1}} \times 0.25997 + \exp_{\mathrm{C8orf49}} \times 0.22279$. Patients were classified into two clusters using the cutoff value of the median risk score. Kaplan-Meier survival analysis indicated that the 5-year survival rates for low- and high-risk groups were more than 0.9 and 0.6, respectively ($p < 0.001$). The area under the curve in ROC analysis was 0.753, suggesting that this signature has a promising power in predicting the clinical outcome of patients with endometrial carcinoma ($p < 0.001$, sensitivity: 0.847, specificity: 0.393) (**Figure 3**).

Clinical information of patients with endometrial carcinoma is shown in **Table 1**. We used the chi-square test to estimate the correlation between risk level and other clinical factors, and found that the risk level was significantly correlated with TNM stage ($p = 0.005$), tumor grade ($p = 0.005$), histological type ($p < 0.001$), neoplasm type ($p = 0.017$), and vital status ($p < 0.001$). This finding indicates that the risk score signature was closely correlated with the above-mentioned clinical parameters (**Table 2**).

To evaluate the predictive power of this risk signature, clinical parameters such as race, age at diagnosis, tumor grade, TNM stage, pathological type, and neoplasm type were included in the survival analysis. As shown in **Figure 4**, aside from tumor grade ($p = 0.004$), TNM stage ($p < 0.001$), and pathological type ($p = 0.006$), type of neoplasm ($p < 0.001$) and risk score ($p < 0.001$) were directly related to prognosis of patients. Multivariate Cox regression analysis showed that only type of neoplasm ($p < 0.001$) and risk score ($p = 0.001$), but not TNM stage ($p = 0.206$), tumor

TABLE 1 | Clinical parameters of endometrial carcinoma patients.

Subgroup	Frequency	Percent
Age		
<60	160	33.9
>=60	312	66.1
Race		
White	342	72.5
Nonwhite	130	27.5
TNM stage		
I + II	344	72.9
III–IV	128	27.1
Tumor grade		
G1 + G2	210	44.5
G3	262	55.5
Histological type		
Endometrioid endometrial adenocarcinoma	361	76.5
Other types	111	23.5
Type of neoplasm		
Tumor free	397	84.1
With tumor	75	15.9
Vital status		
Alive	432	91.5
Dead	40	8.5
Risk level		
Low	242	51.3
High	230	48.7

TABLE 2 | Relationship between risk level and clinical parameters.

Subgroup	Low-risk	High-risk	Total	*P*-value
Age				0.081
<60	91	69	160	
>=60	151	161	312	
Race				0.451
White	179	163	342	
Nonwhite	63	67	130	
TNM stage				0.005
I + II	190	154	344	
III–IV	52	76	128	
Tumor grade				0.005
G1 + G2	123	87	210	
G3	119	143	262	
Histological type				<0.001*
Endometrioid endometrial adenocarcinoma	214	147	361	
Other types	28	83	111	
Type of neoplasm				0.017
Tumor free	213	184	397	
With tumor	29	46	75	
Vital status				<0.001*
Alive	235	197	432	
Dead	7	33	40	

*$p < 0.05$.

grade ($p = 0.558$), and tumor pathological type ($p = 0.576$), were statistically independent predictive factors of poorer prognosis for endometrial cancer (**Figure 5**).

Protein-Protein Network Analyses

To better understand the mechanisms underlying the function of the four lncRNAs, protein-protein interactions of mRNAs involved in the ceRNA network of these four lncRNAs were constructed using the STRING website. In this PPI network, MEF2C has the closet connection with other proteins (**Figure 6**).

DISCUSSION

Endometrial cancer is one of the three leading gynecologic tumors. The Cancer statistics of 2019 revealed 61,880 new cases and 12,160 deaths in United States. (1). Several tumor markers such as CA125, HE4, CA199, and CEA are clinically used for the diagnosis of endometrial cancer. However, the pathological process of the occurrence and development of endometrial cancer is still unclear. More precise preoperative staging and preoperative diagnosis demand better pathophysiological development and new tumor markers of endometrial cancer. Epigenetics of genes, especially lncRNAs, have been recently used for the study of endometrial cancer. Although most lncRNAs lack the capacity of coding protein, many other functions of lncRNAs have been found in endometrial cancer, including epithelial-to-mesenchymal transition (4). But due to the technical limitations, functional studies of lncRNAs are not easy in comparison with those of coding RNAs. ceRNA hypothesis provided a new solution for achieving better functional studies of lncRNAs. It proposed that lncRNA can regulate miRNA abundance by binding and sequestering them. As such, lncRNAs can regulate the expression of target mRNAs. Thus, it has been shown that an efficient way to infer the potential function of lncRNAs is by studying their relationship with miRNAs and mRNAs, whose functions have been annotated. Taken advantage of that, we mapped the ceRNA network in endometrial cancer which could provide new insights to explore the mechanism of it.

In this research, we analyzed the differentially expressed genes to develop ceRNA network and investigated the molecular events that facilitate the development of endometrial carcinoma. Using univariate and multivariate Cox regression analyses, a signature based on four lncRNAs was developed that showed promising outcomes with respect to the prediction of the patient's overall survival. This signature was closely correlated with the TNM stage and tumor grade clinical parameters and served as an independent factor, like neoplasm cancer status and unlike TNM stage. Some clinical studies have suggested the association between diabetes as well as hypertension with the outcome of patients with endometrial cancer (23, 24); however, we did not perform statistical analysis of diabetes and hypertension in the present study, as more than 50% values were missing.

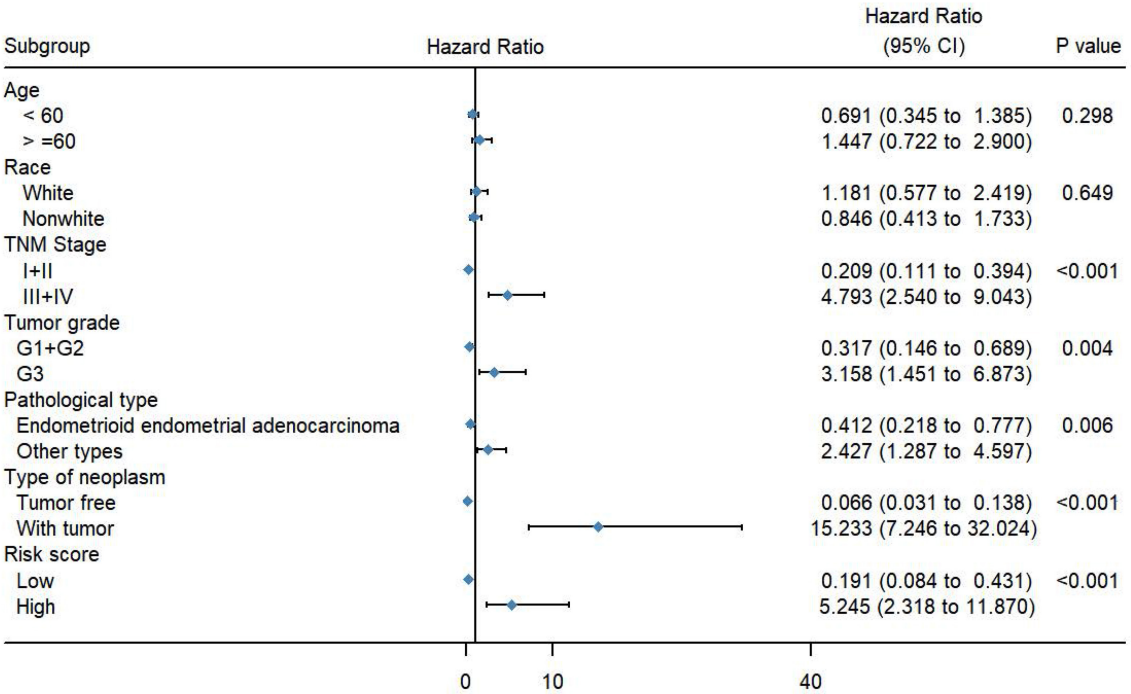


FIGURE 4 | Forest map of clinical characters in univariate analysis. The coordinate of diamond represents the odds ratio. Univariate Cox regression analysis was performed. Subgroup with a value of $p < 0.05$ was considered statistically significant.

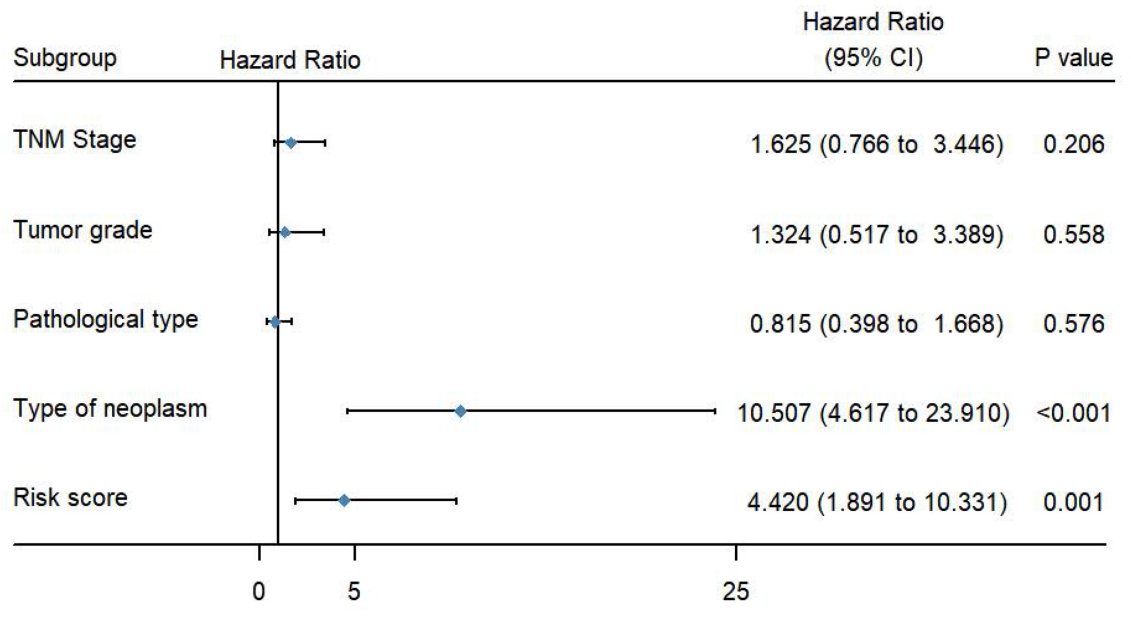


FIGURE 5 | Forest map of clinical characters in multivariate analysis. The coordinate of the blue diamond represents the odds ratio. Multivariate Cox regression analysis was performed. Subgroup with a value of $p < 0.05$ was considered statistically significant.

In general, the survival outcome for patients with endometrial cancer is mainly predicted by two elements, namely, TNM stage and type of neoplasm, but a quantifiable index is lacking.

Several studies have shown that the expression of hormone receptors such as estrogen and progesterone receptors is a favorable independent prognostic factor (25, 26). In addition,

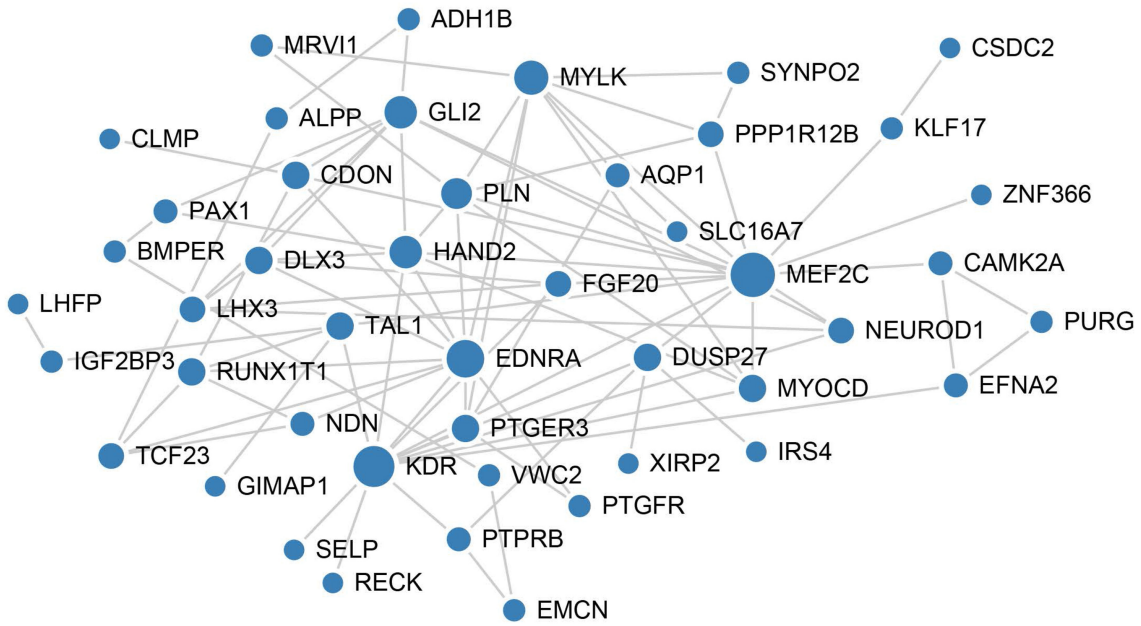


FIGURE 6 | PPI network analysis of mRNAs involved in the ceRNA network of these four lncRNAs. The size of each node represents the degree of this gene.

hormonal therapy was considered as a supplemental therapy in clinical setting. Mutation of the well-known gene P53 is associated with poor clinical outcome, and the overall survival of patients with endometrial cancer with alterations in p53 gene expression was much lower than that of patients with the wild-type p53 (27). Moreover, the overexpression of human epidermal growth factor receptor 2 (HER2) gene is an independent prognostic factor associated with poor overall survival (28).

An efficient and sensitive marker that predicts the outcome of endometrial cancer is still lacking. In this study, we identified a signature based on LINC00491, LINC00483, ADARB2-AS1, and C8orf49 to discriminate patients with endometrial cancer that are at increased risk for poor outcome in combination with the information related to TNM stage and type of neoplasm.

The results of this analysis showed that the expressions of four lncRNAs (LINC00491, LINC00483, ADARB2-AS1, and C8orf49) were markedly different between endometrial cancer tissues and normal endometrial tissues. Many of these genes are incompletely studied. C8orf49 is also called as GATA4 downstream membrane gene (G4DM). GATA4 (GATA transcription factor 4) is a zinc-finger transcription factor involved in the development of heart and adult cardiomyocytes. Mutations in GATA4 gene may cause congenital heart diseases (29) such as the tetralogy of Fallot, atrial septal defect, ventricular septal defect, atrioventricular septal defect, and dilated cardiomyopathy. A key feature of GATA4 is its two-zinc finger domain, which binds to the specific region of the target gene. In serval cancer types, GATA4 serves as a potential tumor suppressor, and hypermethylation and hypomethylation of GATA4 are closely related to the malignant behavior of cancers (30, 31). GATA4 may also be used as a biomarker for ovarian cancer (32). The expression of GATA4 may change during cardiocyte differentiation through the effect of the transcription of the target gene. GATA4 gene is also expressed in the uterus, and C8orf49 is one of the target genes of GATA4. Therefore, C8orf49 may play an important role in the differentiation of endometrial cancer cells. In addition, studies have indicated that ADARB2-AS1, as an open-reading frame, may contribute to the risk of pancreatic ductal adenocarcinoma (PDAC). Along with other seven lncRNAs, ADARB2-AS1 showed better accuracy than the standard clinical and radiologic features in distinguishing aggressive/malignant IPMNs (33). However, the underlying mechanism is unclear.

It is well known that hub genes play critical roles in biological networks. Therefore, node degrees of lncRNA involved in this ceRNA network were calculated. A lncRNA with a node degree >5 was considered as hub lncRNA. In this study, total of 15 lncRNAs were identified with high degree in the ceRNA network. C8orf49 which has prognostic value also act as hub lncRNA in endometrial cancer. This suggests that C8orf49 may play critical roles in the origin and development of endometrial cancer. Here, we demonstrate for the first time the construction of a ceRNA network in endometrial cancer to reveal the molecular mechanism that facilitates the development of endometrial cancer. A signature based on LINC00491, LINC00483, ADARB2-AS1, and C8orf49 was identified as a biomarker to discriminate between patients with high and poor risk outcome. The lncRNAs involved in this signature may serve as therapeutic targets for precision medicine in endometrial cancer. Further studies are warranted to explore the biological function and reveal the molecular mechanism underlying the role of LINC00491, LINC00483, ADARB2-AS1, and C8orf49 in endometrial cancer.

CONCLUSION

This study focused on a ceRNA network to provide a novel perspective and insight into endometrial cancer and suggested that the signature based on LINC00491, LINC00483, ADARB2-AS1, and C8orf49 could serve as an independent prognostic biomarker in endometrial cancer.

ETHICS STATEMENT

High-throughput sequencing-counts (HTSeq-counts) and miRNA sequencing profiles were obtained from the TCGA data portal, which is a publicly available dataset. Therefore, no ethics approval is needed.

AUTHOR CONTRIBUTIONS

LX constructed ceRNA network using R software. QM analyzed the data using SPSS. YW download data from TCGA. XS and BW used photoshop and illustration software. JS and CZ organized data. JW perform GO and KEGG analysis. HH perform PPI network. MX designed this study.

FUNDING

This work was supported by National Key Research and Development Program of China (NO:2016YFC1303100).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00448/full#supplementary-material

Supplement Figure 1 | Heatmap of differentially expressed RNAs. The one on the left is the heatmap of 50 randomly selected differentially expressed mRNAs. The center one is the heatmap of 50 differentially expressed lncRNAs, while the one on the right is the heatmap of 50 randomly selected differentially expressed miRNAs. Orange indicates high-level RNA expression, whereas blue indicates low expression. The first row of each map is the type of each sample. Pink represents normal samples, while blue represents cancerous samples.

Supplement Figure 2 | All lncRNA node degree analysis reveals specific properties of the ceRNA network.

Supplement Table 1 | ceRNA network of endometrial cancer.

REFERENCES

1. Siegel RL, Miller K, Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. *CA Cancer J Clin*. (2019) 69:7–34. doi: 10.3322/caac.21551
2. Leslie KK, Thiel KW, Goodheart MJ, De Geest K, Jia Y, Yang S. Endometrial Cancer. *Obstet Gynecol Clin North Am*. (2012) 39:255–68. doi: 10.1016/j.ogc.2012.04.001
3. Dotters DJ. Preoperative CA 125 in endometrial cancer: is it useful? *Am J Obstet Gynecol*. (2000) 182:1328–34. doi: 10.1067/mob.2000.106251
4. Frei KA, Kinkel K. Staging endometrial cancer: role of magnetic resonance imaging. *J Magn Reson Imaging*. (2001) 13:850–5. doi: 10.1002/jmri.1121
5. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the rosetta stone of a hidden RNA language? *Cell*. (2011) 146:353–8. doi: 10.1016/j.cell.2011.07.014
6. Iyer MK, Niknafs YS, Malik R, Singhal U, Sahu A, Hosono Y, et al. The landscape of long noncoding RNAs in the human transcriptome. *Nat Genet*. (2015) 47:199–208. doi: 10.1038/ng.3192
7. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. (2000) 100:57–70. doi: 10.1016/S0092-8674(00)81683-9
8. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. (2011) 144:646–74. doi: 10.1016/j.cell.2011.02.013
9. Barsyte-Lovejoy D, Lau SK, Boutros PC, Khosravi F, Jurisica I, Andrulis IL, et al. The c-Myc oncogene directly induces the H19 noncoding RNA by allele-specific binding to potentiate tumorigenesis. *Cancer Res*. (2006) 66:5330–7. doi: 10.1158/0008-5472.CAN-06-0037
10. Huarte M, Guttman M, Feldser D, Garber M, Koziol MJ, Kenzelmann-Broz D, et al. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell*. (2010) 142:409–19. doi: 10.1016/j.cell.2010.06.040
11. Tomczak K, Czerwinska P, Wiznerowicz M. The cancer genome atlas (TCGA): an immeasurable source of knowledge. *Contemp Oncol (Pozn)*. (2015) 19:A68–77. doi: 10.5114/wo.2014.47136
12. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. (2010) 26:139–40. doi: 10.1093/bioinformatics/btp616
13. Jeggari A, Marks DS, Larsson E. miRcode: a map of putative microRNA target sites in the long non-coding transcriptome. *Bioinformatics*. (2012) 28:2062–3. doi: 10.1093/bioinformatics/bts344
14. Agarwal V, Bell GW, Nam J-W, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *Elife*. (2015) 4:e05005. doi: 10.7554/eLife.05005
15. Le TD, Zhang J, Liu L, Li J. Computational methods for identifying miRNA sponge interactions. *Brief Bioinform*. (2017) 18:577–90. doi: 10.1186/s12859-017-1467-5
16. Paci P, Colombo T, Farina L. Computational analysis identifies a sponge interaction network between long non-coding RNAs and messenger RNAs in human breast cancer. *BMC Syst Biol*. (2014) 8:83. doi: 10.1186/1752-0509-8-83
17. Sumazin P, Yang X, Chiu H-S, Chung W-J, Iyer A, Llobet-Navas D, et al. An extensive microRNA-mediated network of RNA-RNA interactions regulates established oncogenic pathways in glioblastoma. *Cell*. (2011) 147:370–81. doi: 10.1016/j.cell.2011.09.041
18. Hornakova A, List M, Vreeken J, Schulz MH. JAMI-fast computation of conditional mutual information for ceRNA network analysis. *Bioinformatics*. (2018) 1:2. doi: 10.1093/bioinformatics/bty221
19. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS*. (2012) 16:284–7. doi: 10.1089/omi.2011.0118
20. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Res*. (2017) 45:D362–8. doi: 10.1093/nar/gkw937
21. Goel MK, Khanna P, Kishore J. Understanding survival analysis: Kaplan-Meier estimate. *Int J Ayurveda Res*. (2010) 1:274–8. doi: 10.4103/0974-7788.76794
22. Stel VS, Dekker FW, Tripepi G, Zoccali C, Jager KJ. Survival analysis II: Cox regression. *Nephron Clin Pract*. (2011) 119:c255–60. doi: 10.1159/000328916
23. Folsom AR, Anderson KE, Sweeney C, Jacobs DR Jr. Diabetes as a risk factor for death following endometrial cancer. *Gynecol Oncol*. (2004) 94:740–5. doi: 10.1016/j.ygyno.2004.06.027
24. Aune D, Sen A, Vatten LJ. Hypertension and the risk of endometrial cancer: a systematic review and meta-analysis of case-control and cohort studies. *Sci Rep*. (2017) 7:44808. doi: 10.1038/srep44808
25. Creasman WT, Soper JT, McCarty KS Jr., McCarty KS Sr, Hinshaw W, Clarke-Pearson DL. Influence of cytoplasmic steroid receptor content on

prognosis of early stage endometrial carcinoma. *Am J Obstet Gynecol*. (1985) 151:922–32. doi: 10.1016/0002-9378(85)90671-4
26. Palmer DC, Muir IM, Alexander AI, Cauchi M, Bennett RC, Quinn MA. The prognostic importance of steroid receptors in endometrial carcinoma. *Obstet Gynecol*. (1988) 72(3 Pt 1):388–93.
27. Lee EJ, Kim TJ, Kim DS, Choi CH, Lee JW, Lee JH, et al. p53 alteration independently predicts poor outcomes in patients with endometrial cancer: a clinicopathologic study of 131 cases and literature review. *Gynecol Oncol*. (2010) 116:533–8. doi: 10.1016/j.ygyno.2009.11.018
28. Morrison C, Zanagnolo V, Ramirez N, Cohn DE, Kelbick N, Copeland L, et al. HER-2 is an independent prognostic factor in endometrial cancer: association with outcome in a large cohort of surgically staged patients. *J Clin Oncol*. (2006) 24:2376–85. doi: 10.1200/JCO.2005.03.4827
29. Garg V, Kathiriya IS, Barnes R, Schluterman MK, King IN, Butler CA, et al. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature*. (2003) 424:443–7. doi: 10.1038/nature01827
30. Guo M, Akiyama Y, House MG, Hooker CM, Heath E, Gabrielson E, et al. Hypermethylation of the GATA genes in lung cancer. *Clin Cancer Res*. (2004) 10:7917–24. doi: 10.1158/1078-0432.CCR-04-1140
31. Chmelarova M, Dvorakova E, Spacek J, Laco J, Palicka V. Importance of promoter methylation of GATA4 gene in epithelial ovarian cancer. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. (2013) 157:294–7. doi: 10.5507/bp.2013.079
32. Bubancova I, Kovarikova H, Laco J, Ruszova E, Dvorak O, Palicka V, et al. Next-generation sequencing approach in methylation analysis of HNF1B and GATA4 genes: searching for biomarkers in ovarian cancer. *Int J Mol Sci*. (2017) 18:E474. doi: 10.3390/ijms18020474
33. Permuth JB, Chen DT, Yoder SJ, Li J, Smith AT, Choi JW, et al. Linc-ing circulating long non-coding RNAs to the diagnosis and malignant prediction of intraductal papillary mucinous neoplasms of the pancreas. *Sci Rep*. (2017) 7:10484. doi: 10.1038/s41598-017-09754-5

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 26 February 2019
doi: 10.3389/fonc.2019.00061

A Micro-Costing Study of Screening for Lynch Syndrome-Associated Pathogenic Variants in an Unselected Endometrial Cancer Population: Cheap as NGS Chips?

Neil A. J. Ryan[1,2], *Niall J. Davison*[3], *Katherine Payne*[3], *Anne Cole*[4], *D. Gareth Evans*[2,4,5] *and Emma J. Crosbie*[1,5,6*]

[1] Gynaecological Oncology Research Group, Division of Cancer Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, The University of Manchester, Manchester, United Kingdom, [2] Division of Evolution and Genomic Medicine, School of Medical Sciences, Faculty of Biology, Medicine and Health, The University of Manchester, St Mary's Hospital, Manchester, United Kingdom, [3] Manchester Centre for Health Economics, Division of Population Health, Health Services Research and Primary Care, The University of Manchester, Manchester, United Kingdom, [4] Manchester Centre for Genomic Medicine, St Mary's Hospital, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, United Kingdom, [5] Division of Cancer Sciences, Faculty of Biology, Medicine and Health, NIHR Manchester Biomedical Research Centre, School of Medical Sciences, The University of Manchester, Manchester, United Kingdom, [6] Department of Obstetrics and Gynaecology, St Mary's Hospital, Manchester University NHS Foundation Trust, Manchester Academic Health Science Centre, Manchester, United Kingdom

OPEN ACCESS

Edited by:
Massimo Nabissi, University of Camerino, Italy

Reviewed by:
Margaret Liang, University of Alabama at Birmingham, United States
Suzana Arenhart Pessini, Universidade Federal do Rio Grande do Sul (UFRGS), Brazil

***Correspondence:**
Emma J. Crosbie
emma.crosbie@manchester.ac.uk

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *14 November 2018*
Accepted: *21 January 2019*
Published: *26 February 2019*

Citation:
Ryan NAJ, Davison NJ, Payne K, Cole A, Evans DG and Crosbie EJ (2019) A Micro-Costing Study of Screening for Lynch Syndrome-Associated Pathogenic Variants in an Unselected Endometrial Cancer Population: Cheap as NGS Chips? Front. Oncol. 9:61. doi: 10.3389/fonc.2019.00061

Background: Lynch syndrome is the most common inherited cause of endometrial cancer. Identifying individuals affected by Lynch syndrome enables risk-reducing interventions including colorectal surveillance, and cascade testing of relatives.

Methods: We conducted a micro-costing study of screening all women with endometrial cancer for Lynch syndrome using one of four diagnostic strategies combining tumor microsatellite instability testing (MSI), immunohistochemistry (IHC), and/or *MLH1* methylation testing, and germline next generation sequencing (NGS). Resource use (consumables, capital equipment, and staff) was identified through direct observation and laboratory protocols. Published sources were used to identify unit costs to calculate a per-patient cost (£; 2017) of each testing strategy, assuming a National Health Service (NHS) perspective.

Results: Tumor triage with MSI and reflex *MLH1* methylation testing followed by germline NGS of women with likely Lynch syndrome was the cheapest strategy at £42.01 per case. Tumor triage with IHC and reflex *MLH1* methylation testing of MLH1 protein-deficient cancers followed by NGS of women with likely Lynch syndrome cost £45.68. Tumor triage with MSI followed by NGS of all women found to have tumor microsatellite instability cost £78.95. Immediate germline NGS of all women with endometrial cancer cost £176.24. The cost of NGS was affected by the skills and time needed to interpret results (£44.55/patient).

Conclusion: This study identified the cost of reflex screening all women with endometrial cancer for Lynch syndrome, which can be used in a model-based cost-effectiveness analysis to understand the added value of introducing reflex screening into clinical practice.

Keywords: micro-costing, Lynch syndrome, endometrial cancer, genetic testing, screening

INTRODUCTION

Lynch syndrome is an inherited predisposition to a constellation of different cancers, of which colorectal and endometrial cancer are the most common (1). Estimates of the prevalence of Lynch syndrome among the general population are as high as 300 per 100,000 (2). Lynch syndrome confers a lifetime risk of endometrial cancer of 30 to 40% (3). Endometrial cancer may be the sentinel event in women with Lynch syndrome, providing an early diagnostic opportunity (4). Those found to have Lynch syndrome are offered risk-reducing interventions including colorectal surveillance to reduce cancer-specific mortality (5). A diagnosis of Lynch syndrome enables cascade testing within families and its identification in those who are yet to develop cancer (6). Identified women may be offered prophylactic surgery to reduce their risk of gynecological cancer (7).

Lynch syndrome arises from germline pathogenic variants within the highly conserved mismatch repair (MMR) system. The molecular characteristics of Lynch syndrome-associated cancers enable tumor-based triage and targeted germline sequencing of the MMR genes, commonly performed by next generation sequencing (NGS). A Lynch syndrome-associated tumor classically shows aberrant expression of associated MMR proteins, MLH1, MSH2, MSH6 and/or PMS2, and microsatellite instability (MSI) (8). Loss of MLH1 expression through somatic methylation of the *MLH1* promotor region is a common sporadic event in endometrial cancer; *MLH1* methylation testing is therefore an effective way of reducing the number of women with MLH1 loss by IHC or whose tumors are MSI-H from expensive germline testing (9).

The National Institute for Health and Care Excellence (NICE) recommends universal screening for Lynch syndrome in people with colorectal cancer (10). Universal screening was identified to be a cost-effective use of healthcare resources because of the number of colorectal cancers prevented in family members who are also found to carry Lynch syndrome (11). Screening women with endometrial cancer provides a further opportunity to save lives from Lynch syndrome-associated cancer (12), however, the costs associated with this screening strategy are not known. The aim of this study was to identify and quantify the resource use and costs associated with different diagnostic strategies relevant to screening for Lynch syndrome in an unselected endometrial cancer population.

Abbreviations: NGS, Next generation sequencing; MSI, Microsatellite instability; MSI-H, Microsatellite instability high; IHC, Immunohistochemistry; MMR, Mismatch repair; NICE, National Institute for Health and Care Excellence; NHS, National Health Service, England.

METHODS

A micro-costing study was performed to identify the resource use and cost per patient of four diagnostic testing strategies for Lynch syndrome in an unselected endometrial cancer population. The study assumed the perspective of the National Health Service (NHS) in England. The direct costs associated with providing each diagnostic testing strategy were identified. The time horizon for identifying the relevant resources use in this study started with the process of gaining informed consent for any Lynch syndrome testing and ended with generating a report of the final diagnostic test result.

Diagnostic Testing Strategies

Four Lynch syndrome testing technologies that reflect current and emerging national clinical practice (10) were included in this study: microsatellite instability (MSI) testing; immunohistochemistry (IHC); *MLH1* promoter hypermethylation pyrosequencing (methylation) testing; and next generation sequencing (NGS) (13). The technologies used to produce each diagnostic test were conceptualized into representative clinical pathways using a decision tree to provide a structured approach to represent how each technology would form part of a diagnostic testing strategy for a defined population of women with suspicion of Lynch syndrome. The relevant outcome of testing was defined as either Lynch syndrome diagnosed or not diagnosed. The four diagnostic testing strategies were:

Strategy 1: Initial tumor triage with MSI followed by germline NGS testing for pathogenic variants of the MMR genes for all those found to have microsatellite instability (MSI-H).
Strategy 2: Initial tumor triage with MSI followed by reflex *MLH1* methylation testing for MSI-H tumors, and germline NGS testing for women where the tumor *MLH1* methylation test shows no hypermethylation.
Strategy 3: Initial tumor triage with IHC followed by reflex *MLH1* methylation testing for tumors with MLH1 loss. Germline NGS testing for pathogenic variants of the MMR genes for all women whose tumors show MSH2, MSH6 or PMS2 loss, or MLH1 loss where the *MLH1* methylation test shows no hypermethylation.
Strategy 4: No initial tumor triage. All women with endometrial cancer undergo direct germline NGS testing for pathogenic variants of the MMR genes.

The decision tree (**Figure 1**) was conceptualized through discussion with a panel of 10 local and national experts. These experts included two consultant histopathologists, three senior

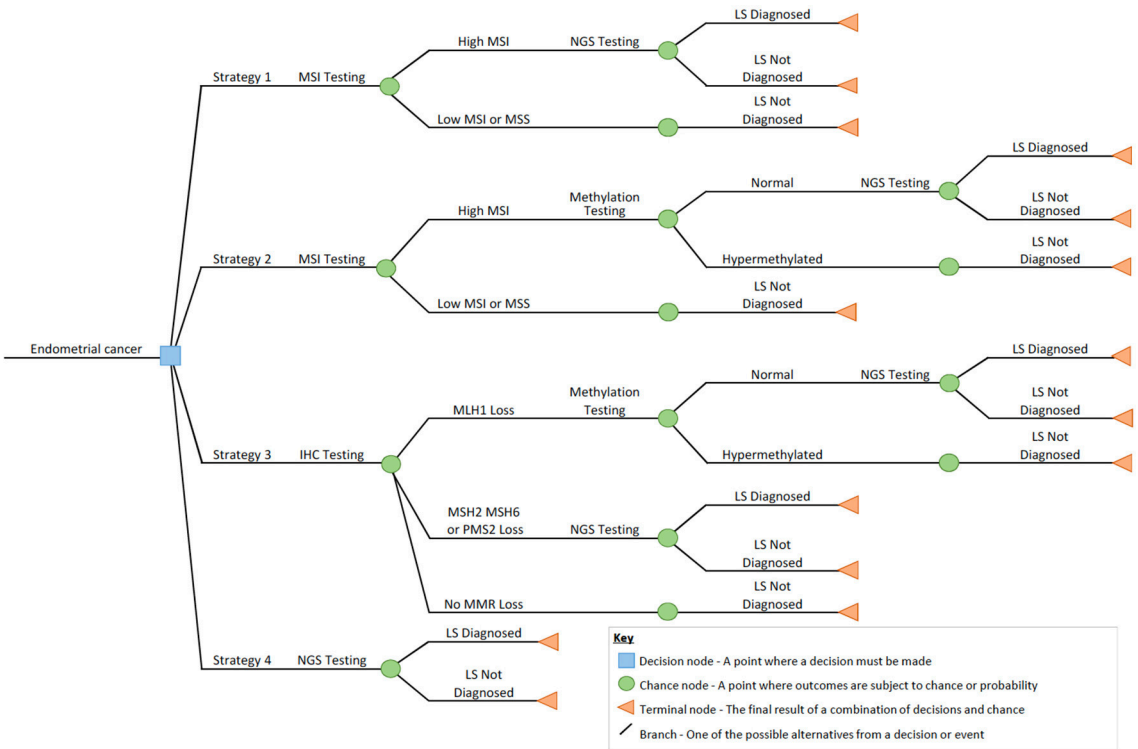


FIGURE 1 | Decision tree outlining the diagnostic strategies for screening endometrial cancer for Lynch syndrome in this study.

(>band 8a Agenda For Change pay scale) clinical laboratory scientists, two consultant gynecological oncology surgeons, two consultant clinical geneticists, and one consultant genetic pathologist. The decision tree represented the proportion of cases testing positive with each technology and subsequent impact on the need to conduct further testing. The input values for the decision tree were informed by a pragmatic review of the literature (see **Supplementary Appendix 1**), which identified the relevant studies to inform the probability of a positive or negative test result in each scenario.

Identifying Resource Use

The use of resources was identified for each diagnostic testing strategy, assuming the NHS perspective. Resource use included clinical and laboratory staff time, capital equipment, and laboratory consumables.

Clinical and laboratory staff time was collected using a prospective study at a large tertiary genetics and gynecology oncology surgical referral center in the North West of England between 2015 and 2017. The study was approved by the North West Research Ethics Committee (15/NW/0733) and all patients gave written, informed consent to participate. Non-participant direct observation was used to identify the time members of staff dedicated to each element of the diagnostic test process. A single observer recorded timings with a stopwatch over multiple rounds of observation between 2015 and 2017, using a structured data collection tool and recording information on sample batch size and staff pay grade. Participant direct observation was used to record the process of obtaining informed consent for Lynch syndrome testing. Because this was done as part of a research study, all participants were consented prior to any testing. The consent process included discussion about the various tumor tests (MSI, IHC, methylation testing) and indicative germline Lynch syndrome testing.

Hospital information systems were used to derive resource use on capital equipment including diagnostic platforms. Total capital throughput for a given piece of equipment was identified as the total annual number of samples it processed. Total Lynch syndrome-associated capital throughput for a given piece of equipment was identified as the total annual number of Lynch syndrome-associated samples it processed, assuming all newly diagnosed endometrial cancer patients were offered testing.

Consumables were identified from laboratory standard operating procedures and through completion of a structured data collection form by seven laboratory staff who routinely test patients for Lynch syndrome, including biomedical technicians, a consultant clinical scientist, a principal clinical scientist, and clinical genetics technicians.

Collating Unit Costs

The unit costs (UK sterling; £) for consumables and equipment were extracted from published list prices, or hospital invoices where list prices were unavailable. The unit costs for staff labor were defined as cost per minute using the midpoints of salary

grades as per NHS Agenda for Change or the British Medical Association's Hospital Doctor pay scales (see **Table S4**). The price year for unit costs was standardized at 2017. **Table S2** shows the complete list of unit costs and sources.

Data Analysis

All analysis was carried out using Microsoft Corporation software program Microsoft Office Excel 2011. The base case analysis calculated the direct medical costs of each of the four diagnostic testing strategies shown in **Figure 1**. The costs were calculated for testing a single sample by multiplying the relevant unit cost (see **Table S2**) with the relevant items and quantities of resource use.

One-way sensitivity analysis was used to identify the impact of using different estimates for the probability of a positive or negative test result for each diagnostic testing strategy. The ranges of the probability input values were informed by a pragmatic literature search (**Supplementary Appendix 1, Table S1**) and used to assess the effect different test outcomes would have on the overall cost of each strategy. Two separate scenario analyses explored the impact of the timing of consent on the cost of each diagnostic testing strategy. Two scenarios were explored to understand the cost if consent was taken before any testing, compared with a scenario in which consent was only taken for those needing germline NGS testing.

RESULTS

Table 1 shows the individual quantities of resource use for MSI, IHC, *MLH1* methylation testing, and NGS, which were used to calculate the total costs incurred per patient for each of the four diagnostic testing strategies.

Tumor triage with MSI and reflex *MLH1* methylation testing followed by germline NGS of women with likely Lynch syndrome (Strategy 2) was the cheapest at £42.01 per patient. Tumor triage with IHC and reflex *MLH1* methylation testing of MLH1 protein-deficient cancers followed by NGS of women with likely Lynch syndrome (Strategy 3) cost £45.68. Tumor triage with MSI followed by NGS of all women found to have MSI-H tumors (Strategy 1) cost £78.95. Immediate germline NGS of all women with endometrial cancer (Strategy 4) cost £176.24.

The cost of consenting a woman for Lynch syndrome testing was calculated from 269 directly observed episodes. Two women declined Lynch syndrome testing. On average, the process of gaining consent took 7 min and 39 s (SD: 5 min, 16 s) of consultant time. This cost an average of £5.32.

Immediate, unselected germline NGS testing for pathogenic variants of the MMR genes in all women with endometrial cancer was the most expensive testing strategy. Consumables were an expensive component, costing £115.14 overall, including DNA extraction. As germline DNA is required, it was assumed that all samples for NGS would require *de-novo* DNA extraction from blood. Equipment costs were expensive for NGS, at £5.43 per sample tested. Labor costs were also relatively high due to the complexity of data interpretation, costing £50.35 per sample.

Strategies one to three involved the use of tumor-based triage with IHC and MSI. Tumor based triage by IHC was cheaper than MSI (£21.17 vs. £27.67). The most expensive resource was consumables (£12.23 vs. £19.19 for IHC and MSI, respectively). Labor costs were similar (£8.51 vs. £8.30 for IHC and MSI, respectively); this was despite the need for a consultant grade doctor to interpret the IHC results, because the per-sample time was relatively short. Equipment costs were more expensive for IHC at £0.43 per sample due to use of a dedicated staining platform and associated maintenance costs. These costs were cheaper for MSI testing at £0.18 per sample, using a commercially available kit.

MLH1 methylation testing was a component of diagnostic strategies two and three, and cost £20.60 and £28.41, respectively, when needed. Methylation testing was cheaper in the context of Strategy two because DNA extraction had already been done for the initial MSI testing. Methylation testing included labor costs at £3.94 (including DNA extraction) or £2.07 (excluding DNA extraction), and equipment costs at £0.23 per sample. Therefore, assuming 30% of endometrial tumors are MSI-H, methylation testing saves £36.95 per patient tested by this strategy because it removes the need for expensive germline NGS by the majority. Incorporating methylation testing in Strategy three (assuming 35% of samples show MMR loss, of which 27% is due to loss of MLH1) reduces the cost of this strategy by £35.21 per patient.

Sensitivity Analysis

One-way sensitivity analysis explored the potential variation in the cost of each diagnostic testing strategy using pessimistic and optimistic values for the probability of a positive test result and need for subsequent testing (see **Supplementary Appendix 2, Table S3**). Depending on the source of data, between 22 to 30% of cases in Strategy one require subsequent germline NGS testing (14, 15). The incorporation of *MLH1* methylation testing in Strategy two reduces this proportion to 5–10% of cases (14–16). For Strategy three, MLH1 loss is observed in 16–27% of cases (15, 17), but ∼93% of these are due to *MLH1* hypermethylation, meaning that just 7% of those women with MLH1 loss by IHC require germline NGS testing for Lynch syndrome (15). Non-MLH1 protein loss by IHC is seen in 6–8% endometrial tumors and all of these require germline NGS testing (**Table S3**).

The range of women who were found to carry a pathogenic variant associated with Lynch syndrome in Strategy 2 is between 1 and 3% of cases (14, 15). For Strategy three, 11% of those with MLH1 loss and no *MLH1* hypermethylation tested by NGS will have a pathogenic variant in *MLH1*, according to current literature (15). Twenty percent of those with MSH2, MSH6, or PMS2 loss by IHC will be found to carry pathogenic variants of *MSH2*, *MSH6,* or *PMS2* (15). Varying the proportions of women requiring subsequent tests in the decision tree, based on pessimistic and optimistic values for the probability of a positive test result in, showed no significant impact on the expected costs for each diagnostic testing strategy.

A scenario analysis explored the impact of the timing of taking consent for Lynch syndrome testing. If consent was only taken at the point of germline NGS testing, the overall cost for Strategy one is cheaper at £75.22 per patient. The overall cost of Strategies two, three, and four would be £36.95, £40.89, and

TABLE 1 | Resource use and unit costs.

Activity	Staff member	Median staff time per sample (hh:mm:ss)°	Source of timing (number of samples)	Staff cost per sample*	Consumable cost per sample (items considered)+	Equipment cost per sample (items considered)^
ALL						
Consent for Lynch syndrome testing	BMA: Consultant Yr 3	00:07:39	Direct observation ($n = 269$)	£5.32		
MICROSATELLITE INSTABILITY						
Preparation of shavings (>20% tumor)	NHS: 5	00:00:06	Direct observation ($n = 130$)	£0.02		
Preparation of shavings (<20% tumor)	NHS: 5	00:00:37	Direct observation ($n = 60$)	£0.14		
DNA extraction#	NHS: 5	00:02:28	Direct observation ($n = 16$)	£0.54		
DNA extraction#	NA	00:00:00	Automated	£0.00		
Clean between sections	NHS: 5	00:01:36	Direct observation ($n = 13$)	£0.35		
Paperwork	NHS: 6	00:03:43	Direct observation ($n = 13$)	£0.97		
Preparation of reagents	NHS: 4	00:01:25	Direct observation ($n = 48$)	£0.26		
0.2ml tubes: label and check	NHS: 4	00:00:42	Direct observation ($n = 48$)	£0.13		
Add reagents and control set up	NHS: 4	00:00:11	Direct observation ($n = 48$)	£0.03		
Spin and place in thermal cycler	NHS: 2	00:00:12	Direct observation ($n = 48$)	£0.03		
PCR cycle 1	NA	00:00:00	Automated	£0.00		
PCR cycle 2	NA	00:00:00	Automated	£0.00		
PCR soak	NA	00:00:00	Direct observation ($n = 16$)	£0.00		
Analysis	NHS: 7	00:01:40	Direct observation ($n = 39$)	£0.52		
Consumables and equipment	–	–	–	–	£19.19 (4)	£0.18 (2)
IMMUNOHISTOCHEMISTRY						
Slide myotome MMR	NHS: 5	00:00:44	Direct observation ($n = 48$)	£0.16		
Labeling	NHS: 5	00:01:20	Direct observation ($n = 48$)	£0.29		
Bake	NHS: 5	00:07:30	Direct observation ($n = 48$)	£1.63		
Loading ultra	NHS: 5	00:00:23	Direct observation ($n = 48$)	£0.08		
Automated IHC MLH1	NA	00:00:00	Automated	£0.00		
Automated IHC MSH2	NA	00:00:00	Automated	£0.00		
Automated IHC MSH6	NA	00:00:00	Automated	£0.00		
Automated IHC PMS2	NA	00:00:00	Automated	£0.00		
Unloading ultra	NHS: 5	00:00:21	Direct observation ($n = 48$)	£0.08		
Wash	NHS: 5	00:00:45	Direct observation ($n = 48$)	£0.16		
Dehydration and clear	NHS: 5	00:00:23	Direct observation ($n = 48$)	£0.09		
Slips	NA	00:00:00	Direct observation ($n = 48$)	£0.00		
Checking (1/3 of slides)	NHS: 5	00:00:02	Direct observation ($n = 48$)	£0.01		
Checking blocks	NHS: 5	00:00:06	Direct observation ($n = 48$)	£0.02		
Scoring slides	BMA: Consultant Yr 3	00:00:58	Direct observation ($n = 48$)	£0.68		
Consumables and equipment	–	–	–	–	£12.23 (8)	£0.43 (1)

(Continued)

TABLE 1 | Continued

Activity	Staff member	Median staff time per sample (hh:mm:ss)°	Source of timing (number of samples)	Staff cost per sample*	Consumable cost per sample (items considered)+	Equipment cost per sample (items considered)^
***MLH1* METHYLATION PYROSEQUENCING**						
DNA extraction#	NHS: 5	00:02:28	Direct observation ($n = 16$)	£0.54		
DNA extraction#	NA	00:00:00	Automated	£0.00		
FFPE bisulphate kit preparation	NHS: 4	00:03:23	Direct observation ($n = 70$)	£0.61		
Lysis of FFPE slice	NHS: 4	00:01:35	Direct observation ($n = 70$)	£0.29		
Bisulphate conversion of DNA	NHS: 4	00:03:06	Direct observation ($n = 70$)	£0.56		
DNA purification and elution	NHS: 4	00:04:05	Direct observation ($n = 70$)	£0.74		
PCR amplification	NHS: 4	00:04:21	Direct observation ($n = 70$)	£0.79		
PCR amplification	NA	00:00:00	Automated	£0.00		
Pyrosequencing	NA	00:00:00	Automated	£0.00		
Data analysis and genotyping	NHS: 6	00:01:34	Direct observation ($n = 42$)	£0.41		
Consumables and equipment	–	–	–	–	£24.25 (11)	£0.23 (3)
NEXT GENERATION PANEL SEQUENCING OF *MLH1*, *MSH2*, and *MSH6*						
DNA extraction#	NHS: 5	00:02:28	Direct observation ($n = 16$)	£0.54		
DNA extraction#	NA	00:00:00	Automated	£0.00		
Long range PCR	NHS: 4	00:02:27	Direct observation ($n = 40$)	£0.44		
SequalPrep (Gel transfer)	NHS: 5	00:01:42	Direct observation ($n = 40$)	£0.37		
SequalPrep (Gel)	NHS: 5	00:02:32	Direct observation ($n = 40$)	£0.55		
SequalPrep (Part 1)	NHS: 5	00:01:35	Direct observation ($n = 40$)	£0.34		
SequalPrep (Part 2)	NHS: 5	00:02:08	Direct observation ($n = 40$)	£0.46		
Pooling of patient plates	NHS: 6	00:06:35	Direct observation ($n = 40$)	£1.72		
Library preparation	NHS: 6	00:01:09	Direct observation ($n = 40$)	£0.30		
Thermal-cycling on PCR machine	NA	00:00:00	Automated	£0.00		
PCR clean-up and Qubit check	NHS: 6	00:01:54	Direct observation ($n = 290$)	£0.50		
Library normalization and library pooling	NHS: 6	00:02:12	Direct observation ($n = 290$)	£0.57		
Sequencing	NA	00:00:00	Automated	£0.00		
First analysis	NHS: 5	00:47:00	Direct observation ($n = 48$)	£10.24		
Repeat analysis and reporting	NHS: 7	01:29:00	Direct observation ($n = 48$)	£27.77		
Authorisation of report	NHS: 8A	00:17:00	Direct observation ($n = 48$)	£6.54		
Consumables and equipment	–	–	–	–	£115.14 (45)	£5.43 (2)

*Staff costs taken from NHS and BMA pay scales (see **Appendix 3 Supplementary Materials**).*

+*Consumables and equipment costs taken from laboratory invoices and manufacturer list prices.*

^*Equipment costs per sample consider estimated equipment life span and percentage use for endometrial cancer lynch syndrome diagnostics*

#*DNA extraction would only needed to be done once per somatic or genomic sample.*

°*Some activities include periods of automation but only hands-on time is recorded.*

£176.24, respectively. Therefore, taking consent only at the point of germline NGS testing saved the Strategy cost by around £4.52 per person, on average (range £3.73–£5.06).

DISCUSSION

This study presents the first comprehensive micro-costing analysis of diagnostic strategies for Lynch syndrome testing in endometrial cancer. In total, four pathways were quantified, reflecting the diagnostic strategies relevant to current clinical practice in the UK. The expected costs were £42.01 or £45.68 per case, respectively if MSI or IHC were used for tumor triage and reflex *MLH1* methylation testing was followed by germline NGS of women with likely Lynch syndrome. Immediate germline NGS for all women with endometrial cancer cost £176.24.

Goverde et al. (18) cites estimates close to our calculated costs for three of the diagnostic strategies (MSI €89, IHC €135, *MLH1* hypermethylation €99). However, the cost of NGS was considerably more expensive than our calculation, at €2152 per test (18). Our findings indicate the cost of Lynch syndrome testing in endometrial cancer is sometimes considerably cheaper than previously described. Published cost effectiveness studies to date may have overestimated the cost of Lynch syndrome testing in clinical practice (18–20). In a published model-based cost-effectiveness analysis of screening for Lynch Syndrome in people with colorectal cancer, the estimated unit cost for IHC was £210 and £202 for MSI and £136 per test for *MLH1* hypermethylation. Using NGS for four MMR gene NGS was estimated to cost between £650 and £860 (21). These unit costs were derived from expert estimates from the UK Genetic Testing Network (21). Three model-based cost-effectiveness analysis of unselected endometrial cancer screening for Lynch syndrome base their analysis on these estimates or on insurance charges (18–20). Two of these published studies concluded that using tumor triage with IHC and reflex *MLH1* methylation testing of MLH1 protein-deficient cancers followed by NGS of women with likely Lynch syndrome (our strategy 3) was cost effective for Lynch syndrome screening, despite using higher costs in their modeling(18, 20). One study that used the highest estimated cost for the diagnostic tests indicated that Lynch syndrome screening was not cost-effective (22). These findings are consistent with the observation of Grosse (23) who indicated that the assumed cost of the diagnostic strategy is a key driver of the relative cost-effectiveness of testing for Lynch syndrome (23).

Ours is the first micro-costing study of Lynch syndrome testing in endometrial cancer. Previous micro-costing studies of genetic-based tests have only measured the costs of unselected sequencing of samples (akin to our strategy 4), without quantifying the impact of using tumor based triage (24, 25). Griffith et al (26) micro-costed two gene (*MLH1*/*MSH2*) mutational screening at £1212.17, but this study pre-dates NGS technology and is therefore no longer relevant to clinical practice (26).

Our study was set within a gynecological oncology center comparable with other centers in the UK. It benefited from prospective recruitment and therefore direct observation of 269 patient consent episodes. All but two of 269 patients agreed to Lynch syndrome testing. This is higher than expected from the literature and may reflect testing within a publically-funded healthcare system rather than one based on health insurance, where a positive test result is likely to impact future insurance premiums (27). Taking informed consent is a variable process as each patient encounter is unique; understanding the uncertainty of the process was possible using the multiple observations. We also used direct observations for all non-automated step in the laboratory testing process. The decision tree was conceptualized by a panel of experts with representation from across the diagnostic pathway, therefore reflecting current clinical practice. The sensitivity analysis drew on multiple high-quality studies sourced through an extensive literature search.

To the best of our knowledge, ours may be the first study to micro-cost NGS testing for any indication. Our micro-costing study indicated that analysis of raw NGS output is time consuming and requires considerable expertise. Indeed, 80% of labor costs, and 25% of the overall costs for NGS, were spent on data analysis. It is departmental policy for all NGS results to be analyzed twice, first by an agenda for change (AFC) band 5 and then a band 7 member of staff. Reports are then authorized by a senior member of staff (band 8a). This is for quality assurance purposes and is in keeping with international recommendations (28).

Another key finding was the impact of the timing of patient consent on the overall costs of testing. Consent is fundamental to germline genetic testing since patients have the absolute right to refuse to be tested (29). However, the point at which consent is sought has a considerable impact on the overall cost of Lynch syndrome testing. Consent taken prior to any testing would add this cost uniformly to all strategies. However, if consent were taken only at the point of germline NGS analysis, only a small proportion of women (5–30%) would need to be consented. Somatic tumor analyses are commonplace in histopathology, for example p53 IHC. Consent is not taken for such tests because they are integral to accurate diagnosis, as well as informing prognosis and treatment planning. Crucially such tests make inferences about cancer biology and not the individual's genome. Such an argument could easily be applied for tumor-based Lynch syndrome testing as such tests merely stratify an individual's risk of having Lynch syndrome and do not diagnose a germline condition. Moving the consent to the point of germline testing would require an additional face-to-face meeting with the patient. This could take place in the context of routine cancer follow-up, therefore mitigating the need for an additional appointment and its associated costs. However, any impact of moving consent to the point of germline testing on uptake and health state utility cannot be ascertained from our data, since all patient consents were taken at recruitment into the study, before any testing was carried out.

Our study is limited by the fact that all observations originate from a single site and therefore may not be generalizable. Their application outside England is difficult to assess given

geographically distinct populations, diverse health systems and variable costs. We have not accounted for failed tests that need to be repeated at additional cost. It was also not possible to analyse capital costs like heating, lighting, and rent. Capital costs are not insignificant, however the confidentiality implicit in private finance initiatives prevented their incorporation in our analysis. Therefore, the true cost of Lynch syndrome testing is likely to lie somewhere between the cost identified by this micro-costing study, which was as thorough as possible but not exhaustive in its pursuit of all applied costs, and the well-sourced expert estimates, which over-estimate costs to ensure the service does not operate at a net loss (30).

A further limitation of our work is that the proportion of positive test results used in our decision tree originated from populations studied outside the UK and therefore may not be fully representative of the local situation. This is of particular concern given that such patients were tested within insurance-based healthcare systems, potentiating a selection bias in which high risk individuals decline testing to avoid would-be increased premiums. This limitation is unavoidable given the complete lack of UK data relating to the prevalence of Lynch syndrome in endometrial cancer patients.

The extrapolation of our data to inform Lynch syndrome testing in colorectal cancer patients is problematic given their use of BRAF V600E as a proxy of *MLH1* promoter hypermethylation in tumor-based triage (31). Furthermore, it is not clear if the proportions used in our decision tree are transferable to a colorectal cancer population. Nonetheless, the costs of IHC, MSI, and NGS testing (as opposed to the cost of testing strategies) are directly transferable and could be used to improve the robustness of model-based cost effectiveness analysis of Lynch syndrome testing in colorectal cancer.

Regarding tumor-based triage, a strategy using MSI analysis is marginally cheaper than one using IHC as the primary test (£42.01 vs. £45.68). A £3.67 saving per tumor would save the NHS over £33,000 annually, were all 9,000 new diagnoses of endometrial cancer tested for Lynch syndrome. However, the choice of tumor triage is complex and beyond a simple cost comparison; this is especially the case when the cost difference is marginal. Both methods have equitable sensitivity and specificity according to the literature (11). IHC has the advantage of identifying the likely mutated gene, which can aid the subsequent interpretation of sequencing data (32). IHC can be performed in most histopathology departments, whereas MSI requires specialist laboratories. IHC is thought to be more sensitive in the case of those carrying a *MSH6* pathogenic variant, where tumors may not be MSI-H (33). However, interpretation of MMR expression patterns requires consultant pathology expertise, and only MSI can identify missense pathogenic variants in MMR genes whereby the protein is expressed but is not functional (34). The choice of tumor triage depends on the availability of local services, expertise, and infrastructure; these data do not infer that any one strategy is clinically superior to another for the diagnosis of Lynch syndrome.

The importance of our work is its ability to inform healthcare policy. There is a growing call for screening all endometrial cancer patients for Lynch syndrome (12, 35). A potential barrier to the transition from expert opinion to clinical application is cost. To date there have been no micro-costing data available to inform policy makers as to the actual costs of Lynch syndrome testing in the UK. As outlined above, the current estimated costs might be prohibitively high and thus impede the implementation of Lynch syndrome testing in endometrial cancer. Our data should prompt healthcare providers to look at this again.

CONCLUSION

We present a micro-costing study for Lynch syndrome testing in unselected endometrial cancer patients from a large tertiary referral center in the North West of England. The use of tumor triage with MSI and reflex *MLH1* methylation testing is the cheapest strategy at £42.01 per case. Substituting MSI for IHC as the initial tumor-based triage increases costs marginally to £45.68. Moving the point of consent for Lynch syndrome testing to just before germline testing reduces the costs of those strategies that incorporate tumor triage. NGS panel testing is considerably cheaper than current estimates at £176.24 per test. The next phase is to use these estimates in model-based cost-effectiveness analysis to understand the relative value of a national Lynch syndrome-testing programme for endometrial cancer.

AUTHOR CONTRIBUTIONS

NR recruited to the study and collected data. NR and ND analyzed data and wrote the first draft of the manuscript. AC collected data. EC and KP designed the study, supervised study execution and contributed to data interpretation. DE provided study oversight. EC was Chief Investigator and study guarantor. All authors provided critical comment, edited the manuscript, and approved its final version.

FUNDING

NR is a Doctoral Medical Research Council (MRC) Research Fellow (MR/M018431/1), DE an National Institute for Health Research (NIHR) Senior Investigator (NF-SI-0513-10076), EC an (NIHR) Clinician Scientist (NIHR-CS-012-009), and their work is supported through the NIHR Manchester Biomedical Research Centre (IS-BRC-1215-20007). This article presents independent research funded by the NIHR and MRC. The views expressed are those of the authors and not necessarily those of the MRC, NHS, NIHR, or the Department of Health.

ACKNOWLEDGMENTS

We are very grateful to all the patients and clinical staff who kindly agreed to take part in this study. Special thanks goes to Dr. Andrew Wallace, Ms. Shona Esquibel, Mrs. Catherine Higgins,

Ms. Sancha Bunstone, Dr. Simon Tobi, Ms. Emma Hobson, and Ms. Megan Adaway who provided access to their laboratories and allowed us to time their activities. We would like to thank Dr. James Bolton, Dr. Rhona McVey, Dr. Ian Frayling, Prof Richard Edmondson, and Dr. Fiona Lalloo for their expert advice.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00061/full#supplementary-material

REFERENCES

1. Lynch HT, Lynch PM, Lanspa SJ, Snyder CL, Lynch JF, Boland CR. Review of the lynch syndrome: history, molecular genetics, screening, differential diagnosis, and medicolegal ramifications. *Clin Genet*. (2009) 76:1–18. doi: 10.1111/j.1399-0004.2009.01230.x
2. Hampel H, la Chapelle de A. The search for unaffected individuals with lynch syndrome: do the ends justify the means? *Cancer Prev Res*. (2011) 4:1–5. doi: 10.1158/1940-6207.CAPR-10-0345
3. Barrow E, Hill J, Evans DG. Cancer risk in lynch syndrome. *Fam cancer* (2013) 12:229–40. doi: 10.1007/s10689-013-9615-1
4. Lu KH, Dinh M, Kohlmann W, Watson P, Green J, Syngal S, et al. Gynecologic cancer as a "Sentinel cancer" for women with hereditary nonpolyposis colorectal cancer syndrome. *Obstet Gynecol*. (2005) 105:569–74. doi: 10.1097/01.AOG.0000154885.44002.ae
5. Moller P, Seppälä T, Bernstein I, Holinski-Feder E, Sala P, Evans DG, et al. Cancer incidence and survival in lynch syndrome patients receiving colonoscopic and gynaecological surveillance: first report from the prospective lynch syndrome database. *Gut* (2017) 66:464–72. doi: 10.1136/gutjnl-2015-309675
6. Hampel H. Genetic counseling and cascade genetic testing in lynch syndrome. *Fam Cancer* (2016) 15:423–7. doi: 10.1007/s10689-016-9893-5
7. Schmeler KM, Lynch HT, Chen L-M, Munsell MF, Soliman PT, Clark MB, et al. Prophylactic surgery to reduce the risk of gynecologic cancers in the lynch syndrome. *N Engl J Med*. (2006) 354:261–9. doi: 10.1056/NEJMoa052627
8. Broaddus RR, Lynch HT, Chen L-M, Daniels MS, Conrad P, Munsell MF, et al. Pathologic features of endometrial carcinoma associated with HNPCC. *Cancer* (2006) 106:87–94. doi: 10.1002/cncr.21560
9. Esteller M, Levine R, Baylin S, Ellenson L, Herman J. MLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. *Oncogene* (1998) 17:2413–7. doi: 10.1038/sj.onc.1202178
10. The National Institute for Health and Care Excellence. Molecular testing strategies for lynch syndrome in people with colorectal cancer. *Diagnostic Guideline* (2017) 27:1–37. Available online at: https://www.nice.org.uk/guidance/dg27
11. Snowsill T, Huxley N, Hoyle M, Jones-Hughes T, Coelho H, Cooper C, et al. A systematic review and economic evaluation of diagnostic strategies for lynch syndrome. *Health Technol Assess*. (2014) 18:1–406. doi: 10.3310/hta18580
12. Vasen HFA, Blanco I, Aktan-Collan K, Gopie JP, Alonso A, Aretz S, et al. Revised guidelines for the clinical management of lynch syndrome (HNPCC): recommendations by a group of european experts. *Gut* (2013) 62:812–23. doi: 10.1136/gutjnl-2012-304356
13. Tafe LJ. Targeted next-generation sequencing for hereditary cancer syndromes: a focus on lynch syndrome and associated endometrial cancer. *J Mol Diagn*. (2015) 17:472–82. doi: 10.1016/j.jmoldx.2015.06.001
14. Hampel H, Frankel W, Panescu J, Lockman J, Sotamaa K, Fix D, et al. Screening for lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. *Cancer Res*. (2006) 66:7810–7. doi: 10.1158/0008-5472.CAN-06-1114
15. Goodfellow PJ, Billingsley CC, Lankes HA, Ali S, Cohn DE, Broaddus RJ, et al. Combined microsatellite instability, MLH1 methylation analysis, and immunohistochemistry for lynch syndrome screening in endometrial cancers from GOG210: an NRG oncology and gynecologic oncology group study. *J Clin Oncol*. (2015) 33:4301–8. doi: 10.1200/JCO.2015.63.9518
16. Zauber NP, Denehy TR, Taylor RR, Ongcapin EH, Marotta SP, Sabbath-Solitare M, et al. Microsatellite instability and DNA methylation of endometrial tumors and clinical features in young women compared with older women. *Int J Gynecol Cancer*. (2010) 20:1549–56.
17. Joehlin-Price AS, Perrino CM, Stephens J, Backes FJ, Goodfellow PJ, Cohn DE, et al. Mismatch repair protein expression in 1049 endometrial carcinomas, associations with body mass index, and other clinicopathologic variables. *Gynecol Oncol*. (2014) 133:43–7. doi: 10.1016/j.ygyno.2014.01.017
18. Goverde A, Spaander MC, van Doorn HC, Dubbink HJ, van den Ouweland AM, Tops CM, et al. Cost-effectiveness of routine screening for lynch syndrome in endometrial cancer patients up to 70years of age. *Gynecol Oncol*. (2016) 143:453–9. doi: 10.1016/j.ygyno.2016.10.008
19. Kwon JS, Scott JL, Gilks CB, Daniels MS, Sun CC, Lu KH. Testing women with endometrial cancer to detect lynch syndrome. *J Clin Oncol*. (2011) 29:2247–52. doi: 10.1200/JCO.2010.32.9979
20. Resnick K, Straughn JM, Backes F, Hampel H, Matthews KS, Cohn DE. Lynch syndrome screening strategies among newly diagnosed endometrial cancer patients. *Obstet Gynecol*. (2009) 114:530–6. doi: 10.1097/AOG.0b013e3181b11ecc
21. Snowsill T, Coelho H, Huxley N, Jones-Hughes T, Briscoe S, Frayling IM, et al. Molecular testing for lynch syndrome in people with colorectal cancer: systematic reviews and economic evaluation. *Health Technol Assess*. (2017) 21:1–238. doi: 10.3310/hta21510
22. Kwon JS, Sun CC, Peterson SK, White KG, Daniels MS, Boyd-Rogers SG, et al. Cost-effectiveness analysis of prevention strategies for gynecologic cancers in lynch syndrome. *Cancer* (2008) 113:326–35. doi: 10.1002/cncr.23554
23. Grosse SD. When is genomic testing cost-effective? testing for lynch syndrome in patients with newly-diagnosed colorectal cancer and their relatives. *Healthcare* (2015) 3:860–78. doi: 10.3390/healthcare3040860
24. Sabatini LM, Mathews C, Ptak D, Doshi S, Tynan K, Hegde MR, et al. Genomic sequencing procedure microcosting analysis and health economic cost-impact analysis: a report of the association for molecular pathology. *J Mol Diagn*. (2016) 18:319–28. doi: 10.1016/j.jmoldx.2015.11.010
25. Costa S, Regier DA, Meissner B, Cromwell I, Ben-Neriah S, Chavez E, et al. A time-and-motion approach to micro-costing of high-throughput genomic assays. *Curr Oncol*. (2016) 23:304–13. doi: 10.3747/co.23.2987
26. Griffith GL, Tudor-Edwards R, Gray J, Butler R, Wilkinson C, Turner J, et al. A micro costing of NHS cancer genetic services. *Br J Cancer* (2005) 92:60–71. doi: 10.1038/sj.bjc.6602270
27. Batte BAL, Bruegl AS, Daniels MS, Ring KL, Dempsey KM, Djordjevic B, et al. Consequences of universal MSI/IHC in screening endometrial cancer patients for lynch syndrome. *Gynecol Oncol*. (2014) 134:319–25. doi: 10.1016/j.ygyno.2014.06.009
28. Mueller CR, Elles RG. Quality issues in molecular genetic testing. In: Kristoffersson U, Schmidtke J, Cassiman JJ, editors. *Quality Issues in Clinical Genetic Services*. Dordrecht: Springer (2010), p. 297–303. doi: 10.1007/978-90-481-3919-4_30
29. Ayuso C, Millán JM, Mancheño M, Dal-Ré R. Informed consent for whole-genome sequencing studies in the clinical setting. Proposed recommendations on essential content and process. *Eur J Hum Genet*. (2013) 21:1054–9. doi: 10.1038/ejhg.2012.297
30. Adams CP, Brantner VV. Estimating the cost of new drug development: is it really 802 million dollars? *Health Aff*. (2006) 25:420–8. doi: 10.1377/hlthaff.25.2.420
31. Parsons MT, Buchanan DD, Thompson B, Young JP, Spurdle AB. Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumour features for MMR variant

classification. *J Med Genet*. (2012) 49:151–7. doi: 10.1136/jmedgenet-2011-100714
32. Rasmussen LJ, Heinen CD, Royer-Pokora B, Drost M, Tavtigian S, Hofstra RM, et al. Pathological assessment of mismatch repair gene variants in lynch syndrome: past, present, and future. *Hum Mutat*. (2012) 33:1617–25. doi: 10.1002/humu.22168
33. Wu Y, Berends MJ, Mensink RG, Kempinga C, Sijmons RH, van Der Zee AG, et al. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. *Am J Hum Genet*. (1999) 65:1291–8. doi: 10.1086/302612
34. Shia J, Holck S, Depetris G, Greenson JK, Klimstra DS. Lynch syndrome-associated neoplasms: a discussion on histopathology and immunohistochemistry. *Fam Cancer* (2013) 12:241–60. doi: 10.1007/s10689-013-9612-4
35. Committee on Practice Bulletins-Gynecology, Society of Gynecologic Oncology. ACOG practice bulletin No. 147: lynch syndrome. *Obstetr gynecol*. (2014) 124:1042–54. doi: 10.1097/01.AOG.0000456325.50739.72

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 27 March 2019
doi: 10.3389/fonc.2019.00182

Assessing the Role of Selenium in Endometrial Cancer Risk: A Mendelian Randomization Study

Pik Fang Kho[1], Dylan M. Glubb[1], Deborah J. Thompson[2], Amanda B. Spurdle[1] and Tracy A. O'Mara[1] on Behalf of the Endometrial Cancer Association Consortium[†]*

[1] *Molecular Cancer Epidemiology Group, Genetics and Computational Biology Department, QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia,* [2] *Department of Public Health and Primary Care, Centre for Cancer Genetic Epidemiology, University of Cambridge, Cambridge, United Kingdom*

Endometrial cancer is the most commonly diagnosed gynecological cancer in developed countries. Based on evidence from observational studies which suggest selenium inhibits the development of several cancers (including lung and prostate cancer), selenium supplementation has been touted as a potential cancer preventative agent. However, randomized controlled trials have not reported benefit for selenium supplementation in reducing cancer risk. For endometrial cancer, limited observational studies have been conducted assessing whether selenium intake, or blood selenium levels, associated with reduced risk, and no randomized controlled trials have been conducted. We performed a two-sample Mendelian randomization analysis to examine the relationship between selenium levels (using a composite measure of blood and toenail selenium) and endometrial cancer risk, using summary statistics for four genetic variants associated with selenium levels at genome-wide significance levels ($P < 5 \times 10^{-8}$), from a study of 12,906 endometrial cancer cases and 108,979 controls, all of European ancestry. Inverse variance weighted (IVW) analysis indicated no evidence of a causal role for selenium levels in endometrial cancer development (OR per unit increase in selenium levels Z-score = 0.99, 95% CI = 0.87–1.14). Similar results were observed for sensitivity analyses robust to the presence of unknown pleiotropy (OR per unit increase in selenium levels Z-score = 0.98, 95% CI 0.89–1.08 for weighted median; OR per unit increase in selenium levels Z-score = 0.90, 95% CI = 0.53–1.50 for MR-Egger). In conclusion, these results do not support the use of selenium supplementation to prevent endometrial cancer.

Keywords: Mendelian randomization, endometrial cancer, toenail selenium, circulating selenium, genome-wide association study

OPEN ACCESS

Edited by:
Massimo Nabissi,
University of Camerino, Italy

Reviewed by:
James Yarmolinsky,
University of Bristol, United Kingdom
Carolina Bonilla,
University of São Paulo, Brazil

****Correspondence:***
Tracy A. O'Mara
tracy.omara@qimrberghofer.edu.au

[†]Full authorship details for Endometrial Cancer Association Consortium are provided in the Supplementary Note

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *28 November 2018*
Accepted: *04 March 2019*
Published: *27 March 2019*

Citation:
Kho PF, Glubb DM, Thompson DJ, Spurdle AB and O'Mara TA (2019) Assessing the Role of Selenium in Endometrial Cancer Risk: A Mendelian Randomization Study. Front. Oncol. 9:182. doi: 10.3389/fonc.2019.00182

INTRODUCTION

Endometrial cancer is the most commonly diagnosed cancer of the female reproductive system in developed countries (1). Unlike breast and cervical cancers where a screening program is available to the general population, there is currently no available screening test for endometrial cancer and diagnosis relies on biopsy in symptomatic patients (2). Furthermore, the incidence of endometrial cancer is rising (3), highlighting the need for preventative measures. Selenium has received considerable attention as a possible cancer preventive agent [reviewed in (4)]. While randomized controlled trials have shown no benefit for selenium supplementation in reducing cancer risk over a period of up to 8 years (5), some observational longitudinal studies assessing

selenium intake or selenium levels, over a period up to 25 years, have shown an inverse association between selenium and cancer risk [reviewed in (4)]. Thus, although findings from the longitudinal studies have been inconsistent (4), they may provide insight into the longer term effects of selenium exposure. A recent meta-analysis examining the association between selenium intake (dietary and supplemental) and overall cancer risk, has suggested that there was a reduction in cancer incidence among people consuming more than the recommended daily allowance of selenium (55 μg/day; RR = 0.96, 95% CI = 0.92–0.99) (6).

Very few studies have assessed the effects of selenium on endometrial cancer. In terms of cellular studies, it has been shown that a selenium metabolite can inhibit endometrial cancer cell proliferation, potentially through disruption of estrogen signaling (7). Findings from human studies, however, have been more equivocal. A population-based, case-control observational study of 417 endometrial cancer cases and 395 controls specifically assessed the role of dietary and supplemental selenium intake (as measured by questionnaire in the 6 months prior to diagnosis or enrolment as a control) in endometrial cancer development (8). In a comparison of the highest (≥103.2 μg) and lowest (<72.4 μg) selenium quartiles, this study did not support an association between selenium intake and endometrial cancer risk (OR = 0.74, 95% CI = 0.47–1.17) (8). Two small case-control studies ($n < 100$) have assessed serum selenium levels in endometrial cancer cases and controls. Sundstrom et al. (9) reported lower blood selenium levels in 64 cases as compared to 61 non-cancer controls, with an average of 1.01 ± 0.05 v 1.40 ± 0.08 μmol/L blood selenium in cases and controls, respectively ($P < 0.001$). A subsequent study of 35 endometrial cancer cases and 32 non-cancer controls reported a similar finding (average of 1.14 ± 0.04 vs. 1.26 ± 0.03 μmol/L blood selenium in cases and controls, respectively, $P < 0.01$) (10). Inconsistent results from these observational studies may be due to small sample sizes (8–10), reverse causation bias (9, 10), recall bias and measurement error in the dietary assessment (8). No prospective studies have examined the association of pre-diagnostic selenium levels with endometrial cancer risk. Thus, the role of selenium in endometrial cancer development remains inconclusive.

As no intervention study has yet been performed to explore the role of selenium in endometrial cancer risk, we employed a two-sample Mendelian randomization approach which uses germline genetic variants associated with selenium levels to proxy for selenium exposure (11). These germline genetic variants are largely independent from environment or lifestyle factors, and are established prior to disease onset, thus analyses using these genetic variants as instrumental variables are less susceptible to biases from confounding and reverse causation. Further, genetic effects on exposure of interest are lifelong, and hence it is comparable to a lifelong randomized controlled trial.

MATERIALS AND METHODS

Summary statistics for 12 genetic variants associated with selenium levels at genome-wide significance ($P < 5 \times 10^{-8}$) were extracted from a genome-wide association study (GWAS) meta-analysis of circulating selenium levels [$n = 5{,}477$; (12)] and toenail selenium levels [$n = 4{,}162$; (13)] in European-ancestry individuals. These variants were at two separate genetic loci; 5q14 (9 variants) and 21q22 (3 variants). To analyze the effect of selenium exposure on endometrial cancer risk, we used summary statistics from the Endometrial Cancer Association Consortium (ECAC) GWAS of 12,906 endometrial cancer cases and 108,979 controls of European descent (14). One of the 5q14 selenium-associated genetic variants, rs558133, was excluded because it was not assessed by the ECAC GWAS (it does not appear on the 1,000 Genomes v3 reference panel) and no proxy with $r^2 > 0.8$ could be found. These potential instrumental variables were pruned for linkage disequilibrium (LD; $r^2 < 0.05$) and four selenium-associated genetic variants (two independent variants per locus) remained as instrumental variables. We used PhenoScanner v2 (15) to explore the possibility of horizontal pleiotropy among the instrumental variables and their highly correlated variants ($r^2 > 0.8$). Specifically, we examined traits associated with known risk factors of endometrial cancer (i.e., body mass index, age at menarche, age at menopause, postmenopausal serum estradiol levels, nulliparity, infertility, and insulin levels) in the published literature at $P < 7.14 \times 10^{-3}$ (i.e., 0.05/number of known risk factors explored, $n = 7$); none of these instrumental variables were associated with these traits.

The reported effect for circulating and toenail selenium instrumental variables was expressed in Z-score units per effect allele. For the purpose of Mendelian randomization analysis, Z-scores were converted to beta and standard error values using the following equations, as per Taylor et al. (16), where N is the sample size, eaf is the effect allele frequency, and SE is the standard error of converted beta:

$$Beta = \frac{Z - score}{\sqrt{N}} \times \frac{1}{\sqrt{eaf\left(1 - eaf\right)}}$$

$$SE = \frac{Beta}{Z - score}$$

Converted selenium level summary statistics for these instrumental variables and their association with endometrial cancer risk are shown in **Table 1**. Because summary statistics were expressed in Z-scores, neither the converted beta values for associations of genetic variants with selenium levels nor the effect sizes from the Mendelian randomization analysis have interpretable units, however they do provide the direction and statistical strength of associations.

Individual Wald-type ratios for each of the instrumental variables were determined as a ratio of instrumental variable-endometrial cancer regression over the instrumental variable-selenium levels regression (17). Individual Wald-type ratios were meta-analyzed using the inverse variance weighted (IVW) approach. A random effect model was used to account for heterogeneity. The IVW approach assumes that instrumental variables do not exhibit horizontal pleiotropy (where a single genetic variant has simultaneous effects on other phenotypes that affect the outcome independently of the exposure of interest) or, if this is violated, that the horizontal pleiotropy is "balanced" across all instrumental variables. Thus, we implemented sensitivity analyses that are more robust to pleiotropy when it is "unbalanced" (i.e., exhibiting directional pleiotropy): (i) weighted

TABLE 1 | Genetic associations with selenium levels and endometrial cancer risk.

Instrumental variables	Chr:Pos*	$R^{2\dagger}$	EA	OA	EAF_{Se}	*Z*-score	$Beta_{Se}$	SE_{Se}	P_{Se}	EAF_{EC}	$Beta_{EC}$	SE_{EC}	P_{EC}
rs1789953	chr21:44482936	0.04	T	C	0.14	5.52	0.16	0.03	3.4×10^{-8}	0.13	−0.04	0.02	0.12
rs6586282	chr21:44478497		T	C	0.17	−5.89	−0.16	0.03	3.96×10^{-9}	0.17	−0.04	0.02	0.04
rs6859667	chr5:78745042	0.03	T	C	0.96	−6.92	−0.36	0.05	4.4×10^{-12}	0.96	0.02	0.04	0.54
rs921943	chr5:78316476		T	C	0.29	13.14	0.29	0.02	1.9×10^{-39}	0.29	0.00	0.02	0.90

**from hg19; † pairwise LD in Europeans (1000 Genomes) provided for instrumental variables at the same locus; Se, Selenium; EC, Endometrial cancer; EA, Effect allele; OA, Other allele; EAF, Effect allele frequency from each GWAS; Beta, effect size; SE, Standard error; P, P-value. $Beta_{EC}$ and SE_{EC} are the natural log odds ratio of endometrial cancer risk and associated standard error, respectively. Estimates for Selenium levels have been taken from (13) and estimates for EC from (14).*

TABLE 2 | F statistics and Individual Wald-type ratios for all instrumental variables.

Instrumental variables	F statistic	$Beta_{Se\text{-}EC}$	$SE_{Se\text{-}EC}$	$P_{Se\text{-}EC}$
rs1789953	34.07	−0.22	0.14	0.12
rs6586282	36.88	0.26	0.13	0.04
rs6859667	19.24	−0.07	0.11	0.54
rs921943	44.55	−0.01	0.06	0.89

Se, Selenium; EC, Endometrial cancer; Beta, effect size in standard deviation unit; SE, Standard error; P, P value.

median analysis, which provides valid causal estimate even when up to 50% of the weight comes from instrumental variables with horizontal pleiotropic effects (18); and (ii) random effect MR-Egger analysis, which provides valid pleiotropy-corrected causal estimates even if all instrumental variables are invalid (19). MR-Egger analysis corrects for the directional pleiotropy by introducing an intercept which captures the average pleiotropic effects of all included variants on the outcome. An exponentiated MR-Egger intercept that deviates from 1 is an indicator of directional pleiotropy. It should also be noted that the validity of IVW and MR-Egger regression estimates rely on satisfaction of the InSIDE (instrument strength independent of direct effect) assumption where the instrument strength does not correlate with the horizontal pleiotropic effects on the outcome (19).

To assess the strength of the instruments, F statistics and the proportion of variance (R^2) in circulating and toenail selenium explained by instrumental variables were calculated as per Rees et al. (20) and Yarmolinsky et al. (21). We used the I^2_{GX} (22) statistic to assess weak instrument bias for MR-Egger analysis using the "MendelianRandomization" package in R (23). This statistic quantifies the regression dilution bias due to violation of the NO Measurement Error (NOME; genetic associations with exposure of interest are measured without error) assumption. An I^2_{GX} statistic approaching 1 indicates that violation of the NOME assumption does not substantially dilute the effect estimates of MR-Egger analysis toward a null association. Unless otherwise stated, Mendelian randomization analyses were performed using the "TwoSampleMR" package in R (24).

RESULTS

The combined multi-allelic instrument explained 2.9% of the variation in circulating and toenail selenium levels. Individual Wald-type ratios and F statistics for instrumental variables are presented in **Table 2**. F statistics for these instrumental variables were all >10 (range 19.24–44.55) indicating instruments were unlikely to suffer from weak instrument bias. Mendelian randomization analysis did not support an association between selenium levels and endometrial cancer risk using the IVW method (OR per unit increase in selenium levels Z-score = 0.99, 95% CI = 0.87–1.14, $P = 0.93$). We found limited evidence for heterogeneity amongst the individual causal estimates for the included variants by Cochran's Q statistic (25) (Cochrain's Q statistics = 7.22, $P = 0.07$). The exponentiated intercept of MR-Egger regression was 1.03 (95% CI = 0.91–1.16, $P = 0.72$) and therefore provided no evidence of directional pleiotropy across the multi-allelic instrument. Further, the I^2_{GX} statistic, quantifying weak instrument bias in the context of MR-Egger, was minimal (I^2_{GX}= 92%). This suggests that any potential bias toward a null association as a result of NOME violation is ≤8%. Association estimates from sensitivity analyses (MR-Egger regression and weighted median analysis) were consistent with that reported by IVW analysis (OR per unit increase in selenium levels Z-score = 0.90, 95% CI = 0.53–1.50, $P = 0.72$ for MR-Egger; OR per unit increase in selenium levels Z-score = 0.98, 95% CI = 0.89–1.08, $P = 0.70$ for weighted median).

DISCUSSION

To our knowledge, this is the first Mendelian randomization study evaluating the effect of selenium on endometrial cancer. This analysis does not support a causal relationship between selenium levels and endometrial cancer risk. However, given the fact that the combined multi-allelic instrument explains a small amount of the variance in circulating and toenail selenium levels (<3%), the power to detect a causal association in Mendelian randomization analysis may be limited and thus, we cannot rule out the possibility that genetically predicted selenium levels have some effect on endometrial cancer risk. This analysis should be revisited when more genome-wide significant selenium variants are identified from future, larger GWAS studies. Further, statistical power for Mendelian randomization analyses may also be increased through the use of more precise effect estimates from larger GWAS of endometrial cancer.

The validity of Mendelian randomization analysis holds under the condition that three important assumptions are fulfilled.

These assumptions require that genetic variants chosen as instrumental variables are:

1. Strongly associated with the exposure of interest
2. Not associated with any confounder(s) that affects the relationship between the exposure of interest and outcome
3. Not associated with outcome, independent of the exposure (i.e., no horizontal pleiotropy).

Our instrumental variables have high F-statistics (>10), thus fulfilling assumption 1. Assumptions 2 and 3 are difficult to validate. We have attempted to minimize violation of assumption 2 by scanning associations of instrumental variables from the literature, finding none of the instrumental variables to be associated with known endometrial cancer risk factors. However, we are limited in exploring this assumption by the GWAS that have been conducted for these risk factors, and we cannot discount the possibility that associations between these variants and unknown endometrial cancer risk factors may exist. Sensitivity testing (by MR-Egger regression and weighted median analysis) has been used to address assumption 3 and we have not found evidence that this assumption has been violated. However, given the limitations of these tests (e.g., the low statistical power of the MR-Egger intercept test, discussed below), we cannot rule out this possibility.

The strengths of our study include incorporation of multiple selenium level-associated genetic variants as a multi-allelic instrument to maximize the variation in selenium levels explained; and use of the largest available GWAS datasets to provide the greatest statistical power possible. Limitations of this study include use of instrumental variables from mixed gender GWAS which were assessed in female-only endometrial cancer GWAS. Although both selenium GWASs controlled for the effect of sex, we cannot not exclude the possibility that there is a residual effect of this covariate which may violate the assumption that instrumental variables are strongly associated with the exposure. A potential limitation of two-sample Mendelian randomization is that by using two different GWAS sample sets to obtain the instrumental variable-exposure and -outcome effect, population stratification may have confounded the observed associations despite all populations being of European descent. Weaknesses of the MR-Egger regression sensitivity analysis performed in our study include its relatively lower statistical power as compared to the IVW and weighted median analysis methods, and its vulnerability to weak instrument bias which may bias MR-Egger regression toward the null (19). However, we assessed the extent to which weak instrument bias may have affected our MR-Egger results using the I^2_{GX} statistic, and found it to be negligible.

The identification of preventative agents for cancer is an attractive avenue of research because unlike other approaches for disease prevention, such as lifestyle changes, taking a dietary supplement (e.g., selenium) should be considerably easier to implement. Candidate dietary supplements can be identified by observational studies; however, moving these candidates through to human use requires the establishment of expensive randomized controlled trials. For example, a recent prostate cancer prevention trial, examining the benefit of selenium and/or vitamin E supplement on cancer risk, failed because of adverse effects and lack of efficacy, at a cost of >US$110 million (26, 27); whereas, a subsequent Mendelian randomization study was able to recapitulate the results of this trial using publicly available GWAS data (21).

In conclusion, Mendelian randomization analysis provided no support for selenium supplementation in the prevention of endometrial cancer. More generally, these findings further highlight the value of Mendelian randomization for rapidly excluding proposed interventions that are unlikely to be successful, prior to the initiation of expensive and lengthy trials. This approach could allow resources to be targeted toward trials of alternative interventions with more promising genetic evidence.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because Data are available from the authors of the original papers on request. Requests to access the datasets should be directed to Tracy O'Mara, tracy.omara@qimrberghofer.edu.au.

AUTHOR CONTRIBUTIONS

PK, DG, DT, AS, and TO conception or design of the work; the acquisition, analysis and interpretation of data for the work, drafting the manuscript, and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors were involved in revision of the manuscript and provide final approval of the version to be published.

FUNDING

This work was supported by a National Health and Medical Research Council (NHMRC) Project Grant (APP1109286). PK is supported by an Australian Government Research Training Program Ph.D. Scholarship and QIMR Berghofer Postgraduate Top-Up Scholarship, TO is supported by an NHMRC Early Career Fellowship (APP1111246), AS is supported by an NHMRC Senior Research Fellowship (APP1061779).

ACKNOWLEDGMENTS

Full funding and acknowledgments for ECAC can be found in the **Supplementary Note**.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00182/full#supplementary-material

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. (2015) 136:E359–86. doi: 10.1002/ijc.29210
2. Braun MM, Overbeek-Wager EA, Grumbo RJ. Diagnosis and management of endometrial cancer. *Am Fam Physician*. (2016) 93:468–74.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. (2018) 68:394–424. doi: 10.3322/caac.21492
4. Vinceti M, Filippini T, Del Giovane C, Dennert G, Zwahlen M, Brinkman M, et al. Selenium for preventing cancer. *Cochrane Database Syst Rev*. (2018) 1:CD005195. doi: 10.1002/14651858.CD005195.pub4
5. Reid ME, Duffield-Lillico AJ, Garland L, Turnbull BW, Clark LC, Marshall JR. Selenium supplementation and lung cancer incidence: an update of the nutritional prevention of cancer trial. *Cancer Epidemiol Biomarkers Prev*. (2002) 11:1285–91.
6. Kuria A, Fang X, Li M, Han H, He J, Aaseth JO, et al. Does dietary intake of selenium protect against cancer? A systematic review and meta-analysis of population-based prospective studies. *Crit Rev Food Sci Nutr*. (2018). doi: 10.1080/10408398.2018.1548427. [Epub ahead of print].
7. Shah YM, Al-Dhaheri M, Dong Y, Ip C, Jones FE, Rowan BG. Selenium disrupts estrogen receptor (alpha) signaling and potentiates tamoxifen antagonism in endometrial cancer cells and tamoxifen-resistant breast cancer cells. *Mol Cancer Ther*. (2005) 4:1239–49. doi: 10.1158/1535-7163.MCT-05-0046
8. Gifkins D, Olson SH, Demissie K, Lu SE, Kong AN, Bandera EV. Total and individual antioxidant intake and endometrial cancer risk: results from a population-based case-control study in New Jersey. *Cancer Causes Control*. (2012) 23:887–95. doi: 10.1007/s10552-012-9958-1
9. Sundstrom H, Yrjanheikki E, Kauppila A. Low serum selenium concentration in patients with cervical or endometrial cancer. *Int J Gynaecol Obstet*. (1984) 22:35–40. doi: 10.1016/0020-7292(84)90101-2
10. Sundstrom H, Ylikorkala O, Kauppila A. Serum selenium and thromboxane in patients with gynaecological cancer. *Carcinogenesis*. (1986) 7:1051–2. doi: 10.1093/carcin/7.7.1051
11. Burgess S, Scott RA, Timpson NJ, Davey Smith G, Thompson SG, Consortium E-I. Using published data in Mendelian randomization: a blueprint for efficient identification of causal risk factors. *Eur J Epidemiol*. (2015) 30:543–52. doi: 10.1007/s10654-015-0011-z
12. Evans DM, Zhu G, Dy V, Heath AC, Madden PA, Kemp JP, et al. Genome-wide association study identifies loci affecting blood copper, selenium and zinc. *Hum Mol Genet*. (2013) 22:3998–4006. doi: 10.1093/hmg/ddt239
13. Cornelis MC, Fornage M, Foy M, Xun P, Gladyshev VN, Morris S, et al. Genome-wide association study of selenium concentrations. *Hum Mol Genet*. (2015) 24:1469–77. doi: 10.1093/hmg/ddu546
14. O'Mara TA, Glubb DM, Amant F, Annibali D, Ashton K, Attia J, et al. Identification of nine new susceptibility loci for endometrial cancer. *Nat Commun*. (2018) 9:3166. doi: 10.1038/s41467-018-05427-7
15. Staley JR, Blackshaw J, Kamat MA, Ellis S, Surendran P, Sun BB, et al. PhenoScanner: a database of human genotype-phenotype associations. *Bioinformatics*. (2016) 32:3207–9. doi: 10.1093/bioinformatics/btw373
16. Taylor AE, Burgess S, Ware JJ, Gage SH, Richards JB, Davey Smith G, et al. Investigating causality in the association between 25(OH)D and schizophrenia. *Sci Rep*. (2016) 6:26496. doi: 10.1038/srep26496
17. Lawlor DA, Harbord RM, Sterne JA, Timpson N, Davey Smith G. Mendelian randomization: using genes as instruments for making causal inferences in epidemiology. *Stat Med*. (2008) 27:1133–63. doi: 10.1002/sim.3034
18. Bowden J, Davey Smith G, Haycock PC, Burgess S. Consistent estimation in mendelian randomization with some invalid instruments using a weighted median estimator. *Genet Epidemiol*. (2016) 40:304–14. doi: 10.1002/gepi.21965
19. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int J Epidemiol*. (2015) 44:512–25. doi: 10.1093/ije/dyv080
20. Rees JMB, Wood AM, Burgess S. Extending the MR-Egger method for multivariable Mendelian randomization to correct for both measured and unmeasured pleiotropy. *Stat Med*. (2017) 36:4705–18. doi: 10.1002/sim.7492
21. Yarmolinsky J, Bonilla C, Haycock PC, Langdon RJQ, Lotta LA, Langenberg C, et al. Circulating selenium and prostate cancer risk: a mendelian randomization analysis. *J Natl Cancer Inst*. (2018) 110:1035–8. doi: 10.1093/jnci/djy081
22. Bowden J, Del Greco MF, Minelli C, Davey Smith G, Sheehan NA, Thompson JR. Assessing the suitability of summary data for two-sample Mendelian randomization analyses using MR-Egger regression: the role of the I2 statistic. *Int J Epidemiol*. (2016) 45:1961–74. doi: 10.1093/ije/dyw220
23. Yavorska OO, Burgess S. MendelianRandomization: an R package for performing Mendelian randomization analyses using summarized data. *Int J Epidemiol*. (2017) 46:1734–9. doi: 10.1093/ije/dyx034
24. Hemani G, Zheng J, Elsworth B, Wade KH, Haberland V, Baird D, et al. The MR-Base platform supports systematic causal inference across the human phenome. *eLife*. (2018) 7:e34408. doi: 10.7554/eLife.34408
25. Bowden J, Del Greco MF, Minelli C, Davey Smith G, Sheehan N, Thompson J. A framework for the investigation of pleiotropy in two-sample summary data Mendelian randomization. *Stat Med*. (2017) 36:1783–802. doi: 10.1002/sim.7221
26. Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, et al. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA*. (2009) 301:39–51. doi: 10.1001/jama.2008.864
27. Klein EA, Thompson IMJr, Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, et al. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA*. (2011) 306:1549–56. doi: 10.1001/jama.2011.1437

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 21 June 2019
doi: 10.3389/fonc.2019.00526

miRNAs as Candidate Biomarker for the Accurate Detection of Atypical Endometrial Hyperplasia/Endometrial Intraepithelial Neoplasia

Simona Giglio[1], Viviana Annibali[2†], Roberto Cirombella[1], Omar Faruq[1], Stefano Volinia[3], Claudia De Vitis[1], Margherita Pesce[1], Donatella Caserta[4], Angela Pettinato[5], Filippo Fraggetta[5] and Andrea Vecchione[1]**

[1] Department of Clinical and Molecular Medicine, "La Sapienza" University, Sant'Andrea Hospital, Rome, Italy, [2] Department of Neurosciences, Mental Health and Sensory Organs, Centre for Experimental Neurological Therapies (CENTERS), "La Sapienza" University, Sant'Andrea Hospital, Rome, Italy, [3] Department of Internal Medicine, Biosystems Analysis, LTTA, Department of Morphology, Surgery and Experimental Medicine, Università Degli Studi, Ferrara, Italy, [4] Department of Medical-Surgical Sciences and Translational Medicine, "La Sapienza" University, Sant'Andrea Hospital, Rome, Italy, [5] Pathology Unit, Cannizzaro Hospital, Catania, Italy

Endometrial cancer is the most common gynecologic malignancy in developed countries. Estrogen-dependent tumors (type I, endometrioid) account for 80% of cases and non-estrogen-dependent (type II, non-endometrioid) account for the rest. Endometrial cancer type I is generally thought to develop via precursor lesions along with the increasing accumulation of molecular genetic alterations. Endometrial hyperplasia with atypia/Endometrial Intraepithelial Neoplasia is the least common type of hyperplasia but it is the type most likely to progress to type I cancer, whereas endometrial hyperplasia without atypia rarely progresses to carcinoma. MicroRNAs are a class of small, non-coding, single-stranded RNAs that negatively regulate gene expression mainly binding to 3′-untranslated region of target mRNAs. In the current study, we identified a microRNAs signature (miR-205, miR-146a, miR-1260b) able to discriminate between atypical and typical endometrial hyperplasia in two independent cohorts of patients. The identification of molecular markers that can distinguish between these two distinct pathological conditions is considered to be highly useful for the clinical management of patients because hyperplasia with an atypical change is associated with a higher risk of developing cancer. We show that the combination of miR-205, −146a, and −1260b has the best predictive power in discriminating these two conditions (>90%). With the aim to find a biological role for these three microRNAs, we focused our attention on a common putative target involved in endometrial carcinogenesis: the oncosuppressor gene SMAD4. We showed that miRs-146a,−205, and−1260b directly target SMAD4 and their enforced expression induced proliferation and migration of Endometrioid Cancer derived cell lines, Hec1a cells. These data suggest that microRNAs-mediated impairment of the TGF-β pathway, due to inhibition of its effector molecule SMAD4, is a relevant molecular alteration in endometrial carcinoma development.

OPEN ACCESS

Edited by:
Jian-Jun Wei,
Northwestern University, United States

Reviewed by:
Tamara Louise Kalir,
Icahn School of Medicine at Mount Sinai, United States
Massimo Nabissi,
University of Camerino, Italy

***Correspondence:**
Simona Giglio
simona.giglio@uniroma1.it
Andrea Vecchione
andrea.vecchione@uniroma1.it

†Present Address:
Viviana Annibali,
Merck, Rome, Italy

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *03 December 2018*
Accepted: *30 May 2019*
Published: *21 June 2019*

Citation:
Giglio S, Annibali V, Cirombella R, Faruq O, Volinia S, De Vitis C, Pesce M, Caserta D, Pettinato A, Fraggetta F and Vecchione A (2019) miRNAs as Candidate Biomarker for the Accurate Detection of Atypical Endometrial Hyperplasia/Endometrial Intraepithelial Neoplasia. Front. Oncol. 9:526. doi: 10.3389/fonc.2019.00526

Our findings show a potential diagnostic role of this microRNAs signature for the accurate diagnosis of Endometrial hyperplasia with atypia/Endometrial Intraepithelial Neoplasia and improve the understanding of their pivotal role in SMAD4 regulation.

Keywords: microRNAs, endometrial hyperplasia, endometrial cancer, biomarkers, SMAD4, TGF-β pathway

INTRODUCTION

Worldwide, endometrial cancer (EC) represents 4% of all cancers in women and is the most common malignant tumor of the female genital tract in industrialized countries (1). The etiology of EC is not yet fully understood, although there is some evidence that molecular modifications and hormonal influences contribute to its initiation and progression (2).

Endometrial cancer is divided into two major classes: estrogen-dependent tumors (type I, endometrioid endometrial carcinomas) that represent 80% of cases and non-estrogen-dependent (type II, non-endometrioid endometrial carcinomas) that account for the rest. EC type I is thought to develop via precursor lesions along with the increasing accumulation of molecular genetic aberration (3, 4).

Recently, the World Health Organization classification of tumors (WHO) (5) divided endometrial hyperplasias into two categories: hyperplasia without atypia (Benign Hyperplasia, BH) and atypical hyperplasia/endometrioid intraepithelial neoplasia: (AH/EIN) (5).

Indeed, AH/EIN is most likely to progress to type I endometrial carcinoma (∼30%), and has been reported to be associated with invasive EC in 62% of endometrial biopsy (6), whereas BH rarely progresses to EC (<5%) (7). Therefore, discerning between these two entities has significant clinical implications (8).

Unfortunately, recognition of atypia in endometrial hyperplasia is subjective among pathologists with a low inter observer reproducibility (<50% in almost all studies) (9, 10).

Although the recent two-tier classification of these entities by WHO (5, 11) improved reproducibility, management of endometrial pre-cancers is compromised by a longstanding debate.

MicroRNAs (miRs) are a class of small, non-coding, single-stranded RNAs that negatively regulate gene expression mainly binding to 3′-untranslated region (UTR) of target mRNAs at the post-transcriptional level (12). Several studies showed that they are important in many biological processes, thus their aberrant expressions are closely associated with the development, invasion, metastasis, and prognosis of various cancers, including EC (13–17).

Up until now, several miR signatures have been documented in either normal or neoplastic endometrium, but the role of miRs in endometrial hyperplasia with or without atypia remains poorly understood (18–22). In the present study, we investigate the hypothesis that changes in miRs may represent useful biomarkers for the diagnosis of AH/EIN.

MATERIALS AND METHODS

Endometrial Tissue Samples and Patients

Eighty-five archived formalin-fixed, paraffin embedded (FFPE) tissue blocks of BH (41 cases), and AH/EIN (44 cases), were obtained from the Pathology Department of Sant'Andrea Hospital and Ospedale Cannizzaro, Catania from 2004 to 2013. Patient's age ranged from 37 to 84 years, with a median of 56 years. This study was authorized by the institutional ethics committee board at S. Andrea Hospital Rome, Italy (Aut. #168/03). Written informed consent was obtained from all patients enrolled.

The selected cases were randomly divided into a training set (23 BH, 19 AH/EIN) and into a validation set (21 BH, 22 AH/EIN).

Hyperplasia was macro or laser-microdissected, were appropriate, for this study.

RNA Extraction

Total RNA, including miRs fraction, was extracted from FFPE tissues using the High Pure miRNA isolation kit (Roche) according to the manufacturer's instructions. RNAs concentration were assessed using Nanodrop (ThermoScientific).

Affymetrix Gene Chip miRNA Array

RNA quality and purity were assessed with the use of the RNA 6000 Nano assay on Agilent 2100 Bioanalyzer (Agilent). Briefly, 500 ng of total RNA was labeled using FlashTag Biotin HSR (Genisphere LLC) and hybridized to GeneChip® miRNA 2.0 Arrays. The arrays were stained in the Fluidics Station 450 and then scanned on the GeneChip® Scanner 3000 (Affymetrix, USA).

Microarray Data Analysis

The statistical analysis was performed by Transcriptome Analysis Console (TAC) software (Thermo Fisher Scientific).

To survey outliers that could disturb the dataset, a Principal Component Analysis (implemented by means of R statistical software) was performed and its visualization, which led to the knowledge of which subjects needed to be excluded from the dataset. MicroRNA probe outliers were defined from the manufacturer's instructions (Affymetrix, USA), and further analysis included data summarization, normalization, and quality control using the web-based miRNA QC Tool software (Affymetrix).

The microarray data has been submitted and assigned a GEO omnibus accession number GSE85105.

Reverse Transcription and Quantitative Real-Time PCRs

Each sample was reverse-transcribed using miRNA miRCURY LNA Universal RT kit (Exiqon) according to the manufacturer's protocol.

Reverse transcription and quantitative real-time PCRs were performed for miRNAs using miRCURY LNA Universal RT microRNA PCR LNA primers set with miRCURY LNA cDNA Synthesis Kit II and ExiLENT Syber Green master mix, in triplicate (Exiqon). RNU48 (U48) was used to normalize input total small RNA.

Expression of each miR was presented as the ratio between miR and RNU48 (RQ). The relative miRs expression was calculated using the $\Delta\Delta C$t method. At least three separate experiments were performed, and each sample was assayed in triplicate.

Cells Transfections

Pre-designed Pre-miR (miR Precursors) for each miR was obtained from Ambion (ThermoFisher Scientific). A negative-control miRNA mimic [Pre-miR miRNA negative #1 (Ambion, ThermoFisher Scientific)] was used to address the specificity of the observed effect to the specific miR sequence. Cells were transfected using Lipofectamine RNAimax (ThermoFisher Scientific).

Protein Extraction, Western Blotting, and Antibodies

Hec1a cell lines were obtained from ATCC (Atcc, HTB-112) and cultured according to the manufacturer's protocol. Total cell extracts with RIPA buffer (Sigma Aldrich) were collected at 24 h and analyzed by western blot to assess proteins expression levels. Briefly, Hec1a cells were rinsed in ice-cold PBS and subsequently lysed in ice-cold RIPA lysis buffer (Sigma Aldrich) and Complete inhibitor (Roche). Proteins were analyzed on pre-cast polyacrylamide gel (Bio-Rad), transferred onto nitrocellulose membranes (Bio-Rad) and blocked with 5% BSA (Sigma Aldrich), and incubated with specific primary antibodies.

Polyclonal antibody against SMAD4 (sc-7966, Santa Cruz Biotechnology) diluted 1:200. Monoclonal antibody against PAX2 (aJ1589a, Abgent) diluted 1:1,000. Monoclonal antibody against Pten (560002, BD Biosciences) diluted 1:1,000.

To normalize protein loading, membranes were probed for 1 h at room temperature with an anti-vinculin antibody (sc- 25336, Santa Cruz Biotechnology).

Secondary antibodies (labeled HRP anti-rabbit or anti-mouse, Bio-Rad) were incubated for 45 min at room temperature and revealed with chemiluminescent ECL method (Bio-Rad).

Digital images of autoradiography were acquired with ChemiDOC XRS (Bio-Rad).

Plasmids and Constructs

The 3′UTR of the SMAD4 gene was obtained from GeneArt Gene Synthesis (Invitrogen, Thermo Fisher Scientific) by cloning 900bp of SMAD4 3′UTR into pMir-vector (Promega) giving rise to the pMir-3′UTRSMAD4 construct.

Site-direct mutagenesis into the miR-205, miR146a, and miR-1260b binding sites of the SMAD4 gene 3′UTR were introduced using GeneArt Site-Directed Mutagenesis PLUS System kit (Thermo Fisher Scientific) according to manufacturer's instructions. The primers used were:

FP 5′CTTCACCTGTTATGTAcctgccAATCATTCCAGT GC3′

RP 5′GCACTGGAATGATTggcaggTACATAACAGGTG AAG3′

FP 5′GCTGATTTTAAAGGCAGAGAAccgtcgAAAGTTA ATTCACC3′

RP 5′GGTGAATTAACTTTcgacggTTCTCTGCCTTTA AAATCAGC3′

FP 5′GTTATTCCTAGTGacccgtTGTTGATGAAGTAT ACTTTTCCCC3′

RP 5′GGGGAAAAGTATACTTCATCAACAacgggtCACTA GGAATAAC3

Luciferase Activity Assays

Hec1a cells were cultured in 12-well-plates and transfected with 500 ng of pMir-3′UTRSMAD4 wt or mutated plasmid or pMir control vector together with 50 ng of β-GAL vector and 50 pmoles of pre-miR-205, pre-miR-146a, pre-miR-1260b, or pre-miR-negative control#1 (Thermo Fisher Scientific). Transfections were carried out using Lipofectamine 2000 and OPTI-MEM as recommended by the manufacturer (Thermo Fisher Scientific). At 48 h after transfection, luciferase activity was measured using the Luciferase Reporter Assay (Promega). Each transfection was repeated twice in triplicate. Transfection efficiency was corrected to β-GAL expression in all cases.

Cell Proliferation Assay

Cell proliferation was measured using Muse® Count & Viability Assay Kit and Muse® Cell Analyzer as recommended by the manufacturer (Merck Millipore). Cells were transfected with each pre-miR and the pre-miR-negative control#1 into a 35 mm dish as described below and incubated for 72 h. Three independent experiments were performed in duplicate.

Transwell Migration Assay

The migration ability of Hec1A cells was determined in a Boyden Chamber. Twenty-four hours after transfection, the cells were seeded into 8 μm Transwells (6.5 mm diameter, Corning) at 5×104 cells well with serum-free culture medium. Medium containing 10% FBS was added into the lower chamber and served as the chemoattractant. After incubation for 24 h, the cells remaining on the upper surface of the filter were removed by gently wiping with a cotton swab. The cells migrated through the filter were fixed with methanol, stained with MGG quick staining (Bio Optica), and visualized by an inverted fluorescence microscope.

Statistical Analysis

Data were expressed as the mean ± SD from at least three independent experiments. Statistical analysis between two samples was performed using Student's *t*-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance (ANOVA).

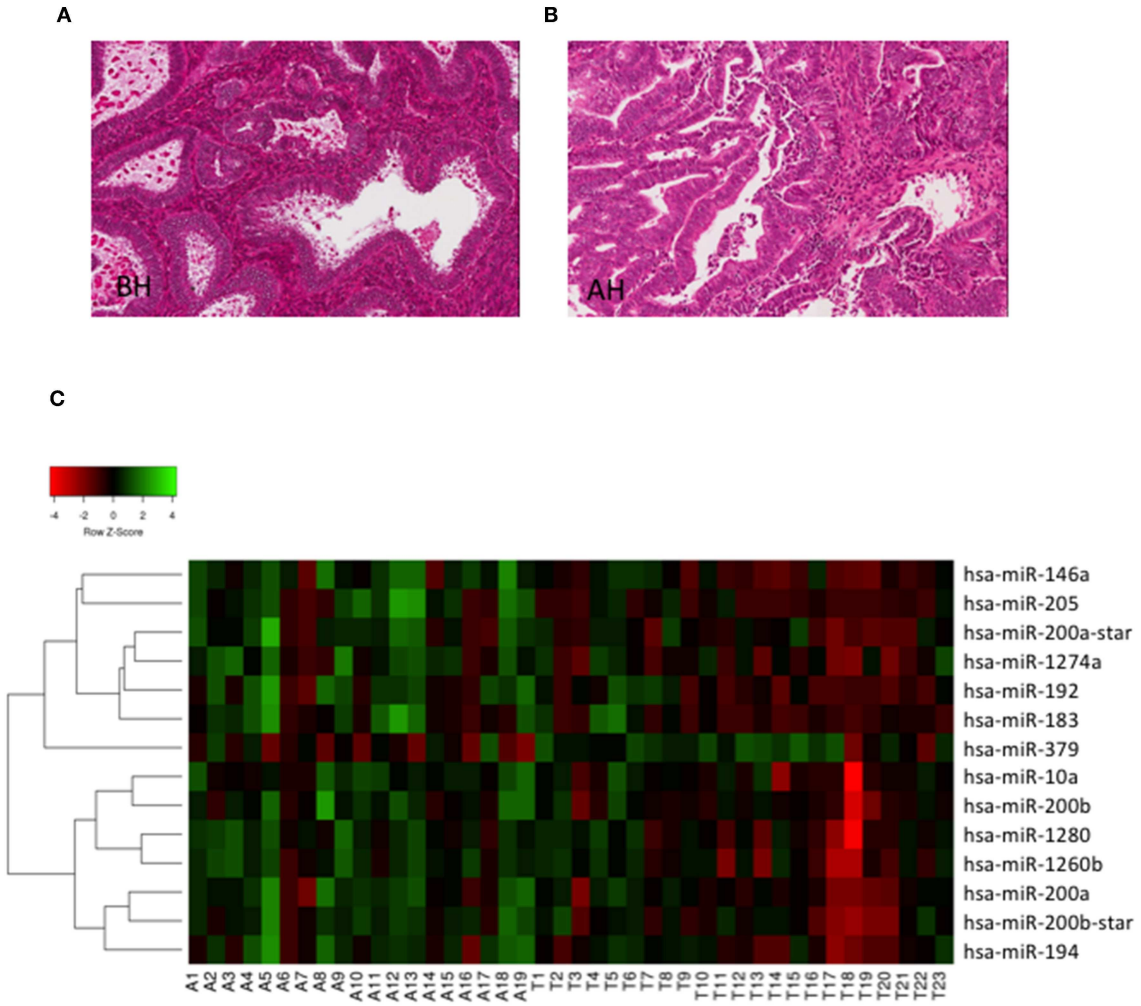


FIGURE 1 | Hierarchical clustering. **(A,B)** Representative H/E stain images of BH and AH (Magnification 20X). **(C)** Heat Map depicting hierarchical cluster analysis of the 14 miRs differentially expressed between AH/EIN (A) and BH (T) identified by microarrays analysis.

The diagnostic ability of miRs-205, −146a, and −1260b in diagnosing atypical hyperplasia was examined via the area under the corresponding receiver operating characteristic curve (AUC). All statistical analyses were performed using Graph Pad Prism software (GraphPad software). $p < 0.05$ was considered statistically significant.

RESULTS

Dysregulated miRs in BH vs. AH/EIN

To investigate whether miRs could discriminate BH from AH/EIN, we analyzed the expression of 1,105 human miRs (miRbase version 15) in the training set (23 BH, 19 AH/EIN). Examples of BH and AH are presented in **Figures 1A,B**.

As shown in **Figure 1C**, we could identify 14 differentially expressed miRs capable of discriminating BH from AH/EIN (FC $\geq$ 1.5, $p \leq 0.05$). In particular, 13 miRs were upregulated (miRs-205,-146a, −200b_star, −1274a, −1260b, −200b, −200a, −192, −183, −10, −194, and −200a_star) and 1 (miR-379) was downregulated in AH/EIN compared to BH samples.

Using multiple logistic regression, the statistical significant variables (Age, BMI, Parity, miR values) were assessed in univariate analysis and investigated comparing BH to AH/EIN. No significant correlation was observed (data not shown).

Differentially expressed miRs were then validated in an independent validation set (21 BH, 22 AH/EIN). Out of the 14 miRs initially identified, we could confirm three miRs all up-regulated (miR-205, −146a, and −1260b) (**Figure 2A**), suggesting that these miRs could discriminate between the two groups.

To assess the ability of each miR to differentiate between AH/EIN and BH, receiver-operating characteristic curves (ROC) were constructed and the area under the curve (AUC) was calculated. Univariate analysis for each individual miR showed an AUC of 0.8 [95% confidence interval (CI) = 0.66–0.93 p = 0.0009] for miR-205, an AUC of 0.8 (95% CI = 0.68–0.94 p =

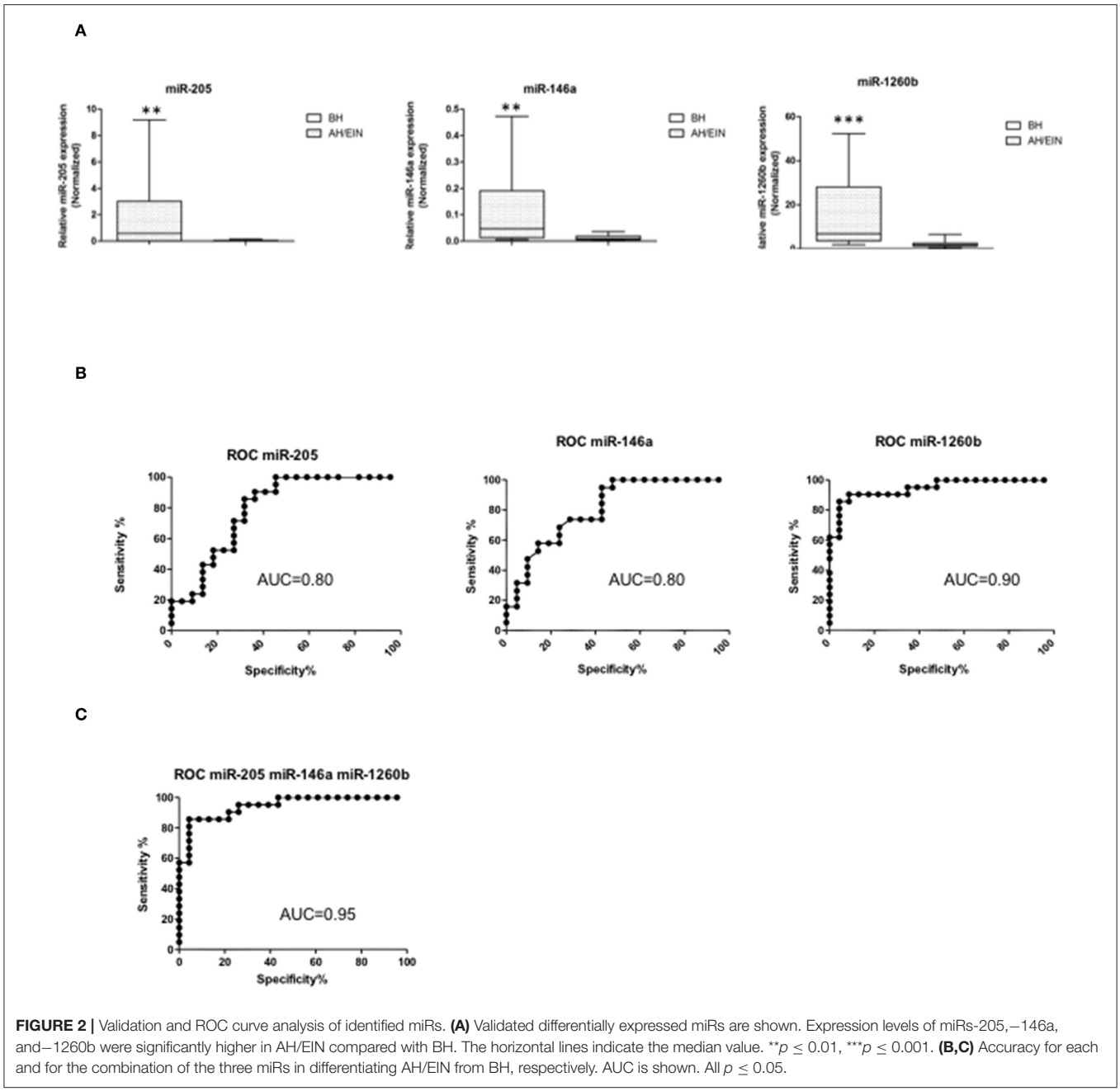


FIGURE 2 | Validation and ROC curve analysis of identified miRs. **(A)** Validated differentially expressed miRs are shown. Expression levels of miRs-205,−146a, and−1260b were significantly higher in AH/EIN compared with BH. The horizontal lines indicate the median value. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. **(B,C)** Accuracy for each and for the combination of the three miRs in differentiating AH/EIN from BH, respectively. AUC is shown. All $p \leq 0.05$.

0.0008) for miR-146a, and an AUC of 0.9 (95% CI = 0.88–1.01 $p < 0.0001$) for miR-1260b, respectively (**Figure 2B**). Performing a multivariate analysis for the combination of the three miRs, we observed an AUC of 0.95 (95% CI 0.88–1.01 $p < 0.0001$) showing that these three miRs have a high predictive power in discriminating AH/EIN from BH (**Figure 2C**).

SMAD4 Is a Target of miRs-205, 146a, and 1260b

To investigate a biological role for these miRs in AH/EIN, we searched different prediction algorithms. We found that highly conserved binding sites for each of these miRs were present in the mRNA of the oncosuppressor gene SMAD4, which has been shown to be down-modulated in EC (23).

We used endometrial cancer-derived cell lines Hec1a and tested the endogenous expression of these miRs.

QRT-PCR analysis showed that Hec1a cells expressed detectable amounts of each miRs (**Figure 3A**). To investigate the effects of these miRs on SMAD4 expression, we transiently transfected pre-miRs-146a, −205, and −1260b or control into Hec1a cells.

In miRs transfected cells, we observed a significant suppression of SMAD4 compared to control (**Figure 3B**). To further confirm this observation, we transiently transfected

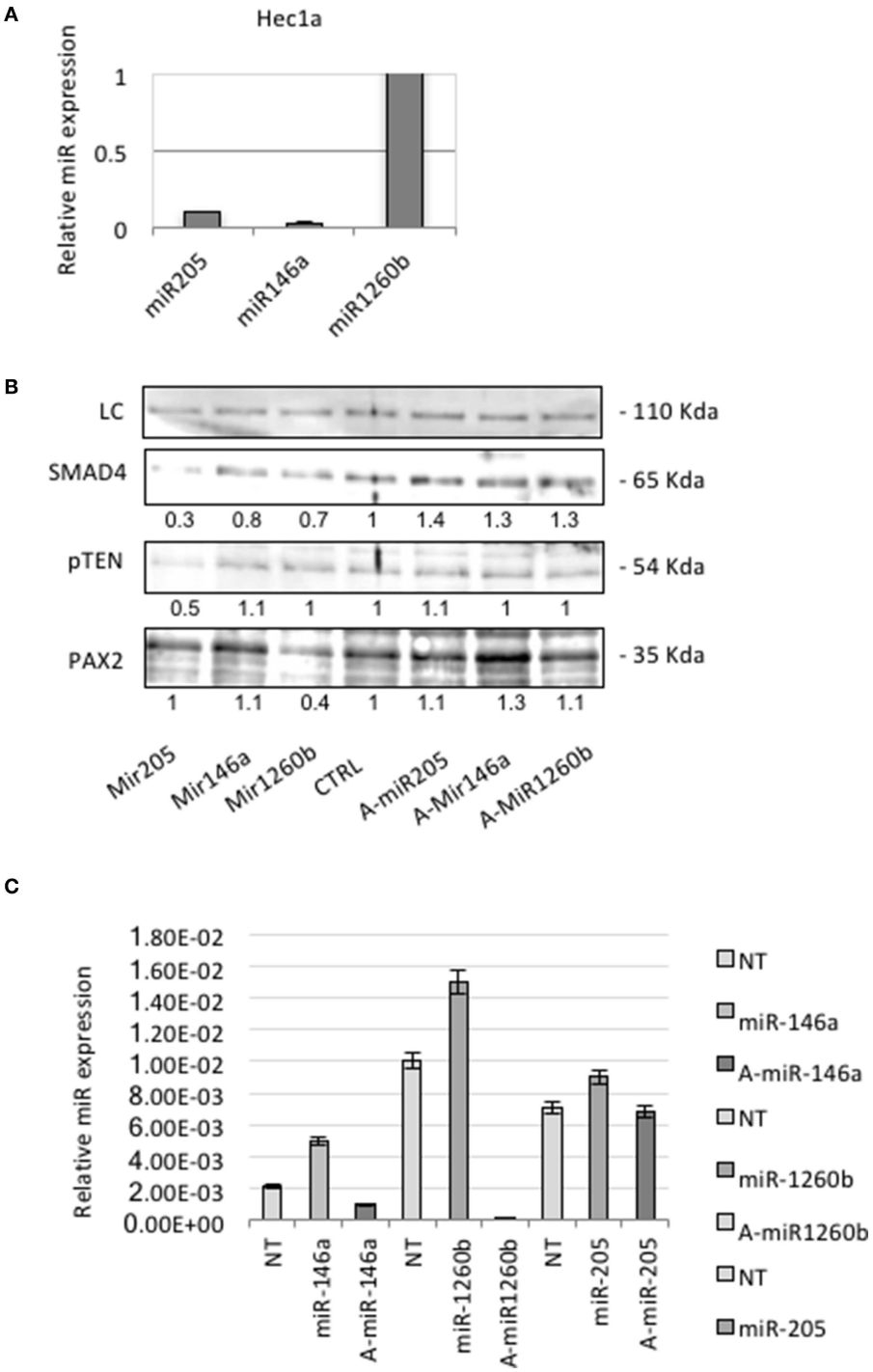


FIGURE 3 | miRs-205,−146a, and−1260b regulate SMAD4 levels. **(A)** Expression levels of each miR were assessed in Hec1a cell line. Relative expression of miRs-205,−146a, and−1260b is reported. Each Ct value is normalized to RNU48. **(B)** Hec1a cells transfected as showed. The Smad4 protein appears as a band at approximately 60 kDa. Actin (∼40 kDa) was used as a loading control LC. Densitometry value is reported under each line. **(C)** RT-PCR verification of the transfection efficiency of Hec1a cells transfected in **B**. Each bar shows miR expression normalized to RNU48 ±SD of three independent experiments.

Hec1a cells with antagomirs. This resulted in an increase of SMAD4 levels, compared to control (**Figures 3B,C**), as expected.

Co-loss of both PTEN and PAX2 has been reported in AH/EIN and it has been regarded as the reference markers for its diagnosis (24).

Therefore, we analyzed Pax2 levels by Western blot and we observed a strong protein reduction in miR-1260b transfected cells. There is no evidence of interaction between these two players, but miR binding sites algorithm prediction showed that other Pax family members are the putative target of miR-1260b. We also found that pTEN was downregulated in miR-205 transfected cells confirming previous evidence showing the potential role of miR-205 in regulating PTEN in endometrial tissue (25).

Although by different means, Smad4 could be a target of these miRs, one can argue that miR-146a, miR-205, and miR-1260b interact with other unknown targets that down-regulate Smad4 protein levels (18–22) (**Figure 4A**). To address this concern, we performed a luciferase reporter assay, cloning a 900 bp 3′UTR of human SMAD4 into a pMIR vector (p3′UTRSmad4pMir). Therefore, Hec1a cells were transfected either with miR-146a, miR-205, mir-1260b, or the pre-miR-control, and p3′UTRSmad4pMir vector. As shown in **Figure 4B**, all miRs decrease luciferase activity of the p3′UTRSmad4pMir compared to control, showing that each miR has a direct effect on their target in this cell lines (**Figure 4B**). To determine this direct miR-target interaction, we constructed a plasmid with mutagenesis of the three seed sequences (**Figure 4C**). As expected, we observed only a slight effect on luciferase activity when we compared the wild-type vector with the p3′UTRSmad4pMir mutants in the presence of each miRs overexpressed, showing that the modification of the seed sequence is enough to block the function of each miR (**Figure 4C**).

SMAD4 Repression by miRs-205, 146a, and 1260b Induces Proliferation and Migration in Hec1a Cell Lines

Smad4 is involved in the signal transduction pathway of the transforming growth factor ß (TGF-ß) that acts as a tumor suppressor gene in several cancers (26, 27). To gain further insights into how dysregulation of these miRs may play a role in endometrial cancer cells, we performed different assays to study the biological effects of the interaction between these miRs and their target Smad4 into endometrial adenocarcinoma-derived cell lines Hec1a.

First, we tested cells proliferation. Cells transfected with pre-miRs-146a, −205, or −1260b showed a higher rate of proliferation compared with non-treated or control transfected cells (**Figure 5A**). Cells transfected with anti-miRs-146a, −205, or −1260b showed a reduction of proliferation compared to control (**Figure 5B**).

To better understand how the dysregulation of these miRs may change the behavior of endometrial cancer-derived cell line, we examined the influence of Smad4 knockdown on Hec1a cell migration. We found a significant increase in the migration capability in pre-miR-205, −146a, or −1260b transfected cells compared to control, showing that these miRs positively regulate the migration of cultured endometrial cancer cells (**Figures 5C,D**).

Since miRs may have multiple targets, to ensure that Smad4 mediated the observed effects, we repeated the assay using a specific Smad4 siRNA.

We confirm significant down-regulation of Smad4 through qRT-PCR (**Figure 5E**). Next, we observed that Hec1a cells transfected with Smad4 siRNA displayed a higher proliferation and migration rate respect to controls (**Figures 5C,F**).

DISCUSSION

Identification of molecular markers that can differentiate between AH/EIN and BH are considered to be highly useful for clinical management of patients because hyperplasia with atypical change and/or Endometrial Intraepithelial Neoplasia are associated with a higher risk to progress to cancer (4).

Since there aren't reference markers, the diagnosis is based only on histological features, such as the presence of nucleoli and other atypical characteristics, which are not consistently associated with that diagnosis (9).

Although the new WHO classification is more likely to successfully identify premalignant lesions, the low interobserver reproducibility among gynecological pathologist in diagnosing atypical hyperplasia/EIN should be improved (6, 10, 28–30).

Atypical endometrial hyperplasia/EIN and EC shares several molecular alterations with each other, including microsatellite instability, PAX2 inactivation, mutation of PTEN, KRAS, and CTNNB1 (β-catenin), but there is not a linear accumulation of mutational events leading to cancer (31). Identifying the disease-related miRs will improve the diagnosis and understanding of pathogenesis of these lesions.

Since over 50% of miRs reside in cancer-associated genomic regions, they have been indicated to play an important role as diagnostic biomarkers (13, 32, 33).

Most of the miRs studies on endometrium have been focused on the identification of their implications in EC development, almost neglecting their possible diagnostic role in precursor lesions (34). In fact, several authors showed an altered expression of miRs that may discriminate EC from non-atypical or atypical hyperplasia (9, 14, 21, 35–40).

In particular, expression of five miRs (miRs-182, 183, 200a, 200c, and 205) was significantly higher in EC when compared with complex atypical hyperplasia, simple hyperplasia (SH) and normal endometrial tissue ($P < 0.05$, respectively) (41).

To our knowledge, our study is the first to identify a miRs signature able to discriminate between atypical hyperplasia/EIN and benign endometrial hyperplasia with the capability to better distinguish between low- and high- risk lesions. Identification of miR-target genes and pathways to understand the molecular basis of endometrial cancer pathogenesis is a major challenge, as there are numerous pathways that drive cancer. Accordingly, in this study, we proposed a novel miR-based classification method to categorize the high risk pre-cancerous endometrial lesions.

In fact, we showed a high predictive power, above 90%, using a three miRs-signature (miRs-146a, −205, and −1260b) in distinguishing between non-atypical and atypical

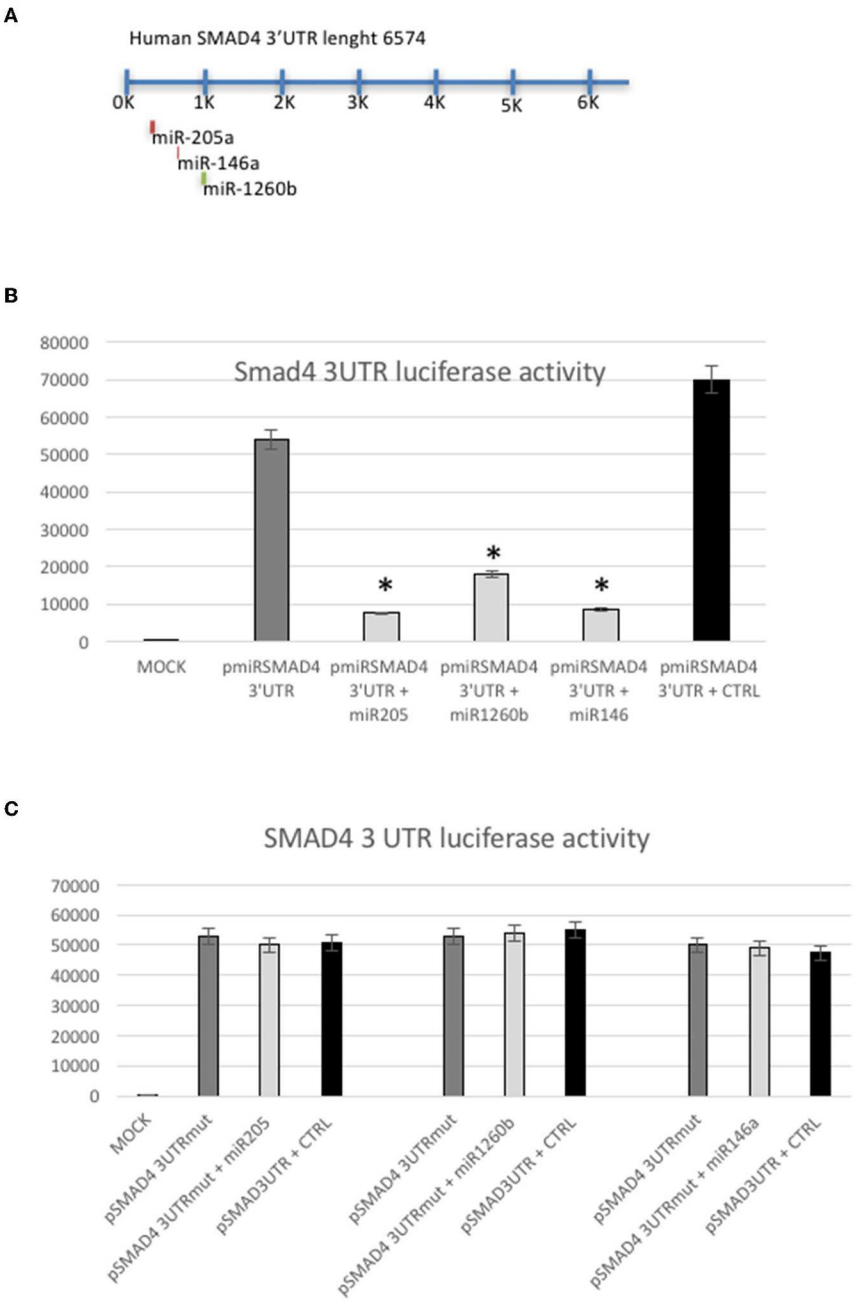


FIGURE 4 | SMAD4 is a target of miRs-205, −146a, and −1260b. **(A)** Schematic representation of predicted miRs binding sites in the SMAD4 3′UTR. **(B)** Luciferase reporter assay in Hec1a cells co-transfected with the reporter gene containing the SMAD4 3′UTR alone (pmiRSMAD4 3′UTR) (gray bar), the SMAD4 3′UTR and miRs-205, −1260b, and −146a (light gray bars), respectively and the negative control (CTRL) (black bar). Each reporter plasmid was transfected three times, and each sample was assayed in triplicate. **(C)** Luciferase reporter assay performed in Hec1a cells co-transfected with the reporter gene containing the SMAD4 3′UTR mutated (pmiRSMAD4 3′UTR-Mut) in the miRs-205, −146a, or −1260b seed sequences (gray bar) alone, the pmiRSMAD4 3′UTR-Mut in each mir seed sequence and miRs-205, −1260b, and −146a (light gray bars), respectively and the negative control (CTRL) (black bar). Bars indicate Firefly Luciferase activity normalized to β-Gal activity ±SD. *$p \leq 0.05$ compared to control (CTRL) transfected cells.

hyperplasia/EIN provides a supplementary diagnostic tool when required.

Interestingly, a previous study conducted by Snowdon and colleagues examined a miRs profile in atypical hyperplasia compared to normal proliferative controls. The microarray expression profile shares some important similarities with our data. MiRs-146a, miR-200a, miR-200b, miR-200b-star, and miR-205 resulted up-regulated and miR-542-5p down-regulated in

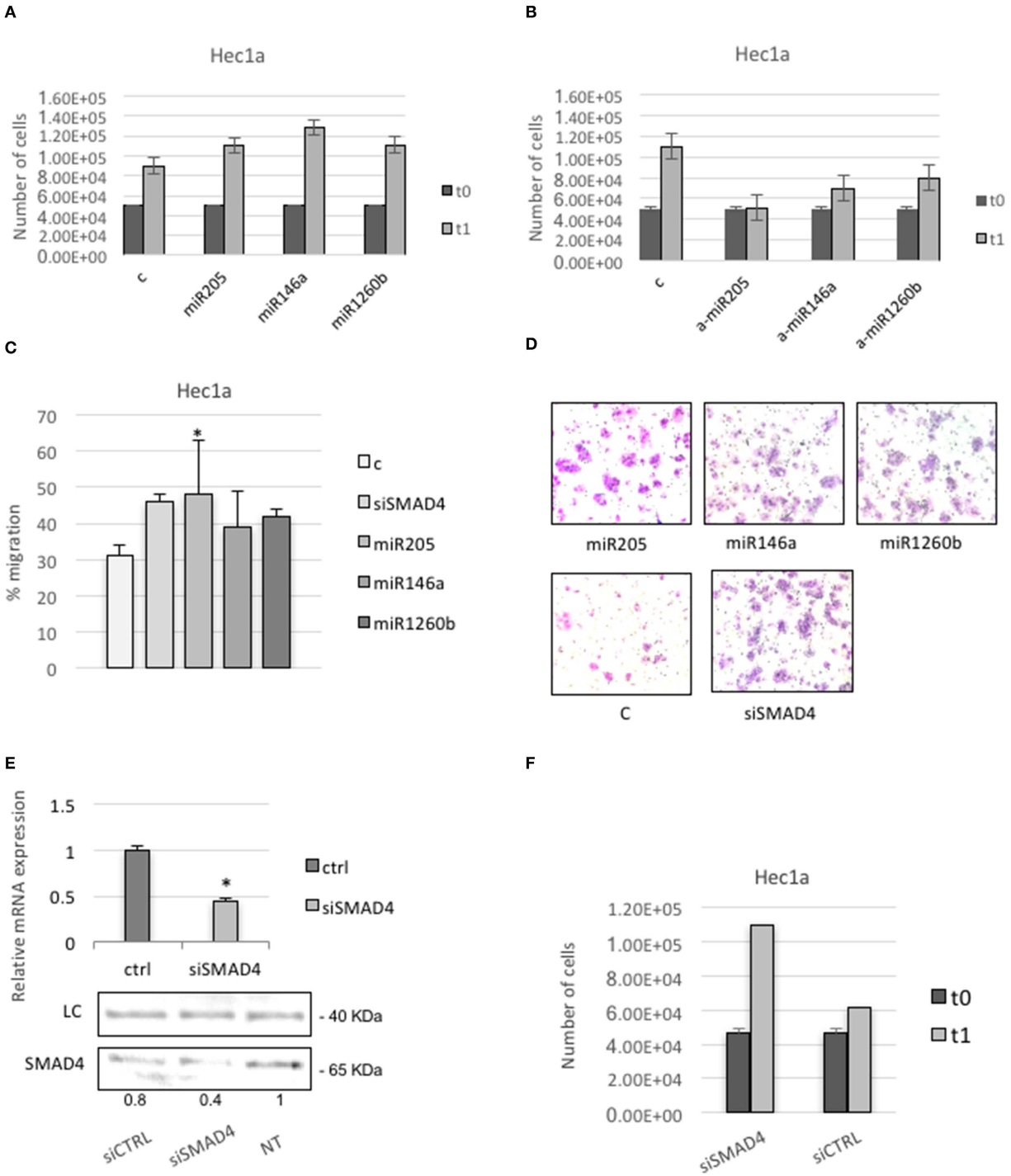


FIGURE 5 | SMAD4 regulation by miRs-205, −146a, and −1260b induces proliferation and migration in Hec1a cells. **(A,B)** Hec1a cells proliferation was measured at 72 h. Cells were transiently transfected with negative control #1 **(C)** and miRs-205, −146a, −1260b in **A** or anti-miRs-205, −146a, and −1260b (a-miRs) in **B**. **(C)** Transwell migration assay shows that overexpression of miRs-205, −146a, −1260b, and siSMAD4 enhance cell migration ability of Hec1a cells. **(D)** Representative photographs of the Transwell migration assay is shown. All data are presented as mean ± SD, $^*p < 0.05$. **(E,F)** Effects of siSMAD4 expression in Hec1a cells. **(E)** RT-PCR (upper panel) and western blot analysis (lower panel) are shown. LC (loading control, β-actin). PCR bars depict SMAD4 expression in control (ctrl) and si transfected cells normalized to β-actin. **(F)** Measure of Hec1a cells proliferation in siSMAD4 and SiCTRL transfected cells is reported. Data represent the mean (from three independent experiments) ±SD. $^*P < 0.05$.

atypical hyperplasia vs. normal endometrium, adding further emphasis to our results (22). The up-regulation of miR-200 family members and miR-205 in EC is observed among different studies, indicating that these miRs may play a role in driving oncogenesis in the endometrium (21, 35, 37, 42, 43).

Some authors showed that miR-205 is a negative prognostic marker for EC and its levels were significantly increased in endometrial cancer cell lines and endometrial tumors compared to normal tissues (25, 26). Mir-205 is directly involved in PTEN regulation that represents one of the most commonly investigated markers implicated in endometrial tumorigenesis (44). Our results have enforced this effect indicating a remarkable influence of miR-205 on regulating essential target genes involved in different signal pathways in endometrial cells. On the other hand, Lacey et al. have shown that a loss of expression of PTEN status was not associated with progression risk of endometrial hyperplasia (45).

Even if EC seems to be characterized by elevated expression of miR-205, a recent study conducted by Wilczynski showed that higher levels of miR-205 may be a marker of an early stage disease and is associated with a more favorable prognosis, whereas patients with lower levels of miR-205 had worse survival (42).

No evidence has revealed the dysregulation of miRs-1260b and −146a in EC, suggesting that up-regulation of these miRs may be specific of AH/EIN.

Interestingly, authors found that a single nucleotide polymorphism (SNP) rs2910164 G>C within miR-146a is associated with the increased risk of gastric cancer and papillary thyroid carcinoma (46, 47). A recent publication showed that overexpression of miR-146a inhibited cell proliferation, enhanced apoptosis, and increased sensitivity to chemotherapy drugs in epithelial ovarian cancers cells showing, therefore, that the role of miR-146a is still to be elucidated (48).

MiR-1260b has been found to be highly expressed in the prostate, renal cell, and in colorectal carcinomas (49, 52). Recently, it was demonstrated that in Hepatocellular carcinoma, MiR-1260b promotes cell migration and invasion through the G-protein signaling 22 (50).

All of this evidence confirmed that our identified miRs regulate genes involved in different signal pathways that may trigger the endometrial cellular transformation.

Thus, we investigated a possible common pathway that could be regulated by these miRs and could be implicated in cellular transformation, and we found that they target SMAD4. Also noteworthy are several other reports, which demonstrated a direct interaction of these miRs and this transcript (51–53).

Smad4 is a gene implicated in several cancers, including EC, albeit its role in endometrial carcinogenesis is yet not clear (54–56).

Impairment of the Smad pathway results in escape from growth inhibition and leads to the promotion of cell proliferation, contributing to carcinogenesis (57).

The disturbances in Smad proteins expression and/or differences in their intracellular distribution, that trigger a TGF-β signaling pathway deregulation, it was reported in endometrial carcinomas, but it is still not well-understood (58). The region within 18q21 where Smad4 is located is frequently deleted in endometrial carcinomas, showing its involvement in EC, however, an immunohistochemical study showed that inactivation of this gene occurs infrequently in this tumor.

Changes in the expression of the TGF-β signaling cascade in type I ECs seem to be associated mainly with deregulation of TGF-β receptors and SMAD expression at the protein level, indicating SMAD4 as a central molecule of this pathway (59, 60).

Thus, the potential pathogenic role of SMAD4 in endometrial hyperplasia is supported by our finding, albeit further studies are required to understand its biological and diagnostic role in this environment.

Our results clearly demonstrated that overexpression of miRs-146a, −205, and −1260b induced Hec1a proliferation and migration through SMAD4 inhibition, providing an insight into the possible mechanisms underlying the function of these miRs in endometrial hyperplasia.

Thus, our work highlights the relevance of miRs in regulating cellular processes that may ultimately lead to tumorigenesis.

Taken together these results strongly show that miRs-205, −146a, and −1260b contribute to enhancing proliferation and migration properties of endometrial cancer cells through Smad4 inhibition.

In conclusion, distinguishing between hyperplasia and true pre-cancerous lesions has significant clinical implications because distinct endometrial pre-cancerous conditions require intervention. Thus, we proposed a three miR-signature (146a −205, −1260b) as a potential biomarker for diagnosis of atypical endometrial hyperplasia/EIN that could have a significant impact on treatment decisions. Furthermore, the regulatory capability of these three miRs on cell proliferation and migration, possibly through impairment of TGF-β signaling Smad4-mediated, highlights their crucial role in endometrial hyperplasia outcome.

Although we believe that this study represents a step forward in investigating the molecular relationship between miR deregulation and EIN lesions, we analyzed a relatively small group of patients, and therefore, a prospective analysis is needed to strengthen the accuracy of our results.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of name of guidelines, name of committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the IRB committee.

AUTHOR CONTRIBUTIONS

SG and AV: study conception and design and drafting the manuscript. SG, VA, RC, OF, CD, and MP: methodology. SG, SV, and AV: analysis and interpretation of data. DC, AP, FF, and AV: access to clinical data. SG, DC, AP, FF, and AV: critical revision.

FUNDING

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC IG 16862 to AV) and La Sapienza University Fondi Ateneo 2017 to AV.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. *CA Cancer J Clin*. (2015) 65:5–29. doi: 10.3322/caac.21254
2. Di Cristofano A, Ellenson LH. Endometrial carcinoma. *Annu Rev Pathol*. (2007) 2:57–87. doi: 10.1146/annurev.pathol.2.010506.091905
3. Murali R, Soslow RA, Weigelt B. Classification of endometrial carcinoma: more than two types. *Lancet Oncol*. (2014) 15:268–78. doi: 10.1016/S1470-2045(13)70591-6
4. Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol*. (1983) 15:10–7. doi: 10.1016/0090-8258(83)90111-7
5. Kurman RJ, Carcangiu ML, Herrington CS, Young RH. *WHO Classification of Tumours of Female Reproductive Organs*. Lyon: IARC (2014).
6. Trimble CL, Kauderer J, Zaino R, Silverberg S, Lim PC, Burke JJ, et al. Concurrent endometrial carcinoma in women with a biopsy diagnosis of atypical endometrial hyperplasia: a gynecologic oncology group study. *Cancer*. (2006) 106:812–9. doi: 10.1002/cncr.21650
7. Kurman RJ, Kaminski PF, Norris HJ. The behavior of endometrial hyperplasia. A long-term study of "untreated" hyperplasia in 170 patients. *Cancer*. (1985) 56:403–12. doi: 10.1002/1097-0142(19850715)56:2<403::AID-CNCR2820560233>3.0.CO;2-X
8. Emons G, Beckmann MW, Schmidt D, Mallmann P. New WHO classification of endometrial hyperplasias. *Geburtsh Frauenheilk*. (2015) 75:135–6. doi: 10.1055/s-0034-1396256
9. Kendall BS, Ronnett BM, Isacson C, Cho KR, Hedrick L, Diener-West M, et al. Reproducibility of the diagnosis of endometrial hyperplasia, atypical hyperplasia and well-differentiated carcinoma. *Am J Surg Pathol*. (1998) 22:1012–9. doi: 10.1097/00000478-199808000-00012
10. Bergeron C, Nogales FF, Masseroli M, Abeler V, Duvillard P, Müller-Holzner E, et al. A multicentric European study testing the reproducibility of the WHO classification of endometrial hyperplasia with a proposal of a simplified working classification for biopsy and curettage specimens. *Am J Surg Pathol*. (1999) 23:1102–8. doi: 10.1097/00000478-199909000-00014
11. Ordi J, Bergeron C, Hardisson D, McCluggage WG, Hollema H, Felix A, et al. Reproducibility of current classifications of endometrial endometrioid glandular proliferations: further evidence supporting a simplified classification. *Histopathology*. (2014) 64:284–92. doi: 10.1111/his.12249
12. Ambros V. The functions of animal microRNAs. *Nature*. (2004) 431:350–5. doi: 10.1038/nature02871
13. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer*. (2006) 6:857–66. doi: 10.1038/nrc1997
14. Boren T, Xiong Y, Hakam A, Wenham R, Apte S, Wei Z, et al. MicroRNAs and their messenger RNAs associated with endometrial carcinogenesis. *Gynecol Oncol*. (2008) 110:206–15. doi: 10.1016/j.ygyno.2008.03.023
15. Giglio S, Vecchione A. Role of microRNAs in the molecular diagnosis of cancer. *JNAI*. (2010) 1:e4. doi: 10.4081/jnai.2010.1659
16. Hayes J, Peruzzi PP, Lawler S. MicroRNAs in cancer: biomarkers, functions and therapy. *Trends Mol. Med*. (2014) 20:460–9. doi: 10.1016/j.molmed.2014.06.005
17. Jayaraman M, Radhakrishnan R, Mathews CA, Yan M, Husain S, Moxley KM, et al. Identification of novel diagnostic and prognostic miRNA signatures in endometrial cancer. *Genes Cancer*. (2017) 8:566–76. doi: 10.18632/genesandcancer.144
18. Kaur S, Lotsari JE, Al-Sohaily S, Warusavitarne J, Kohonen-Corish MR, Peltomäki P. Identification of subgroup-specific miRNA, patterns by epigenetic profiling of sporadic and Lynch syndrome-associated colorectal and endometrial carcinoma. *Clin Epigene*. (2015) 7:20. doi: 10.1186/s13148-015-0059-3
19. Xu YY, Wu HJ, Ma HD. microRNA-503 suppresses proliferation and cell-cycle progression of endometrioid endometrial cancer by negatively regulating cyclin D1. *FEBS J*. (2013) 280:3768–79. doi: 10.1111/febs.12365
20. Lee TS, Jeon HW, Kim YB. Aberrant microRNA expression in endometrial carcinoma using formalin-fixed paraffin-embedded (FFPE) tissues. *PLoS ONE*. (2013) 8:e81421. doi: 10.1371/journal.pone.0081421
21. Lee H, Choi HJ, Kang CS, Lee HJ, Lee WS, Park CS. Expression of miRNAs and PTEN in endometrial specimens ranging from histologically normal to hyperplasia and endometrial adenocarcinoma. *Mod Pathol*. (2012) 25:1508–15. doi: 10.1038/modpathol.2012.111
22. Snowdon J, Zhang X, Childs T, Tron VA, Feilotter H. The microRNA-200 family is upregulated in endometrial carcinoma. *PLoS ONE*. (2011) 6:e22828. doi: 10.1371/journal.pone.0022828
23. Piestrzeniewicz-Ulanska D, Brys M, Semczuk A, Jakowicki JA, Krajewska WM. Expression and intracellular localization of Smad proteins in human endometrial cancer. *Oncol Rep*. (2003) 10:1539–44. doi: 10.3892/or.10.5.1539
24. Monte NM, Webster KA, Neuberg D, Dressler GR, Mutter GL. Joint loss of PAX2 and PTEN expression in endometrial precancers and cancer. *Cancer Res*. (2010) 70:6225–32. doi: 10.1158/0008-5472.CAN-10-0149
25. Karaayvaz M, Zhang C, Liang S, Shroyer KR, Ju J. Prognostic significance of miR-205 in endometrial cancer. *PLoS ONE*. (2012) 7:e35158. doi: 10.1371/journal.pone.0035158
26. Tabruyn SP, Hansen S, Ojeda-Fernández ML, Bovy N, Zarrabeitia R, Recio-Poveda L, et al. MiR-205 is downregulated in hereditary hemorrhagic telangiectasia and impairs TGF-beta signaling pathways in endothelial cells.*Angiogenesis*. (2013) 16:877–87. doi: 10.1007/s10456-013-9362-9
27. Zhong H, Wang HR, Yang S, Zhong JH, Wang T, Wang C, et al. Targeting Smad4 links microRNA-146a to the TGF-beta pathway during retinoid acid induction in the acute promyelocytic leukemia cell line. *Int J Hematol*. (2010) 92:129–35. doi: 10.1007/s12185-010-0626-5
28. Skov BG, Broholm H, Engel U, Franzmann MB, Nielsen AL, Lauritzen AF, et al. Comparison of the reproducibility of the WHO classifications of 1975 and 1994 of endometrial hyperplasia. *Int J Gynecol Pathol*. (1997) 16:33–7. doi: 10.1097/00004347-199701000-00006
29. Zaino RJ, Kauderer J, Trimble CL. Reproducibility of the diagnosis of atypical endometrial hyperplasia a gynecologic oncology group study. *Cancer*. (2006) 106:804–11. doi: 10.1002/cncr.21649
30. Raffone A, Travaglino A, Saccone G. Endometrial hyperplasia and progression to cancer: which classification system stratifies the risk better? A systematic review and meta-analysis. *Arch Gynecol Obstet*. (2019) 299:1233–42. doi: 10.1007/s00404-019-05103-1
31. Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, Shen H, et al. Cancer Genome Atlas Research Network. Integrated genomic characterization of endometrial carcinoma. *Nature*. (2013) 497:67–73. doi: 10.1038/nature12113
32. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring, therapeutics. A comprehensive review. *EMBO Mol Med*. (2012) 4:143–59. doi: 10.1002/emmm.201100209
33. Croce CM. Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet*. (2009) 10:704–14. doi: 10.1038/nrg2634
34. Ratner ES, Tuck D, Richter C, Nallur S, Patel RM, Schultz V, et al. MicroRNA signatures differentiate uterine cancer tumor subtypes. *Gynecol Oncol*. (2010) 118:251–7. doi: 10.1016/j.ygyno.2010.05.010
35. Pan Q, Chegini N. MicroRNA signature and regulatory functions in the endometrium during normal and disease states. *Semin Reprod Med*. (2008) 26:479–93. doi: 10.1055/s-0028-1096128
36. Cohn DE, Fabbri M, Valeri N, Alder H, Ivanov I, Liu CG, et al. Comprehensive miRNA profiling of surgically staged endometrial cancer. *Am J Obstet Gynecol*. (2010) 202:651–8. doi: 10.1016/j.ajog.2010.02.051
37. Hiroki E, Akahira J, Suzuki F, Nagase S, Ito K, Suzuki T, et al. Changes in microRNA expression levels correlate with clinicopathological features and prognoses in endometrial serous adenocarcinomas. *Cancer Sci*. (2010) 101:241–9. doi: 10.1111/j.1349-7006.2009.01385.x
38. Wu W, Lin Z, Zhuang Z, Liang X. Expression profile of mammalian microRNAs in endometrioid adenocarcinoma. *Eur J Cancer Prev*. (2009) 18:50–5. doi: 10.1097/CEJ.0b013e328305a07a
39. Pan Q, Luo X, Toloubeydokhti T, Chegini N. The expression profile of micro-RNA in endometrium and endometriosis and the influence of ovarian steroids on their expression. *Mol Hum Reprod*. (2007) 13:797–806. doi: 10.1093/molehr/gam063
40. Chung TK, Cheung TH, Huen NY, Wong KW, Lo KW, Yim SF, et al. Dysregulated microRNAs and their predicted targets associated with endometrioid endometrial adenocarcinoma in Hong Kong women. *Int J Cancer*. (2009) 124:1358–65. doi: 10.1002/ijc.24071

41. Lee JW, Park YA, Choi JJ, Lee YY, Kim CJ, Choi C, et al. The expression of the miRNA-200 family in endometrial endometrioid carcinoma. *Gynecol Oncol*. (2011) 120:56–62. doi: 10.1016/j.ygyno.2010.09.022
42. Wilczynski M, Danielska J, Dzieniecka M, Szymanska B, Wojciechowski M, Malinowski A. Prognostic and clinical significance of miRNA-205 in endometrioid endometrial cancer. *PLoS ONE*. (2016) 11:e0164687. doi: 10.1371/journal.pone.0164687
43. Zhang G, Hou X, Li Y, Zhao M. MiR-205 inhibits cell apoptosis by targeting phosphatase and tensin homolog deleted on chromosome ten in endometrial cancer Ishikawa cells. *BMC Cancer*. (2014) 14:440. doi: 10.1186/1471-2407-14-440
44. Mutter GL, Lin MC, Fitzgerald JT, Kum JB, Baak JP, Lees JA, Weng LP, Eng C. Altered PTEN expression as a diagnostic marker for the earliest endometrial precancers. *J Natl Cancer Inst*. (2000) 92:924–30. doi: 10.1093/jnci/92.11.924
45. Lacey JV Jr., Mutter GL, Ronnett BM. PTEN expression in endometrial biopsies as a marker of progression to endometrial carcinoma. *Cancer Res*. (2008) 68:6014–20. doi: 10.1158/0008-5472.CAN-08-1154
46. Xia ZG, Yin HF, Long Y, Cheng L, Yu LJ, Guo WJ, et al. Genetic variant of miR-146a rs2910164 C>G and gastric cancer susceptibility. *Oncotarget*. (2016) 7:34316–21. doi: 10.18632/oncotarget.8814
47. Jazdzewski K, Murray EL, Franssila K, Jarzab B, Schoenberg DR, de la Chapelle A. Common SNP in pre-miR-146a decreases mature miR expression and predisposes to papillary thyroid carcinoma. *Proc Natl Acad Sci USA*. (2008) 105:7269–74. doi: 10.1073/pnas.0802682105
48. Cui Y, She K, Tian D, Zhang P, Xin X. MiR-146a inhibits proliferation and enhances chemosensitivity in epithelial ovarian cancer via reduction of SOD2. *Oncol Res*. (2016) 23:275–82. doi: 10.3727/096504016X14562725373798
49. Liu DR, Guan QL, Gao MT, Jiang L, Kang HX. MiR-1260b is a potential prognostic biomarker in colorectal cancer. *Med Sci Monit*. (2016) 22:2417–23. doi: 10.12659/MSM.898733
50. Li X, Song H, Liu Z, Bi Y. miR-1260b promotes cell migration and invasion of hepatocellular carcinoma by targeting the regulator of G-protein signaling. *Biotechnol Lett*. (2018) 40:57–62. doi: 10.1007/s10529-017-2455-6
51. Xiao B, Zhu ED, Li N, Lu DS, Li W, Li BS, et al. Increased miR-146a in gastric cancer directly targets SMAD4 and is involved in modulating cell proliferation and apoptosis. *Oncol Rep*. (2012) 27:559–66. doi: 10.3892/or.2011.1514
52. Hirata H, Hinoda Y, Shahryari V, Deng G, Tanaka Y, Tabatabai ZL, et al. Genistein downregulates onco-miR-1260b and upregulates sFRP1 and Smad4 via demethylation and histone modification in prostate cancer cells. *Br J Cancer*. (2014) 110:1645–54. doi: 10.1038/bjc.2014.48
53. Li J, Hu K, Gong G. Upregulation of MiR-205 transcriptionally suppresses SMAD4 and PTEN and contributes to human ovarian cancer progression. *Sci Rep*. (2017) 7:41330. doi: 10.1038/srep41330
54. Hahn SA, Schmiegel WH. Recent discoveries in cancer genetics of exocrine pancreatic neoplasia. *Digestion*. (1998) 59:493–501. doi: 10.1159/000007526
55. Kang YK, Kim WH, Jang JJ. Expression of G1-S modulators (p53, p16, p27, cyclin D1, Rb) and Smad4/Dpc4 in intrahepatic cholangiocarcinoma. *Hum Pathol*. (2002) 33:877–83. doi: 10.1053/hupa.2002.127444
56. Liu FS, Chen JT, Hsieh YT, Ho ES, Hung MJ, Lu CH, et al. Loss of Smad4 protein expression occurs infrequently in endometrial carcinomas. *Int J Gynecol Pathol*. (2003) 22:347–52. doi: 10.1097/01.pgp.0000092131.88121.0a
57. Principe Daniel R, Doll Jennifer A, Jessica B, Barbara J, Munshi Hidayatullah G, Laurent B, et al. TGF-β: duality of function between tumor prevention and carcinogenesis. *J Natl Cancer Inst*. (2014) 106:djt369. doi: 10.1093/jnci/djt369
58. Piestrzeniewicz-Ulanska D, Brys M, Semczuk A, Rechberger T, Jakowicki JA, Krajewska WM. TGF-beta signaling is disrupted in endometrioid-type endometrial carcinomas. *Gynecol Oncol*. (2004) 95:173–80. doi: 10.1016/j.ygyno.2004.06.032
59. Ijichi H, Otsuka M, Tateishi K, Ikenoue T, Kawakami T, Kanai F, et al. Smad4-independent regulation of p21/WAF1 by transforming growth factor-beta. *Oncogene*. (2004) 23:1043–51. doi: 10.1038/sj.onc.1207222
60. Zeng Y, Zhu J, Shen D, Qin H, Lei Z, Li W, et al. Repression of Smad4 by miR-205 moderates TGF-β-induced epithelial-mesenchymal transition in A549 cell lines. *Int J Oncol*. (2016). 49:700–8. doi: 10.3892/ijo.2016.3547

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 29 January 2019
doi: 10.3389/fonc.2019.00027

Long Non-coding RNA CDKN2B Antisense RNA 1 Gene Contributes to Paclitaxel Resistance in Endometrial Carcinoma

Chao Shang[1], *Cheng N. Ao*[2], *Chi C. Cheong*[2] *and Lirong Meng*[2]*

[1] Department of Neurobiology, School of Life Science, China Medical University, Shenyang, China, [2] School of Health Sciences, Macao Polytechnic Institute, Macau, China

Endometrial cancer (EC) is the most common malignancy of the female reproductive tract. In this study, we clarified the clinical significance of CDKN2B antisense RNA 1 (CDKN2B-AS) gene, and its effects on paclitaxel sensitivity in EC. Firstly, CDKN2B-AS gene was highly expressed in EC tissues and cell lines. The high-expression of CDKN2B-AS gene was associated with high pathological grade and low paclitaxel sensitivity of EC tissues. Knockdown of CDKN2B-AS gene sensitized Ishikawa/PA and HEC1A/PA cells to paclitaxel, and promoted paclitaxel-induced cytotoxicity. Secondly, the low-expression of miR-125a-5p was closely associated with low paclitaxel sensitivity of EC cells, and up-regulation of miR-125a-5p could increase paclitaxel sensitivity of Ishikawa/PA and HEC1A/PA cells. MiR-125a-5p also mediated the suppressive effects of knockdown of CDKN2B-AS on paclitaxel resistance in EC cells. Thirdly, B-cell lymphoma-2 (Bcl2) and Multidrug Resistance-Associated Protein 4 (MRP4) genes were target genes of miR-125a-5p, which modulated paclitaxel resistance of Ishikawa/PA and HEC1A/PA cells through targeted silencing Bcl2 and MRP4. In conclusion, high-expression of CDKN2B-AS is associated with a poor response to paclitaxel of EC patients, and knockdown of CDKN2B-AS inhibits paclitaxel resistance through miR-125a-5p-Bcl2/MRP4 pathway in EC patients. Our findings help elucidate the molecular mechanisms of chemoresistance in EC patients.

Keywords: non-coding RNA, endometrial carcinoma, CDKN2B antisense RNA 1, miR-125a-5p, chemotherapy, paclitaxel

OPEN ACCESS

Edited by:
Massimo Nabissi, University of Camerino, Italy

Reviewed by:
Amanda Lee Hudson, Bill Walsh Translational Cancer Research Laboratory, Australia
Luigi Formisano, Vanderbilt University Medical Center, United States

***Correspondence:**
Lirong Meng
lrmeng@ipm.edu.mo

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *11 August 2018*
Accepted: *10 January 2019*
Published: *29 January 2019*

Citation:
Shang C, Ao CN, Cheong CC and Meng L (2019) Long Non-coding RNA CDKN2B Antisense RNA 1 Gene Contributes to Paclitaxel Resistance in Endometrial Carcinoma. Front. Oncol. 9:27. doi: 10.3389/fonc.2019.00027

INTRODUCTION

Endometrial cancer (EC) is one of the most common malignancy of the female reproductive tract and is increasing in incidence. The mortality of EC is next to ovarian and cervical cancer, and that is currently increasing year-by-year (1, 2). Chemotherapy is extensively used for treatment of EC, and it can significantly improve the prognosis and inhibit the recurrence and metastasis (3). Drug resistance reduces the sensitivity to chemotherapeutic drugs, and contributes a barrier, leading to treatment failure of EC. Accordingly, that is pivotal to identify the therapeutic target, re-sensitizing EC to chemotherapeutic drugs and its underlying mechanism. Several scholars have proposed diverse hypotheses, accounting for chemotherapy failure of EC patients, including DNA repair deregulation, aberrant function of efflux pumps, imbalance of signaling pathway, and so on Brasseur et al. (4), Shang et al. (5), and Liu et al. (6).

In recent years, non-coding RNAs (ncRNAs) have become a hotspot in the research of life science, especially in oncology, involving long non-coding RNAs (lncRNAs) and short non-coding RNA (e.g., microRNAs). Recent studies reported that some ncRNAs could be prognostic and diagnostic biomarkers, as well as being therapeutic targets for tumors (7–9). Increasing evidences have indicated that ncRNAs are involved in formation and progress of chemotherapy resistance, including EC (10–12). Our previous study revealed that Tumor Suppressor Candidate 7 (TUSC7) gene could specifically combine and silence miR-23b, and then inhibit the resistance of cisplatin and taxol in EC (12). Our preliminary experiments showed that CDKN2B antisense RNA 1 (CDKN2B-AS) gene was associated with paclitaxel resistance of EC, thus, we attempted to study the molecular mechanism of CDKN2B-AS triggered regulation for chemotherapy resistance of EC.

CDKN2B antisense RNA 1 (CDKN2B-AS) gene is a lncRNA gene identified by Pasmant et al. during the genetic study of a melanoma-neural system tumor family in 2007, that also named Antisense Non-coding RNA In The INK4 Locus (ANRIL) (13). Recent studies revealed that the CDKN2B-AS gene was up-regulated and acted as an oncogene in several malignant tumors, such as breast cancer, prostate cancer, and cervical cancer (14–17). At present, there is no report on CDKN2B-AS gene associated with EC. It is well-known that lncRNA could be associated with microRNA to regulate its expression and function. This study demonstrated that CDKN2B-AS could specifically silence miR-125a-5p expression of in EC cells.

MiR-125a-5p originates from 5′ end of pre-miR-125a and belongs to miR-125 family. Accumulating evidences found that miR-125a-5p was down-regulated in a variety of tumors and participated in the tumorigenesis and malignant progression of tumors by regulating its target genes to play the role of a tumor suppressor gene (18–20). The expression and potential function of miR-125a-5p in EC patients have still remained unclear. Our findings confirmed that B-cell lymphoma-2 (Bcl2) and Multidrug Resistance-Associated Protein 4 (MRP4) were the target genes of miR-125a-5p.

It is widely accepted that B-cell lymphoma-2 (Bcl2) gene is an important member of Bcl2 family and can inhibit apoptosis and promote cell survival, while Bcl2 has abnormal expression or function in almost all tumors. Defect to the Bcl-2 gene has been identified as a cause of resistance to cancer treatments. Bcl2 was negatively regulated by lncRNA GAS5 and contributed to doxorubicin resistance of bladder transitional cell carcinoma (21). Notch3-specific inhibition reduced the expression of Bcl2 and reversed paclitaxel resistance of ovarian cancer (22). Chon et al. reported that knockdown of Bcl2 antagonist of cell death (BAD) pathway increased the cisplatin resistance of Ishikawa and HEC1-A cells (23). However, it is not clear enough whether Bcl2 is involved in paclitaxel resistance of EC.

MRP4 gene belongs to ATP-binding cassette (ABC) transporters superfamily, that also named ATP Binding Cassette Subfamily C Member 4 (ABCC4). MRP4 can expel the chemotherapeutic drugs from the cells before they work, so as to reduce the damage of the chemotherapeutic drugs to the cells. (24). Overexpression of MRP4 mediated the acquired docetaxel resistance, targeting MRP4 treatment which re-sensitized docetaxel-resistant prostate cancer cells to docetaxel chemotherapy (25). The roles of MRP4 on chemotherapy resistance of EC need to be further studied.

Therefore, this study explored the clinical significance of CDKN2B-AS expression, and clarified the mechanism of CDKN2B-AS, contributing to paclitaxel resistance through miR-125a-5p/Bcl2&MRP4 pathway in EC.

MATERIALS AND METHODS

Clinical Specimens

In this study, 87 cases of EC patients were diagnosed and treated at Shengjing Hospital of China Medical University (Shenyang, China) from October 2015 to November 2016. The paracancerous normal endometrium tissue (PNET) and EC tissue specimens were obtained through hysteroscopy accompanied with biopsy, and diagnosed by two pathologists, in which the complete clinical data were collected as well. All the patients were not treated with radiotherapy or chemotherapy before diagnosis. The EC tissues specimens without treatment were used to detect the expression level of CDKN2B-AS and miR-125a-5p in EC patients. After diagnosis, all EC patients were treated with paclitaxel. After two or three cycles of chemotherapy, the curative effects were verified according to hysteroscopy with biopsy and imaging detection, and all EC patients were divided into two groups, including sensitive group ($n = 36$) and insensitive group ($n = 51$).

This study was conducted in accordance with the Declaration of Helsinki, and was approved by the Ethics Committee of Shengjing Hospital of China Medical University, and written informed consent was obtained from all participants as well.

Cell Lines and Culture

Human endometrial cell lines (HEC-251), human EC cell lines (Ishikawa, HEC-1A), and human embryonic kidney cell lines (HEK293T) were obtained from the Cell Resource Center of Chinese Academy of Medical Sciences (Beijing, China). Paclitaxel-resistant EC cell lines (Ishikawa/PA and HEC1A/PA cell lines) were set up previously from parental cell lines (Ishikawa, HEC-1A), and stored in our laboratory (12). Those cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum (FBS; Shanghai ExCell Biology, Inc., Shanghai, China) in a 95% air/5% CO_2 incubator at 37°C.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted using TRNzol reagent (TIANGEN, Beijing, China) and reversely transcribed into cDNA using lnRcute lncRNA First-Strand cDNA Synthesis Kit (TIANGEN, Beijing, China). The expression level of CDKN2B-AS was examined using an lnRcute lncRNA qPCR Detection Kit (TIANGEN, Beijing, China) in accordance with manufacturer's instructions. The sense primer of CDKN2B-AS was 5′-TGCTCT ATCCGCCAATCAGG-3′ and its antisense primer was 5′-GGG CCTCAGTGGCACATACC-3′ (26), in which the specificity was

checked, that could not be used to amplify CDKN2B gene. The expression level of miR-125a-5p was examined with Taqman Universal Master Mix II (Life Technologies, Carlsbad, CA, USA). The relative expression levels of CDKN2B-AS and miR-125a-5p were calculated using $2^{-\Delta\Delta CT}$ method after normalization with reference genes (β-actin and U6).

Cells Transfection

The inhibitor of CDKN2B-AS (smart silencer-CDKN2B-AS, ss-CDKN2B-AS) and its negative control (ss-NC) were designed and synthesized by Ribobio Co. (Guangzhou, China), and transfected into EC cells via HiPerFect reagent (QIAGEN, Hilden, Nordrhein-Westfalen, Germany) in a 6-well-culture plate in accordance with the manufacturer's instructions. The stable transfected cells were selected using Geneticin (Sigma-Aldrich, St Louis, MO, USA).

The agonist and antagonist of miR-125a-5p (agomiR-125a-5p and antagomiR-125a-5p), as well as their negative controls (agomiR-NC and antagomiR-NC) were synthesized by GenePharma Co. Ltd. (Shanghai, China). The expression plasmid of Bcl2 and MRP4 (pUC-Bcl2 and pUC-MRP4) and their negative control (pUC-NC) were synthesized by Cyagen Inc. (Santa Clara, CA, USA). The microRNAs and plasmids were transiently transfected into EC cells using HiPerFect reagent.

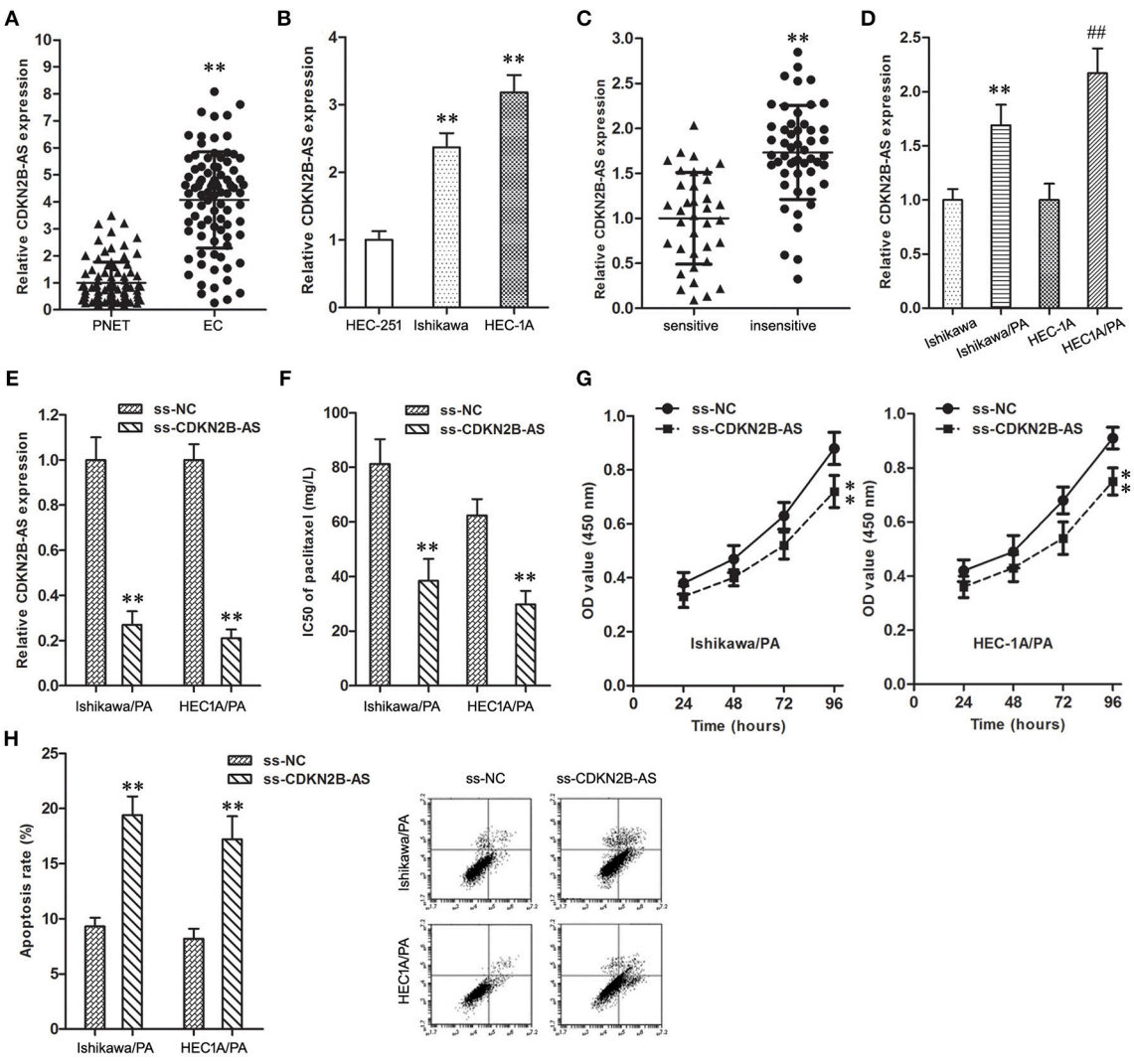


FIGURE 1 | High-expression of CDKN2B-AS was correlated with poor response to paclitaxel in EC**. (A)** The expression of CDKN2B-AS gene in PNET and EC specimens. $^{**}P < 0.01$ vs. PNET group. **(B)** The expression of CDKN2B-AS gene in HEC-251, Ishikawa and HEC-1A cells. $^{**}P < 0.01$ vs. HEC-251 group. **(C)** The expression of CDKN2B-AS gene in paclitaxel sensitive and insensitive EC specimens. $^{**}P < 0.01$ vs. sensitive group. **(D)** The expression of CDKN2B-AS gene in Ishikawa, Ishikawa/PA, HEC1A and HEC-1A/PA cells. $^{**}P < 0.01$ vs. Ishikawa group; $^{\#\#}P < 0.01$ vs. HEC1A group. **(E)** The expression of CDKN2B-AS gene in Ishikawa/PA and HEC-1A/PA cells. $^{**}P < 0.01$ vs. ss-NC group. **(F)** The IC50 of paclitaxel in Ishikawa/PA and HEC-1A/PA cells. $^{**}P < 0.01$ vs. ss-NC group. **(G)** The cell viability of Ishikawa/PA and HEC-1A/PA cells under treating with 10 mg/L paclitaxel. $^{**}P < 0.01$ vs. ss-NC group. **(H)** The cell apoptosis of Ishikawa/PA and HEC-1A/PA cells under treating with 10 mg/L paclitaxel. $^{**}P < 0.01$ vs. ss-NC group.

Cell Proliferation Assay

Enhanced Cell Counting Kit-8 (Beyotime Institute of Biotechnology, Beijing, China) was applied to examine cell proliferation. The cells in logarithmic growth phase were digested with trypsin, washed by phosphate-buffered saline (PBS), and suspended in the culture medium. Then, 2,000 cells in 100 μl medium were added into one pore of 96-well plates, 10 μl enhanced CCK-8 solution was added, and incubated for 1 h. The value of optical density was detected with the help of an MK3 microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at the wavelength of 450 nm.

Cell Apoptosis Detection

Annexin V-FITC/PI Apoptosis Detection Kit (Jiancheng, Nanjing, Jiangsu, China) was used to detect cell apoptosis rate according to the manufacturer's instructions. In addition, 2×10^5 cells were re-suspended in 500 μl binding buffer, 5 μl Annexin V-FITC and 5 μl Propidium iodide (PI) were added, and incubated at 25°C for 10 min. The apoptosis rate was detected and analyzed by FACScan flow cytometry with Diva 8.0 software (Becton Dickinson, Franklin Lakes, NJ, USA). The apoptosis rate was presented as the percentage of cells with FITC-Annexin V positive/PI negative in the right lower quadrant.

Drug Sensitivity Assay

The Ishikawa/PA and HEC1A/PA cells were treated with paclitaxel (10, 20, 50, 100, and 150 mg/L) (12). The cell viability was examined after 24 h. Then, the half maximal inhibitory concentration (IC50) of paclitaxel was calculated according to their dose-response curve.

Western Blotting

Protein of cells was extracted using a Protein Extraction Kit (Beyotime Institute of Biotechnology, Beijing, China), and quantified by using a Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Beijing, China). Protein (30 μg) was separated by polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. PVDF membrane was blocked with Tween-Tris-buffered saline (TTBS), containing 5% non-fat milk at 25°C for 2 h, and hybridized with Bcl2 and MRP4 antibodies (#2872 and #12705; Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After that, PVDF membrane was incubated with the second antibody at 25°C for 2 h. The PVDF membrane was treated with Chemiluminescent reagent (TIANGEN, Beijing, China) to visualize the bands. Then, the bands were analyzed by ImageJ software (NIH, Bethesda, MD, USA).

RNA Pull-Down Assay

The biotinylated probes for CDKN2B-AS and miR-125a-5p (bio-CDKN2B-AS-W and bio-miR-125a-5p-W, containing wild-type binding site), as well as their negative controls (bio-CDKN2B-AS-M and bio-miR-125a-5p-M, containing mutant binding site) were synthetized by GenePharma Co. Ltd. (Shanghai, China). Probes were dissolved in the buffer and incubated with Dynabeads M-280 Streptavidin (Thermo Fisher Scientific, Waltham, MA, USA) for 10 min at 25°C to form probe-coated beads. Those probe-coated beads were incubated with the lysates from Ishikawa/PA and HEC1A/PA cells, and eluted with the washing buffer. The pulled down RNAs were detected by RT-qPCR.

RNA Immunoprecipitation (RIP) Assay

RIP was assayed using a Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore Sigma, Burlington, MA, USA) according to the manufacturer's instructions and a previous study (27). Whole-cell lysate from Ishikawa/PA and HEC1A/PA cells was incubated with RIP buffer, containing magnetic beads conjugated with human anti-Ago2 antibody or negative control normal mouse IgG. Samples were incubated with Proteinase K, and immunoprecipitated RNA was isolated. The RNA concentration was measured by a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, MA, USA) and the quality of RNA was assessed by using a bio-analyzer (Agilent, Santa Clara, CA, USA). Furthermore, purified RNAs were extracted and analyzed by RT-qPCR to demonstrate the presence of the binding targets.

Luciferase Reporter Assay

The luciferase reporter plasmids (Bcl2-W and MRP4-W, containing wild-type binding site; Bcl2-M and MRP4-M, containing mutant binding site) were synthesized by the GenScript Co. (Piscataway, NJ, USA). HEK293T cells were co-transfected with the luciferase reporter plasmids and microRNAs, respectively. The Luciferase Reporter Kit (Beyotime Institute of Biotechnology, Beijing, China) was applied to detect the luciferase activity after 48 h in accordance with manufacturer's instructions.

Statistical Analysis

Each experiment was repeated five times, the data were presented as mean ± standard deviation (SD), and analyzed with Student's *t*-test and one-way analysis of variance (ANOVA) using SPSS 22.0

TABLE 1 | The correlation analysis between the expression of ANRIL gene and the clinicopathological characteristics of 87 EC tissues.

Factors		Case	Relative expression of CDKN2B-AS	*P*
Age(years)	>54	43	4.01 ± 1.23	0.257
	≤54	44	4.13 ± 1.38	
Pathological grading	I–II	55	3.81 ± 1.09	0.011*
	III	32	4.52 ± 1.42	
Estrogen receptor	Negative	39	3.85 ± 1.36	0.195
	Positive	48	4.25 ± 1.48	
Progesterone receptor	Negative	41	3.78 ± 1.22	0.056
	Positive	46	4.33 ± 1.41	
Chemotherapy	Sensitive	36	2.84 ± 1.43	< 0.001**
	Insensitive	51	4.92 ± 1.47	

*$P < 0.05$, **$P < 0.01$.

software (IBM, Armonk, NY, USA). If the $P < 0.05$, the difference was statistically significant.

RESULTS

The Expression Level of CDKN2B-AS Was Up-Regulated in EC

CDKN2B-AS gene in EC specimens was up-regulated in comparison with matched PNET specimens (**Figure 1A**). In addition, the expression level of CDKN2B-AS gene in Ishikawa and HEC-1A cells was significantly higher than that in HEC-251 cells (**Figure 1B**).

As shown in **Table 1**, the high-expression of CDKN2B-AS showed a positive correlation with high pathological grade of EC, however, that was not correlated with other clinical characteristics, including patient's age, estrogen receptor, and progesterone receptor. These findings suggested that CDKN2B-AS gene was involved in the progress of EC.

High-Expression of CDKN2B-AS Was Correlated With Poor Response to Paclitaxel in EC

Furthermore, the over-expression of CDKN2B-AS was associated with the low paclitaxel sensitivity of EC patients (**Table 1** and **Figure 1C**), and the expression level of CDKN2B-AS in Ishikawa/PA and HEC1A/PA cells was significantly higher than that in Ishikawa and HEC1A cells (**Figure 1D**), which preliminarily confirmed that CDKN2B-AS participated in the genesis of chemotherapy resistance in EC cells.

To verify the roles of CDKN2B-AS on chemotherapy resistance, the expression level of CDKN2B-AS was knockdown in Ishikawa/PA and HEC1A/PA cells to carry out loss-of-function assays (**Figure 1E**). Knockdown of CDKN2B-AS decreased the IC50 of paclitaxel from 81.29 and 62.37 mg/L to 38.41 and 29.74 mg/L in Ishikawa/PA and HEC-1A/PA cells, respectively, (**Figure 1F**), which certified that knockdown of CDKN2B-AS sensitized Ishikawa/PA and HEC-1A/PA cells to paclitaxel. Moreover, under treating with paclitaxel (10 mg/L), knockdown of CDKN2B-AS depressed cell viability (**Figure 1G**) and promoted apoptosis in Ishikawa/PA and HEC-1A/PA cells (**Figure 1H**). Besides, knockdown of CDKN2B-AS gene significantly increased paclitaxel-induced cytotoxicity.

CDKN2B-AS Silenced the Expression Level of miR-125a-5p in EC Cells

The online databases (TargetScan 7.1 and Starbase 2.0) predicted a specific combination between CDKN2B-AS and miR-125a-5p (**Figure 2A**). Then, the expression level of miR-125a-5p in EC specimens was down-regulated in comparison with matched PNET specimens (**Figure 2B**), and analysis of the co-expression patterns showed a negative correlation between CDKN2B-AS and miR-125a-5p in EC cells (**Figure 2C**, $r = -0.5609$, $P < 0.001$). Knockdown of CDKN2B-AS significantly up-regulated the expression level of miR-125a-5p in Ishikawa/PA and HEC-1A/PA cells (**Figure 2D**). In addition, RNA pull-down assay identified CDKN2B-AS could be combined with bio-miR-125a-5p-W probe, except for bio-miR-125a-5p-M (**Figure 2E**). Similarly, miR-125a-5p could be combined with bio-CDKN2B-AS-W probe, while that was not observed for bio-CDKN2B-AS-M (**Figure 2F**). Furthermore, RIP experiments confirmed that CDKN2B-AS and miR-125a-5p were both enriched in anti-Ago2 group (**Figures 2G,H**), and they were in a RNA-induced silencing complex (RISC). These results indicated that CDKN2B-AS and miR-125a-5p were associated with Ago-2 protein in a RISC complex, and CDKN2B-AS decreased the expression level of miR-125a-5p in a RISC-dependent manner, which was a classical regulatory manner of lncRNAs, regulating microRNAs.

Up-Regulation of the Expression Level of miR-125a-5p Inhibited Paclitaxel Resistance in EC

The expression level of miR-125a-5p in resistant EC patients was down-regulated in comparison with sensitive patients (**Figure 3A**). Similarly, compared with Ishikawa and HEC1A cells, the expression level of miR-125a-5p was also down-regulated in Ishikawa/PA and HEC1A/PA cells (**Figure 3B**).

To identify the role of miR-125a-5p on paclitaxel resistance, the expression level of miR-125a-5p was up-regulated in Ishikawa/PA and HEC1A/PA cells to carry out gain-of-function assays (**Figure 3C**). Additionally, up-regulation of miR-125a-5p decreased the IC50 of paclitaxel from 78.68 to 63.24 mg/L to 36.17 and 27.52 mg/L in Ishikawa/PA and HEC-1A/PA cells, respectively, (**Figure 3D**), which certified that up-regulation of miR-125a-5p sensitized Ishikawa/PA and HEC-1A/PA cells to paclitaxel. Moreover, under treatment with paclitaxel (10 mg/L), up-regulation of miR-125a-5p depressed cell viability (**Figure 3E**), and promoted apoptosis in Ishikawa/PA and HEC-1A/PA cells (**Figure 3F**). Up-regulation of miR-125a-5p also significantly increased paclitaxel-induced cytotoxicity.

MiR-125a-5p Mediated the Chemoresistance-Suppressive Effects of Knockdown of CDKN2B-AS in EC Cells

To determine whether the chemoresistance-suppressive effects of CDKN2B-AS knockdown were mediated by miR-125a-5p, antagomiR-125a-5p was transfected into stable ss-CDKN2B-AS cells (**Figure 4A**). Co-transfection of ss-CDKN2B-AS with antagomiR-125a-5p showed the lower paclitaxel sensitivity compared with co-transfection of ss-CDKN2B-AS with antagomiR-NC, and transfection with antagomiR-125a-5p rescued the inhibitory effects of ss-CDKN2B-AS on paclitaxel resistance (**Figures 4B–D**). Based on the above-mentioned results, we confirmed that miR-125a-5p mediates suppressive effects of CDKN2B-AS knockdown on paclitaxel resistance in EC cells.

Bcl2 and MRP4 Are Target Genes of miR-125a-5p in EC Cells

Based on information obtained from the online database TargetScan 7.1, Bcl2 and MRP4 might be targets of miR-125a-5 (**Figure 5A**). Firstly, up-regulation of miR-125a-5p remarkably

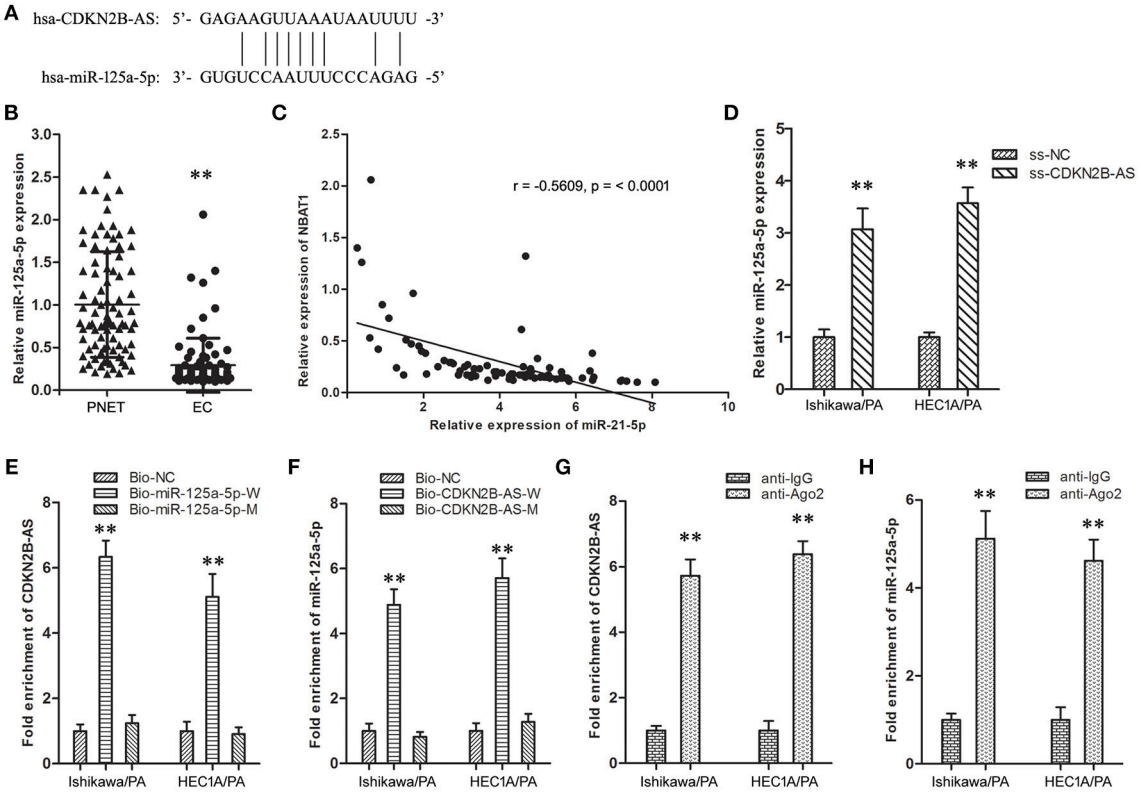


FIGURE 2 | CDKN2B-AS silenced specifically miR-125a-5p expression of in EC cells**. (A)** The predicted miR-125a-5p binding site in the CDKN2B-AS sequence. Short vertical lines indicated complementary nucleotides. **(B)** The expression of CDKN2B-AS gene in PNET and EC specimens. $^{**}P < 0.01$ vs. PNET group. **(C)** The co-expression patterns analysis between CDKN2B-AS and miR-125a-5p in EC. **(D)** The expression of miR-125a-5p gene in Ishikawa/PA and HEC-1A/PA cells. $^{**}P < 0.01$ vs. ss-NC group. **(E)** Detection of CDKN2B-AS using qRT-PCR in the sample pulled down by biotinylated miR-125a-5p. $^{**}P < 0.01$ vs. Bio-NC group. **(F)** Detection of miR-125a-5p using qRT-PCR in the sample pulled down by biotinylated CDKN2B-AS. $^{**}P < 0.01$ vs. Bio-NC group. **(G)** Detection of CDKN2B-AS using qRT-PCR in RNA immunoprecipitation complex. $^{**}P < 0.01$ vs. anti-IgG group. **(H)** Detection of miR-125a-5p using qRT-PCR in RNA immunoprecipitation complex. $^{**}P < 0.01$ vs. anti-IgG group.

depressed the expression level of Bcl2 and MRP4 in Ishikawa/PA and HEC-1A/PA cells (**Figure 5B**). Then, the specific binding sites of miR-125a-5p in the 3′UTR of Bcl2 and MRP4 were confirmed by luciferase reporter assay. In the Bcl2-W group, the luciferase activity of co-transfection with agomiR-125a-5p was inhibited, while no significant change was observed in their NC group; in the Bcl2-M group, the luciferase activity remained unchanged (**Figure 5C**). In addition, similar results were observed in MRP4 (**Figure 5D**). The results confirmed our prediction that Bcl2 and MRP4 are targets of miR-125a-5p in EC cells.

Enhancement of Bcl2 and MRP4 Partially Reversed the Suppressive Effects of Up-Regulation of miR-125a-5p on Paclitaxel Resistance in EC Cells

To uncover whether Bcl2 and MRP4 could reverse the chemoresistance-suppressive effects of up-regulation of miR-125a-5p in EC cells, the cells were co-transfected with agomiR-125a-5p, as well as pUC-Bcl2 or pUC-MRP4. Western blot analysis demonstrated that pUC-Bcl2 could up-regulate the expression level of Bcl2 in agomiR-125a-5p + pUC-Bcl2 group compared with agomiR-125a-5p + pUC-NC group (**Figure 6A**). In addition, similar results were achieved with pUC-MRP4 transfection (**Figure 6B**).

Drug sensitivity assay showed that up-regulation of miR-125a-5p sensitized Ishikawa/PA and HEC-1A/PA cells to paclitaxel, and over-expression of Bcl2 promoted the paclitaxel resistance and notably decreased miR-125a-5p-triggered increasing in paclitaxel sensitivity (**Figure 6C**). Similarly, over-expression of MRP4 also partially rescued the promotive effects of miR-125a-5p's on paclitaxel sensitivity (**Figure 6D**). Enhancement of Bcl2 and MRP4 could partially reverse the suppressive effects of up-regulation of miR-125a-5p on paclitaxel resistance in EC cells. These results illustrated that miR-125a-5p suppressed paclitaxel resistance by targeted silencing Bcl2 and MRP4 in EC cells.

DISCUSSION

Recent studies reported that lncRNAs were involved in the formation and maintenance of chemotherapy being resistance in

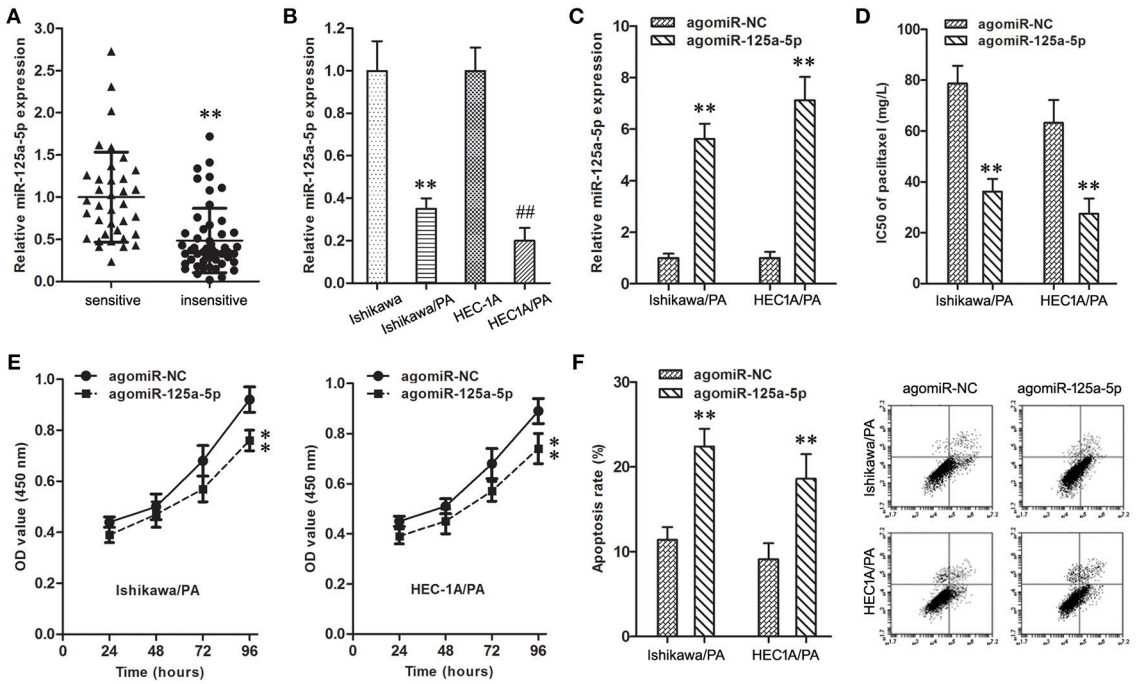


FIGURE 3 | Up-regulation of miR-125a-5p inhibited paclitaxel resistance in EC**. (A)** The expression of miR-125a-5p gene in paclitaxel sensitive and insensitive EC specimens. $^{**}P < 0.01$ vs. sensitive group. **(B)** The expression of miR-125a-5p gene in Ishikawa, Ishikawa/PA, HEC1A and HEC-1A/PA cells. $^{**}P < 0.01$ vs. Ishikawa group; $^{\#\#}P < 0.01$ vs. HEC1A group. **(C)** The expression of miR-125a-5p gene in Ishikawa/PA and HEC-1A/PA cells. $^{**}P < 0.01$ vs. agomiR-NC group. **(D)** The IC50 of paclitaxel in Ishikawa/PA and HEC-1A/PA cells. $^{**}P < 0.01$ agomiR-NC group. **(E)** The cell viability of Ishikawa/PA and HEC-1A/PA cells under treating with 10 mg/L paclitaxel. $^{**}P < 0.01$ vs. agomiR-NC group. **(F)** The cell apoptosis of Ishikawa/PA and HEC-1A/PA cells under treating with 10 mg/L paclitaxel. $^{**}P < 0.01$ vs. agomiR-NC group.

almost all malignant tumors, including EC. For example, KCNQ1 Opposite Strand/Antisense Transcript 1 (KCNQ1OT1) was closely associated with insensitivity of lung adenocarcinoma, and also knockdown of KCNQ1OT1 depressed the expression level of Multidrug Resistance Protein 1 (MDR1) and the paclitaxel resistance (28). Urothelial carcinoma associated 1 (UCA1) is an independent prognostic biomarker, which contributed to adriamycin resistance in gastric cancer (29).

Paclitaxel is a broad-spectrum antitumor drug that is used in chemotherapy for a variety of tumors, including EC. Paclitaxel is isolated from the bark of the Pacific yew tree, and it can bind to tubulin and inhibit the disassembly of microtubules, thereby causing the obstacle of cell division and the death of tumor cells. However, paclitaxel resistance is a major factor associated with treatment failure.

In this study, CDKN2B-AS gene was highly expressed in EC tissues and cell lines, and its over-expression was positively associated with high pathological grade of EC, which suggested that CDKN2B-AS gene was involved in the progress of EC. Furthermore, the over-expression of CDKN2B-AS was associated with the low sensitivity to paclitaxel of EC patients, which preliminarily confirmed that CDKN2B-AS participated in the genesis of chemotherapy resistance in EC. Lan et al. reported that knockdown of CDKN2B-AS inhibited the development of multidrug resistance in gastric cancer cells to paclitaxel or 5-fluorouracil (30). CDKN2B-AS also contributed to paclitaxel resistance of lung adenocarcinoma A549 cells (31).

To verify the roles of CDKN2B-AS on paclitaxel resistance, the impacts of CDKN2B-AS on paclitaxel resistance in EC were examined by a series of loss-of-function assays. Knockdown of CDKN2B-AS decreased IC50 of paclitaxel in Ishikawa/PA and HEC-1A/PA cells, and also promoted the cytotoxicity induced by paclitaxel, which showed that knockdown of CDKN2B-AS increased paclitaxel sensitivity in EC cells.

Recently, lncRNAs were proposed to act as miRNA sponges or competitive endogenous RNAs (ceRNAs), forming extensive regulatory networks, thereby negatively regulating miRNA expression (32). For instance, LINC00161 sensitized the osteosarcoma cells to cisplatin through sponging miR-645 (33); lncRNA AC023115.3 acted as a ceRNA for miR-26a to inhibit cisplatin resistance of glioma cells (34). Using online bioinformatics databases, we predicted a negative regulatory relationship between CDKN2B-AS and miR-125a-5p. Furthermore, non-coding RNAs can ordinarily form ribonucleoprotein (RNP) complexes with their partner proteins to exert their functions and miRNAs assembling with Argonaute (Ago) family proteins into an effective complex called RISC, mediating silencing the target gene (35, 36).

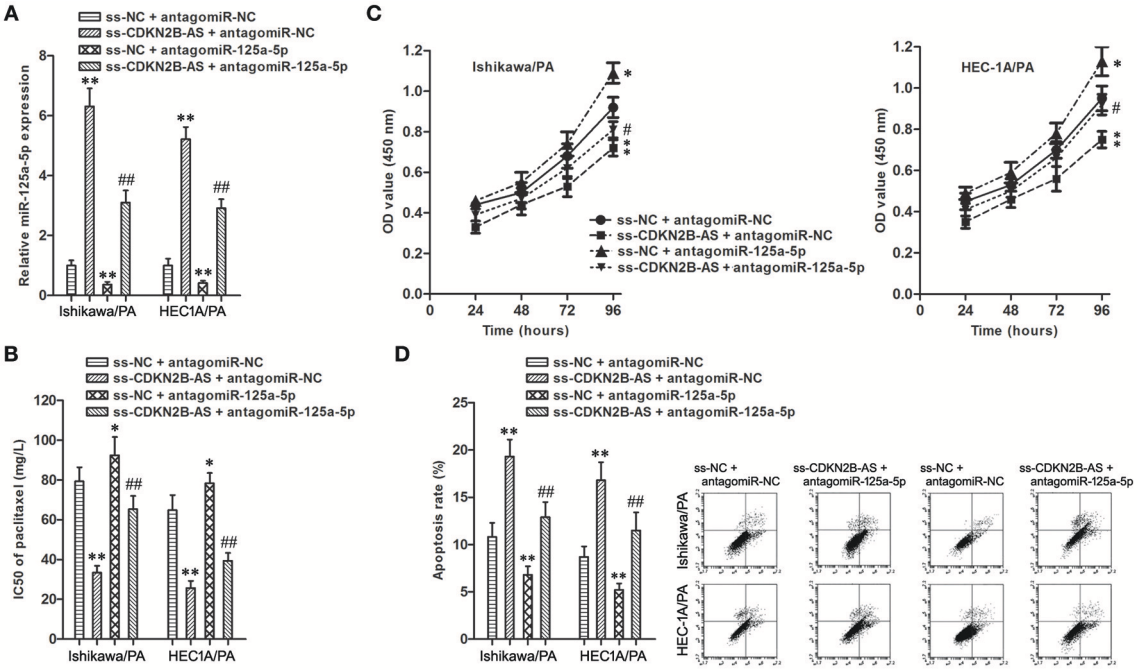


FIGURE 4 | miR-125a-5p mediated the chemoresistance-suppressive effects of CDKN2B-AS knockdown in EC cells**. (A)** The expression of miR-125a-5p gene in Ishikawa/PA and HEC-1A/PA cells. $^{**}P < 0.01$ vs. ss-NC + antagomiR-NC group; ## $P < 0.01$ vs. ss-NC + antagomiR-125a-5p group. **(B)** The IC50 of paclitaxel in Ishikawa/PA and HEC-1A/PA cells. $^{**}P < 0.01$ agomiR-NC group. $^{*}P < 0.05$ vs. ss-NC + antagomiR-NC group; $^{**}P < 0.01$ vs. ss-NC + antagomiR-NC group; ##$P < 0.01$ vs. ss-NC + antagomiR-125a-5p group. **(C)** The cell viability of Ishikawa/PA and HEC-1A/PA cells under treating with 10 mg/L paclitaxel. $^{**}P < 0.01$ vs. agomiR-NC group. $^{*}P < 0.05$ vs. ss-NC + antagomiR-NC group; $^{**}P < 0.01$ vs. ss-NC + antagomiR-NC group; #$P < 0.05$ vs. ss-NC + antagomiR-125a-5p group. **(D)** The cell apoptosis of Ishikawa/PA and HEC-1A/PA cells under treating with 10 mg/L paclitaxel. $^{**}P < 0.01$ vs. ss-NC + antagomiR-NC group; ##$P < 0.01$ vs. ss-NC + antagomiR-125a-5p group.

A limited number of researches on miR-125a-5p and chemotherapy resistance were conducted. To date, only one literature reported that up-regulation of miR-125a-3p could advance docetaxel sensitivity of breast cancer cells through modulation of BRCA1 signaling (37). Little attention has been paid to the miR-125a-5p-associated chemotherapy effect in EC, especially to paclitaxel. Therefore, the correlation between the expression of miR-125a-5p and paclitaxel resistance in EC was here examined, and the results revealed that its low-expression was related to poor paclitaxel response of EC patients. Furthermore, enhanced miR-125a-5p decreased IC50 of paclitaxel in Ishikawa/PA and HEC-1A/PA cells, and promoted the paclitaxel-induced cytotoxicity, which showed that over-expression of miR-125a-5p could increase paclitaxel sensitivity in EC cells.

We attempted to explore whether the influence of CDKN2B-AS on paclitaxel resistance is mediated by miR-125a-5p in EC. In detail, the regulatory relationship between CDKN2B-AS and miR-125a-5p was confirmed based on the following results: (1) Knockdown of CDKN2B-AS significantly increased the expression level of miR-125a-5p in Ishikawa/PA and HEC-1A/PA cells; (2) RNA pull-down assay revealed CDKN2B-AS function via interaction with miR-125a-5p; (3) RIP experiment confirmed that CDKN2B-AS and miR-125a-5p were associated with Ago-2 protein in a RISC complex, and suggested that CDKN2B-AS decreased the expression level of miR-125a-5p in a RISC-dependent manner; (4) miR-125a-5p deficiency rescued the inhibitory effect of knockdown of CDKN2B-AS on paclitaxel resistance. Based on the above-mentioned achievements, we confirmed that miR-125a-5p mediates the suppressive effects of knockdown of CDKN2B-AS on paclitaxel resistance in EC cells.

As microRNAs play their roles through regulating their target genes, such as miR-16-1 and FUBP1 (38), we predicted that Bcl2 and MRP4 genes might be miR-125a-5p's target genes based on the online bioinformatics database. In addition, Tong et al. reported that miR-125a-5p could regulate cytobiological phenotypes of colon cancer via targeting Bcl2 (20). A series of following gain-of-function experiments, such as luciferase reporter assay and Western blotting, demonstrated that Bcl2 and MRP4 genes were the target genes of miR-125a-5p. To sum up, we speculate that miR-125a-5p might modulate the paclitaxel resistance of EC cells through targeted silencing Bcl2 and MRP4.

To verify this hypothesis, knockdown of Bcl2 and MRP4 by miR-125a-5p enhancement was rescued by transfection with pUC-Bcl2 and pUC-MRP4. The following experiments found that up-regulation of Bcl2 and MRP4 separately reversed the regulatory roles of miR-125a-5p on paclitaxel resistance in Ishikawa/PA and HEC-1A/PA cells. Besides, miR-125a-5p might promote cell apoptosis and reduce paclitaxel discharge

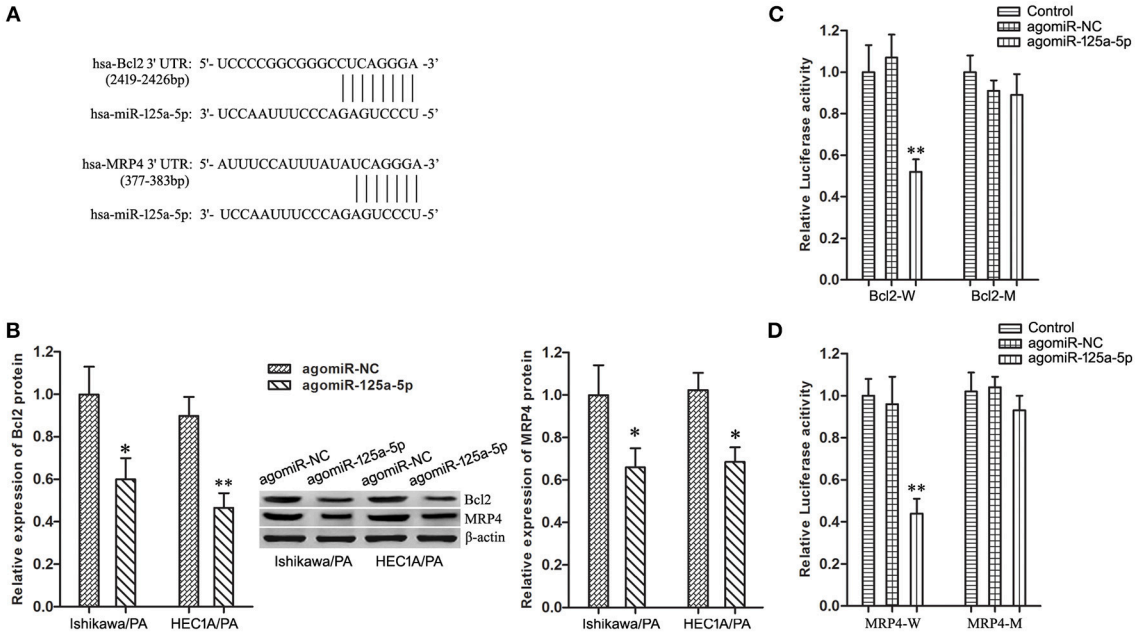


FIGURE 5 | Bcl2 and MRP4 are target genes of miR-125a-5p in EC cells**. (A)** The predicted miR-125a-5p binding site in the 3′UTR of Bcl2 and MRP4 mRNA. Short vertical lines indicated complementary nucleotides. **(B)** The expression of Bcl2 and MRP4 protein in Ishikawa/PA and HEC-1A/PA cells. *$P < 0.05$ vs. agomiR-NC group. **$P < 0.01$ vs. agomiR-NC group. **(C)** The relative luciferase reporter assay of HEK 293T cells co-transfected with Bcl2-W or Bcl2-M and agomiR-125a-5p or agomiR-NC. **$P < 0.01$ vs. Bcl2-W + agomiR-NC group. **(D)** The relative luciferase reporter assay of HEK 293T cells co-transfected with MRP4-W or MRP4-M and agomiR-125a-5p or agomiR-NC. **$P < 0.01$ vs. MRP4-W + agomiR-NC group.

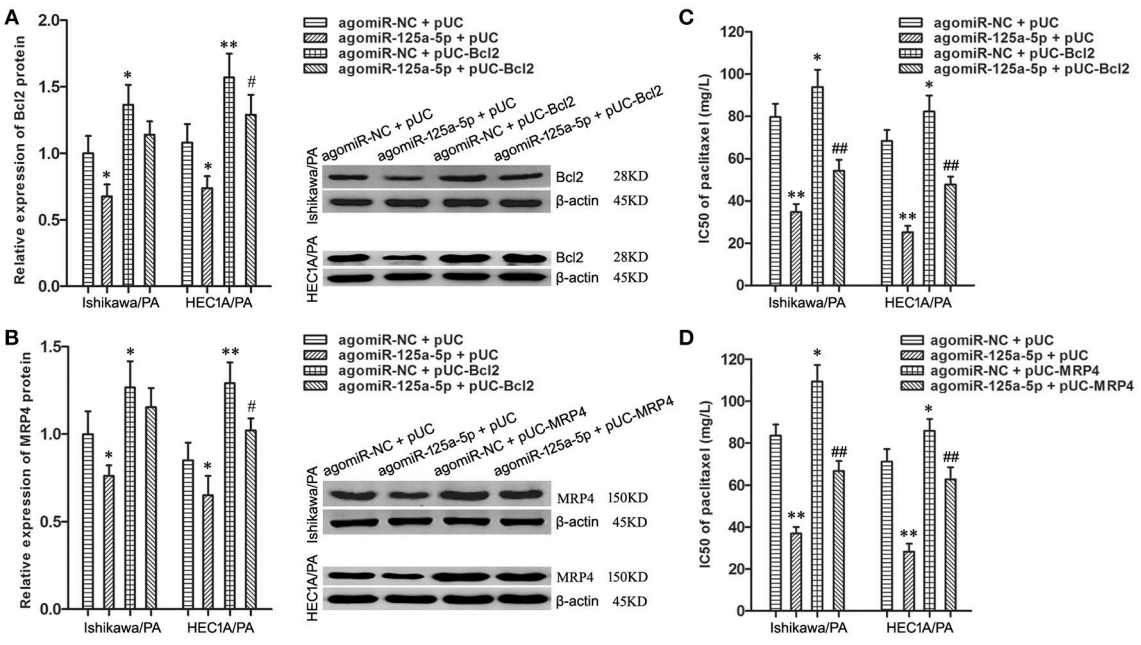


FIGURE 6 | Enhancement of Bcl2 and MRP4 partially reversed the suppressive effects of up-regulation of miR-125a-5p on paclitaxel resistance in EC cells. **(A)** The expression of Bcl2 protein in Ishikawa/PA and HEC-1A/PA cells. *$P < 0.05$ vs. agomiR-NC + pUC group; **$P < 0.01$ vs. agomiR-NC + pUC group; #$P < 0.05$ vs. agomiR-125a-5p + pUC group. **(B)** The expression of MRP4 protein in Ishikawa/PA and HEC-1A/PA cells. *$P < 0.05$ vs. agomiR-NC + pUC group; **$P < 0.01$ vs. agomiR-NC + pUC group; #$P < 0.05$ vs. agomiR-125a-5p + pUC group. **(C,D)**: The IC50 of paclitaxel in Ishikawa/PA and HEC-1A/PA cells. *$P < 0.05$ vs. agomiR-NC + pUC group; **$P < 0.01$ vs. agomiR-NC + pUC group; ##$P < 0.01$ vs. agomiR-125a-5p + pUC group.

through silencing targeted Bcl2 and MRP4, and then re-sensitize Ishikawa/PA and HEC-1A/PA cells to paclitaxel.

To sum up, CDKN2B-AS is specifically combined with miR-125a-5p and also down-regulates its expression level in EC, weakening the ability of miR-125a-5p to silence its target genes, and then up-regulates the expression of Bcl2 and MRP. Additionally, Bcl2 can inhibit apoptosis and promote cell survival from paclitaxel-induced cytotoxicity. Moreover, an overexpressed MRP4 pumps paclitaxel out of EC cells, and reduced intracellular paclitaxel concentration and paclitaxel-induced chemotherapy damage. Accordingly, CDKN2B-AS can desensitize EC cells into paclitaxel, and contribute to the formation of chemotherapy resistance through miR-125a-5p/Bcl2 and miR-125a-5p/MRP4 pathways.

In conclusion, high-expression of CDKN2B-AS is associated with a poor response to paclitaxel of EC patients, and knockdown of CDKN2B-AS inhibits paclitaxel resistance through miR-125a-5p-Bcl2/MRP4 pathway in EC. Our findings helped elucidate the underlying mechanism of chemotherapeutic resistance in EC patients.

AUTHOR CONTRIBUTIONS

LM and CS conceived and designed the project. CS and CA completed the experiments and acquired the data. CA and CC analyzed the data. All authors read and approved the final version of this manuscript.

FUNDING

This study was funded by the Foundation of Macau Polytechnic Institute (RP/ESS-03/2016).

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics 2018. *CA Cancer J Clin*. (2018) 68:7–30. doi: 10.3322/caac.21442
2. Yamagami W, Nagase S, Takahashi F, Ino K, Hachisuga T, Aoki D, et al. Clinical statistics of gynecologic cancers in Japan. *J Gynecol Oncol*. (2017) 28:e32. doi: 10.3802/jgo.2017.28.e32
3. Suri V, Arora A. Management of endometrial cancer: a review. *Rev Recent Clin Trials* (2015) 10:309–16. doi: 10.2174/1574887110666150923115228
4. Brasseur K, Gévry N, Asselin E. Chemoresistance and targeted therapies in ovarian and endometrial cancers. *Oncotarget* (2017) 8:4008–42. doi: 10.18632/oncotarget.14021
5. Shang C, Lang B, Meng LR. Blocking NOTCH pathway can enhance the effect of EGFR inhibitor through targeting CD133+ endometrial cancer cells. *Cancer Biol Ther*. (2018) 19:113–19. doi: 10.1080/15384047.2016.1250985
6. Liu Z, Xie D, Zhang H. Long non-coding RNA neuroblastoma-associated transcript 1 gene inhibits malignant cellular phenotypes of bladder cancer through miR-21/SOCS6 axis. *Cell Death Dis*. (2018) 9:1042. doi: 10.1038/s41419-018-1090-z
7. Zheng P, Li H, Xu P, Wang X, Shi Z, Han Q, et al. High lncRNA HULC expression is associated with poor prognosis and promotes tumor progression by regulating epithelial-mesenchymal transition in prostate cancer. *Arch Med Sci*. (2018) 14:679–86. doi: 10.5114/aoms.2017.69147
8. Sun L, Su Y, Liu X, Xu M, Chen X, Zhu Y, et al. Serum and exosome long non-coding RNAs as potential biomarkers for hepatocellular carcinoma. *J Cancer* (2018) 9:2631–39. doi: 10.7150/jca.24978
9. Yang S, Sun Z, Zhou Q, Wang W, Wang G, Song J, et al. MicroRNAs, long non-coding RNAs, and circular RNAs: potential tumor biomarkers and targets for colorectal cancer. *Cancer Manag Res*. (2018) 10:2249–57. doi: 10.2147/CMAR.S166308
10. Shang C, Tang W, Pan C, Hu X, Hong Y. Long non-coding RNA TUSC7 inhibits temozolomide resistance by targeting miR-10a in glioblastoma. *Cancer Chemother Pharmacol*. (2018) 81:671–8. doi: 10.1007/s00280-018-3522-y
11. Zhang J, Zhao B, Chen X, Wang Z, Xu H, Huang B. Silence of long non-coding RNA NEAT1 inhibits malignant biological behaviors and chemotherapy resistance in gastric cancer. *Pathol Oncol Res*. (2018) 24:109–13. doi: 10.1007/s12253-017-0233-3
12. Shang C, Lang B, Ao CN, Meng L. Long non-coding RNA tumor suppressor candidate 7 advances chemotherapy sensitivity of endometrial carcinoma through targeted silencing of miR-23b. *Tumour Biol*. (2017) 39:1010428317707883. doi: 10.1177/1010428317707883
13. Pasmant E, Laurendeau I, Héron D, Vidaud M, Vidaud D, Bièche I. Characterization of a germ-line deletion, including the entire INK4/ARF locus, in a melanoma-neural system tumor family: identification of ANRIL, an antisense non-coding RNA whose expression coclusters with ARF. *Cancer Res*. (2007) 67:3963–9. doi: 10.1158/0008-5472.CAN-06-2004
14. Xu ST, Xu JH, Zheng ZR, Zhao QQ, Zeng XS, Cheng SX, et al. Long non-coding RNA ANRIL promotes carcinogenesis via sponging miR-199a in triple-negative breast cancer. *Biomed Pharmacother*. (2017) 96:14–21. doi: 10.1016/j.biopha.2017.09.107
15. Zhao B, Lu YL, Yang Y, Hu LB, Bai Y, Li RQ, et al. Overexpression of lncRNA ANRIL promoted the proliferation and migration of prostate cancer cells via regulating let-7a/TGF-β1/Smad signaling pathway. *Cancer Biomark*. (2018) 21:613–20. doi: 10.3233/CBM-170683
16. Zhang JJ, Wang DD, Du CX, Wang Y. Long non-coding RNA ANRIL promotes cervical cancer development by acting as a sponge of miR-186. *Oncol Res*. (2017) 22: 345–52. doi: 10.3727/096504017X14953948675449
17. Ma J, Li T, Han X, Yuan H. Knockdown of LncRNA ANRIL suppresses cell proliferation, metastasis, and invasion via regulating miR-122-5p expression in hepatocellular carcinoma. *J Cancer Res Clin Oncol*. (2018) 144:205–214. doi: 10.1007/s00432-017-2543-y
18. Zhong L, Sun S, Shi J, Cao F, Han X, Chen Z. MicroRNA-125a-5p plays a role as a tumor suppressor in lung carcinoma cells by directly targeting STAT3. *Tumour Biol*. (2017) 39:1010428317697579. doi: 10.1177/1010428317697579
19. Zhang Y, Xue C, Zhu X, Zhu X, Xian H, Huang Z. Suppression of microRNA-125a-5p upregulates the TAZ-EGFR signaling pathway and promotes retinoblastoma proliferation. *Cell Signal*. (2016) 28:850–60. doi: 10.1016/j.cellsig.2016.04.002
20. Tong Z, Liu N, Lin L, Guo X, Yang D, Zhang Q. miR-125a-5p inhibits cell proliferation and induces apoptosis in colon cancer via targeting Bcl2, Bcl2L12 and MCL1. *Biomed Pharmacother*. (2015) 75:129–36. doi: 10.1016/j.biopha.2015.07.036
21. Zhang H, Guo Y, Song Y, Shang C. Long non-coding RNA GAS5 inhibits malignant proliferation and chemotherapy resistance to doxorubicin in bladder transitional cell carcinoma. *Cancer Chemother Pharmacol*. (2017) 79:49–55. doi: 10.1007/s00280-016-3194-4
22. Kang H, Jeong JY, Song JY, Kim TH, Kim G, Huh JH, et al. Notch3-specific inhibition using siRNA knockdown or GSI sensitizes paclitaxel-resistant ovarian cancer cells. *Mol Carcinog*. (2016) 55:1196–209. doi: 10.1002/mc.22363
23. Chon HS, Marchion DC, Xiong Y, Chen N, Bicaku E, Stickles XB, et al. The BCL2 antagonist of cell death pathway influences endometrial cancer cell sensitivity to cisplatin. *Gynecol Oncol*. (2012) 124:119–24. doi: 10.1016/j.ygyno.2011.09.020
24. Kochel TJ, Reader JC, Ma X, Kundu N, Fulton AM. Multiple drug resistance-associated protein (MRP4) exports prostaglandin E2 (PGE2) and contributes to metastasis in basal/triple negative breast cancer. *Oncotarget* (2017) 8:6540–54. doi: 10.18632/oncotarget.14145
25. Li YF, Ji HH, Zhang ZL, Zhang TT, Gan W, Zhang SF. Targeting MRP4 expression by anti-androgen treatment reverses MRP4-mediated docetaxel

resistance in castration-resistant prostate cancer. *Oncol Lett*. (2017) 14:1748–56. doi: 10.3892/ol.2017.6357
26. Kotake Y, Nakagawa T, Kitagawa K, Suzuki S, Liu N, Kitagawa M, Xiong Y. Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15(INK4B) tumor suppressor gene. *Oncogene* (2011) 30:1956–62. doi: 10.1038/onc.2010.568
27. Shang C, Guo Y, Hong Y, Xue YX. Long non-coding RNA TUSC7, a target of miR-23b, plays tumor-suppressing roles in human gliomas. *Front Cell Neurosci*. (2016) 10:235. doi: 10.3389/fncel.2016.00235
28. Ren K, Xu R, Huang J, Zhao J, Shi W. Knockdown of long non-coding RNA KCNQ1OT1 depressed chemoresistance to paclitaxel in lung adenocarcinoma. *Cancer Chemother Pharmacol*. (2017) 80:243–50. doi: 10.1007/s00280-017-3356-z
29. Shang C, Guo Y, Zhang J, Huang B. Silence of long non-coding RNA UCA1 inhibits malignant proliferation and chemotherapy resistance to adriamycin in gastric cancer. *Cancer Chemother Pharmacol*. (2016) 77:1061–7. doi: 10.1007/s00280-016-3029-3
30. Lan WG, Xu DH, Xu C, Ding CL, Ning FL, Zhou YL, et al. Silencing of long non-coding RNA ANRIL inhibits the development of multidrug resistance in gastric cancer cells. *Oncol Rep*. (2016) 36:263–70. doi: 10.3892/or.2016.4771
31. Xu R, Mao Y, Chen K, He W, Shi W, Han Y. The long non-coding RNA ANRIL acts as an oncogene and contributes to paclitaxel resistance of lung adenocarcinoma A549 cells. *Oncotarget* (2017) 8:39177–84. doi: 10.18632/oncotarget.16640
32. Thomson DW, Dinger ME. Endogenous microRNA sponges: evidence and controversy. *Nat Rev Genet*. (2016) 17:272–83. doi: 10.1038/nrg.2016.20
33. Wang Y, Zhang L, Zheng X, Zhong W, Tian X, Yin B, et al. Long non-coding RNA LINC00161 sensitises osteosarcoma cells to cisplatin-induced apoptosis by regulating the miR-645-IFIT2 axis. *Cancer Lett*. (2016) 382:137–46. doi: 10.1016/j.canlet.2016.08.024
34. Ma B, Yuan Z, Zhang L, Lv P, Yang T, Gao J, et al. Long non-coding RNA AC023115.3 suppresses chemoresistance of glioblastoma by reducing autophagy. *Biochim Biophys Acta* (2017) 1864:1393–404. doi: 10.1016/j.bbamcr.2017.05.008
35. Kobayashi H, Tomari Y. RISC assembly: Coordination between small RNAs and Argonaute proteins. *Biochim Biophys Acta* (2016) 1859:71–81. doi: 10.1016/j.bbagrm.2015.08.007
36. Liu X, Zheng J, Xue Y, Yu H, Gong W, Wang P, et al. PIWIL3/OIP5-AS1/miR-367-3p/CEBPA feedback loop regulates the biological behavior of glioma cells. *Theranostics* (2018) 8:1084–105. doi: 10.7150/thno.21740
37. Xu X, Lv YG, Yan CY, Yi J, Ling R. Enforced expression of hsa-miR-125a-3p in breast cancer cells potentiates docetaxel sensitivity via modulation of BRCA1 signaling. *Biochem Biophys Res Commun*. (2016) 479:893–900. doi: 10.1016/j.bbrc.2016.09.087
38. Zhao D, Zhang Y, Song L. MiR-16-1 targeted silences far upstream element binding protein 1 to advance the chemosensitivity to adriamycin in gastric cancer. *Pathol Oncol Res*. (2018) 24:483–88. doi: 10.1007/s12253-017-0263-x

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 17 May 2019
doi: 10.3389/fonc.2019.00344

Telomerase and Telomeres in Endometrial Cancer

Rafah A. A. Alnafakh[1,2], *Meera Adishesh*[1,2], *Lucy Button*[1,2], *Gabriele Saretzki*[3] *and Dharani K. Hapangama*[1,2]*

[1] *Liverpool Women's Hospital NHS Foundation Trust, Liverpool, United Kingdom,* [2] *Department of Women's and Children's Health, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom,* [3] *The Ageing Biology Centre and Institute for Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, United Kingdom*

Telomeres at the termini of human chromosomes are shortened with each round of cell division due to the "end replication problem" as well as oxidative stress. During carcinogenesis, cells acquire or retain mechanisms to maintain telomeres to avoid initiation of cellular senescence or apoptosis and halting cell division by critically short telomeres. The unique reverse transcriptase enzyme complex, telomerase, catalyzes the maintenance of telomeres but most human somatic cells do not have sufficient telomerase activity to prevent telomere shortening. Tissues with high and prolonged replicative potential demonstrate adequate cellular telomerase activity to prevent telomere erosion, and high telomerase activity appears to be a critical feature of most (80–90%) epithelial cancers, including endometrial cancer. Endometrial cancers regress in response to progesterone which is frequently used to treat advanced endometrial cancer. Endometrial telomerase is inhibited by progestogens and deciphering telomere and telomerase biology in endometrial cancer is therefore important, as targeting telomerase (a downstream target of progestogens) in endometrial cancer may provide novel and more effective therapeutic avenues. This review aims to examine the available evidence for the role and importance of telomere and telomerase biology in endometrial cancer.

Keywords: endometrial cancer, telomere, telomerase, endometrium, TERRA, TRAP, hTERT, hTERC

OPEN ACCESS

Edited by:
Paola Gehrig,
University of North Carolina at Chapel Hill, United States

Reviewed by:
Marilyn Huang,
Sylvester Comprehensive Cancer Center, United States
Saori Furuta,
University of Toledo, United States

***Correspondence:**
Dharani K. Hapangama
dharani@liv.ac.uk

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *08 January 2019*
Accepted: *15 April 2019*
Published: *17 May 2019*

Citation:
Alnafakh RAA, Adishesh M, Button L, Saretzki G and Hapangama DK (2019) Telomerase and Telomeres in Endometrial Cancer. Front. Oncol. 9:344. doi: 10.3389/fonc.2019.00344

INTRODUCTION

Telomeres are specialized structures that are found at the ends of linear chromosomes, containing a tandemly repeated specific DNA sequence and associated protective proteins. The protective function of telomeres in preventing the loss of genomic DNA in proliferating cells is well-established (1–3). As telomeres shorten with each cell division, critically short telomeres initiate cellular senescence or an apoptotic pathway, leading to cessation of cell division, therefore telomere shortening is a major tumor suppressor mechanism (4, 5). In addition, oxidative stress is an important additional cause for telomere shortening (6, 7). Telomerase is a unique reverse transcriptase enzyme (8) that is able to add repetitive telomeric sequences *de novo* onto telomeric ends (9) that are continually lost during DNA replication due to oxidative stress and the "end replication problem" in mitotic cells. Thus, telomerase prevents shortening and maintains telomeres. However, most human somatic cells do not have significant levels of telomerase activity whereas cells, such as embryonic stem cells and most cancer cells exhibit high telomerase activity while adult tissue stem cells are potentially able to up-regulate telomerase upon activation (10–12).

Human endometrium is a unique somatic organ that contains a relatively high yet dynamic pattern of telomerase activity that changes according to the menstrual cycle, correlating with endometrial cellular proliferation (13, 14). Further evidence from benign endometrium also suggests that telomerase activity is a fundamental requirement for endometrial cell proliferation and survival (15). The involvement of telomerase in most cancer-related cellular abnormalities in cell fate regulatory pathways prompted many studies into telomerase and telomeres in a variety of cancers including endometrial cancer (16–18).

Endometrial cancer is the fourth common cancer in women in the UK and is the commonest gynecological cancer (CRUK). Increasing obesity and longevity have both caused the incidence of EC to increase at an alarming rate. For example, in the United Kingdom, the incidence of EC increased by more than 40% since 1993. European estimates predict a 100% increase in the incidence by 2025 not only in older post-menopausal women but also in younger women (19). Figures from the UK report that mortality associated with EC has risen by 21% over the last decade in an era of improving survival rates for most other cancers, highlighting the inequality and lack of translation of advances in cancer research to EC (CRUK) (20). The survival rates for high-grade EC are exceptionally poor, similar to ovarian cancer; and the traditional surgical treatment is associated with significant morbidity and mortality for many women even when presented with early disease due to frequently occurring co-morbidities and obesity (21). Urgent novel therapeutic options are therefore needed to prevent, treat as well as to avoid progression of EC.

Although EC is an important disease with a significant clinical and economic consequence, the molecular biology of endometrial carcinogenesis is not well-described or understood when compared with other female-specific malignancies, such as breast or ovarian cancer. Human endometrium is a unique organ with a massive regenerative potential (22) and is the main target organ for ovarian steroid hormone action (23). While being a hormonally responsive tissue, endometrium responds rather differently to the same steroid hormones than other hormone responsive organs, such as breast tissue (23, 24). This has made it difficult to translate the pioneering discoveries made in other cancers to EC management and therapy. Unlike most other somatic tissue, benign endometrial tissue demonstrate high telomerase activity, and telomerase has a pivotal functional role in healthy endometrial cell proliferation (14, 15). High telomerase activity is observed in most epithelial cancers, and the carcinogenesis process in those tissues involved ectopic expression of telomerase components and genetic alterations, such as activation mutations in promotors of the vital genes. In the endometrium however, the high telomerase activity is a feature even without being associated with driver mutations. It is therefore intriguing to explore the distinctive endometrial telomerase biology relevant to EC and we hypothesize EC to have a unique telomerase biology that is different to the other cancers. Furthermore, EC is a hormone driven disease and advanced and recurrent ECs are treated with progesterone which regress these tumors albeit without extending survival (24). It is therefore of particular interest to examine telomerase as a downstream progesterone target in the endometrium (15) which can be manipulated for therapeutic utility in progesterone resistant ECs. This review therefore focuses on the significance and role of telomerase and telomere biology in EC, highlighting recent findings proposing some aspects of telomerase biology as potential therapeutic targets for EC (25).

METHOD

We performed systematic PubMed (Medline) and Ovid searches using a combination of relevant controlled vocabulary terms and free-text terms related to telomeres and telomerase. The key words used included: telomerase, telomeres, telomere length, telomerase reverse transcriptase (TERT), telomeric RNA component (TERC), shelterin proteins, telomerase associated proteins, with endometrium, endometriosis, endometrial hyperplasia, endometrial cancer (EC), endometrial carcinomas, uterine cancer, cancers. All studies investigating telomerase or telomere biology in endometrium in women or animals or respective cell lines, either primary cells or tissue explants in culture, and published from database inception until December 2018, were included in this review.

TELOMERES

Structure

Human telomeres consist of a repetitive TTAGGG hexanucleotide sequence bound by six-proteins forming the shelterin complex [(26) **Figure 1**]. In normal somatic cells the average length of telomeres is around 5–15 kilobases and they shorten *in vitro* by 30–200 base pairs (bp) during every cell division depending on the cell type and environmental conditions (34). Under increased oxidative stress telomere shortening rate per cell division can increase substantially, up-to 500 bp (6).

Most of the non-coding telomeric DNA is double-stranded whilst the terminal nucleotides (nt) form the single stranded 3′ G-rich overhang, which serves as the primer for telomerase action (35) and also protect telomeres from being recognized as DNA damage. This forms a D-loop (Displacement loop) facilitating repetitive DNA sequences to be added by telomerase (36).

Another mechanism to protect telomeres from being recognized as DNA damage is the formation a t-*loop,* which is a specific higher order conformation. This large duplex loop-back structure is formed via invasion of the single-stranded telomeric 3′ overhang into the double stranded telomeric repeat array (37). The authors suggested that the t-loops are the basic mechanism by which the telomeric nucleoprotein complex sequesters chromosome ends from the DNA damage pathway, preventing inappropriate DNA repair and telomerase action (37).

The shelterin complex (**Figure 1**) includes telomeric repeat binding factor 1 and 2 (TRF1 and TRF2), which are homodimeric proteins that bind specifically to double-strand telomeric DNA (27, 37). In contrast, Protection of telomeres 1(POT1) binds to the single-stranded region of the telomere (28) and forms a heterodimer with TPP1 (38). The Repressor/activator protein 1 (RAP1) is recruited through its relation with TRF2 (31) and TRF1-interacting protein 2 (TIN2) is the central part of

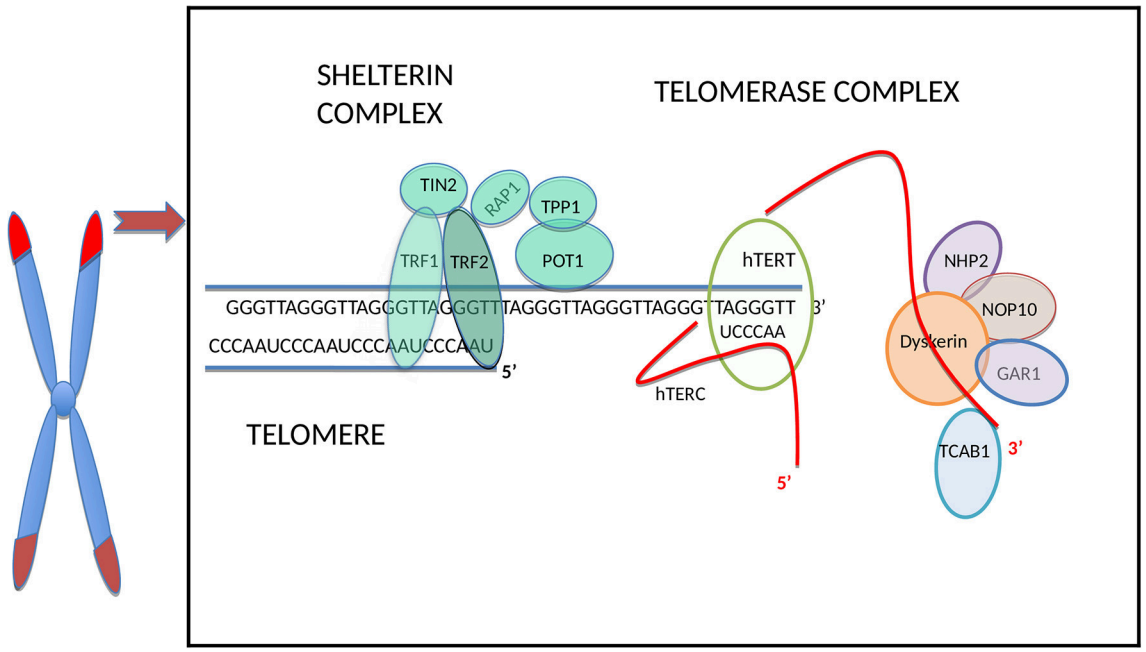


FIGURE 1 | Schematic illustration of the telomere and main telomerase complex components. The human telomere and telomerase enzyme complex (only one half of the dimeric holoenzyme complex is shown for clarity), adapted from Hapangama et al. (14). From all sheltrin proteins only telomere repeat binding factors 1 (TRF1) and 2 (TRF2) (27) bind directly to the double-stranded telomeric sequence, and protection of telomeres protein-1 (POT1) (28) binds to the single-stranded overhang; hence these are termed as telomere binding proteins and they interact with remaining shelterin proteins TIN2 (binds to TRF1 and TRF2) (29, 30), RAP1 (binds to TRF2) (31) and TPP1 (binds to POT1) (32). The TERC H/ACA region located at the 3′ end binds to dyskerin and the other telomerase associated proteins: NOP10, NHP2, and GAR1 (14). The hTERC at the 3′ end binds also to telomerase Cajal body protein 1 (TCAB1) (33).

the shelterin complex (29) and it interacts with TRF1, TRF2 (30), and POT1/TPP1 (32) to assure structural integrity of the complex. Removal of individual shelterin proteins has been shown to stimulate a DNA damage response (DDR) pathway: TRF1 prevents the stimulation of both ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) pathways (39); TRF2 and RAP1 inhibit the activation of the ATM pathway (40, 41) and homology-directed recombination (HDR) (42) while TPP1 bound POT1 (POT1a/b in mouse) inhibit the ATR pathway (43). TRF2 plays a vital role in facilitating this t-loop formation (44). Super-resolution fluorescence light microscopy visualization of the t-loop has shown that the strand invasion point can be located at almost any point along the duplex DNA, resulting in highly variable t-loops sizes (45).

Functions of Telomeres

The main function of telomeres is to protect chromosomal ends from degradation and end-to end-fusion (1) as well as to prevent the ends of chromosomes being recognized as DNA damage by the DNA damage response machinery of the cell (37). However, when telomeres are critically short, they activate the apoptosis/senescence pathways, thereby preventing genetic material being lost by inhibiting inappropriate continuous DNA replication in the context of short telomeres. The telomere structure described above, prevents inappropriate DNA repair at these sites, for example the loop conformation (D-loop) masks the single stranded terminal DNA and enables its protection from the DNA damage response pathway (37).

The shelterin complex supports the chromosome protective function of telomeres and stabilization of telomere lengths, and the complex interaction of shelterin proteins at the chromosomal ends have a key role in telomere maintenance via a negative feedback loop which also has an inhibitory effect on the telomerase enzyme (46).

In cells which have replicative capability, telomere shortening can lead to chromosomal instability by promoting end-to-end fusions leading to multiple chromosomal aberrations, such as breakages, fusions, and translocations rendering the genome aneuploid and therefore promoting carcinogenesis. To maintain telomere length, the homeostasis mechanism that involves telomerase, uses both TRF1 and TRF2 as negative regulators that stabilize and limit telomere length elongation (47, 48). Overexpression of both TRF1 and TRF2 was reported to cause telomere shortening (47) and this could be due to the binding of TRF1 and TRF2 along the length of the double stranded telomeric repeat array which measures telomere length as demonstrated in yeast (47, 48). POT1 can either facilitate or inhibit telomerase accessing telomeres depending on its position relative to the DNA 3′-end (49). Examining the high-resolution crystal structure of the human POT1-TTAGGGTTAG complex suggested that it would not be elongated by telomerase. When POT1 is bound at one telomeric repeat before the 3′-end, leaving an 8-nucleotide 3′-tail, the resulting complex is elongated with increased activity

and processivity (50). Replication protein A (RPA) is another ssDNA binding protein which has an important role in telomere replication by facilitating telomerase enzyme at the telomeres (51, 52). It also recruits the ATR-ATRIP protein kinase complex to DNA damage sites and initiates the checkpoint signaling (53, 54). Collectively, the available evidence demonstrates that shelterin and other telomere-binding proteins are involved in the regulation of telomere length.

Gene regulation is another reported function of telomeres but with limited evidence available for it. Telomeric attrition extensively alters expression of some genes, and the difference in expression of genes proximal to telomeres may result from chromatin modifications, a conserved phenomenon termed as *telomere position effect* (TPE). TPE is a silencing mechanism spreading from the telomeres toward subtelomeric regions (55). In humans, only a limited number of endogenous genes (e.g., ISG15) has been mentioned to be affected by TPE (56, 57), however, microarray data suggests that the expression of many other genes close to telomeres to be also altered with the aid of a telomere length-dependent and DNA damage-independent mechanism, and this is known as *telomere position effect–over long distance* (TPE-OLD) (58). For example, the looping of chromosomes brought long telomeres closer to some genes which are over 10 Mb away from the telomere, but these same loci were completely separated from the telomeres when the telomeres were short (58). Further microarray data supports the notion that telomere length-dependent chromosome conformation can affect the transcription of non-subtelomeric genes (58). At the genome-wide level, the effect of this mechanism on gene expression has been proposed to occur earlier than replicative senescence and that could potentially explain the increased incidence of age-related pathologies that are associated with old age without necessarily imposing a DNA damage signal from a critically-short telomere (59, 60).

Telomere length is the main determinant of a cell's replicative life span. Dysfunctional telomeres which result from either progressive telomere shortening, internal DNA damage (61) or shelterin complex loss, provoke a strong DNA damage response and genomic instability (62). A plethora of experimental data has shown that tumorigenesis can be caused by genome instability resulting from telomere shortening (4, 63). Nevertheless, in late generation telomerase knock-out mouse models, telomere attrition was also a tumor suppressor mechanism through the induction of replicative senescence or apoptosis that repress tumorigenesis. Telomere shortening and telomere uncapping in metazoans stimulate ATM/ATR kinases to phosphorylate downstream kinases CHK1 and CHK2, which initiate p53-dependent replicative senescence and apoptosis pathways which inhibit tumor formation (4).

TERRAs (Telomeric Repeat Containing RNAs)

Telomeres were initially thought to be transcriptionally silent, but recently they have been found to be transcribed into telomeric repeat containing, long non-coding RNAs, termed TERRAs (64). TERRAs have a role on telomere regulation and also regulate telomeric access of telomerase as described below in more detail.

REGULATION OF TELOMERE LENGTH AND TELOMERE MAINTENANCE MECHANISMS (FIGURE 2)

The most widely known classical telomere maintenance mechanism is dependent on telomerase reverse transcriptase activity. However, another telomerase-independent telomere maintaining pathway has been described in cells that do not have measurable telomerase activity, termed alternative lengthening of telomeres (ALT) pathway (69). TERRAs also have a role in telomere length regulation by mainly managing telomeric access of telomerase.

Telomerase

Structure of Telomerase (Figure 1)

Telomerase, the only RNA dependent DNA polymerase in mammals, was first discovered in protozoans in 1985 (70), and subsequent studies demonstrated mammalian/human species in 1989 (71). The telomerase holoenzyme contains three core components: the RNA component harboring the template region for telomere synthesis (hTR or hTERC), a catalytic protein with reverse transcriptase activity, hTERT (72) as well as dyskerin (**Figure 1**). However, only the RNA component (TERC) and the catalytic subunit (TERT) are necessary and sufficient for *in vitro* telomerase activity (73). **Table 1** lists some of the well-known telomerase associated proteins.

Telomerase RNA component (hTERC or hTR)

The human telomerase RNA (TERC or hTR) consists of 451 nt and is an essential constituent of the telomerase catalytic core complex. Although the length is variable among eukaryotes, the structure of TERC remains conserved. For example, the length ranges from ∼150 nt in ciliates, 400–600 nt in vertebrates to ∼1,300 nt in yeast (114). Additionally, in ciliates, polymerase III transcribes the telomerase RNA (115), whereas it is RNA polymerase II in yeast and vertebrates (116).

Vertebrate TERC's secondary structure has four conserved elements: a pseudoknot domain (CR2/CR3), a CR4/CR5 (conserved region 4 and conserved region 5) domain, box H/ACA (CR6/CR8) domain and a CR7 domain (114, 117). The proximal template/pseudoknot domain and the distal CR4/5 domain represent the essential regions of TERC for telomerase activity (118).

As mentioned before, an active telomerase enzyme can be generated by combining the two RNA domains from the TERC subunit with the TERT protein on oligodeoxynucleotide substrates *in vitro* (73, 119–121). The human/vertebrate TERC has a third, conserved component, the H/ACA domain located at the 3′ end that has homologies to small nucleolar (sno) and small Cajal body-specific (sca) RNAs. The TERC H/ACA region binds to telomerase associated proteins, such as dyskerin, NOP10, NHP2, and GAR1 (14), and this region is essential

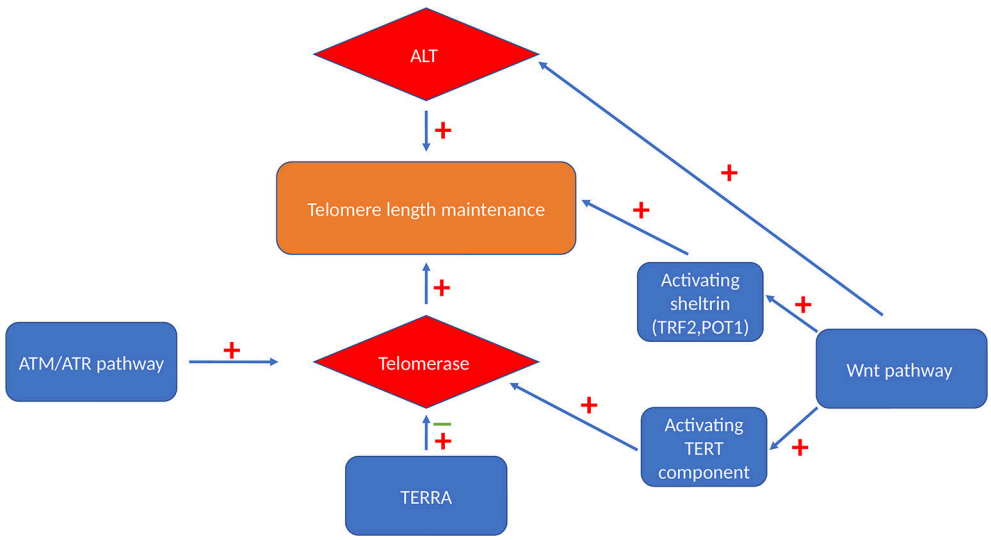


FIGURE 2 | Telomere maintenance mechanisms. Cells can maintain their telomeres via either telomerase-dependent pathway or a telomerase-independent ALT pathway. Activated Wnt signaling pathway can maintain telomere length by activating both these maintenance mechanism and by maintaining the level of TRF2 and POT1 sheltrin components that are essential for telomere protection (65). ATM and ATR also have stimulatory effect on telomerase enzyme via triggering its recruitment and enhancing the assembly of this enzyme (66). TERRA binds independently to hTERC and hTERT telomerase subunits with an inhibitory effect on human telomerase enzyme (67) or it acts as a recruiter of telomerase enzyme rather than an inhibitor (68).

for telomerase biogenesis, and are important for RNA stability. Additionally, in the 3′ stem-loop of the H/ACA, there is another domain, the Cajal body localization box (CAB), for binding the telomerase Cajal body protein 1 (TCAB1) (33). Mutations in the H/ACA region decrease TERC accumulation, whereas mutations in the CAB cause TERC to accumulate in nucleoli instead of Cajal bodies (122, 123). Although this mutant TERC has the capacity of forming catalytically active telomerase *in vivo*, it is highly impaired in telomere elongation because of the decreased association of telomerase with telomeres (124). This result emphasizes that sub-nuclear localization of telomerase as an important regulatory mechanism for the homeostasis of telomere length in human cells (124). TERC therefore, not only provides the template, which identifies the telomere repeat sequence, but it also comprises motifs, which are crucial to reconstitute telomerase activity (125). Furthermore, it plays a role in stability, maturation, accumulation, and functional assembly of the telomerase holo-enzyme.

hTERT

TERT is the catalytic component of the telomerase enzyme and as described above, together with TERC, it is essential for telomerase activity and thus for the maintenance of telomere length, chromosomal stability, and cellular immortality. The human TERT gene (hTERT) is located at chromosome 5p15, and encompasses more than 37 kb and contains 16 exons (126). The TERT protein consists of four conserved structural domains, the telomerase essential N-terminal (TEN) domain, the telomerase RNA binding domain (TRBD), the central catalytic reverse transcription (RT) domain, and the C-terminal extension (CTE). Mutations in the RT conserved residues prevent telomerase enzymatic activity *in vitro* (127). These mutated TERT proteins fail to maintain telomere lengths *in vivo* (128), and many of these mutations have been identified in individuals with telomere-mediated disorders or telomeropathies (129). As already stated above, telomerase activity can be reconstituted by hTERC and hTERT co-expression in yeast and mammalian extracts (73, 130). Telomerase activity is established in *Saccharomyces cerevisiae* via reconstitution of telomerase by hTERC and hTERT co-expression (130). Therefore, hTERC and hTERT are the minimal requirement for telomerase activity (72). However, biochemical telomerase activity as measured by the telomere repeat amplification protocol (TRAP) assay does not always mean that the enzyme has necessarily telomere elongation capacity *in vivo*. This was demonstrated when the hTERT protein was modified by attaching a hemagglutinin (HA) epitope tag to the C terminus: while the catalytic activity of telomerase enzyme remained unaffected telomere maintenance function was lost *in vivo* due to loss of access to the telomere (131). Telomerase associated proteins are also essential for the full biological function of the enzyme but hTERT is the primary determinant of enzyme activity in most cells (120, 132).

Dyskerin

Dyskerin is a highly conserved, nucleolar, 514-amino-acid long protein, also known as NAP57 in rat (133) or Cbf5 in yeast (134) and has been proposed to be the third core component of the telomerase holoenzyme. Dyskerin is an essential member of the telomerase complex (but not required for biochemical telomerase activity as stated above); it binds to the telomerase RNA component (TERC) and participates in stabilizing the telomerase enzymatic complex (135). It is a pseudouridine synthase, encoded by the *DKC1* locus at Xq28 (136), which is

TABLE 1 | Telomerase associated proteins [adapted from Hapangama et al. (14)].

Protein	Function in Cancer
hTERT ASSOCIATED PROTEINS	
Hsp 90, P23	Hsp90 is an essential modulator for the proper folding and stabilization of several client proteins and it is a major contributor to carcinogenesis. Hsp90 and P23 act together to regulate telomerase DNA binding. Since heat shock protein 90 (Hsp90) client proteins have major cancer biological hallmarks, targeting Hsp90 provides the prospect for simultaneous disturbance of multiple oncogenic pathways. In triple-negative breast cancer, inhibition of Hsp90 has shown to be a promising therapeutic avenue (74–76)
Protein 14-3-3	These proteins are involved in regulating multiple cellular functions via their interaction with phosphorylated partners. An elevated level of 14-3-3 proteins facilitates tumor progression in a variety of malignancies. The observations of Seimiya et al. identified the 14-3-3 signaling proteins as human TERT (hTERT)-binding partners and suggested that 14-3-3 improves nuclear localization of TERT. A dominant-negative 14-3-3 redistributed hTERT into the cytoplasm, which was normally localized in the nucleus (77)
DHX36 (DEAH-Box Helicase 36)	It mediates AU-rich element mRNA degradation and as a resolvase for G-quadruplex DNA *in vitro* (78, 79). It involves in TERT stabilization and Correction of the positioning of the template domain of hTERT (80), it also Regulates p53 Pre-mRNA 3′-End Processing Following UV-Induced DNA Damage (81) and Prevents migration of colon cancer cells (82)
Pontin and reptin	Pontin and Reptin are conserved proteins belong to AAA + ATPases family, they have a role in various cellular processes that are critical for oncogenesis, such as transcriptional regulation, chromatin remodeling, DNA damage signaling and repair, assembly of macromolecular complexes, regulation of cell cycle/mitotic progression, and cellular motility, all of which contribute to their central roles in activating cell proliferation and survival (83–85). They also act together in telomerase assembly. Pontin and/or Reptin implicated in cancers of the esophagus, stomach, colon, and pancreas (86–90) Their exact functions are still entirely unclear as they interact with many molecular complexes with vastly various downstream effectors, with overexpression relating to factors, such as response to treatment, prognosis and outcome, reviewed in (91) Pontin and reptin have a well-established role in hepatocellular carcinoma (HCC), both were overexpressed in HCC tissues and associated with poor outcome (92, 93) Pontin and/or Reptin expression in both non-small cell lung cancer (NLSCLC) and small cell lung cancer (SCLC) with potential use as biomarkers in lung cancer (94–98). Pontin identified in screens of biomarker/autoantigen panels in breast cancer (99, 100) and both proteins are essential in cancers of white blood cells, resulting in lymphomas and leukemia (101)
hTERC ASSOCIATED PROTEINS	
Dyskerin	Dyskerin is one of H/ACA ribonucleoproteins (RNPs) which also include (NOP10, NHP2, and GAR1) (102), it is suggested in rRNA modification and processing, impaired dyskerin function in X-DC patients and DKC1 hypomorphic mutant model causes a decrease in the protein production which results in a reduction in tumor suppressor proteins (P53 and P27) reviewed in Montanaro (103). Dyskerin binds to the telomerase RNA component (TERC); thus dyskerin allows TERC stabilization and enhances telomerase activity. As a consequence, impaired dyskerin reviewed in Montanaro (103) Dyskerin protects from genetic instability. Loss and gain of dyskerin function may play critical roles in tumorigenesis (104)
NOP10	NOP10 as an H/ACA RNP contributes to telomerase enzyme assembly and stabilization, post-transcriptional processing of nascent ribosomal RNA and pre-mRNA splicing. Therefore, it is essential for ribosome biogenesis, pre-mRNA splicing, and telomere maintenance (105, 106) NOP10 mRNA level was reported to be decreased in patients with chronic lymphocytic leukemia (CLL) relative to controls (105)
NHP2	NHP2 has the same function as other H/ACA RNPs, increased NHP2 protein in gastric and colorectal cancer relative to healthy controls (107) Significant upregulation of the NHP2 protein encoding gene in colonic cancer, specifically those with high clinical stage (108)
GAR1	GAR1 is one of the four H/ACA RNPs. It also involved in telomerase assembly and stabilization, post-transcriptional processing of nascent ribosomal RNA and pre-mRNA splicing. All these RNPs are concentrated in nucleoli and Cajal bodies of mammalian cells, reflecting the location of H/ACA RNPs. GAR1 binds only to Dyskerin and it is crucial for the nucleolar localization and function of the RNP complex. In CLL patients, a significant decrease of GAR1 mRNA level in patients with CLL compared to controls (105)
TEP1 (telomerase protein component 1)	TEP1 is overexpressed in tumor cells compared to normal cells and it contributes to carcinogenesis and progression of renal cell carcinoma, bladder and prostate cancer (109). Additionally, Findings of Kohno study suggest TEP1 plays a role as a tumor suppressor gene in the genesis and progression of human lung cancer (110)
TCAB1 (telomerase and Cajal body protein 1, encoded by WRAP53)	TCAB1 is a subunit of active telomerase and is essential for the telomerase holoenzyme to be accumulated in Cajal bodies and to elongate telomeres (111), so it is involved in Cajal body maintenance, telomere maintenance and ribonucleoprotein biogenesis. Overexpression of TCAB1 seen in head and neck carcinoma clinical specimens as well as in carcinoma cell lines while depletion of TCAB1 decreased cellular proliferation and invasion potential both *in vitro* and *in vivo* (112)
A1/UP1	Findings of Nagata et al. suggested that UP1, a proteolytic product of heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1), can unfold the quadruplex structure of telomeric DNA into a single-stranded structure. Therefore, UP1 may enhance the telomerase activity via unfolding of the quadruplex structure of telomeric DNA and resultant provision of the accessible overhang. The authors assumed that both unfolding and recruitment by hnRNP A1/UP1 contribute to improve telomerase activity and maintain proper telomere length. Thus, hnRNP A1/UP1 may be promising targets to control telomerase activity which is associated with several cancers (113)

Hsp90, heat shock protein 90; CLL, chronic lymphocytic leukemia.

responsible for the conversion of uridine to pseudouridine in non-coding RNAs, a vital step in rRNA and ultimately ribosomal synthesis (103).

Complete dyskerin depletion is lethal in mice, Drosophila (they do not have telomerase activity therefore a non-telomerase related function) and yeast (137–139). In humans, germline mutation in the *DKC1* gene is the causative factor for X-linked dyskeratosis congenita (140).

Functions of Telomerase

Telomerase is a specialized reverse transcriptase, which maintains and elongates telomeres at the 3′-single strand in the absence of a DNA template while using the inherent RNA (TERC) for the template function and is thus a RNA dependent DNA polymerase. In the subsequent S-phase of the cell cycle, the conventional DNA replication machinery can then replicate the complementary C-rich strand. Thus, telomerase ascertains chromosomal stability and cellular proliferation in proliferative somatic cells, tissue progenitor cells and in cancer cells (141). When telomeres shorten beyond a critical threshold length, normal healthy cells in humans which are devoid of telomerase activity, will assimilate a cellular senescence phenotype with an irreversible growth arrest and the classical morphological alterations (142). Somatic human cells lacking measurable telomerase yet expressing certain viral oncoproteins can overcome the senescence checkpoint and continue to proliferate, but they then accumulate chromosomal instability including aneuploidy, polyploidy and chromosomal fusions. On these grounds, high telomerase activity has been assigned a role in maintaining genome stability by preventing telomere shortening. Telomerase fulfills this important role via interaction with many key cellular pathways as detailed below.

ATM/ATR pathway

Ataxia-Telangiectasia Mutated (ATM) and ATM and Rad3 related (ATR) DNA damage response kinases have essential roles in telomerase-mediated telomere maintenance (66). The conserved ATM and ATR family of serine-threonine kinase proteins mediates DNA damage and replication stress checkpoint responses (143, 144), therefore, play a crucial role in DNA repair, cell apoptosis, and cell senescence, and are closely associated with the development and progression of cancer in humans (145, 146). ATM is required for the addition of new repeats onto telomeres by telomerase (147) and evaluation of bulk telomeres in both immortalized human and mouse cells showed that ATM inhibition suppressed elongation of telomeres while ATM stimulation through PARP1 led to an increase in telomere length (147).

Stalled replication forks increased telomerase localization to telomeres in an ATR-dependent manner (66). Additionally, increased telomerase recruitment was observed upon phosphorylation of the shelterin component TRF1 at an ATM/ATR target site (S367) (66) and this led to TRF1 loss from telomeres and may therefore increase replication fork stalling (148). ATM and ATR depletion reduced assembly of the telomerase complex, and ATM was required for telomere elongation in cells expressing POT1ΔOB, an allele of POT1 that causes disruption in telomere length homeostasis (66). Hence from this data it can be concluded that ATM and ATR are involved in triggering telomerase recruitment and facilitating its assembly (66).

WNT pathway

Wnt family proteins are essential for regulating cell proliferation, cell polarity, and cell fate determination during embryonic development and tissue homeostasis (149). A dysregulated Wnt/β-catenin signaling pathway is also associated with human tumourigenesis (149). Due to the intricate relationship of telomeres and telomerase with similar cellular functions, their close interaction is not a surprise. An activated Wnt signaling pathway can reinforce the stability of telomeres by coupling and enhancing the two main telomere maintenance pathways: telomerase-dependent and ALT pathways. A Wnt-mediated telomere protective effect is particularly expected to have an important role during development, in adult stem cell function and oncogenesis (65).

The Wnt pathway may regulate telomere maintenance via its effect on several essential shelterin components, including TRF2 and POT1. Recently, in human somatic and cancer cells as well as in mouse intestinal tissue, activation of canonical Wnt/β-catenin pathway activated TRF2 and also increased telomere protection were demonstrated (65). In mice lacking telomerase, apoptosis of the Wnt-dependent intestinal crypt stem cell niche could be rescued by administration of Wnt agonists (150). Additional evidence demonstrates that the Wnt pathway triggers APC- and β-catenin induced regulation of TRF2 and TCF4 which further regulate TRF1 and POT1 (150, 151).

Further to the enhancement of shelterin protection, the Wnt/β-catenin signaling pathway also activates TERT (152). Importantly, the use of Wnt pathway agonists can rescue telomere uncapping, suppress apoptosis and lead to elevated *Ascl2* transcripts as well as Sox9 protein levels (150) suggesting a therapeutic strategy for some conditions with aberrations in telomerase.

Non-canonical functions of TERT

Non-canonical functions of TERT have been discovered later than telomerase activity, and they also play a role in tumorigenesis, for example via TERT's role in regulating the Wnt signaling as a cofactor for the β-catenin pathway (153). TERT has been shown to be inducible in ischemic brain cells and to prevent apoptosis via a non-telomeric action via shift of the cytosolic free Ca^{2+} into the mitochondria (154). Despite having normal telomere lengths, lack of hTERT impairs the cellular capability to repair damaged DNA and fragmented chromatin (155). TERT also is demonstrated to have RNA dependent RNA polymerase function by interacting with the RNA component of mitochondrial RNA processing endoribonuclease (*RMRP*) and forming ribonucleoprotein complexes. These complexes produce double-stranded (ds) RNAs that serve as substrates for the generation of siRNA which may regulate the expression of other genes related to stem cell biology (156). Further to the above, there are many other additional non-telomeric functions of TERT active in cancer, such as improved DNA repair, increased

apoptosis resistance, changes in chromatin structure and altered gene expression (157).

Hormone Regulation of Telomerase in Hormone Responsive Tissues

There is evidence from multiple studies that telomerase is under the regulation of steroid hormones in hormone responsive tissues. This corroborates with the known direct regulation of cell fate and proliferation in such tissues by steroid hormones, for example the ovarian hormone, estradiol, induces a mitotic response in endometrial epithelial cells (23, 158). In different studies, telomerase is induced by estrogen in various macaque and human cell lines (15, 159, 160). Androgens also upregulate telomerase in an ovarian cancer cell line (161) but progesterones down regulate telomerase in the endometrium (15). ATM silencing also down regulated proteins, such as ChK2, p53, and caspase 3, which were stimulated by the synthetic progestogen, medroxyprogesterone acetate (MPA) (162). This result suggested that MPA exerts its effects via the ATM-Chk2-p53-caspase-3 pathway protecting against carcinogenesis (162). The progestagenic effect on telomerase may also be mediated through this pathway. Hormonal regulation of telomerase in the healthy endometrium was recently reviewed in detail (14).

Telomerase-Related Telomere Regulation by TERRAs

Telomerase regulation by TERRAs has initially been examined in yeast although recent work also suggests a similar regulation in human cells. In yeast cells, TERRAs were found to sequester and direct telomerase to the specific telomeres which were the shortest (68). In addition, TERRA was found to bind to hTERC and hTERT components of telomerase independently, to function as an inhibitor of human telomerase enzyme (67). In telomerase negative cells with shortened telomeres, increase in TERRA levels trigger homology directed repair (HDR) whereas in telomerase positive cells, it results in recruitment of telomerase to the short telomeres (163). Absence of both telomerase and HDR accelerates the cell senescence pathway (164). Due to loss of Rat1 function, in yeast free TERRA accumulates at critically short telomeres which helps in recruiting the telomerase enzyme to that telomere and elongation of that telomere (165).

TERRA was found to be induced in cells with short telomeres and acted as a scaffold for spatial organization of the telomerase components forming a TERRA-telomerase complex which helped in recruitment of telomerase to the telomere of its origin hence TERRA was proposed to be a recruiter of telomerase enzyme rather than an inhibitor (68). Contrary to some *in vitro* studies, in human cancer cells, telomerase-led telomere elongation was not affected by the transcription of the telomere. In these cells, it was suggested that shortening of telomeres may not have been due to telomerase inhibition, but due to impaired replication due to integrity of the chromosomes affected by high levels of TERRAs (166). In general, the interaction of TERRAs and telomerase is complex and might depend on cell type and conditions, such as cell cycle phase, or telomere length.

Telomere Maintenance by Alternative Lengthening of Telomeres (ALT)

Cells can maintain their telomeres *via* a telomerase dependent pathway or a telomerase independent ALT pathway (69). New telomeric DNA is synthesized from a DNA template in ALT (167) by homologous recombination (HR) (168). The template could either be the telomere of another chromosome, another region of the same telomere by t-loop formation or sister telomere recombination.

The first evidence for the presence of an ALT mechanism was described in several immortalized human cell lines that did not have telomerase activity but maintained telomere lengths for hundreds of population doublings, and this mechanism occurs in ∼15% of cancers including osteosarcomas, soft tissue sarcoma subtypes, and some glial brain tumors (169, 170).

In human cells, where ALT activity is elevated to a degree sufficient for telomere length maintenance, telomeres are characterized by their highly heterogeneous length, but the average length (>17 kb) is about double that of most cells where telomeres are elongated by telomerase (171).

Mutations in the ATRX/DAXX chromatin remodeling complex have been observed in cancers and cell lines that use the ALT mechanism, suggesting that ATRX may suppress the ALT pathway (172). In mortal cells or immortal telomerase-positive cells, knockout or knockdown of ATRX does not stimulate ALT (172). However, ATRX loss in SV40-transformed fibroblasts together with one or more unidentified genetic or epigenetic alterations was attributed to either a marked increase in the proportion of cells with an activated ALT (instead of telomerase) or significant decrease in the time taken for ALT activation (172). Loss of ATRX protein and mutations in the ATRX gene are also characteristic features of ALT-immortalized cell lines (172). In addition, ALT is associated with marked genome rearrangements, extensive micronucleation, a defective G2/M checkpoint and alteration in double-strand break (DSB) repair (173).

ROLE OF TELOMERES AND TELOMERASE IN PRE-MALIGNANT AND MALIGNANT PROLIFERATIVE DISORDERS

Alteration of Telomere Biology in Premalignant Conditions and in Cancers

Limitless proliferation is a cardinal feature of cancer cells, whist increased proliferation is common to all premalignant changes including hyperplasia. The excessive proliferation observed in these malignant/premalignant conditions is maintained by avoiding senescence and crisis/apoptosis. Senescence/apoptosis exist as barriers for mitosis, thus they are tumor suppressor mechanisms in normal cells, which are regulated intricately by telomeres and checkpoint activation (**Figure 3**). The unrestricted proliferation of cancer cells is therefore thought to be sustained by telomere maintenance mechanisms which were detailed above. Since high telomerase activity is reported in over 85% of cancers, telomerase dependent telomere lengthening is believed

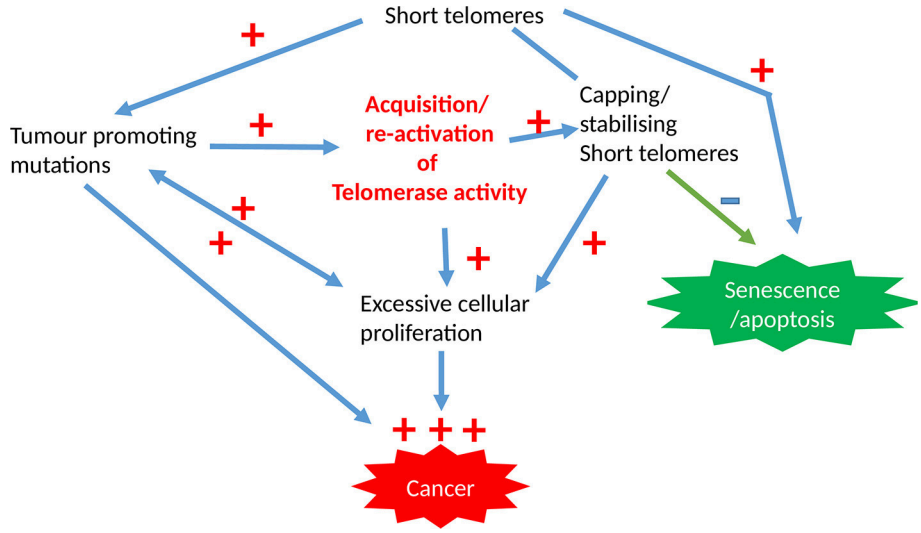


FIGURE 3 | The involvement of telomeres and telomerase activity in epithelial cancers. The initial acquisition of tumor promoting mutations is promoted by short dysfunctional telomeres which are subsequently stabilized by high telomerase activity levels that is characteristic for most cancer cells, with the overall result being pre-requisite for unregulated proliferation capacity.

to be the most common telomere maintenance mechanism relevant to carcinogenesis.

Evidence for Altered Telomere Lengths in Cancers

During ongoing proliferation in normal somatic cells without telomerase or other telomere-maintenance mechanisms, telomeres shorten until reaching a certain minimal length. Beyond this, when tumor suppressor checkpoints, such as p53 are functioning, senescence or apoptosis can be induced. In contrast, when p53 or other important DNA damage checkpoints are not functioning, cells can enter a crisis state where ongoing proliferation promotes further telomere shortening and telomere dysfunction (174). This can cause various genomic instabilities, such as end-to-end fusion of telomeres resulting in anaphase bridges in subsequent cell division cycles. Most of these cells usually die due to apoptosis and gross genomic instabilities. However, some rare cells acquire mutations in the TERT promoter that increase telomerase activity resulting in re-stabilization of telomeres, Importantly, as long as telomeres are capped and protected, they can be rather short and this situation is frequently found in epithelial cancer cells compared with adjacent healthy tissue. Several studies using telomere PNA-FISH have shown that breast, prostate, and pancreatic cancers are associated with telomere shortening (175–177). Furthermore, around 40 to 97% of colorectal tumors have shorter telomeres compared with normal tissue, and telomere shortening is therefore considered to be one of the early events in tumourigenesis (178, 179).

However, importantly, acquiring telomerase activity can stabilize even short telomeres in genetically unstable cells and provide sufficient capping for them to attain an unlimited proliferation potential. Thereby, telomerase re-activation conserves genomic mutations and instabilities and contributes further to tumourigenesis (**Figure 2**).

Significant telomere length shortening results in end-to-end fusion, thus increasing the potential for genome instability and carcinogenesis. There are few other generic associations which lead to telomere attrition, such as oxidative stress, lifestyle choices, environmental factors, smoking and obesity (180) and some of these also increase the risk of developing a variety of cancers. Telomere shortening can influence the progression of premalignant breast tissue to malignancy and premalignant breast lesions had short telomeres leading to non-clonal chromosome aberrations (181).

Meta-analyses of available studies also revealed that shorter peripheral blood mono-nucleocyte (PBMC) telomeres are associated with a significant increase in the risk of developing cancer (OR = 1.35, 95% CI = 1.14–1.60) than longer telomeres (182, 183). Shorter PBMC telomeres could be related to oxidative stress endured by an organism, which is in agreement with the established mediatory role that oxidative stress plays between inflammation and cancer (184). When PBMC mean telomere lengths were prospectively studied in the general population in Denmark, shorter telomere lengths were also associated with decreased survival after cancer rather than the cancer risk itself (185). Another systematic review has also reported a consistent inverse relationship between age and PBMC telomere length (186).

Telomere dysfunction may also be a resultant of altered telomere-associated proteins that are also essential for regular end-capping function (187, 188). For example, mutations in the C-terminal of POT1 can initiate genomic instability permissive for tumourigenesis (189). TRF1 flox/flox × K5-Cre transgenic mice, do not have TRF1 in stratified epithelia. These mice demised perinatally and showed skin hyperpigmentation and epithelial dysplasia and were associated with telomere initiated DNA damage, p53/p21 and p16 pathway activation and *in vivo* cell cycle arrest. Deficiency of p53 rescues mouse survival but

causes increase in the incidence of squamous cell carcinomas (39). Alteration of the levels of TRF1, TRF2, TIN2, and POT1 has also been described in some human tumors (190). A dysregulated expression of TRF1, RAP1, and TPP1 has been reported in patients with chronic lymphocytic leukemia (191). Likewise, TIN2, TRF1, and TRF2 mutations have been associated with some cases of Dyskeratosis congenita and aplastic anemia (192–195) and both these conditions increase the risk of developing some cancers. Defects in shelterin components naturally cause dysregulation of telomere homeostasis as explained above. This may operate as a tumor suppressor mechanism when it initiates the p53/pRb pathways which in turn triggers senescence and prevents the tumorigenesis process. Alternatively, it can contribute to carcinogenesis with the fusion of dysfunctional telomeres or fusion between dysfunctional telomeres and double strand breaks which trigger breakage-fusion-bridge cycles (196). In hepatocellular carcinomas, longer telomeres, increased hTERT expression and higher levels of TRF2 protein as "stemness markers" were associated with poorer prognosis and more chromosomal instability (197). Further studies have confirmed that different causal factors, such as hepatitis B and C, and alcohol lead to telomere dysfunction in hepatic cells hence initiating the carcinogenesis process (198). A significant decrease in POT1 and RAP1 protein levels are described in familial papillary thyroid cancers (199). TP53 disruption in hematological malignancies has been associated with the downregulation of expression in shelterin genes and severe telomere dysfunction and genomic instability (200). Therefore, genetic mutations resulting in functional alterations in the essential components of the telomerase enzyme or shelterin components may repress telomerase activity and thus shorter telomeres will be the consequence. The available evidence also suggests a concerted dysregulation in the expression of shelterin genes and protein levels with the commonly observed removal of cellular tumor suppressor mechanisms in premalignant conditions can lead to alteration in telomere lengths that can trigger the tumourigenesis process.

Evidence for Altered Telomerase in Cancers

Polymorphism in genes of the telomerase complex

Such as *hTERT* and *hTERC* has been reported to affect individual susceptibility to cancers (201, 202). Variants in chromosome 5p15, the region that harbors the *hTERT* gene, have been identified by Genome-wide association studies (GWAS) to be associated with the risk of bladder, pancreas, brain, testicular, breast, prostate, skin, and lung cancers (203–207).

hTERT promotor mutations

Tumors with high *hTERT* promoter mutation frequencies have almost always originated in tissues with relatively low cell turnover rates. Contrastingly, tissues with rapid cell turnover seem to have different mechanisms to elongate telomeres and seem less likely to benefit from activating *hTERT* expression by mutations (208). Mutations that result in increased hTERT expression, telomerase activity or longer telomere lengths have been identified in cancers of the central nervous system, thyroid, bladder, liver, tongue, adipose tissue and skin (208–210). In thyroid cancers, when *hTERT* and *BRAF* mutations coexist, such tumors express high levels of *hTERT* (211).

Common inherited variants of telomere related genes, such as *TERC*, *TERT*, and rare *POT1* mutations have been found to be associated with higher risk of developing gliomas. *TERT* promoter and *ATRX* mutations were found to be the most recurrent somatic events which led to glioma associated lengthening of telomeres (212).

A high frequency of *hTERT* promoter mutations was also reported in follicular cell-derived thyroid carcinomas (213). An over-representation of *hTERT* promoter mutations had been detected in advanced thyroid cancers and these mutations were more prevalent in advanced disease (51%) compared with well-differentiated tumors (22%). Thus, *hTERT* promoter mutations have been suggested as biomarkers of tumor progression (213). *hTERT* promoter mutations usually cause an increased expression of the *hTERT* gene and paradoxically, these mutations were reported to occur together with short telomeres in tissues with low-rates of self-renewal and were also associated with poor patient survival in primary melanomas (210). Tissue stem cells are reported to have active telomerase and daughter cells produced by these switch off telomerase upon differentiation, and subsequent reactivation of telomerase in these tissues have been proposed to be the reason for the observed short telomeres in thyroid cancers with high telomerase expression (210). Rachakonda et al. showed that mutations of the *hTERT* promoter were also the most common somatic lesions in bladder cancer (214). The authors also found that a common polymorphism rs2853669 in the *hTERT* promoter acts as modulator of the mutations effect on survival and disease recurrence. The patients with the mutations had poor survival outcome in the absence but not in the presence of the variant allele of the polymorphism. The mutations without the presence of the variant allele were markedly correlated with tumor recurrence in patients with non-invasive and invasive T1 bladder tumors (214). Polymorphisms in the *hTERT* gene were also associated with an increased lung cancer risk in the Chinese Han population (215).

Telomerase activity in cancers

The early observation that telomerase activity is absent in most human somatic tissues during differentiation but strongly upregulated in tumors, agrees with the hypothesis that telomerase playing an important role in the carcinogenesis process (216). In pancreatic ductal cell carcinoma, levels of telomerase activity were higher compared to other types of pancreatic cancer and benign pancreatic tissues (217). In gastric cancers, tumors with high telomerase activity had poorer prognosis and the authors concluded that detecting telomerase activity might be useful as a prognostic indicator of clinical outcome (217). Telomerase activity was also detected in 90% of head and neck squamous cell cancers, in 100% hyperplastic squamous epithelium but not in normal mucosa (218). Colorectal cancers with high telomerase activity had poorer prognosis in spite of curative surgery in apparently disease free patients, thus the survival seems to have been associated with the level of telomerase activity (219). A systematic analysis of telomerase activity levels in many cancer

types performed by Bacchetti and Shay in 1997 demonstrate high telomerase being a common observation in most of them (220).

hTERC alterations in cancer: Recent work has proposed that hTERC maturation involves the poly(A)-specific ribonuclease (PARN) which is localized in the nucleolus and in the Cajal body (CB). The enzyme trims hTERC precursors by removing poly (A) tails and may be involved in impairment of telomerase activity (221). Individuals with biallelic PARN mutations and PARN-deficient cells showed a reduction of expression of genes encoding several key telomerase components, such as TERC, and DKC1. These cells also have critically short telomeres (222). Improper hTERC processing and telomere dysfunction in premalignant diseases, such as Pontocerebellar Hypoplasia 7 (PCH7) and dyskeratosis congenita had been proposed to have a mechanistic link (221). *hTERC* amplification was associated with the aggressive progression of cervical cancer, and authors suggested that hTERC may serve as a surrogate marker for cancer progression and form a potential therapeutic target for cervical cancer (223). However, it is important to appreciate that most cervical cancers initiated in a background of persistent papilloma virus infection in the transformed epithelial cells. hTERC over-expression has been reported in many other cancers including prostate (224); breast (225); and oral squamous cell carcinoma (226).

Dyskerin alterations in cancer: Dyskeratosis congenita is a rare multisystemic syndrome characterized by low telomerase activity already during development and consequently, shorter telomeres in many tissues resulting in a high susceptibility to develop a subset of cancers, therefore, wild type dyskerin protein has been suggested to act as a tumor suppressor. Conversely, wild-type dyskerin protein is upregulated in a number of human cancers, such as in breast, prostate, colon and hepatocellular carcinomas (108, 227–229) and in these cancers, high levels of dyskerin were associated with an aggressive histopathological feature and poor prognosis (229). Acute loss of dyskerin function by RNA interference led to marked reduction of steady-state levels of H/ACA RNAs, disruption of the morphology and repression of anchorage-independent growth of telomerase-positive and telomerase-negative human cell lines. The levels of dyskerin in cancer cells modulate telomerase activity through the regulation of TERC levels, independently of TERT expression (227). The function of telomerase associated proteins in cancer is summarized in **Table 1**. Dyskerin might also contribute to tumor development through mechanisms where the presence of cellular telomerase activity is not essential, and which may be only partially dependent upon the protein's role in rRNA processing (104).

ENDOMETRIUM

The endometrium is the inner mucosal lining of the uterus that contains several cell types including tissue specific epithelial and stromal cells, as well as leucocytes and blood vessels (22, 230–233). It is the primary target organ for ovarian steroid hormone action (24) and healthy human endometrium is characterized by its regenerative and remodeling capacity that undergoes repetitive monthly cycles of proliferation, secretory changes, break-down and regeneration. These cycles of changes occur ∼400 times in a female's reproductive life (22, 230) and are regulated by ovarian steroid hormones (23). Telomerase activity as well as mean telomere length change according to ovarian cycle in whole healthy endometrial samples (15, 234) suggesting an ovarian regulation and correlation with proliferative activity (15). Epithelial cells demonstrated significantly higher telomerase activity, but contrastingly, shorter telomeres compared with stromal cells across the cycle (14, 15) (**Figure 4**). In the endometrium, Estrogen upregulates telomerase activity. Whilst progesterone inhibits telomerase activity and *hTERT* expression (15). The telomere and telomerase biology of normal endometrium has recently been reviewed in detail (14).

The Role of Telomeres and Telomerase in Benign Endometrial Disorders (Table 2)

The role of telomeres and telomerase in benign endometrial disorders was recently reviewed in detail in Hapangama et al (14). There are various benign gynecological disorders, such as endometriosis (243), recurrent reproductive failure, subfertility with reported abnormal telomerase activity and telomere length aberrations (13, 235). High telomerase activity, high hTERT mRNA and protein levels with longer mean endometrial telomere lengths are characteristics of the eutopic secretory endometrium (13, 235, 242, 244), whereas epithelial cells of ectopic lesions also demonstrated longer mean telomere length (15).

The progesterone dominant window of implantation in healthy women has shown virtually no hTERT immunoreactivity (235) and lowest telomerase activity (13, 234). However, immunostaining for hTERT was significantly and differentially increased in various endometrial cellular compartments in women with recurrent reproductive failure (235). These observations suggest that particular aberrations in cellular proliferation or causative dysregulation of telomerase to be important in endometrial pathologies. Furthermore, normal telomerase activity seems to play a pivotal functional role in ensuring normal endometrial function.

Alteration of Telomere Biology in Endometrial Premalignant Conditions and in Endometrial Cancer

Endometrial Hyperplasia

Endometrial epithelial hyper-proliferation with increased glandular to stromal cell ratio is defined as endometrial hyperplasia. Pathogenesis of endometrial hyperplasia is virtually always associated with relative predominance of the mitotic estrogen signal, due to direct excess of Estrogen or due to insufficient levels of progesterone (24). Anovulatory cycles in premenopausal women, extra-ovarian aromatization of adrenal androgens in to estrogenic compounds in obese women and iatrogenic interventions, such as Tamoxifen and Estrogen only hormonal replacement therapy are common examples

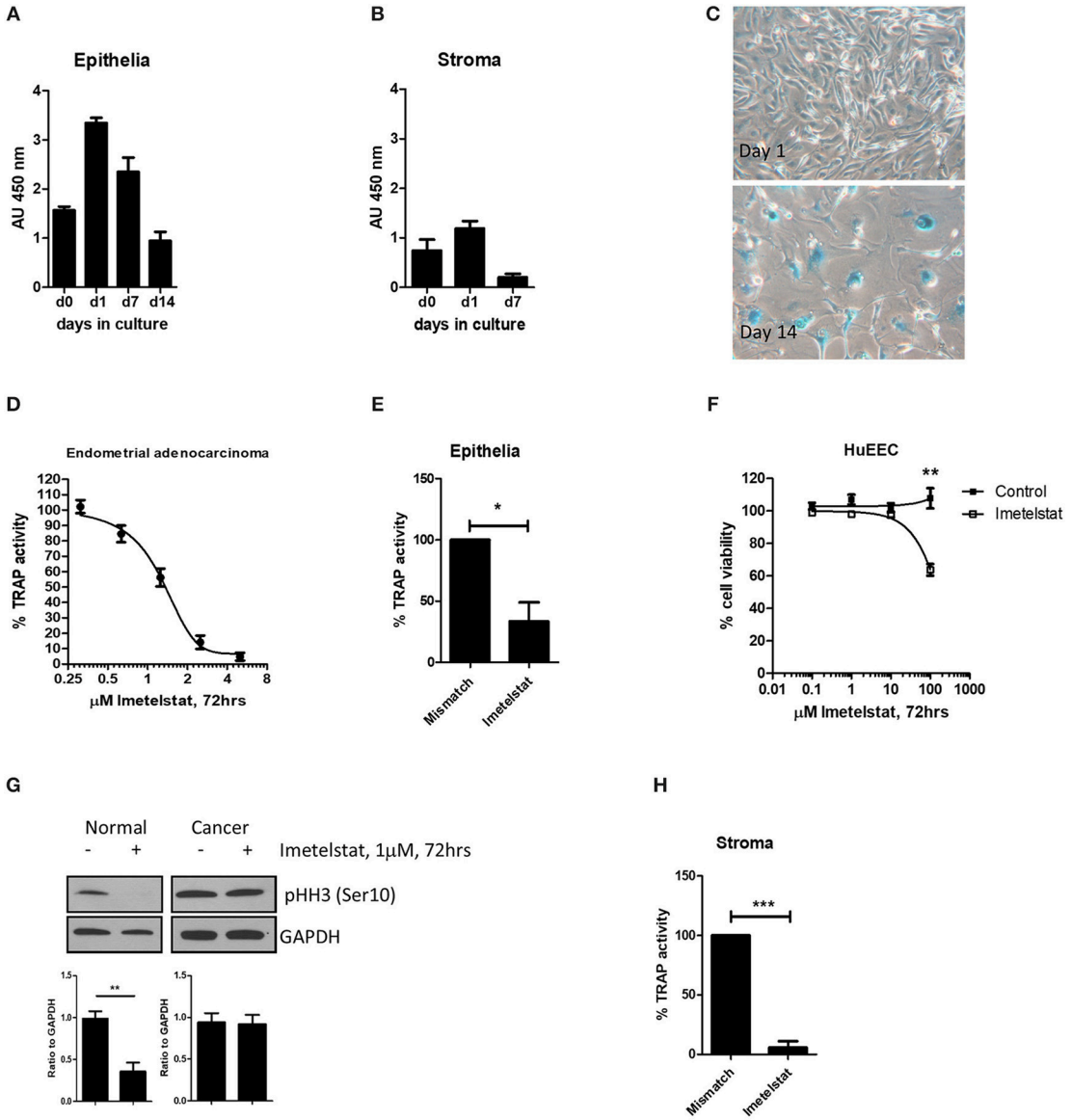


FIGURE 4 | From Valentijn et al. (15). Telomerase inhibitor, Imetelstat affects Telomerase Activity and cell proliferation, but not viability of endometrial epithelial cells. **(A)** Epithelial cells and **(B)** stromal cells were maintained in monolayer culture for the indicated times prior to harvesting for TRAP assay. For each time point, $n \geq 4$; Patient group 2. **(C)** Epithelial cells maintained in long-term culture had a phenotype consistent with senescence. Note the enlarged cells and positive blue stain for β-galactosidase in the micrographs (representative of $n = 5$). **(D)** Epithelial cells were isolated from an adenocarcinoma of the human endometrium and maintained in culture as a cell line. The cells were treated with the concentrations of Imetelstat indicated for 72 h prior to TRAP. TRAP activity is expressed as a percentage relative to the activity of the mismatch control (mean ± SEM for $n = 3$ separate experiments). **(E)** Epithelial cells were maintained in culture for up to 3 days and then treated with 1 μM Imetelstat or mismatch control oligonucleotide for a further 72 h prior to TRAP assay. TRAP activity is expressed as a percentage of the mismatch control ($n = 4$). *T*-test, $^{*}p = 0.02$. **(F)** EEC ($n = 5$) were directly seeded into 96-well dishes, allowed to attach and treated the next day with Imetelstat or the mismatch control at the concentrations indicated for 72 h. Cell viability was assessed by MTT assay. Note significant loss in cell viability at 100 μM (Mann Whitney test, $p = 0.002$). **(G)** Cultures of normal epithelial cells and an adenocarcinoma of the endometrium treated with Imetelstat or mismatch control as before, and immunoblotted for phospho-H3 [phosphohistone H3 (Ser10)]. Histone H3 is only phosphorylated on Ser 10 during mitosis. Shown is a representative blot (top) of normal epithelial cells ($n = 5$) and the adenocarcinoma (representative of two separate experiments) and densitometric analysis (bottom). *T*-test, $^{**}p = 0.009$. **(H)** Stromal cells were grown for 24 h and then treated with 1 μM Imetelstat or mismatch control oligonucleotide for 72 h prior to TRAP assay. Telomerase activity is expressed as a percentage of the mismatch control. *T*-test, $^{***}p = 0.0004$. This previously published figure in human reproduction (15) is reused with permission.

of conditions related to endometrial hyperplasia. Importantly, the premalignant endometrial hyperplasia, which includes the category of atypical hyperplasia/endometrial intraepithelial neoplasia according to the 2014 World Health Organization (WHO) classification is the typical precursor of endometrioid endometrial cancers (245).

Alterations in telomere lengths in endometrial hyperplasia

The involvement of telomere shortening in chromosomal instability has been associated with the initiation of carcinogenesis (246). There are only 2 studies that have examined telomere lengths in endometrial hyperplasia. A study using a telomere-FISH (telo-FISH) assay to measure telomere lengths, compared chromosomal arm loss or gain in premalignant endometrial lesions with normal endometrium, and reported telomere lengths to be stable with the pathological transformation in endometrial hyperplasia and in endometrial carcinoma (247). Albeit using a small sample size, the authors conclude that unlike in cervical precancerous lesions, endometrial hyperplasia did not have widespread chromosomal alterations, implying that endometrial carcinogenesis involves mechanisms distinct from those of cervical carcinogenesis, which is almost always induced by viral infection (247). However, close scrutiny of the data presented on different endometrial hyperplasia subtypes suggested that atypical endometrial hyperplasia may be associated with higher telomere length heterogeneity. This may be also suggestive of the involvement of ALT mechanism in this premalignant condition, but larger studies are needed to confirm the ALT mechanism in the true pre-malignant endometrial hyperplasia subtype with atypia. Importantly, the analysis method utilized in the Maida study did not allow inter-patient comparison of tissues samples (of different women) but was only suitable to compare adjacent cells of a single tissue sample. Therefore, the study presented insufficient data to conclude if there was a definite change in the telomere length in precancerous endometrial hyperplasia when compared with either normal or cancerous endometrium.

By using a three-dimensional (3D) imaging technique, a specific 3D arrangement of telomeres was revealed in tumor cell nuclei (248). Unlike the non-overlapping nature of telomeres in normal nuclei, telomeres of cancer nuclei have the tendency to form aggregates (248). Different numbers and sizes of such telomere aggregates can be found in tumor nuclei (248). Telomere aggregate formation does not depend on telomere length and telomerase activity (249).

The existence of telomere aggregates in precancerous lesions, such as in human cervical intraepithelial neoplasia supports the notion that changes in the organization of the 3D nucleus may facilitate tumorigenesis (250). The "telomere-driven genome-instability" can happen as a result of the close contiguity of telomeres forming aggregates of different numbers and sizes that increase the risk of end-to-end telomeric fusions followed by cycles of breakage-bridge-fusion (249). A significantly increased number of telomere aggregates was observed in atypical hyperplastic cells in a mouse models which is also a specific feature of cancer cells. Moreover, the *PTEN* heterozygous mouse model further demonstrated that 3D telomere architectural changes occur before the complete loss of *PTEN* and prior to the development of histological characteristics of atypical hyperplasia and endometrial carcinoma (251). Therefore, the presence of telomere aggregates in hyperproliferative lesions with atypical nuclei may render them to be precancerous changes. Further studies including larger sample size and both types of endometrial hyperplasia are warranted to examine and conclude on changes in telomere length in precancerous endometrial hyperplasia lesions.

Telomerase in endometrial hyperplasia

High hTERT levels and elevated telomerase activity were reported in all types of endometrial hyperplasia, including simple, complex and complex with atypia subtypes (252–256). This early observation prompted some investigators to propose that telomerase activity measured by TRAP assay to be a suitable tool to screen the endometria of post-menopausal women with post-menopausal bleeding (257). The authors proposed that this method will determine endometrial premalignant and malignant conditions (257) from benign endometrium, since telomerase activity was rarely detected in normal post-menopausal women, while the majority of endometrial hyperplasia and cancers contained high telomerase activity. However, there are other studies that reported a lack of measurable telomerase activity by TRAP assay in benign endometrial hyperplasia (258). Further work also found that it was possible to use hTERT immunohistochemical (IHC) analysis (259) as a marker for premalignant (atypical) endometrial hyperplasia. However, it is difficult to conclude on the diagnostic feasibility of telomerase activity or hTERT protein (IHC) in endometrial hyperplasia considering these studies, because of the inadequate sample sizes which were only $n = 12$ atypical endometrial hyperplasia in Brustmann (259) and $n = 18$ simple and atypical endometrial hyperplasia in Maida et al. (257) and Brustmann (259). In addition, the studies did not clarify whether the existence of endometrial hyperplasia cells were confirmed in the analyzed samples, particularly with TRAP assay and since endometrial hyperplasia can co-exist with either normal or cancerous endometrium, this may affect the results. Progesterone is one of the main current pharmacological therapies for treating endometrial hyperplasia (24) and telomerase being a (albeit indirect) downstream target of progesterone in the endometrium is of interest. This justifies future studies exploring the therapeutic utility of directly targeting telomerase in the treatment of endometrial hyperplasia.

Endometrial Cancer

Traditionally, EC had been divided into two major groups: estrogen-dependent, type-I (endometrioid type) and estrogen-independent, type-II (non-endometrioid), with the former accounting for 80% of ECs. Five-years survival rates are exceptionally poor for advanced type-I and type-II (high grade) EC at 23% which is a far worse rate than for most other common cancers, such as breast cancer (CRUK). However, the recent trend had been to apply for an alternative classification system that more accurately defines ECs into prognostically distinct molecular subtypes that reflect the underlying molecular alterations with well-described underlying genomic aberrations (260). EC is a disease of post-menopausal women, however, obesity associated unopposed estrogen action is an established cause for the trend toward increasing incidence of this cancer even in younger women (23, 24, 261). ECs are hormone responsive tumors and even high grade ECs retain some

TABLE 2 | Published literature on telomerase biology in benign endometrial disorders, telomerase, and telomere length.

TA/hTERT/TL	Title	References	No. of samples	Methods	Conclusions
hTERT/TL	Endometrial telomerase shows specific expression patterns in different types of reproductive failure	(235)	Control group ($n = 15$), idiopathic recurrent loss of empty gestational sacs ($n = 10$), miscarriage following identification of fetal cardiac activity ($n = 10$) and recurrent implantation failure ($n = 10$)	IHC (telomerase protein level) real-time PCR (TL)	In recurrent reproductive failure samples, the immunostaining for telomerase was significantly high in various endometrial cellular compartments and this indicates that there are specific alterations occur in the regulation of endometrial cell fate are associated with recurrent reproductive failure various types
hTERT/TL/TA	Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length	(13)	Group 1: healthy fertile ($n = 27$), group 2 symptomatic endometriosis ($n = 29$)	IHC (Telomerase and ERβ) qPCR (Mean TL), TRAP (TA)	Either weak or absent telomerase immunoreactivity was observed in the endometria of fertile healthy women throughout the luteal phase. Increased telomerase protein level (IHC) during the implantation window and the premenstrual endometria of women with endometriosis. The mean TL were significantly longer in endometria of women with endometriosis during the implantation window This study suggested that aberrant expression of telomerase in endometrium alters the cell fate and enhances the cellular proliferation and that leads to the occurrence of endometriosis
hTERT	The expression levels of stem cell markers importin13, c-kit, CD146, and telomerase are decreased in endometrial polyps	(236)	Control (proliferative phase $n = 20$ and secretory $n = 20$), Endometrial polyp (proliferative phase $n = 20$ and secretory $n = 20$)	IHC (Telomerase protein)	In endometrial polyp tissue, the level of telomerase was decreased in comparison with normal endometrial tissue
hTERT	Enhanced differentiation and clonogenicity of human endometrial polyp stem cells	(237)	Endometrial polyp ($n = 6$)	Quantitative RT-PCR (TERT)	No telomerase reverse transcriptase (TERT) expression was noted in endometrial polyp tissue
hTERT	Aberrant Telomerase Expression in the Endometrium of Infertile Women with Deep Endometriosis	(238)	Control group: Fertile women without endometriosis ($n = 44$) and Infertile women with endometriosis ($n = 25$) from which endometrium and endometriotic peritoneal lesions of the same patient were taken in the late luteal phase of the cycle	qRT-PCR (hTERT and GAPDH mRNA) based on TaqMan methodology	Telomerase (hTERT mRNA) level is associated with the development and progression of endometriosis
hTERT	The Status of Telomerase Enzyme Activity in Benign and Malignant Gynaecologic Pathologies	(239)	Benign endometrial tissue ($n = 7$): six endometrial polyps and one irregular proliferative-phase endometrium; endometriosic ectopic samples ($n = 13$) and endometrial cancer ($n = 6$)	Real-time reverse transcriptase polymerase chain reaction RT-PCR (hTERT mRNA)	hTERT was positive only in the irregular proliferative phase endometrium (14.2%) and hTERT was also positive in one of 13 endometriosis ectopic specimens (7.7%)
TA, hTERT, TL	Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis	(15)	Group 1 ($n = 85$) endometrial and matched blood, group 2 ($n = 74$) healthy endometrial biopsies (not on hormonal treatment) group 3 ($n = 5$) endometrial biopsies on medroxyprogesterone acetate (MPA) for contraception group 4 ($n = 10$) matched endometriotic ectopic and euotopic, group 5 ($n = 22$) healthy women in mid-secretory phase before ($n = 8$), and after administering 200 mg mifepristone ($n = 14$)	TRAP (TA), qPCR and Q-FISH (TL), immunoblotting (histone H3) (cell proliferation), 3D-culture (assess the ability of EECs to form spheroids, IHC (TERT and Ki67)	High TA and short TLs were observed in proliferating EECs *in vivo* and *in vitro*. In mid-secretory phase endometrial tissue where progesterone is dominant, TL was significantly shorter in comparison with the proliferative phase. Progestagen treatment repressed EEC TA *in vivo* and reduced endometrial TA in explants and *in vitro* cultures compared with non-treated cells

(Continued)

TABLE 2 | Continued

TA/hTERT/TL	Title	References	No. of samples	Methods	Conclusions
hTERT	Endometrial expression of telomerase, progesterone, and estrogen receptors during the implantation window in patients with recurrent implantation failure	(240)	Endometrial biopsies fertile ($n = 30$) and RIF ($n = 30$)	qRT-PCR (TERT, ER alpha and PR), western blotting and IHC (TERT and ER alpha)	Expression of endometrial telomerase was substantially increased as ER alpha decreased in women with RIF during the implantation window.
TA	Does telomerase activity have an effect on infertility in patients with endometriosis?	(241)	Healthy control ($n = 16$), endometriosis infertile ($n = 14$) and fertile ($n = 17$)	PCR (TA)	In peripheric blood analysis, telomerase activity is useless as a biomarker. Telomerase activity is absent in cystic wall and that suggesting a high differentiation of endometriosis tissue and that might be considered as a cause of low malignancy risk. Whereas, telomerase activity is high in the eutopic endometrium of the infertile group which may be the possible reason of endometriosis-related infertility.
hTERT, TA	Increased telomerase activity and human telomerase reverse transcriptase mRNA expression in the endometrium of patients with endometriosis	(242)	Healthy control ($n = 30$), endometriosis ($n = 30$)	qRT-PCR (hTERT), TRAP (TA)	In the endometrium of endometriosis patients, the hTERT mRNA is overexpressed and telomerase activity is increased suggesting that the replication potential of endometrial cells might be crucial in the pathogenesis of endometriosis

TA, teelomerase activity; TL, telomere length; IHC, immunohistochemistery; TRAP, telomeric repeat amplification protocol; hTERT, human telomerase reverse transcriptase; Q-FISH, quantitative fluorescent in situ hybridization; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; EECs, endometrial epithelial cells; RIF, recurrent implantation failure.

hormone responsiveness as depicted by the expression of steroid hormone receptors (261).

Evidence for telomere alterations in endometrial cancer (Figure 5) (Table 3)

A study in 1992 found that endometrial adenocarcinomas have reduced telomeric repeat sequences suggesting shorter telomeres compared with normal tissue (262). A decade later a second study demonstrated changes in telomere lengths in 17/23 (73.9%) of endometrial cancers using a Southern blot technique (269). Another study by Menon and Simha (273), using the same telomere restriction fragment (TRF) measurement, found that mean TRF lengths became shortened when normal endometrium underwent neoplastic changes (273). A study which used a telomere-oligonucleotide ligation assay demonstrated erosion of the telomere overhang length, rather than overall telomere length, and proposed that this might play a role in endometrial carcinogenesis and may be related to tumor aggressiveness (274). All these studies utilized techniques that assess the average telomere length values of a tissue sample. However, when endometrial samples were harvested and frozen, they did not examine if the proportion of the endometrial sample examined for telomere length actually contained cancerous cells. Subsequently, 12 years ago, Maida et al. (247) employed a telomere-FISH (telo-FISH) assay that assessed the relative telomere length in normal and pathological cells in intact tissue at the cellular level and no significant difference was found between the telomere length of normal endometrium and endometrial cancer (247). That study however did not specify the normal cell type that they used as the control (stromal/epithelium) and included only adenocarcinomas (Type I). A similar, but slightly modified version of telomere chromogenic *in situ* hybridization method was subsequently used by Akbay et al. and the authors demonstrated a significant telomere shortening in both type I and type II endometrial cancers in comparison with normal stromal cells (270). They also reported that the adjacent normal stromal cells were compared with epithelial cancer cells to demonstrate telomere shortening only in type II cancers. The authors expanded the study to confirm their hypothesis in a rodent model. These animals were generated with shortened telomeres to show that telomere attrition contributes to the initiation of type II endometrial cancers and progression of Type I endometrial cancers (270). This is of interest, but caution should be taken when interpreting these results, as the endometrial stromal cells are known to possess longer telomeres when compared even with healthy epithelial cells (14, 15) and that has been hypothesized to be due to the difference in the cell proliferation rates, telomerase activity levels and different regulation of telomere maintenance in these two cell types (14). Therefore, the data may simply reflect cell type specific difference in relative telomere lengths but not demonstrating a true endometrial cancer associated change in telomere lengths. Hashimoto et al. (274) found that endometrial cancers show short 3′ single-strand telomeric overhang length compared to normal endometrium (274). They also found that poorly differentiated cancers or deeply invading endometrial cancers had a longer overhang length in comparison with well-differentiated cancers

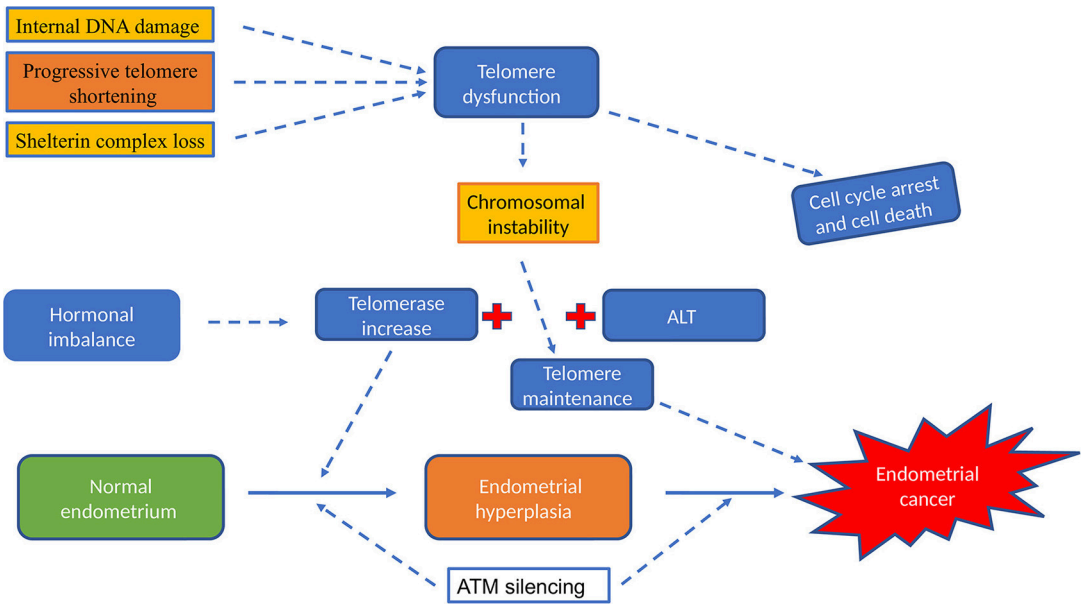


FIGURE 5 | The role of telomere and telomerase activity in endometrial cancer. Hormonal imbalance (excess of Estrogen or insufficient levels of progesterone) will increase telomerase and elevate telomerase activity was described in all types of endometrial hyperplasia and in endometrial cancer. Dysfunctional telomeres results in genomic instability, the first step in endometrial carcinogenesis. Telomerase dependent pathway is the most widely reported classical telomere length maintenance mechanism but ALT pathway; telomerase independent telomere maintenance was described in some cancer types that lack telomerase activity.

or superficial invading cancers and this may suggest that the 3′ overhang may have a role in tumor progression (274).

A recent paper that considered germline genetic variants in a genome wide association study (GWAS) as instrumental variables to appraise the causal relevance of telomere length for the risk of cancer, demonstrated that their predicted increase in telomere lengths was strongly associated with some specific cancers, such as gliomas, low grade serous ovarian cancers, lung adenocarcinomas, neuroblastomas, bladder cancers, melanomas, testicular cancers, and also endometrial cancers (275). However, this study did not measure the exact telomere length of the tissue of origin of cancers but assumed the particular genetic variance may promote longer telomere lengths. With that assumption, the authors calculated a stronger association of presumed longer telomere lengths and rarer cancers and cancers with a lower stem cell division rate (275). However, this data should be considered with caution, since age associated tissue/cell specific telomere length change is a well-established fact but that was not considered by the authors. Therefore, the postulated prediction in telomere length change may be relevant to the effect of genetic variants that were examined, in increasing cancer risk, but it does not provide direct or compelling evidence for a role for tissue telomere length change in endometrial carcinogenesis. When telomere lengths were estimated for cancer cohorts in The Cancer Genome Atlas (TCGA) dataset; sarcomas, testicular germ cell tumors and low grade gliomas were associated with longer telomeres whilst cervical and endometrial cancers had shortest average telomere length (276). This observation has also been explained as a result of some tumors having high telomerase activity, thus shorter telomere lengths that are stabilized [e.g., in testicular tumors (277)], and others have long telomere lengths accompanied by increased activity of the ALT mechanism (e.g., in low grade gliomas and sarcomas). Longer telomere length in PBMC has also been associated with a significantly increased risk of endometrial cancer in a group of Caucasian Americans (272). Since endometrial cancers are known to have high telomerase activity, the ALT mechanism is less likely to be active in those cancers. Considering the above evidence, it is likely that endometrial cancers have relatively shorter telomere lengths that are maintained by high telomerase activity compared with normal tissue. Further studies are warranted to examine subtype specific telomere length aberrations and the relationship of telomere lengths with the telomerase activity in the different types of endometrial cancers.

The protein and/or mRNA levels of the most conserved out of all shelterin proteins, POT1 (26) were increased in many different cancers including gastric, thyroid, breast (199, 278, 279) and in endometrial cancers (280). Higher levels of point mutations in the POT1 gene were observed in endometrial cancers, revealing that genetic variations in *POT1* may lead to carcinogenesis in the endometrium (280). Simultaneous conditional inactivation of the shelterin protein POT1a with the tumor suppressor p53 in endometrial epithelial cells in a murine model, induced type II metastatic adenocarcinomas in 100% of the animals by 15 months (281). This suggests that telomere dysfunction and loss of tumor suppressor genes can produce Type II endometrial cancers. This will obviously need to be accompanied by telomerase re-activation observed in most endometrial cancers supporting the cancer-associated increased cellular proliferation. The loss of POT1 proteins

activates ATR (282) and ATR activation requires Replication Protein A (RPA), which binds single stranded (ss) DNA (282); the POT1-TPP1 heterodimer protects telomere ends from being detected as DNA damage by excluding RPA from binding telomeric ssDNA. Therefore, the loss of POT1 described in endometrial cancer may cause inappropriate telomere access of telomerase resulting in compromised telomere capping and sustained telomere dysfunction facilitating genetic instability.

There are no published studies examining the expression or function of other shelterin proteins or TERRAs in EC to date.

Evidence for a role of telomerase in endometrial cancer (Figures 5, 6)

Kyo et al. examining 13 endometrial cancers and 5 cell lines derived from endometrial cancers using a Telomerase Repeated Amplification Protocol (TRAP) assay reported that 92% of cancer samples displayed detectable telomerase activity (263). At that point in time, the general consensus was that only specialized cells or cancer cells would have detectable telomerase activity. A year later, the same group increased their endometrial samples to 17, included 60 normal endometrial samples, and reported that being a somatic organ, the benign human endometrium, expresses dynamic levels of telomerase activity (measured by TRAP assay), with the highest levels observed in the late proliferative phase endometrium which was comparable to endometrial cancer. They also indicated that endometrial telomerase levels are closely associated with proliferation and likely to be regulated by estrogen (264). During the same year, Saito et al. examined a larger and more diverse endometrial cancer sample set and reported that activation of telomerase was found in most of these cancers, similar to the reports on gastric, prostate, bladder, and skin cancers (252, 283–286). Saito et al. further confirmed the earlier work by Kyo et al. that 28/30 endometrial cancers had high telomerase activity and late proliferative phase to have the highest telomerase activity levels in the benign endometrial samples. Additionally, the authors found that endometrial hyperplasia demonstrated high telomerase activity similar to cancer, whereas no activity was detected in healthy post-menopausal endometria with or without bleeding problems, indicating telomerase activity to be a suitable diagnostic test for identifying post-menopausal endometrial pathology (252). The authors also noted that telomerase activity was increased by estrogen which induced cell proliferation and was reduced in progesterone dominant conditions, indicative of an ovarian steroid hormonal regulation. The finding of high telomerase activity in endometrial cancers has been subsequently confirmed by many other groups (15, 25, 159, 239, 247, 267–269). In addition to the high telomerase activity measured by the gold standard test, the TRAP assay, some authors studied expression levels of components of the telomerase holoenzyme using qPCR to detect gene expression levels. They concluded that hTERT levels correlated well with TRAP assay data (159, 268) and both seem to be related to endometrial epithelial proliferation (15). In a relatively small study, Bonatz et al. (287) have shown a significant correlation between higher telomerase activity and higher International Federation of Gynecology and Obstetrics (FIGO) stage and grade, suggesting that telomerase activity is increased in advanced stages of endometrial cancer (287). In their study, Wang et al showed that 82% of their endometrial cancer samples had telomerase activity but they did not find any correlation between telomere lengths and telomerase activity in different gynaecologic cancers (cervical, ovarian and endometrial) (269).

Detection of hTERT mRNA in peripheral blood (PBMCs) has been reported to be significantly higher in women with EC compared to patients with benign uterine diseases and healthy controls. Using a relatively moderate sample size ($n = 56$ patients with endometrial cancer, $n = 40$ patients with benign uterine diseases and $n = 40$ healthy control) the authors claimed that the exact levels of hTERT mRNA will demarcate those with metastatic disease thus may be useful in stratifying patients for adjunctive therapy (288). This claim needs to be confirmed in a future study which includes an adequate sample size.

Recently, in two progesterone responsive and progesterone-insensitive human endometrial cancer cell lines (162), ATM protein was shown by reverse-phase protein array (RPPA) to participate in progesterone stimulation to suppress carcinogenesis in the endometrium (162). Additionally, a progressive loss of ATM levels from hyperplasia to the lowest levels was observed in type 1 endometrial cancer lesions and there was a negative relationship of the pathological grades and ATM levels (162).

Activating *hTERT* promotor mutations do not usually occur in a background of loss of the tumor suppressor protein ARID1A (289). Recent data suggest that ARID1A negatively regulates *hTERT* transcription and telomerase activity; while induction of ARID1A represses transcription and histones via occupying SIN3A and H3K9me3 sites (290). ARID1A is a member of the SWI/SNF chromatin remodeling complex, and it is frequently mutated in endometrial adenocarcinoma (291), therefore it is conceivable how hTERT might be upregulated in the endometrial cancer with loss of ARID1A.

In endometrial cancer cell lines, telomerase activity and expression of hTERT were both increased by estrogen in an estrogen receptor alpha (ERα) dependent and estrogen responsive element (ERE) dependent effect in the *hTERT* promoter (292). Additionally, a previous study showed that estrogen also induced telomerase activity via post-transcriptional Akt dependent phosphorylation of hTERT in human ovarian cancer cell lines (293).

Lehner et al. (268) compared hTERT mRNA levels and telomerase activity using TRAP assay in normal endometrium with endometrial cancer and they concluded that the levels and activity were significantly higher in cancer and low in normal endometrium during the secretory phase of the menstrual cycle as well as in atrophic endometrium (268). Thus, they suggested that quantitative analysis of these parameters may be useful as markers for diagnosis of endometrial cancer.

PTEN regulates telomerase activity, most likely through its known effects on the PI3-kinase/Akt pathway (294). Reconstitution of PTEN in the PTEN-null Ishikawa endometrial cancer cells resulted in inhibition of cell growth and suppression of Akt phosphorylation as well as a parallel decrease in telomerase activity and hTERT mRNA levels (294). At present, there are no reports of different expression levels of other telomerase associated proteins. Interestingly DC, which is associated with an

TABLE 3 | Published literature on telomerase biology in endometrial cancer: telomerase activity and telomere length.

TA/TL/ hTERT	Title	References	No. of samples	Methods	Key findings
TL	Telomere reduction in endometrial adenocarcinoma.	(262)	Normal endometrium and EC samples ($n = 11$) and five endometrial carcinoma cell lines. Note: in this study the normal endometrial samples (control) were taken from areas adjacent to the cancer lesions	The relative number of telomeric repeat sequences in each sample was measured by hybridization of these deoxyribonucleic acids (DNA) to a probe specific for the human telomeric repeat. quantifion of the Hybridization signals were performed by autoradiography and a β-particle detection system	Telomeric repeat sequences were reduced in EC vs. normal endometium (in 10 out of 11 cases) and also reduced in endometrial carcinoma cell lines. the data of this study suggested that Telomeric reduction is a genetic characteristic of many endometrial cancers. Telomere reduction may play an essential role in the genesis and progression of endometrial carcinoma, or it may be a secondary effect of the tumorigenesis process
TA	Telomerase activity in gynecological tumors	(263)	EC cell lines ($n = 5$) EC ($n = 13$)	TRAP assay with dilution assay	5 of 5 EC cell lines displayed strong signals for TA 12 of 13 (92%) ECs positive for TA 4 of 13 ECs classified as high TA No significant correlation between high TA and clinical stage or pathological grade of EC
TA	Telomerase Activity in Human Endometrium	(264)	Normal ($n = 60$) EC ($n = 17$)	TRAP assay Immunohistochemistry	TA regulated during the menstrual cycle Highest TA in late proliferative phase No or faint TA in late secretory phase and during menstruation TA in EC comparable to that in late proliferative phase Low level of TA in post-menopausal samples telomerase enzyme is linked to cellular proliferation and it might be regulated by Estrogen
TA	Proliferation-associated regulation of telomerase activity in human endometrium and its potential implication in early cancer diagnosis	(252)	Normal ($n = 15$) PMB ($n = 6$) Hyperplastic PM ($n = 16$) Non-hyperplastic PM ($n = 9$) EC ($n = 30$)	TRAP assay (TA)	TA detected in 28 of 30 ECs TA demonstrated in all hyperplastic endometrial samples Early-proliferative phase showed no TA Late-proliferative phase showed the strongest TA Late-secretory, early pregnancy and post-menopausal samples showed no TA
TA	Telomerase expression in normal endometrium, endometrial hyperplasia, and endometrial adenocarcinoma.	(253)	Normal endometrium (pre and post-menopausal) ($n = 40$), EH ($n = 17$), EC ($n = 48$)	TRAP assay (TA)	Telomerase activity was detected in 40 of 48 cases of endometrial adenocarcinoma. In this study telomerase activity did not correlated with tumor grade, myometrial invasion, or cancer stage. However, there was a statistical significant association between telomerase activity in benign atrophic endometrium vs. any endometrial abnormality in women 52 years of age or older
TA	Telomerase Activity in Benign Endometrium and Endometrial Carcinoma.	(265)	EC ($n = 20$) Benign endometrium ($n = 14$)	TRAP-eze using PCR Quantitative DNA analysis using Feulgen method	Strong TA detected in 8 of 8 benign, premenopausal endometrial specimens (proliferative $n = 5$; secretory $n = 3$) Weak TA in 6 of 6 post-menopausal samples TA detected in 19 of 20 Ecs No correlation of positive TA with FIGO tumor grade, depth of myometrial invasion, or DNA content in the EC specimens

(Continued)

TABLE 3 | Continued

TA/TL/ hTERT	Title	References	No. of samples	Methods	Key findings
TA	Telomerase activity in human gynecological malignancies.	(266)	EC ($n = 6$) Normal endometria ($n = 8$) Ovarian carcinomas ($n = 13$) Benign ovarian tumors ($n = 5$) Cervical carcinomas ($n = 6$) Normal cervices ($n = 5$)	TRAP assay using PCR	TA was detected in 6 of 6 EC TA was detected in 5 of the 8 normal endometrial samples
TA	Telomerase activity in gynecologic tumors.	(258)	EC ($n = 4$) Ovarian cancer ($n = 16$) Cervical cancer ($n = 16$) Benign (total $n = 8$; endometrial $n = 4$) Normal (total $n = 4$; endometrial $n = 1$)	TRAP assay using PCR	TA activity was detected in all ECs TA was not detected in any benign or normal samples
TA	Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation	(267)	Normal ($n = 52$) EC ($n = 19$)	TRAP assay (stretch PCR) *in situ* RNA hybridization of hTERC Cell culture and MTT assay	TA regulated in menstrual-phase-dependent manner Maximal TA in late proliferative phase Minimal TA in late secretory phase and post-menopausal TA in EC equivalent to that in late proliferative phase TA limited to epithelial glandular cells in proliferative phase Telomerase activation closely associated with cellular proliferative activity Estrogen may play a role in the regulation of TA
TA	Telomerase activity correlates with histo-pathological factors in uterine endometrial carcinoma.	(25)	EC ($n = 35$)	TRAP assay	TA detected in 31 of 35 ECs Of the 31 tumors showing positive TA: 15 tumors had high and 16 had low TA High TA in post-menopausal EC significantly correlated with the presence of pelvic lymph node metastasis and advanced surgical stage
TA	Human telomerase reverse transcriptase as a critical determinant of telomerase activity in normal and malignant endometrial tissues	(159)	Normal ($n = 32$) EC ($n = 23$) EC cell lines ($n = 5$)	TRAP assay	TA detected in 12 of 12 proliferative endometria TA detected in 4 of 13 secretory phase endometria TA detected in 3 of 7 atrophic endometria TA detected in 20 of 23 ECs Approximately 80% of ECs were concordant for positivity or negativity of hTERT expression and TA—suggesting hTERT is a critical factor directing TA in tumors
hTERT, TA	Quantitative analysis of telomerase hTERT mRNA and telomerase activity in endometrioid adenocarcinoma and in normal endometrium	(268)	Normal ($n = 20$) EC ($n = 26$)	RT-PCR of hTERT mRNA TRAP assay	In normal endometrium hTERT mRNA and TA levels were highest in the proliferative phase and relatively low in secretory and atrophic endometrium hTERT mRNA levels and TA levels significantly higher in EC than in normal endometrium
TA/TL	The relationship between telomere length and telomerase activity in gynecologic cancers	(269)	EC ($n = 23$) Ovarian ($n = 15$) Cervical ($n = 14$)	TRAP(EZE) ELISA kit (TA) Southern blot (TL)	TA detected in 18 of 22 ECs There was no detectable relationship between TL and stage of disease, pathologic diagnosis, or TA Rate and strength of telomerase activity increased progressively from clinical Stage I–III

(Continued)

TABLE 3 | Continued

TA/TL/ hTERT	Title	References	No. of samples	Methods	Key findings
TA	Is the telomerase assay useful for screening of endometrial lesions?	(257)	Normal ($n = 82$) EC ($n = 15$) Hyperplasia ($n = 3$)	TRAP assay (TRAP-eze telomerase detection kit)	TA detected in 10 of 15 proliferative phase endometrial samples TA was detected in 5 of 20 secretory phase samples and 1 of 4 samples taken during menstruation TA was exhibited in 3 of 38 samples from the post-menopausal patients TA was detected in 12 of 15 EC pre-operative samples and 15 of 15 post-operative biopsies Lack of TA does not indicate an absence of endometrial lesions
TL	Differential Roles of Telomere Attrition in Type I and II Endometrial Carcinogenesis	(270)	EC ($n = 29$) Normal ($n = 29$)	Evaluated telomere lengths *in situ* using a novel chromogenic method (Telo-CISH) and Southern blot analysis	Telo-CISH demonstrates telomere shortening is a general feature of type I and II endometrial carcinogenesis Southern blot analysis confirmed significant telomere attrition in type I tumors relative to matched normal DNA
TL	Telomere length and genetic analyses in population-based studies of endometrial cancer risk.	(271)	EC ($n = 279$) Matched controls ($n = 791$)	Relative leukocyte TL measured using qPCR based telomere assay from blood sample	No relationship between leukocyte TL and EC
hTERT	The status of telomerase enzyme activity in benign and malignant gynaecologic pathologies.	(239)	EC ($n = 6$) Benign endometrium ($n = 7$) Ovarian ($n = 35$) Cervical ($n = 6$) Placental site trophoblastic tumor tissue ($n = 1$)	hTERT mRNA quantification using RT-PCR (presence of hTERT, not assessing TA)	6 of 6 ECs found to be hTERT positive Benign endometrial tissue samples: 6 endometrial polyps and 1 irregular proliferative-phase endometrium; hTERT positivity was found only in irregular proliferative phase endometrium
TL	Association of leukocyte telomere length in peripheral blood leukocytes with endometrial cancer risk in Caucasian Americans	(272)	EC ($n = 139$) Controls ($n = 139$)	Relative leukocyte TL measured using qPCR based telomere assay from blood sample	Normalized LTL was significantly longer in EC cases than in controls Individuals with long LTL had significantly increased risk of EC compared to those with short LTL

EC, endometrial cancer; EH, endometrial hyperplasia; TA, telomerase activity; TL, telomere length; TRAP, telomeric repeat amplification protocol; hTERT, human telomerase reverse transcriptase; hTERC, human telomerase RNA component.

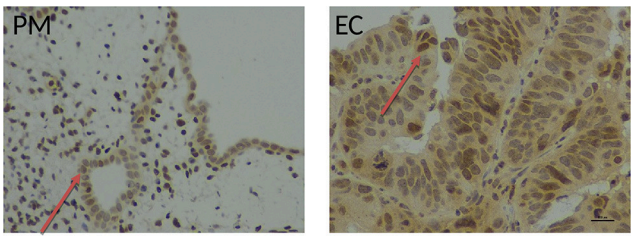


FIGURE 6 | Immunohistochemical staining with an anti-human telomerase antibody in healthy and endometrial tissue samples. Endometrial tissue sections demonstrating hTERT immunostaining in full thickness post-menopausal (PM) section and pipelle biopsy from a patient with endometrial cancer (EC) using a polyclonal rabbit anti-human telomerase antibody (ab27573, Abcam, Cambridge UK), detected with ImmPRESS anti-rabbit polymer and visualization with ImmPACT DAB (Vector Laboratories, Peterborough, UK). Positive nuclear hTERT brownish staining was observed in endometrial normal and cancer glands (red arrow). Magnification ×400, scale bar 10 μm.

increase in the risk of developing some cancer types, has not been reported to be linked with an increased incidence in EC. There are no published studies examining the role of dyskerin in EC to date.

ANTI-TELOMERASE THERAPY

Telomerase was thought to be a suitable target for anti-cancer agents due to the high activity levels seen in most cancers. Available anti-telomerase strategies can be grouped into three main categories: (1) Telomerase inhibitors, (2) telomerase targeted immunotherapy and (3) telomerase directed viral therapy. Imetelstat (GRN163L) is the only clinically applicable specific oligonucleotide telomerase inhibitor (**Figure 4**), which is a water soluble, acid and nuclease resistant compound that forms stable RNA duplexes (295). It prevents the 13-nucleotide region of TERC to form a complex with hTERT. Unfortunately, clinical data for Imetelstat has been disappointing with high toxicity (296). The other anti-telomerase agents are also undergoing clinical trials yet there are no conclusive data yet available for their clinical effectiveness in cancer. For those cancers harboring activating TERT promotor mutations, directed immunotherapies have been proposed as part of a personalized treatment (297). Anti-telomerase therapy and its relevance to cancer was reviewed in detail in several reviews recently (298, 299).

Progestogens remain to be one of the main hormone-based chemotherapeutic agents that are used in early, advanced and recurrent EC with only modest benefit (24). The loss of response to progesterone or progressive disease despite progestogens has been alluded to progesterone-induced down regulation of progesterone receptor (261) and the lack of progesterone receptor expression is a feature of advanced ECs (261). Since telomerase levels are high in most ECs and since telomerase seem to be a downstream target of progesterone in the endometrium, direct telomerase inhibition may have an added benefit in some women with EC. Those with recurrent disease despite progesterone treatment or having PR negative advanced ECs may particularly respond to telomerase inhibition. However, the available limited *in vitro* data may suggest that Imetelstat may reduce telomerase activity but may not cause cell death (**Figure 4**) (15). Since the *in vitro* data has been generated in a mono-cellular 2D culture system comprising of only epithelial cells, thus it may not accurately reflect the *in vivo* response to the medication (158). Further studies using either physiologically more relevant 3D culture systems containing epithelial and stromal cells or animal models are warranted to explore this avenue further before embarking on clinical studies.

CONCLUSION

Telomere and telomerase have an intricate relationship with cancer-related multiple cellular functional pathway aberrations. Collectively, the available evidence suggests that endometrial cancer tissues have relatively short telomeres that are maintained by high telomerase activity. Further studies should shed light into different endometrial cancer subtype-associated changes in telomere length, which might facilitate exploring alternative therapeutic strategies to prevent occurrence and progression or recurrence of this devastating disease. Future studies examining the involvement of various telomere and telomerase associated proteins as prognostic markers that potentially could be used in stratifying patients for adjuvant therapies in endometrial cancer are also warranted. In addition, a comprehensive understanding of the telomere and telomerase biology in endometrial cancer will facilitate assessment of targeting telomerase as a personalized therapeutic strategy in endometrial cancer.

AUTHOR CONTRIBUTIONS

DH conceived the manuscript. RA, MA, LB, and DH prepared the first draft. GS, RA, MA, and DH revised the manuscript critically for important intellectual content and RA, MA, and DH prepared the figures and references. All authors revised and read the manuscript and approved the submitted final version.

FUNDING

The authors would like to acknowledge the support from Wellbeing of Women project grant RG1487 (DH), Higher Committee for Education Development in Iraq (RA), Liverpool Women's Hospital cancer charity (MA).

ACKNOWLEDGMENTS

The authors are grateful for department of Women's and Children's Health of University of Liverpool in supporting this work (RA, MA, and DH).

REFERENCES

1. van Steensel B, Smogorzewska A, de Lange T. TRF2 protects human telomeres from end-to-end fusions. *Cell.* (1998) 92:401–13.
2. Karlseder J, Broccoli D, Dai Y, Hardy S, de Lange T. p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science.* (1999) 283:1321–5.
3. Sandell LL, Zakian V A. Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell.* (1993) 75:729–39.
4. Deng Y, Chan S, Chang S. Telomere dysfunction and tumor suppression-the senescence connection. *Nat Rev Cancer.* (2008) 8:450–8. doi: 10.1038/nrc2393
5. Ohtani N, Mann DJ, Hara E. Cellular senescence: its role in tumor suppression and aging. *Cancer Sci.* (2009) 100:792–7. doi: 10.1111/j.1349-7006.2009.01123.x
6. von Zglinicki T, Saretzki G, Docke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? *Exp Cell Res.* (1995) 220:186–93.
7. von Zglinicki T. Role of oxidative stress in telomere length regulation and replicative senescence. *Ann N Y Acad Sci.* (2000) 908:99–110. doi: 10.1111/j.1749-6632.2000.tb06639
8. Greider CW. Telomerase discovery: the excitement of putting together pieces of the puzzle (Nobel lecture). *Angew Chem Int Ed Engl.* (2010) 49:7422–39. doi: 10.1002/anie.201002408
9. Blackburn EH, Greider CW, Henderson E, Lee MS, Shampay J, Shippen-Lentz D. Recognition and elongation of telomeres by telomerase. *Genome.* (1989) 31:553–60.
10. Wright WE, Piatyszek MA, Rainey WE, Byrd W, Shay JW. Telomerase activity in human germline and embryonic tissues and cells. *Dev Genet.* (1996) 18:173–9.
11. Hiyama E, Hiyama K. Telomere and telomerase in stem cells. *Br J Cancer.* (2007) 96:1020–4. doi: 10.1038/sj.bjc.6603671
12. Khattar E, Kumar P, Liu CY, Akincilar SC, Raju A, Lakshmanan M, et al. Telomerase reverse transcriptase promotes cancer cell proliferation by augmenting tRNA expression. *J Clin Invest.* (2016) 126:4045–60. doi: 10.1172/JCI86042
13. Hapangama DK, Turner MA, Drury JA, Quenby S, Saretzki G, Martin-Ruiz C, et al. Endometriosis is associated with aberrant endometrial expression of telomerase and increased telomere length. *Hum Reprod.* (2008) 23:1511–9. doi: 10.1093/humrep/den172
14. Hapangama DK, Kamal A, Saretzki G. Implications of telomeres and telomerase in endometrial pathology. *Hum Reprod Update.* (2017) 23:166–87. doi: 10.1093/humupd/dmw044
15. Valentijn AJ, Saretzki G, Tempest N, Critchley HO, Hapangama DK. Human endometrial epithelial telomerase is important for epithelial proliferation and glandular formation with potential implications in endometriosis. *Hum Reprod.* (2015) 30:2816–28. doi: 10.1093/humrep/dev267
16. Minafra M, Laforgia R, Sederino MG, Fedele S, Delvecchio A, Lattarulo S, et al. Study of the role of telomerase in colorectal cancer: preliminary report and literature review. *G Chir.* (2017) 38:213–8. doi: 10.11138/gchir/2017.38.5.213
17. Lu L, Zhang C, Zhu G, Irwin M, Risch H, Menato G, et al. Telomerase expression and telomere length in breast cancer and their associations with adjuvant treatment and disease outcome. *Breast Cancer Res.* (2011) 13:R56. doi: 10.1186/bcr2893
18. Graham MK, Meeker A. Telomeres and telomerase in prostate cancer development and therapy. *Nat Rev Urol.* (2017) 14:607–19. doi: 10.1038/nrurol.2017.104
19. Lindemann K, Eskild A, Vatten LJ, Bray F. Endometrial cancer incidence trends in Norway during 1953–2007 and predictions for 2008–2027. *Int J Cancer.* (2010) 127:2661–8. doi: 10.1002/ijc.25267
20. Uterine Cancer Statistics. Available online at: http://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/uterine-cancer. (accessed April 26, 2019).
21. Orekoya O, Samson ME, Trivedi T, Vyas S, Steck SE. The impact of obesity on surgical outcome in endometrial cancer patients: a systematic review. *J Gynecol Surg.* (2016) 32:149–57. doi: 10.1089/gyn.2015.0114
22. Tempest N, Maclean A, Hapangama DK. Endometrial stem cell markers: current concepts and unresolved questions. *Int J Mol Sci.* (2018) 19:E3240. doi: 10.3390/ijms19103240
23. Hapangama DK, Kamal AM, Bulmer JN. Estrogen receptor beta: the guardian of the endometrium. *Hum Reprod Update.* (2015) 21:174–93. doi: 10.1093/humupd/dmu053
24. Kamal A, Tempest N, Parkes C, Alnafakh R, Makrydima S, Adishesh M, et al. Hormones and endometrial carcinogenesis. *Horm Mol Biol Clin Investig.* (2016) 25:129–48. doi: 10.1515/hmbci-2016-0005
25. Ebina Y, Yamada H, Fujino T, Furuta I, Sakuragi N, Yamamoto R, et al. Telomerase activity correlates with histo-pathological factors in uterine endometrial carcinoma. *Int J Cancer.* (1999) 84:529–32.
26. de Lange T. Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev.* (2005) 19:2100–10. doi: 10.1101/gad.1346005
27. Fairall L, Chapman L, Moss H, de Lange T, Rhodes D. Structure of the TRFH dimerization domain of the human telomeric proteins TRF1 and TRF2. *Mol Cell.* (2001) 8:351–61. doi: 10.1016/S1097-2765(01)00321-5
28. Lei M, Podell ER, Cech TR. Structure of human POT1 bound to telomeric single-stranded DNA provides a model for chromosome end-protection. *Nat Struct Mol Biol.* (2004) 11:1223–9. doi: 10.1038/nsmb867
29. Nelson ND, Dodson LM, Escudero L, Sukumar AT, Williams CL, Mihalek I, et al. The C-terminal extension unique to the long isoform of the shelterin component TIN2 enhances its interaction with TRF2 in a phosphorylation- and dyskeratosis congenita cluster-dependent fashion. *Mol Cell Biol.* (2018) 38:e00025-18. doi: 10.1128/MCB.00025-18
30. Ye JZ, Donigian JR, van Overbeek M, Loayza D, Luo Y, Krutchinsky AN, et al. TIN2 binds TRF1 and TRF2 simultaneously and stabilizes the TRF2 complex on telomeres. *J Biol Chem.* (2004) 279:47264–71. doi: 10.1074/jbc.M409047200
31. Palm W, de Lange T. How shelterin protects mammalian telomeres. *Ann Rev Genet.* (2008) 42:301–34. doi: 10.1146/annurev.genet.41.110306.130350
32. Takai KK, Kibe T, Donigian JR, Frescas D, de Lange T. Telomere protection by TPP1/POT1 requires tethering to TIN2. *Mol Cell.* (2011) 44:647–59. doi: 10.1016/j.molcel.2011.08.043
33. Vogan JM, Zhang X, Youmans DT, Regalado SG, Johnson JZ, Hockemeyer D, et al. Minimized human telomerase maintains telomeres and resolves endogenous roles of H/ACA proteins, TCAB1, and Cajal bodies. *Elife.* (2016) 5:e18221. doi: 10.7554/eLife.18221
34. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature.* (1990) 345:458–60.
35. Cimino-Reale G, Pascale E, Battiloro E, Starace G, Verna R, D'Ambrosio E. The length of telomeric G-rich strand 3′-overhang measured by oligonucleotide ligation assay. *Nucleic Acids Res.* (2001) 29:E35. doi: 10.1093/nar/29.7.e35
36. Greider CW. Telomeres. *Curr Opin Cell Biol.* (1991) 3:444–51.
37. Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, et al. Mammalian telomeres end in a large duplex loop. *Cell.* (1999) 97:503–14.
38. O'Connor MS, Safari A, Xin H, Liu D, Songyang Z. A critical role for TPP1 and TIN2 interaction in high-order telomeric complex assembly. *Proc Natl Acad Sci USA.* (2006) 103:11874–9. doi: 10.1073/pnas.0605303103
39. Martinez P, Thanasoula M, Munoz P, Liao C, Tejera A, McNees C, et al. Increased telomere fragility and fusions resulting from TRF1 deficiency lead to degenerative pathologies and increased cancer in mice. *Genes Dev.* (2009) 23:2060–75. doi: 10.1101/gad.543509
40. Karlseder J, Hoke K, Mirzoeva OK, Bakkenist C, Kastan MB, Petrini JHJ, et al. The telomeric protein TRF2 binds the ATM kinase and can inhibit the ATM-dependent DNA damage response. *PLoS Biol.* 2:E240. doi: 10.1371/journal.pbio.0020240
41. Li X, Liu W, Wang H, Yang L, Li Y, Wen H, et al. Rap1 is indispensable for TRF2 function in etoposide-induced DNA damage response in gastric cancer cell line. *Oncogenesis.* (2015) 4:e144. doi: 10.1038/oncsis.2015.1
42. Rai R, Chen Y, Lei M, Chang S. TRF2-RAP1 is required to protect telomeres from engaging in homologous recombination-mediated deletions and fusions. *Nat Commun.* (2016) 7:10881. doi: 10.1038/ncomms10881
43. Kibe T, Zimmermann M, de Lange T. TPP1 blocks an ATR-mediated resection mechanism at telomeres. *Mol Cell.* (2016) 61:236–46. doi: 10.1016/j.molcel.2015.12.016

44. Stansel RM, de Lange T, Griffith JD. T-loop assembly *in vitro* involves binding of TRF2 near the 3′ telomeric overhang. *EMBO J.* (2001) 20:5532–5540. doi: 10.1093/emboj/20.19.5532
45. Doksani Y, Wu JY, de Lange T, Zhuang X. Super-resolution fluorescence imaging of telomeres reveals TRF2-dependent t-loop formation. *Cell.* (2013) 155:345–56. doi: 10.1016/j.cell.2013.09.048
46. Colgin LM, Baran K, Baumann P, Cech TR, Reddel RR. Human POT1 facilitates telomere elongation by telomerase. *Curr Biol.* (2003) 13:942–6. doi: 10.1016/S0960-9822(03)00339-7
47. Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, et al. Control of human telomere length by TRF1 and TRF2. *Mol Cell Biol.* (2000) 20:1659–68. doi: 10.1128/MCB.20.5.1659-1668.2000
48. van Steensel B, de Lange T. Control of telomere length by the human telomeric protein TRF1. *Nature.* (1997) 385:740–3.
49. Bunch JT, Bae NS, Leonardi J, Baumann P. Distinct requirements for Pot1 in limiting telomere length and maintaining chromosome stability. *Mol Cell Biol.* (2005) 25:5567–78. doi: 10.1128/MCB.25.13.5567-5578.2005
50. Lei M, Zaug AJ, Podell ER, Cech TR. Switching human telomerase on and off with hPOT1 protein *in vitro*. *J Biol Chem.* (2005) 280:20449–56. doi: 10.1074/jbc.M502212200
51. Luciano P, Coulon S, Faure V, Corda Y, Bos J, Brill SJ, et al. RPA facilitates telomerase activity at chromosome ends in budding and fission yeasts. *EMBO J.* (2012) 31:2034–46. doi: 10.1038/emboj.2012.40
52. Rubtsova MP, Skvortsov DA, Petruseva IO, Lavrik OI, Spirin PV, Prasolov VS, et al. Replication protein A modulates the activity of human telomerase *in vitro*. *Biochemistry (Mosc).* (2009) 74:92–6. doi: 10.1134/S0006297909010143
53. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science.* (2003) 300:1542–8. doi: 10.1126/science.1083430
54. Haring SJ, Mason AC, Binz SK, Wold MS. Cellular functions of human RPA1. Multiple roles of domains in replication, repair, and checkpoints. *J Biol Chem.* (2008) 283:19095–111. doi: 10.1074/jbc.M800881200
55. Ottaviani A, Gilson E, Magdinier F. Telomeric position effect: from the yeast paradigm to human pathologies? *Biochimie.* (2008) 90:93–107. doi: 10.1016/j.biochi.2007.07.022
56. Lou Z, Wei J, Riethman H, Baur JA, Voglauer R, Shay JW, et al. Telomere length regulates ISG15 expression in human cells. *Aging.* (2009) 1:608–21. doi: 10.18632/aging.100066
57. Boussouar A, Barette C, Nadon R, Saint-Léger A, Broucqsault N, Ottaviani, A., et al. Acacetin and chrysin, two polyphenolic compounds, alleviate telomeric position effect in human cells. *Mol Ther Nucleic Acids.* (2013) 2:e116. doi: 10.1038/mtna.2013.42
58. Robin JD, Ludlow AT, Batten K, Magdinier F, Stadler G, Wagner KR, et al. Telomere position effect: regulation of gene expression with progressive telomere shortening over long distances. *Genes Dev.* (2014) 28:2464–76. doi: 10.1101/gad.251041.114
59. Campisi J. The biology of replicative senescence. *Eur J Cancer.* (1997) 33:703–9.
60. Robin JD, Ludlow AT, Batten K, Gaillard MC, Stadler G, Magdinier F, et al. SORBS2 transcription is activated by telomere position effect-over long distance upon telomere shortening in muscle cells from patients with facioscapulohumeral dystrophy. *Genome Res.* (2015) 25:1781–90. doi: 10.1101/gr.190660.115
61. Hewitt G, Jurk D, Marques FD, Correia-Melo C, Hardy T, Gackowska A, et al. Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nat Commun.* (2012) 3:708. doi: 10.1038/ncomms1708
62. Martinez P, Blasco MA. Role of shelterin in cancer and aging. *Aging Cell.* (2010) 9:653–66. doi: 10.1111/j.1474-9726.2010.00596.x
63. Maser RS, DePinho RA. Connecting chromosomes, crisis, and cancer. *Science.* (2002) 297:565–9. doi: 10.1126/science.297.5581.565
64. Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E, Lingner J. Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science.* (2007) 318:798–801. doi: 10.1126/science.1147182
65. Diala I, Wagner N, Magdinier F, Shkreli M, Sirakov M, Bauwens S, et al. Telomere protection and TRF2 expression are enhanced by the canonical Wnt signalling pathway. *EMBO Rep.* (2013) 14:356–63. doi: 10.1038/embor.2013.16
66. Tong AS, Stern JL, Sfeir A, Kartawinata M, de Lange T, Zhu XD, et al. ATM and ATR signaling regulate the recruitment of human telomerase to telomeres. *Cell Rep.* (2015) 13:1633–46. doi: 10.1016/j.celrep.2015.10.041
67. Redon S, Reichenbach P, Lingner J. The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. *Nucleic Acids Res.* (2010) 38:5797–806. doi: 10.1093/nar/gkq296
68. Cusanelli E, Romero CA, Chartrand P. Telomeric noncoding RNA TERRA is induced by telomere shortening to nucleate telomerase molecules at short telomeres. *Mol Cell.* (2013) 51:780–91. doi: 10.1016/j.molcel.2013.08.029
69. Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA, Reddel RR. Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med.* (1997) 3:1271–4.
70. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell.* (1985) 43:405–13.
71. Morin GB. The human telomere terminal transferase enzyme is a ribonucleoprotein that synthesizes TTAGGG repeats. *Cell.* (1989) 59:521–9.
72. Cong YS, Wright WE, Shay JW. Human telomerase and its regulation. *Microbiol Mol Biol Rev.* (2002) 66:407–25. doi: 10.1128/MMBR.66.3.407-425.2002
73. Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, et al. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. *Nat Genet.* (1997) 17:498–502.
74. Mouradian M, Ma IV, Vicente ED, Kikawa KD, Pardini RS. Docosahexaenoic acid-mediated inhibition of heat shock protein 90-p23 chaperone complex and downstream client proteins in lung and breast cancer. *Nutr Cancer.* (2017) 69:92–104. doi: 10.1080/01635581.2017.1247886
75. Toogun OA, Dezwaan DC, Freeman BC. The hsp90 molecular chaperone modulates multiple telomerase activities. *Mol Cell Biol.* (2008) 28:457–67. doi: 10.1128/MCB.01417-07
76. Mumin NH, Drobnitzky N, Patel A, Lourenco LM, Cahill FF, Jiang Y, et al. Overcoming acquired resistance to HSP90 inhibition by targeting JAK-STAT signalling in triple-negative breast cancer. *BMC Cancer.* (2019) 19:102. doi: 10.1186/s12885-019-5295-z
77. Seimiya H, Sawada H, Muramatsu Y, Shimizu M, Ohko K, Yamane K, et al. Involvement of 14-3-3 proteins in nuclear localization of telomerase. *EMBO J.* (2000) 19:2652–61. doi: 10.1093/emboj/19.11.2652
78. Tran H, Schilling M, Wirbelauer C, Hess D, Nagamine Y. Facilitation of mRNA deadenylation and decay by the exosome-bound, DExH protein RHAU. *Mol Cell.* (2004) 13:101–11. doi: 10.1016/S1097-2765(03)00481-7
79. Vaughn JP, Creacy SD, Routh ED, Joyner-Butt C, Jenkins GS, Pauli S, et al. The DEXH protein product of the DHX36 gene is the major source of tetramolecular quadruplex G4-DNA resolving activity in HeLa cell lysates. *J Biol Chem.* (2005) 280:38117–20. doi: 10.1074/jbc.C500348200
80. Sexton AN, Collins K. The 5' guanosine tracts of human telomerase RNA are recognized by the G-quadruplex binding domain of the RNA helicase DHX36 and function to increase RNA accumulation. *Mol Cell Biol.* (2011) 31:736–43. doi: 10.1128/MCB.01033-10
81. Newman M, Sfaxi R, Saha A, Monchaud D, Teulade-Fichou MP, Vagner S. The G-quadruplex-specific RNA helicase DHX36 regulates p53 pre-mRNA 3′-end processing following UV-induced DNA damage. *J Mol Biol.* (2017) 429:3121–31. doi: 10.1016/j.jmb.2016.11.033
82. Matsumura K, Kawasaki Y, Miyamoto M, Kamoshida Y, Nakamura J, Negishi L, et al. The novel G-quadruplex-containing long non-coding RNA GSEC antagonizes DHX36 and modulates colon cancer cell migration. *Oncogene.* (2017) 36:1191–9. doi: 10.1038/onc.2016.282
83. Gallant P. Control of transcription by pontin and reptin. *Trends Cell Biol.* (2007) 17:187–92. doi: 10.1016/j.tcb.2007.02.005
84. Jha S, Dutta A. RVB1/RVB2: running rings around molecular biology. *Mol Cell.* (2009) 34:521–33. doi: 10.1016/j.molcel.2009.05.016
85. Rosenbaum J, Baek SH, Dutta A, Houry WA, Huber O, Hupp TR, et al. The emergence of the conserved AAA+ ATPases pontin and reptin on the signaling landscape. *Sci Signal.* (2013) 6:mr1. doi: 10.1126/scisignal.2003906

86. Li W, Zeng J, Li Q, Zhao L, Liu T, Bjorkholm M, et al. Reptin is required for the transcription of telomerase reverse transcriptase and over-expressed in gastric cancer. *Mol Cancer.* (2010) 9:132. doi: 10.1186/1476-4598-9-132
87. Lauscher JC, Elezkurtaj S, Dullat S, Lipka S, Grone J, Buhr HJ, et al. Increased pontin expression is a potential predictor for outcome in sporadic colorectal carcinoma. *Oncol Rep.* (2012) 28:1619–24. doi: 10.3892/or.2012.1968
88. Tung CW, Wu MT, Chen YK, Wu CC, Chen WC, Li HP, et al. Identification of biomarkers for esophageal squamous cell carcinoma using feature selection and decision tree methods. *ScientificWorldJournal.* (2013) 2013:782031. doi: 10.1155/2013/782031
89. Taniuchi K, Furihata M, Iwasaki S, Tanaka K, Shimizu T, Saito M, et al. RUVBL1 directly binds actin filaments and induces formation of cell protrusions to promote pancreatic cancer cell invasion. *Int J Oncol.* (2014) 44:1945–54. doi: 10.3892/ijo.2014.2380
90. Cui F, Zan X, Li Y, Sun W, Yang Y, Ping L. Grifola frondosa glycoprotein GFG-3a arrests S phase, alters proteome, and induces apoptosis in human gastric cancer cells. *Nutr Cancer.* (2016) 68:267–79. doi: 10.1080/01635581.2016.1134599
91. Mao YQ, Houry WA. The role of pontin and reptin in cellular physiology and cancer etiology. *Front Mol Biosci.* (2017) 4:58. doi: 10.3389/fmolb.2017.00058
92. Rousseau B, Menard L, Haurie V, Taras D, Blanc JF, Moreau-Gaudry F, et al. Overexpression and role of the ATPase and putative DNA helicase RuvB-like 2 in human hepatocellular carcinoma. *Hepatology.* (2007) 46:1108–18. doi: 10.1002/hep.21770
93. Haurie V, Menard L, Nicou A, Touriol C, Metzler P, Fernandez J, et al. Adenosine triphosphatase pontin is overexpressed in hepatocellular carcinoma and coregulated with reptin through a new posttranslational mechanism. *Hepatology.* (2009) 50:1871–83. doi: 10.1002/hep.23215
94. Dehan E, Ben-Dor A, Liao W, Lipson D, Frimer H, Rienstein S, et al. Chromosomal aberrations and gene expression profiles in non-small cell lung cancer. *Lung Cancer.* (2007) 56:175–84. doi: 10.1016/j.lungcan.2006.12.010
95. Ocak S, Friedman DB, Chen H, Ausborn JA, Hassanein M, Detry B, et al. Discovery of new membrane-associated proteins overexpressed in small-cell lung cancer. *J Thorac Oncol.* (2014) 9:324–36. doi: 10.1097/JTO.0000000000000090
96. Uribarri M, Hormaeche I, Zalacain R, Lopez-Vivanco G, Martinez A, Nagore D, et al. A new biomarker panel in bronchoalveolar lavage for an improved lung cancer diagnosis. *J Thorac Oncol.* (2014) 9:1504–12. doi: 10.1097/JTO.0000000000000282
97. Yuan XS, Wang ZT, Hu YJ, Bao FC, Yuan P, Zhang C, et al. Downregulation of RUVBL1 inhibits proliferation of lung adenocarcinoma cells by G1/S phase cell cycle arrest via multiple mechanisms. *Tumour Biol.* (2016). 37:16015–27. doi: 10.1007/s13277-016-5452-9
98. Velmurugan BK, Yang HH, Sung PJ, Weng CF. Excavatolide B inhibits nonsmall cell lung cancer proliferation by altering peroxisome proliferator activated receptor gamma expression and PTEN/AKT/NF-Kbeta expression. *Environ Toxicol.* (2017) 32:290–301. doi: 10.1002/tox.22235
99. Lacombe J, Mange A, Jarlier M, Bascoul-Mollevi C, Rouanet P, Lamy PJ, et al. Identification and validation of new autoantibodies for the diagnosis of DCIS and node negative early-stage breast cancers. *Int J Cancer.* (2013) 132:1105–13. doi: 10.1002/ijc.27766
100. Lacombe J, Mange A, Bougnoux AC, Prassas I, Solassol J. A multiparametric serum marker panel as a complementary test to mammography for the diagnosis of node-negative early-stage breast cancer and DCIS in young women. *Cancer Epidemiol Biomarkers Prev.* (2014) 23:1834–42. doi: 10.1158/1055-9965.EPI-14-0267
101. Baron BW, Baron RM, Baron JM. The relationship between RUVBL1 (Pontin, TIP49, NMP238) and BCL6 in benign and malignant human lymphoid tissues. *Biochem Biophys Rep.* (2016) 6:1–8. doi: 10.1016/j.bbrep.2016.02.006
102. Lin P, Mobasher ME, Hakakian Y, Kakarla V, Naseem AF, Ziai H, et al. Differential requirements for H/ACA ribonucleoprotein components in cell proliferation and response to DNA damage. *Histochem Cell Biol.* (2015) 144:543–58. doi: 10.1007/s00418-015-1359-6
103. Montanaro L. Dyskerin and cancer: more than telomerase. The defect in mRNA translation helps in explaining how a proliferative defect leads to cancer. *J Pathol.* (2010) 222:345–9. doi: 10.1002/path.2777
104. Alawi F, Lin P. Dyskerin is required for tumor cell growth through mechanisms that are independent of its role in telomerase and only partially related to its function in precursor rRNA processing. *Mol Carcinog.* (2011) 50:334–45. doi: 10.1002/mc.20715
105. Dos Santos PC, Panero J, Stanganelli C, Palau Nagore V, Stella F, Bezares R, et al. Dysregulation of H/ACA ribonucleoprotein components in chronic lymphocytic leukemia. *PLoS ONE.* (2017) 12:e0179883. doi: 10.1371/journal.pone.0179883
106. Meier UT. The many facets of H/ACA ribonucleoproteins. *Chromosoma.* (2005) 114:1–14. doi: 10.1007/s00412-005-0333-9
107. Kim MS, Kim SS, Yoo NJ, Lee SH. Expressional analysis of NOLA1, NOLA2, NOLA3 and DKC1, the core proteins in H/ACA riboproteins, in gastric and colorectal cancers. *Pathology.* (2012) 44:576–7. doi: 10.1097/PAT.0b013e3283583bf6
108. Witkowska A, Gumprecht J, Glogowska-Ligus J, Wystrychowski G, Owczarek A, Stachowicz M, et al. Expression profile of significant immortalization genes in colon cancer. *Int J Mol Med.* (2010) 25:321–9. doi: 10.3892/ijmm_00000348
109. Carrozza F, Santoni M, Piva F, Cheng L, Lopez-Beltran A, Scarpelli M, et al. Emerging immunotherapeutic strategies targeting telomerases in genitourinary tumors. *Crit Rev Oncol Hematol.* (2018) 131:1–6. doi: 10.1016/j.critrevonc.2018.07.008
110. Kohno T, Takahashi M, Manda R, Yokota J. Inactivation of the PTEN/MMAC1/TEP1 gene in human lung cancers. *Genes Chromosomes Cancer.* (1998) 22:152–156. doi: 10.1002/(SICI)1098-2264(199806)22:2<152::AID-GCC10>3.0.CO;2-S
111. Venteicher AS, Artandi SE. TCAB1: driving telomerase to Cajal bodies. *Cell Cycle.* (2009) 8:1329–31. doi: 10.4161/cc.8.9.8288
112. Sun CK, Luo XB, Gou YP, Hu L, Wang K, Li C, et al. TCAB1: a potential target for diagnosis and therapy of head and neck carcinomas. *Mol Cancer.* (2014) 13:180. doi: 10.1186/1476-4598-13-180
113. Nagata T, Takada Y, Ono A, Nagata K, Konishi Y, Nukina T, et al. Elucidation of the mode of interaction in the UP1-telomerase RNA-telomeric DNA ternary complex which serves to recruit telomerase to telomeric DNA and to enhance the telomerase activity. *Nucleic Acids Res.* (2008) 36:6816–24. doi: 10.1093/nar/gkn767
114. Chen JL, Greider CW. Telomerase RNA structure and function: implications for dyskeratosis congenita. *Trends Biochem Sci.* (2004) 29:183–92. doi: 10.1016/j.tibs.2004.02.003
115. Yu GL, Bradley JD, Attardi LD, Blackburn EH. *In vivo* alteration of telomere sequences and senescence caused by mutated Tetrahymena telomerase RNAs. *Nature.* (1990) 344:126–32.
116. Rubtsova MP, Vasilkova DP, Naraykina YV, Dontsova OA. Peculiarities of yeasts and human telomerase RNAs processing. *Acta Nat.* (2016) 8:14–22.
117. Chen JL, Blasco MA, Greider CW. Secondary structure of vertebrate telomerase RNA. *Cell.* (2000) 100:503–14. doi: 10.1016/S0092-8674(00)80687-X
118. Tesmer VM, Ford LP, Holt SE, Frank BC, Yi X, Aisner DL, et al. Two inactive fragments of the integral RNA cooperate to assemble active telomerase with the human protein catalytic subunit (hTERT) *in vitro*. *Mol Cell Biol.* (1999) 19:6207–16.
119. Egan ED, Collins K. Biogenesis of telomerase ribonucleoproteins. *RNA.* (2012) 18:1747–59. doi: 10.1261/rna.034629.112
120. Cristofari G, Lingner J. Telomere length homeostasis requires that telomerase levels are limiting. *EMBO J.* (2006) 25:565–74. doi: 10.1038/sj.emboj.7600952
121. Mitchell JR, Collins K. Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase. *Mol Cell.* (2000) 6:361–71. doi: 10.1016/S1097-2765(00)00036-8
122. Podlevsky JD, Chen JJ. It all comes together at the ends: telomerase structure, function, and biogenesis. *Mutat Res.* (2012) 730:3–11. doi: 10.1016/j.mrfmmm.2011.11.002
123. Venteicher AS, Abreu EB, Meng Z, McCann KE, Terns RM, Veenstra TD, et al. A human telomerase holoenzyme protein required for

Cajal body localization and telomere synthesis. *Science.* (2009) 323:644–8. doi: 10.1126/science.1165357
124. Cristofari G, Adolf E, Reichenbach P, Sikora K, Terns RM, Terns MP, et al. Human telomerase RNA accumulation in Cajal bodies facilitates telomerase recruitment to telomeres and telomere elongation. *Mol Cell.* (2007) 27:882–9. doi: 10.1016/j.molcel.2007.07.020
125. Webb CJ, Zakian VA. Telomerase RNA is more than a DNA template. *RNA Biol.* (2016) 13:683–9. doi: 10.1080/15476286.2016.1191725
126. Wick M, Zubov D, Hagen G. Genomic organization and promoter characterization of the gene encoding the human telomerase reverse transcriptase (hTERT). *Gene.* (1999) 232:97–106.
127. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science.* (1997) 276:561–7.
128. Alder JK, Cogan JD, Brown AF, Anderson CJ, Lawson WE, Lansdorp PM, et al. Ancestral mutation in telomerase causes defects in repeat addition processivity and manifests as familial pulmonary fibrosis. *PLoS Genet.* (2011) 7:e1001352. doi: 10.1371/journal.pgen.1001352
129. Opresko PL, Shay JW. Telomere-associated aging disorders. *Ageing Res Rev.* (2017) 33:52–66. doi: 10.1016/j.arr.2016.05.009
130. Bachand F, Autexier C. Functional regions of human telomerase reverse transcriptase and human telomerase RNA required for telomerase activity and RNA-protein interactions. *Mol Cell Biol.* (2001) 21:1888–97. doi: 10.1128/MCB.21.5.1888-1897.2001
131. Counter CM, Meyerson M, Eaton EN, Ellisen LW, Caddle SD, Haber DA, et al. Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. *Oncogene.* (1998) 16:1217–22. doi: 10.1038/sj.onc.1201882
132. Leao R, Apolonio JD, Lee D, Figueiredo A, Tabori U, Castelo-Branco P. Mechanisms of human telomerase reverse transcriptase (hTERT) regulation: clinical impacts in cancer. *J Biomed Sci.* (2018) 25:22. doi: 10.1186/s12929-018-0422-8
133. Meier UT, Blobel G. NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. *J Cell Biol.* (1994) 127:1505–14. doi: 10.1083/jcb.127.6.1505
134. Wang C, Meier UT. Architecture and assembly of mammalian H/ACA small nucleolar and telomerase ribonucleoproteins. *EMBO J.* (2004) 23:1857–67. doi: 10.1038/sj.emboj.7600181
135. Rocchi L, Barbosa AJ, Onofrillo C, Del Rio A, Montanaro L. Inhibition of human dyskerin as a new approach to target ribosome biogenesis. *PLoS ONE.* (2014) 9:e101971. doi: 10.1371/journal.pone.0101971
136. Kirwan M, Dokal I. Dyskeratosis congenita: a genetic disorder of many faces. *Clin Genet.* (2008) 73:103–12. doi: 10.1111/j.1399-0004.2007.00923.x
137. He J, Navarrete S, Jasinski M, Vulliamy T, Dokal I, Bessler M, et al. Targeted disruption of Dkc1, the gene mutated in X-linked dyskeratosis congenita, causes embryonic lethality in mice. *Oncogene.* (2002) 21:7740–4. doi: 10.1038/sj.onc.1205969
138. Giordano E, Peluso I, Senger S, Furia M. Minifly, a drosophila gene required for ribosome biogenesis. *J Cell Biol.* (1999) 144:1123–33. doi: 10.1083/jcb.144.6.1123
139. Lafontaine DL, Bousquet-Antonelli C, Henry Y, Caizergues-Ferrer M, Tollervey D. The box H + ACA snoRNAs carry Cbf5p, the putative rRNA pseudouridine synthase. *Genes Dev.* (1998) 12:527–37. doi: 10.1101/gad.12.4.527
140. Heiss NS, Knight SW, Vulliamy TJ, Klauck SM, Wiemann S, Mason PJ, et al. X-linked dyskeratosis congenita is caused by mutations in a highly conserved gene with putative nucleolar functions. *Nat Genet.* (1998) 19:32–8. doi: 10.1038/ng0598-32
141. Yuan DZ, Ding XL, Yu HL, Cheng Z, Tang XR, He YP, et al. Progesterone-induced cyclin G1 inhibits the proliferation of endometrial epithelial cell and its possible molecular mechanism. *Horm Metab Res.* (2014) 46:761–7. doi: 10.1055/s-0034-1383647
142. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. *Genes Dev.* (2010) 24:2463–79. doi: 10.1101/gad.1971610
143. Harper JW, Elledge SJ. The DNA damage response: ten years after. *Mol Cell.* (2007) 28:739–45. doi: 10.1016/j.molcel.2007.11.015
144. Jeggo PA, Pearl LH, Carr AM. DNA repair, genome stability and cancer: a historical perspective. *Nat Rev Cancer.* (2016) 16:35–42. doi: 10.1038/nrc.2015.4
145. Smith J, Tho LM, Xu N, Gillespie DA. The ATM-Chk2 and ATR-Chk1 pathways in DNA damage signaling and cancer. *Adv Cancer Res.* (2010) 108:73–112. doi: 10.1016/B978-0-12-380888-2.00003-0
146. Stracker T. H, Roig I, Knobel PA, Marjanovic M. The ATM signaling network in development and disease. *Front Genet.* (2013) 4:37. doi: 10.3389/fgene.2013.00037
147. Lee SS, Bohrson C, Pike AM, Wheelan SJ, Greider CW. ATM kinase is required for telomere elongation in mouse and human cells. *Cell Rep.* (2015) 13:1623–32. doi: 10.1016/j.celrep.2015.10.035
148. Sfeir A, Kosiyatrakul ST, Hockemeyer D, MacRae SL, Karlseder J, Schildkraut CL, et al. Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. *Cell.* (2009) 138:90–103. doi: 10.1016/j.cell.2009.06.021
149. Zhang Y, Toh L, Lau P, Wang X. Human telomerase reverse transcriptase (hTERT) is a novel target of the Wnt/β-catenin pathway in human cancer. *J Biol Chem.* (2012) 287:32494–511. doi: 10.1074/jbc.M112.368282
150. Yang TB, Chen Q, Deng JT, Jagannathan G, Tobias JW, Schultz DC, et al. Mutual reinforcement between telomere capping and canonical Wnt signalling in the intestinal stem cell niche. *Nat Commun.* (2017) 8:14766. doi: 10.1038/ncomms14766
151. Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, et al. Loss of Apc *in vivo* immediately perturbs Wnt signaling, differentiation, and migration. *Genes Dev.* (2004) 18:1385–90. doi: 10.1101/gad.287404
152. Hoffmeyer K, Raggioli A, Rudloff S, Anton R, Hierholzer A, Del Valle I, et al. Wnt/beta-catenin signaling regulates telomerase in stem cells and cancer cells. *Science.* (2012) 336:1549–54. doi: 10.1126/science.1218370
153. Park JI, Venteicher AS, Hong JY, Choi J, Jun S, Shkreli M, et al. Telomerase modulates Wnt signalling by association with target gene chromatin. *Nature.* (2009) 460:66–72. doi: 10.1038/nature08137
154. Kang HJ, Choi YS, Hong SB, Kim KW, Woo RS, Won SJ, et al. Ectopic expression of the catalytic subunit of telomerase protects against brain injury resulting from ischemia and NMDA-induced neurotoxicity. *J Neurosci.* (2004) 24:1280–7. doi: 10.1523/JNEUROSCI.4082-03.2004
155. Masutomi K, Possemato R, Wong JMY, Currier JL, Tothova Z, Manola JB, et al. The telomerase reverse transcriptase regulates chromatin state and DNA damage responses. *Proc Natl Acad Sci USA.* (2005) 102:8222–7. doi: 10.1073/pnas.0503095102
156. Maida Y, Yasukawa M, Furuuchi M, Lassmann T, Possemato R, Okamoto N, et al. An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. *Nature.* (2009) 461:230–5. doi: 10.1038/nature08283
157. Saretzki G. Extra-telomeric functions of human telomerase: cancer, mitochondria and oxidative stress. *Curr Pharm Des.* (2014) 20:6386–03. doi: 10.2174/13816128206614063095606
158. Parkes C, Kamal A, Valentijn AJ, Alnafakh R, Gross SR, Barraclough R, et al. Assessing estrogen-induced proliferative response in an endometrial cancer cell line using a universally applicable methodological guide. *Int J Gynecol Cancer.* (2018) 28:122–33. doi: 10.1097/IGC.0000000000001121
159. Kyo S, Takakura M, Kanaya T, Zhuo W, Fujimoto K, Nishio Y, et al. Estrogen activates telomerase. *Cancer Res.* (1999) 59:5917–21.
160. Vidal JD, Register TC, Gupta M, Cline JM. Estrogen replacement therapy induces telomerase RNA expression in the macaque endometrium. *Fertil Steril.* (2002) 77:601–8. doi: 10.1016/S0015-0282(01)03227-7
161. Nourbakhsh M, Golestani A, Zahrai M, Modarressi MH, Malekpour Z, Karami-Tehrani F. Androgens stimulate telomerase expression, activity and phosphorylation in ovarian adenocarcinoma cells. *Mol Cell Endocrinol.* (2010) 330:10–6. doi: 10.1016/j.mce.2010.07.021
162. Shan W, Wang C, Zhang Z, Luo X, Ning C, Yu Y, et al. ATM may be a protective factor in endometrial carcinogenesis with the progesterone pathway. *Tumour Biol.* (2015) 36:1529–37. doi: 10.1007/s13277-014-2712-4
163. Rippe K, Luke B. TERRA and the state of the telomere. *Nat Struct Mol Biol.* (2015) 22:853–8. doi: 10.1038/nsmb.3078
164. Balk B, Dees M, Bender K, Luke B. The differential processing of telomeres in response to increased telomeric transcription and RNA-DNA hybrid accumulation. *RNA Biol.* (2014) 11:95–100. doi: 10.4161/rna.27798

165. Graf M, Bonetti D, Lockhart A, Serhal K, Kellner V, Maicher A, et al. Telomere length determines TERRA and R-Loop regulation through the cell cycle. *Cell.* (2017) 170:72–85.e14. doi: 10.1016/j.cell.2017.06.006
166. Farnung BO, Brun CM, Arora R, Lorenzi LE, Azzalin CM. Telomerase efficiently elongates highly transcribing telomeres in human cancer cells. *PLoS ONE.* (2012) 7:e35714. doi: 10.1371/journal.pone.0035714
167. Neumann AA, Watson CM, Noble JR, Pickett HA, Tam PP, Reddel RR. Alternative lengthening of telomeres in normal mammalian somatic cells. *Genes Dev.* (2013) 27:18–23. doi: 10.1101/gad.205062.112
168. Dunham MA, Neumann AA, Fasching CL, Reddel RR. Telomere maintenance by recombination in human cells. *Nat Genet.* (2000) 26:447–50. doi: 10.1038/82586
169. Dilley RL, Greenberg RA. ALTernative telomere maintenance and cancer. *Trends Cancer.* (2015) 1:145–56. doi: 10.1016/j.trecan.2015.07.007
170. Min J, Wright WE, Shay JW. Alternative lengthening of telomeres can be maintained by preferential elongation of lagging strands. *Nucleic Acids Res.* (2017) 2615–2628. doi: 10.1093/nar/gkw1295
171. Bryan TM, Englezou A, Gupta J, Bacchetti S, Reddel RR. Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.* (1995) 14:4240–8. doi: 10.1002/j.1460-2075.1995.tb00098.x
172. Napier CE, Huschtscha LI, Harvey A, Bower K, Noble JR, Hendrickson EA, et al. ATRX represses alternative lengthening of telomeres. *Oncotarget.* (2015) 6:16543–58. doi: 10.18632/oncotarget.3846
173. Lovejoy CA, Li W, Reisenweber S, Thongthip S, Bruno J, de Lange T, et al. Loss of ATRX, genome instability, and an altered DNA damage response are hallmarks of the alternative lengthening of telomeres pathway. *PLoS Genet.* (2012) 8:e1002772. doi: 10.1371/journal.pgen.1002772
174. Shay JW, Wright WE. Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis.* (2005) 26:867–74. doi: 10.1093/carcin/bgh296
175. Meeker AK, Hicks JL, Gabrielson E, Strauss WM, De Marzo AM, Argani P. Telomere shortening occurs in subsets of normal breast epithelium as well as *in situ* and invasive carcinoma. *Am J Pathol.* (2004) 164:925–35. doi: 10.1016/S0002-9440(10)63180-X
176. Meeker AK, Hicks JL, Platz EA, March GE, Bennett CJ, Delannoy MJ, et al. Telomere shortening is an early somatic DNA alteration in human prostate tumorigenesis. *Cancer Res.* (2002) 62:6405–9.
177. van Heek NT, Meeker AK, Kern SE, Yeo CJ, Lillemoe KD, Cameron JL, et al. Telomere shortening is nearly universal in pancreatic intraepithelial neoplasia. *Am J Pathol.* (2002) 161:1541–47. doi: 10.1016/S0002-9440(10)64432-X
178. Valls C, Pinol C, Rene JM, Buenestado J, Vinas J. Telomere length is a prognostic factor for overall survival in colorectal cancer. *Colorectal Dis.* (2011) 13:1265–72. doi: 10.1111/j.1463-1318.2010.02433.x
179. Roger L, Jones RE, Heppel NH, Williams GT, Sampson JR, Baird DM. Extensive telomere erosion in the initiation of colorectal adenomas and its association with chromosomal instability. *J Natl Cancer Inst.* (2013) 105:1202–11. doi: 10.1093/jnci/djt191
180. Bojesen SE. Telomeres and human health. *J Intern Med.* (2013) 274:399–413. doi: 10.1111/joim.12083
181. Raynaud CM, Hernandez J, Llorca FP, Nuciforo P, Mathieu MC, Commo F, et al. DNA damage repair and telomere length in normal breast, preneoplastic lesions, and invasive cancer. *Am J Clin Oncol.* (2010) 33:341–5. doi: 10.1097/COC.0b013e3181b0c4c2
182. Ma H, Zhou Z, Wei S, Liu Z, Pooley KA, Dunning AM, et al. Shortened telomere length is associated with increased risk of cancer: a meta-analysis. *PLoS ONE.* (2011) 6:e20466. doi: 10.1371/journal.pone.0020466
183. Wentzensen IM, Mirabello L, Pfeiffer RM, Savage SA. The association of telomere length and cancer: a meta-analysis. *Cancer Epidemiol Biomarkers Prev.* (2011) 20:1238–50. doi: 10.1158/1055-9965.EPI-11-0005
184. Morgillo F, Dallio M, Della Corte CM, Gravina AG, Viscardi G, Loguercio C, et al. Carcinogenesis as a result of multiple inflammatory and oxidative hits: a comprehensive review from tumor microenvironment to gut microbiota. *Neoplasia.* (2018) 20:721–33. doi: 10.1016/j.neo.2018.05.002
185. Weischer M, Nordestgaard BG, Cawthon RM, Freiberg JJ, Tybjaerg-Hansen A, Bojesen SE. Short telomere length, cancer survival, and cancer risk in 47102 individuals. *J Natl Cancer Inst.* (2013) 105:459–68. doi: 10.1093/jnci/djt016
186. Muezzinler A, Zaineddin AK, Brenner H. A systematic review of leukocyte telomere length and age in adults. *Ageing Res Rev.* (2013) 12:509–19. doi: 10.1016/j.arr.2013.01.003
187. Mizuguchi T, Taneja N, Matsuda E, Belton JM, FitzGerald P, Dekker J, et al. Shelterin components mediate genome reorganization in response to replication stress. *Proc Natl Acad Sci USA.* (2017) 114:5479–84. doi: 10.1073/pnas.1705527114
188. Amir M, Kumar V, Mohammad T, Dohare R, Hussain A, Rehman MT, et al. Investigation of deleterious effects of nsSNPs in the POT1 gene: a structural genomics-based approach to understand the mechanism of cancer development. *J Cell Biochem.* (2018) 16:10281–94. doi: 10.1002/jcb.28312
189. Chen C, Gu P, Wu J, Chen X, Niu S, Sun H, et al. Structural insights into POT1-TPP1 interaction and POT1 C-terminal mutations in human cancer. *Nat Commun.* (2017) 8:14929. doi: 10.1038/ncomms14929
190. Blasco MA. Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet.* (2005) 6:611–22. doi: 10.1038/nrg1656
191. Poncet D, Belleville A, t'kint de Roodenbeke C, Roborel de Climens A, Ben Simon E, Merle-Beral H, et al. Changes in the expression of telomere maintenance genes suggest global telomere dysfunction in B-chronic lymphocytic leukemia. *Blood.* (2008) 111:2388–91. doi: 10.1182/blood-2007-09-111245
192. Savage SA, Calado RT, Xin ZT, Ly H, Young NS, Chanock SJ. Genetic variation in telomeric repeat binding factors 1 and 2 in aplastic anemia. *Exp Hematol.* (2006) 34:664–71. doi: 10.1016/j.exphem.2006.02.008
193. Savage SA, Giri N, Baerlocher GM, Orr N, Lansdorp PM, Alter BP. TINF2, a component of the shelterin telomere protection complex, is mutated in dyskeratosis congenita. *Am J Hum Genet.* (2008) 82:501–9. doi: 10.1016/j.ajhg.2007.10.004
194. Tsangaris E, Adams SL, Yoon G, Chitayat D, Lansdorp P, Dokal I, et al. Ataxia and pancytopenia caused by a mutation in TINF2. *Hum Genet.* (2008) 124:507–13. doi: 10.1007/s00439-008-0576-7
195. Walne AJ, Vulliamy T, Beswick R, Kirwan M, Dokal I. TINF2 mutations result in very short telomeres: analysis of a large cohort of patients with dyskeratosis congenita and related bone marrow failure syndromes. *Blood.* (2008) 112:3594–600. doi: 10.1182/blood-2008-05-153445
196. Frias C, Pampalona J, Genesca A, Tusell L. Telomere dysfunction and genome instability. *Front Biosci.* (2012) 17:2181–96. doi: 10.2741/4044
197. Kim H, Yoo JE, Cho JY, Oh BK, Yoon YS, Han HS, et al. Telomere length, TERT and shelterin complex proteins in hepatocellular carcinomas expressing "stemness"-related markers. *J Hepatol.* (2013) 59:746–52. doi: 10.1016/j.jhep.2013.05.011
198. El Idrissi M, Hervieu V, Merle P, Mortreux F, Wattel E. Cause-specific telomere factors deregulation in hepatocellular carcinoma. *J Exp Clin Cancer Res.* (2013) 32:64. doi: 10.1186/1756-9966-32-64
199. Cantara S, Capuano S, Capezzone M, Benigni M, Pisu M, Marchisotta S, et al. Lack of mutations of the telomerase RNA component in familial papillary thyroid cancer with short telomeres. *Thyroid.* (2012) 22:363–8. doi: 10.1089/thy.2011.0109
200. Guieze R, Pages M, Veronese L, Combes P, Lemal R, Gay-Bellile M, et al. Telomere status in chronic lymphocytic leukemia with TP53 disruption. *Oncotarget.* (2016) 7:56976–85. doi: 10.18632/oncotarget.10927
201. Bayram S, Ulger Y, Sumbul AT, Kaya BY, Genc A, Rencuzogullari E, et al. Polymorphisms in human telomerase reverse transcriptase (hTERT) gene and susceptibility to gastric cancer in a Turkish population: hospital-based case-control study. *Gene.* (2016) 585:84–92. doi: 10.1016/j.gene.2016.03.030
202. Polat F, Yilmaz M, Budak Diler S. The association of MYNN and TERC gene polymorphisms and bladder cancer in a Turkish population. *Urol J.* (2018) 16:50–5. doi: 10.22037/uj.v0i0.4083
203. Rafnar T, Sulem P, Stacey SN, Geller F, Gudmundsson J, Sigurdsson A, et al. Sequence variants at the TERT-CLPTM1L locus associate with many cancer types. *Nat Genet.* (2009) 41:221–7. doi: 10.1038/ng.296
204. Kote-Jarai Z, Olama AA, Giles GG, Severi G, Schleutker J, Weischer M, et al. Seven prostate cancer susceptibility loci identified by a multi-stage genome-wide association study. *Nat Genet.* (2011) 43:785–91. doi: 10.1038/ng.882
205. Kote-Jarai Z, Saunders EJ, Leongamornlert DA, Tymrakiewicz M, Dadaev T, Jugurnauth-Little S, et al. Fine-mapping identifies multiple prostate cancer

risk loci at 5p15, one of which associates with TERT expression. *Hum Mol Genet.* (2013) 22:2520–8. doi: 10.1158/1538-7445.AM2013-2546
206. McKay JD, Hung RJ, Gaborieau V, Boffetta P, Chabrier A, Byrnes G, et al. Lung cancer susceptibility locus at 5p15.33. *Nat Genet.* (2008) 40:1404–6. doi: 10.1038/ng.254
207. Petersen GM, Amundadottir L, Fuchs CS, Kraft P, Stolzenberg-Solomon RZ, Jacobs KB, et al. A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet.* (2010) 42:224–8. doi: 10.1038/ng.522
208. Killela PJ, Reitman ZJ, Jiao Y, Bettegowda C, Agrawal N, Diaz LA Jr., et al. TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. *Proc Natl Acad Sci USA.* (2013) 110:6021–6. doi: 10.1073/pnas.1303607110
209. Vinagre J, Almeida A, Populo H, Batista R, Lyra J, Pinto V, et al. Frequency of TERT promoter mutations in human cancers. *Nat Commun.* (2013) 4:2185. doi: 10.1038/ncomms3185
210. Heidenreich B, Rachakonda PS, Hemminki K, Kumar R. TERT promoter mutations in cancer development. *Curr Opin Genet Dev.* (2014) 24:30–7. doi: 10.1016/j.gde.2013.11.005
211. Liu R, Xing M. TERT promoter mutations in thyroid cancer. *Endocr Relat Cancer.* (2016) 23:R143–55. doi: 10.1530/ERC-15-0533
212. Walsh KM, Wiencke JK, Lachance DH, Wiemels JL, Molinaro AM, Eckel-Passow JE, et al. Telomere maintenance and the etiology of adult glioma. *Neuro Oncol.* (2015) 17:1445–52. doi: 10.1093/neuonc/nov082
213. Landa I, Ganly I, Chan TA, Mitsutake N, Matsuse M, Ibrahimpasic T, et al. Frequent somatic TERT promoter mutations in thyroid cancer: higher prevalence in advanced forms of the disease. *J Clin Endocrinol Metab.* (2013) 98:E1562–6. doi: 10.1210/jc.2013-2383
214. Rachakonda PS, Hosen I, de Verdier PJ, Fallah M, Heidenreich B, Ryk C, et al. TERT promoter mutations in bladder cancer affect patient survival and disease recurrence through modification by a common polymorphism. *Proc Natl Acad Sci USA.* (2013) 110:17426–31. doi: 10.1073/pnas.1310522110
215. Gao L, Thakur A, Liang Y, Zhang S, Wang T, Chen T, et al. Polymorphisms in the TERT gene are associated with lung cancer risk in the Chinese Han population. *Eur J Cancer Prev.* (2014) 23:497–501. doi: 10.1097/CEJ.0000000000000086
216. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, et al. Specific association of human telomerase activity with immortal cells and cancer. *Science.* (1994) 266:2011–5. doi: 10.1126/science.7605428
217. Hiyama E, Yamaoka H, Matsunaga T, Hayashi Y, Ando H, Suita S, et al. High expression of telomerase is an independent prognostic indicator of poor outcome in hepatoblastoma. *Br J Cancer.* (2004) 91:972–9. doi: 10.1038/sj.bjc.6602054
218. Mao L, El-Naggar AK, Fan YH, Lee JS, Lippman SM, Kayser S, et al. Telomerase activity in head and neck squamous cell carcinoma and adjacent tissues. *Cancer Res.* (1996) 56:5600–4.
219. Tatsumoto N, Hiyama E, Murakami Y, Imamura Y, Shay JW, Matsuura Y, et al. High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. *Clin Cancer Res.* (2000) 6:2696–701.
220. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer.* (1997) 33:787–91. doi: 10.1016/S0959-8049(97)00062-2
221. Deng T, Huang Y, Weng K, Lin S, Li Y, Shi G, et al. TOE1 acts as a 3′ exonuclease for telomerase RNA and regulates telomere maintenance. *Nucleic Acids Res.* (2018) 47:391–405. doi: 10.1093/nar/gky1019
222. Tummala H, Walne A, Collopy L, Cardoso S, de la Fuente J, Lawson S, et al. Poly(A)-specific ribonuclease deficiency impacts telomere biology and causes dyskeratosis congenita. *J Clin Invest.* (2015) 125:2151–60. doi: 10.1172/JCI78963
223. Zhu Y, Han Y, Tian T, Su P, Jin G, Chen J, et al. MiR-21-5p, miR-34a, and human telomerase RNA component as surrogate markers for cervical cancer progression. *Pathol Res Pract.* (2018) 214:374–9. doi: 10.1016/j.prp.2018.01.001
224. Baena-Del Valle JA, Zheng Q, Esopi DM, Rubenstein M, Hubbard GK, Moncaliano MC, et al. MYC drives overexpression of telomerase RNA (hTR/TERC) in prostate cancer. *J Pathol.* (2018) 244:11–24. doi: 10.1002/path.4980
225. Heng J, Zhang F, Guo X, Tang L, Peng L, Luo X, et al. Integrated analysis of promoter methylation and expression of telomere related genes in breast cancer. *Oncotarget.* 8:25442–54. doi: 10.18632/oncotarget.16036
226. Dorji T, Monti V, Fellegara G, Gabba S, Grazioli V, Repetti E, et al. Gain of hTERC: a genetic marker of malignancy in oral potentially malignant lesions. *Hum Pathol.* 46:1275–81. doi: 10.1016/j.humpath.2015.05.013
227. Montanaro L, Calienni M, Ceccarelli C, Santini D, Taffurelli M, Pileri S, et al. Relationship between dyskerin expression and telomerase activity in human breast cancer. *Cell Oncol.* (2008) 30:483–90. doi: 10.3233/CLO-2008-0436
228. Sieron P, Hader C, Hatina J, Engers R, Wlazlinski A, Muller M, et al. DKC1 overexpression associated with prostate cancer progression. *Br J Cancer.* (2009) 101:1410–6. doi: 10.1038/sj.bjc.6605299
229. Liu B, Zhang J, Huang C, Liu H. Dyskerin overexpression in human hepatocellular carcinoma is associated with advanced clinical stage and poor patient prognosis. *PLoS ONE.* (2012) 7:e43147. doi: 10.1371/journal.pone.0043147
230. Hapangama DK, Bulmer JN. Pathophysiology of heavy menstrual bleeding. *Womens Health (Lond).* (2016) 12:3–13. doi: 10.2217/whe.15.81
231. Biswas Shivhare S, Bulmer JN, Innes BA, Hapangama DK, Lash GE. Menstrual cycle distribution of uterine natural killer cells is altered in heavy menstrual bleeding. *J Reprod Immunol.* (2015) 112:88–94. doi: 10.1016/j.jri.2015.09.001
232. Drury JA, Parkin KL, Coyne L, Giuliani E, Fazleabas AT, Hapangama D K. The dynamic changes in the number of uterine natural killer cells are specific to the eutopic but not to the ectopic endometrium in women and in a baboon model of endometriosis. *Reprod Biol Endocrinol.* (2018) 16:67. doi: 10.1186/s12958-018-0385-3
233. Biswas Shivhare S, Bulmer JN, Innes BA, Hapangama DK, Lash GE. Endometrial vascular development in heavy menstrual bleeding: altered spatio-temporal expression of endothelial cell markers and extracellular matrix components. *Hum Reprod.* (2018) 33:399–410. doi: 10.1093/humrep/dex378
234. Williams CD, Boggess JF, LaMarque LR, Meyer WR, Murray MJ, Fritz MA, et al. A prospective, randomized study of endometrial telomerase during the menstrual cycle. *J Clin Endocrinol Metab.* (2001) 86:3912–7. doi: 10.1210/jcem.86.8.7729
235. Hapangama DK, Turner MA, Drury JA, Martin-Ruiz C, Von Zglinicki T, Farquharson RG, et al. Endometrial telomerase shows specific expression patterns in different types of reproductive failure. *Reprod Biomed Online.* (2008) 17:416–24. doi: 10.1016/S1472-6483(10)60227-1
236. Hu J, Yuan R. The expression levels of stem cell markers importin13, c-kit, CD146, and telomerase are decreased in endometrial polyps. *Med Sci Monit.* (2011)17:BR221–227.
237. Ding DC, Chu TY, Chiou SH, Liu HW. Enhanced differentiation and clonogenicity of human endometrial polyp stem cells. *Differentiation.* (2011) 81:172–80. doi: 10.1016/j.diff.2010.12.002
238. Mafra FA, Christofolini DM, Cavalcanti V, Vilarino FL, André GM, Kato P, et al. Aberrant telomerase expression in the endometrium of infertile women with deep endometriosis. *Arch Med Res.* (2014) 45:31–5. doi: 10.1016/j.arcmed.2013.12.002
239. Gül I, Dündar O, Bodur S, Tunca Y, Tütüncü L. The status of telomerase enzyme activity in benign and malignant gynaecologic pathologies. *Balkan Med J.* (2013) 30:287–92. doi: 10.5152/balkanmedj.2013.7328
240. Long N, Liu N, Liu XL, Li J, Cai BY, Cai X. Endometrial expression of telomerase, progesterone, and estrogen receptors during the implantation window in patients with recurrent implantation failure. *Genet Mol Res.* (2016) 15. doi: 10.4238/gmr.15027849
241. Sofiyeva N, Ekizoglu S, Gezer A, Yilmaz H, Kolomuc Gayretli T, Buyru N, et al. Does telomerase activity have an effect on infertility in patients with endometriosis? *Eur J Obstet Gynecol Reprod Biol.* (2017) 213:116–22. doi: 10.1016/j.ejogrb.2017.04.027
242. Kim CM, Oh YJ, Cho SH, Chung DJ, Hwang JY, Park KH, et al. Increased telomerase activity and human telomerase reverse transcriptase mRNA expression in the endometrium of patients with endometriosis. *Hum Reprod.* (2007) 22:843–9. doi: 10.1093/humrep/del425
243. Sourial S, Tempest N, Hapangama DK. Theories on the pathogenesis of endometriosis. *Int J Reprod Med.* (2014) 2014:179515. doi: 10.1155/2014/179515

244. Hapangama DK, Turner MA, Drury, JA, Quenby S, Hart A, Maddick M, et al. Sustained replication in endometrium of women with endometriosis occurs without evoking a DNA damage response. *Hum Reprod.* (2009) 24:687–96. doi: 10.1093/humrep/den416
245. Emons G, Beckmann MW, Schmidt D, Mallmann P. New WHO classification of endometrial hyperplasias. *Geburtshilfe Frauenheilkd.* (2015) 75:135–6. doi: 10.1055/s-0034-1396256
246. Artandi SE, DePinho RA. Telomeres and telomerase in cancer. *Carcinogenesis.* (2010) 31:9–18. doi: 10.1093/carcin/bgp268
247. Maida Y, Kyo S, Forsyth NR, Takakura M, Sakaguchi J, Mizumoto Y, et al. Distinct telomere length regulation in premalignant cervical and endometrial lesions: implications for the roles of telomeres in uterine carcinogenesis. *J Pathol.* (2006) 210:214–23. doi: 10.1002/path.2038
248. Chuang TC, Moshir S, Garini Y, Chuang AY, Young IT, Vermolen B, et al. The three-dimensional organization of telomeres in the nucleus of mammalian cells. *BMC Biol.* (2004) 2:12. doi: 10.1186/1741-7007-2-12
249. Louis SF, Vermolen BJ, Garini Y, Young IT, Guffei A, Lichtensztejn Z, et al. c-Myc induces chromosomal rearrangements through telomere and chromosome remodeling in the interphase nucleus. *Proc Natl Acad Sci USA.* (2005) 102:9613–8. doi: 10.1073/pnas.0407512102
250. Amiel T, Sharony R, Goldberg-Bittman L, Biron-Shental T, Fejgin MD, Amiel A. Telomere aggregates in amniocytes with karyotype of balanced chromosomal rearrangements. *Hereditas.* (2010) 147:90–3. doi: 10.1111/j.1601-5223.2009.2170.x
251. Danescu A, Herrero Gonzalez S, Di Cristofano A, Mai S, Hombach-Klonisch S. Three-dimensional nuclear telomere architecture changes during endometrial carcinoma development. *Genes Chromosomes Cancer.* (2013) 52:716–32. doi: 10.1002/gcc.22067
252. Saito T, Schneider A, Martel N, Mizumoto H, Bulgay-Moerschel M, Kudo R, et al. Proliferation-associated regulation of telomerase activity in human endometrium and its potential implication in early cancer diagnosis. *Biochem Biophys Res Commun.* (1997) 231:610–4. doi: 10.1006/bbrc.1997.6164
253. Shroyer KR, Stephens JK, Silverberg SG, Markham N, Shroyer AL, Wilson ML, et al. Telomerase expression in normal endometrium, endometrial hyperplasia, and endometrial adenocarcinoma. *Int J Gynecol Pathol.* (1997) 16:225–32. doi: 10.1097/00004347-199707000-00006
254. Mazurek U, Witek A, Olejek A, Paul M, Skalba P, Wilczok T. Expression of telomerase genes as potential marker of neoplastic changes. *Folia Histochem Cytobiol.* (2001) 39 Suppl. 2:183–4.
255. Dong Y, Li T, Liang Y, Zou WZ. Human telomerase P53 estrogen and progesterone receptor expression in endometrial carcinoma. *Beijing Da Xue Xue Bao Yi Xue Ban.* (2004) 36:61–5.
256. Dong Y, Li T, Wang Y, Wu HR, Xie M, Zou WZ. Expression of human telomerase reverse transcriptase and c-myc transcripts in endometrial carcinoma and its precursors. *Zhonghua Bing Li Xue Za Zhi.* (2004) 33:40–3. doi: 10.3760/j.issn:0529-5807.2004.01.010
257. Maida Y, Kyo S, Kanaya T, Wang Z, Tanaka M, Yatabe N, et al. Is the telomerase assay useful for screening of endometrial lesions? *Int J Cancer.* (2002) 100:714–8. doi: 10.1002/ijc.10543
258. Zheng PS, Iwasaka T, Yamasaki F, Ouchida M, Yokoyama M, Nakao Y, et al. Telomerase activity in gynecologic tumors. *Gynecol Oncol.* (1997) 64:171–5. doi: 10.1006/gyno.1996.4523
259. Brustmann H. Immunohistochemical detection of human telomerase reverse transcriptase (hTERT), topoisomerase IIalpha expression, and apoptosis in endometrial adenocarcinoma and atypical hyperplasia. *Int J Gynecol Pathol.* (2005) 24:184–92. doi: 10.1097/01.PGP.0000148338.84887.08
260. McAlpine J, Leon-Castillo A, Bosse T. The rise of a novel classification system for endometrial carcinoma; integration of molecular subclasses. *J Pathol.* (2018) 244:538–49. doi: 10.1002/path.5034
261. Kamal AM, Bulmer JN, DeCruze SB, Stringfellow HF, Martin-Hirsch P, Hapangama DK. Androgen receptors are acquired by healthy postmenopausal endometrial epithelium and their subsequent loss in endometrial cancer is associated with poor survival. *Br J Cancer.* (2016) 114:688–96. doi: 10.1038/bjc.2016.16
262. Smith JK, Yeh G. Telomere reduction in endometrial adenocarcinoma. *Am J Obstetr Gynecol.* (1992) 167:1883–7. doi: 10.1016/0002-9378(92)91791-8
263. Kyo S, Kanaya T, Ishikawa H, Ueno H, Inoue M. Telomerase activity in gynecological tumors. *Clin Cancer Res.* (1996) 2:2023–8.
264. Kyo S, Takakura M, Kohama T, Inoue M. Telomerase activity in human endometrium. *Cancer Res.* (1997) 57:610–4.
265. Brien TP, Kallakury BV, Lowry CV, Ambros RA, Muraca PJ, Malfetano JH, et al. Telomerase activity in benign endometrium and endometrial carcinoma. *Cancer Res.* (1997) 57:2760–4.
266. Gorham H, Yoshida K, Sugino T, Marsh G, Manek S, Charnock M, et al. Telomerase activity in human gynaecological malignancies. *J Clin Pathol.* (1997) 50:501–4.
267. Tanaka M, Kyo S, Takakura M, Kanaya T, Sagawa T, Yamashita K, et al. Expression of telomerase activity in human endometrium is localized to epithelial glandular cells and regulated in a menstrual phase-dependent manner correlated with cell proliferation. *Am J Pathol.* (1998) 153:1985–91. doi: 10.1016/S0002-9440(10)65712-4
268. Lehner R, Enomoto T, McGregor JA, Shroyer AL, Haugen BR, Pugazhenthi U, et al. Quantitative analysis of telomerase hTERT mRNA and telomerase activity in endometrioid adenocarcinoma and in normal endometrium. *Gynecol Oncol.* (2002) 84:120–5. doi: 10.1006/gyno.2001.6474
269. Wang SJ, Sakamoto T, Yasuda Si S, Fukasawa I, Ota Y, Hayashi M, et al. The relationship between telomere length and telomerase activity in gynecologic cancers. *Gynecol Oncol.* 84:81–4. doi: 10.1006/gyno.2001.6483
270. Akbay EA, Contreras CM, Perera SA, Sullivan JP, Broaddus RR, Schorge JO, et al. Differential roles of telomere attrition in type I and II endometrial carcinogenesis. *Am J Pathol.* (2008) 173:536–44. doi: 10.2353/ajpath.2008.071179
271. Prescott J, McGrath M, Lee IM, Buring JE, De Vivo I. Telomere length and genetic analyses in population-based studies of endometrial cancer risk. *Cancer.* (2010) 116:4275–82. doi: 10.1002/cncr.25328
272. Sun Y, Zhang L, Zhao L, Wu X, Gu J. Association of leukocyte telomere length in peripheral blood leukocytes with endometrial cancer risk in Caucasian Americans. *Carcinogenesis.* (2015) 36:1327–32. doi: 10.1093/carcin/bgv133
273. Menon MM, Simha MR. Telomerase and telomere length in normal and malignant human endometrium as prognostic markers. *Indian J Pathol Microbiol.* (2003) 46:394–8.
274. Hashimoto M, Kyo S, Masutomi K, Maida Y, Sakaguchi J, Mizumoto Y, et al. Analysis of telomeric single-strand overhang length in human endometrial cancers. *FEBS Lett.* 579:2959–64. doi: 10.1016/j.febslet.2005.04.021
275. Haycock PC, Burgess S, Nounu A, Zheng J, Okoli GN, Bowden J, et al. Association between telomere length and risk of cancer and non-neoplastic diseases: a Mendelian randomization study. *JAMA Oncol.* (2017) 3:636–51. doi: 10.1001/jamaoncol.2016.5945
276. Barthel FP, Wei W, Tang M, Martinez-Ledesma E, Hu X, Amin SB, et al. Systematic analysis of telomere length and somatic alterations in 31 cancer types. *Nat Genet.* (2017) 49:349–57. doi: 10.1038/ng.3781
277. Albanell J, Bosl GJ, Reuter VE, Engelhardt M, Franco S, Moore MA, et al. Telomerase activity in germ cell cancers and mature teratomas. *J Natl Cancer Inst.* (1999) 91:1321–6. doi: 10.1093/jnci/91.15.1321
278. Wan SM, Tie J, Zhang YF, Guo J, Yang LQ, Wang J, et al. Silencing of the hPOT1 gene by RNA inference promotes apoptosis and inhibits proliferation and aggressive phenotype of gastric cancer cells, likely through up-regulating PinX1 expression. *J Clin Pathol.* (2011) 64:1051–7. doi: 10.1136/jclinpath-2011-200211
279. Kondo T, Oue N, Yoshida K, Mitani Y, Naka K, Nakayama H, et al. Expression of POT1 is associated with tumor stage and telomere length in gastric carcinoma. *Cancer Res.* (2004) 64:523–9. doi: 10.1158/0008-5472.CAN-03-1196
280. Liu F, Pu XY, Huang SG, Xiang GM, Jiang DN, Hou G, et al. Expression of hPOT1 in HeLa cells and the probability of gene variation of hpot1 Exon14 in endometrial cancer are much higher than in other cancers. *Asian Pac J Cancer Prev.* (2012) 13:5659–63. doi: 10.7314/APJCP.2012.13.11.5659
281. Akbay EA, Pena CG, Ruder D, Michel JA, Nakada Y, Pathak S, et al. Cooperation between p53 and the telomere-protecting shelterin component Pot1a in endometrial carcinogenesis. *Oncogene.* (2013) 32:2211–9. doi: 10.1038/onc.2012.232
282. Flynn RL, Chang S, Zou L. RPA and POT1: friends or foes at telomeres? *Cell Cycle.* (2012) 11:652–7. doi: 10.4161/cc.11.4.19061

283. Hiyama E, Yokoyama T, Tatsumoto N, Hiyama K, Imamura Y, Murakami Y, et al. Telomerase activity in gastric cancer. *Cancer Res.* (1995) 55:3258–62.
284. Lin Y, Miyamoto H, Fujinami K, Uemura H, Hosaka M, Iwasaki Y, et al. Telomerase activity in human bladder cancer. *Clin Cancer Res.* (1996) 2:929–32.
285. Sommerfeld HJ, Meeker AK, Piatyszek MA, Bova GS, Shay JW, Coffey DS. Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res.* (1996) 56:218–22.
286. Taylor RS, Ramirez RD, Ogoshi M, Chaffins M, Piatyszek MA, Shay JW. Detection of telomerase activity in malignant and nonmalignant skin conditions. *J Invest Dermatol.* (1996) 106:759–65.
287. Bonatz G, Frahm SO, Klapper W, Helfenstein A, Heidorn K, Jonat W, et al. High telomerase activity is associated with cell cycle deregulation and rapid progression in endometrioid adenocarcinoma of the uterus. *Hum Pathol.* (2001) 32:605–14. doi: 10.1053/hupa.2001.25002
288. Liang J, Yin G, Chen M, Wu A. Detection of hTERT mRNA in peripheral blood and its implication for diagnosis of early stage postoperative endometrial cancer micrometastasis. *Panminerva Med.* (2016) 58:206–10.
289. Wu RC, Wang TL, Shih Ie M. The emerging roles of ARID1A in tumor suppression. *Cancer Biol Ther.* (2014) 15:655–64. doi: 10.4161/cbt.28411
290. Suryo Rahmanto Y, Jung JG, Wu RC, Kobayashi Y, Heaphy CM, Meeker AK, et al. Inactivating ARID1A tumor suppressor enhances TERT transcription and maintains telomere length in cancer cells. *J Biol Chem.* (2016) 291:9690–9. doi: 10.1074/jbc.M115.707612
291. Takeda T, Banno K, Okawa R, Yanokura M, Iijima M, Irie-Kunitomi H, et al. ARID1A gene mutation in ovarian and endometrial cancers. *Oncol Rep.* (2016) 35:607–13. doi: 10.3892/or.2015.4421
292. Boggess JF, Zhou C, Bae-Jump VL, Gehrig PA, Whang YE. Estrogen-receptor-dependent regulation of telomerase activity in human endometrial cancer cell lines. *Gynecol Oncol.* (2006) 103:417–24. doi: 10.1016/j.ygyno.2006.03.032
293. Kimura A, Ohmichi M, Kawagoe J, Kyo S, Mabuchi S, Takahashi T, et al. Induction of hTERT expression and phosphorylation by estrogen via Akt cascade in human ovarian cancer cell lines. *Oncogene.* (2004) 23:4505–15. doi: 10.1038/sj.onc.1207582
294. Zhou C, Bae-Jump VL, Whang YE, Gehrig PA, Boggess JF. The PTEN tumor suppressor inhibits telomerase activity in endometrial cancer cells by decreasing hTERT mRNA levels. *Gynecol Oncol.* (2006) 101:305–10. doi: 10.1016/j.ygyno.2005.10.038
295. Buseman CM, Wright WE, Shay JW. Is telomerase a viable target in cancer? *Mutat Res.* (2012) 730:90–7. doi: 10.1016/j.mrfmmm.2011.07.006
296. Salloum R, Hummel TR, Kumar SS, Dorris K, Li S, Lin T, et al. A molecular biology and phase II study of imetelstat (GRN163L) in children with recurrent or refractory central nervous system malignancies: a pediatric brain tumor consortium study. *J Neurooncol.* (2016) 129:443–51. doi: 10.1007/s11060-016-2189-7
297. Zanetti M. A second chance for telomerase reverse transcriptase in anticancer immunotherapy. *Nat Rev Clin Oncol.* (2017) 14:115–28. doi: 10.1038/nrclinonc.2016.67
298. Ivancich M, Schrank Z, Wojdyla L, Leviskas B, Kuckovic A, Sanjali A, et al. Treating cancer by targeting telomeres and telomerase. *Antioxidants (Basel).* (2017) 6:E15. doi: 10.3390/antiox6010015
299. Jager K, Walter M. Therapeutic targeting of telomerase. *Genes (Basel).* (2016) 7:E39. doi: 10.3390/genes7070039

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

REVIEW
published: 04 June 2019
doi: 10.3389/fonc.2019.00462

PP2A: A Promising Biomarker and Therapeutic Target in Endometrial Cancer

*Michiel Remmerie and Veerle Janssens**

Laboratory of Protein Phosphorylation and Proteomics, Department of Cellular and Molecular Medicine, KU Leuven, Leuven, Belgium

Over the last decade, the use of targeted therapies has immensely increased in the treatment of cancer. However, treatment for endometrial carcinomas (ECs) has lagged behind, although potential molecular markers have been identified. This is particularly problematic for the type II ECs, since these aggressive tumors are usually not responsive toward the current standard therapies. Therefore, type II ECs are responsible for most EC-related deaths, indicating the need for new treatment options. Interestingly, molecular analyses of type II ECs have uncovered frequent genetic alterations (up to 40%) in *PPP2R1A,* encoding the Aα subunit of the tumor suppressive heterotrimeric protein phosphatase type 2A (PP2A). *PPP2R1A* mutations were also reported in type I ECs and other common gynecologic cancers, albeit at much lower frequencies (0–7%). Nevertheless, PP2A inactivation in the latter cancer types is common via other mechanisms, in particular by increased expression of Cancerous Inhibitor of PP2A (CIP2A) and PP2A Methylesterase-1 (PME-1) proteins. In this review, we discuss the therapeutic potential of direct and indirect PP2A targeting compounds, possibly in combination with other anti-cancer drugs, in EC. Furthermore, we investigate the potential of the PP2A status as a predictive and/or prognostic marker for type I and II ECs.

Keywords: phosphatase targeted therapy, PPP2R1A, endometrial cancer, type II endometrial carcinoma, serous endometrial carcinoma, CIP2A, PME-1, PP2A activating drug

OPEN ACCESS

Edited by:
Massimo Nabissi,
University of Camerino, Italy

Reviewed by:
Alessandro Igor Cavalcanti Leal,
Johns Hopkins Medicine, United States
Luigi Formisano,
University of Naples Federico II, Italy

***Correspondence:**
Veerle Janssens
veerle.janssens@kuleuven.be

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *07 December 2018*
Accepted: *14 May 2019*
Published: *04 June 2019*

Citation:
Remmerie M and Janssens V (2019) PP2A: A Promising Biomarker and Therapeutic Target in Endometrial Cancer. Front. Oncol. 9:462. doi: 10.3389/fonc.2019.00462

INTRODUCTION

Treatment options for cancer have advanced immensely throughout history, with mainly one goal: to specifically target the tumor with as little harm as possible for the patient (1–4). During the last decade, molecular characterization of many tumors has brought cancer research another step closer toward this goal (5), providing the keys to unlock the door toward personalized medicine. However, treatment for endometrial cancer seems to lag behind, although potential markers for this disease have been identified and successful precedents using such markers for targeted therapy have been set in other cancers (e.g., lung cancer, chronic myeloid leukemia, breast cancer, melanoma) (6–13).

In this review, we will discuss the potential of the tumor suppressive protein phosphatase type 2A (PP2A) as a new biomarker and therapeutic target for both type I and type II endometrial carcinomas (ECs). We will mainly focus on the potential predictive and prognostic value of *PPP2R1A*, encoding the Aα subunit of PP2A, which is mutated in up to 40% of type II ECs,

while largely being unaffected in type I ECs and other common gynecologic cancers. In the latter cancer types, PP2A dysfunction commonly occurs, however, by other mechanisms of inactivation, stressing the importance of functional PP2A for preventing tumor development and/or progression. Overall, we propose that current, unsatisfactory (type II) EC treatments could be largely improved by taking the PP2A status of the tumor into account since it could be a potential useful indicator for prognosis and therapy response.

WHY TARGETED THERAPIES SHOULD BE THE FOCUS IN TYPE II EC

To date, the standard treatment for all ECs is surgery, followed by adjuvant therapy if necessary (14). This treatment protocol is usually sufficient for the clinically indolent and hormone-responsive type I ECs, which comprise 80% of all ECs. However, the other 20% of ECs are aggressive type II cancers, with serous histology as the most prominent subtype (15, 16). Unfortunately, most of these high-grade tumors are resistant to conventional chemo- and radiation therapies, underscoring the major clinical need for improved treatment regimens for this EC subgroup (17–23). Additionally, due to their late stage detection and metastatic character, surgery is usually not an option for type II ECs, since the cancer has often already spread outside the uterus at the time of diagnosis (24–26). Therefore, not surprisingly, most EC-related deaths are due to the type II cancers, with dismal overall survival rates of generally <30% (25, 27–29).

Despite the above knowledge, patients with type II EC are still treated with largely ineffective chemotherapy regimens, thereby leading to unnecessary physical and economic burdens for the patient. In Japan, for example, one cycle of the commonly used carboplatin/paclitaxel protocol consists of three courses with a cost of ∼2,000 Euros per course (30). Additionally, the medical care costs for managing the side effects were 1.6 times as much as the cost for one course, accumulating in a total cost of ∼9,200 Euros per chemotherapy cycle. A study in the United Kingdom further demonstrated that the total costs (diagnosis/surgery, adjuvant therapy, and further treatment) increased with increasing EC grade (31). Hence, more pre-clinical studies and clinical trials should be focusing on targeted therapies in order to provide more adequate treatment options for patients with high-grade type II EC (32). This view seems particularly justified in light of the fact that successful results have already been obtained in other cancer types and several molecular markers have been identified in EC (33–35). One of the most promising markers in this context is certainly *PPP2R1A*, encoding the Aα subunit of the tumor suppressive phosphatase PP2A.

Abbreviations: Bad, Bcl2-associated death promotor; CIP2A, Cancerous inhibitor of PP2A; EC, Endometrial carcinoma; ERK, Extracellular signal-regulated kinase; GSK-3β, Glycogen synthase kinase 3β; HPV, Human papilloma virus; MAPK, Mitogen-activated protein kinase; MEK, Mitogen-activated protein kinase kinase; mTOR, Mammalian target of rapamycin; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PME-1, PP2A methylesterase 1; PP2A, Protein phosphatase 2A; pRb, Protein retinoblastoma; PTPA, Phosphatase 2A phosphatase activator; SET, Suvar/Enhancer of zeste/Trithorax; SMAP, Small molecule activator of PP2A; SV40, Simian virus 40; TKI, Tyrosine kinase inhibitor.

THE TUMOR SUPPRESSIVE PROTEIN PHOSPHATASE PP2A

Reversible phosphorylation is the key pillar on which signal transduction is built. The enzymes responsible for these post-translational modifications are the protein kinases, which catalyze phosphorylation of proteins, and the protein phosphatases, which, in turn, remove the phosphate group from their substrates (36–38). Importantly, the presence or absence of a phosphate can affect the biological activity of the modified protein, either positively or negatively, depending on the substrate. Like that, kinases and phosphatases act as on/off or off/on switches in cellular signaling. The balanced activities between both enzymes ensures that cellular homeostasis is preserved and that cells can generate the appropriate responses (e.g., proliferation, differentiation, survival, apoptosis…) to specific external stimuli. However, in cancer cells, this balance is genetically disrupted by mutations in key signaling molecules that often directly or indirectly affect kinases and phosphatases, so that signaling pathways will be constitutively activated or inhibited, eventually leading to overall uncontrolled cell growth and survival (39, 40).

Initially, in cancer research, protein kinases got the bulk of the attention, since their over-activation commonly drives oncogenic signaling and their pharmacologic inhibition showed promising clinical potential (6, 38, 41, 42). Furthermore, with more than 500 genes encoding protein kinases, they were thought to be the specific regulators of important oncogenic signaling pathways (43). However, if one accepts that a change in phosphorylation often just reflects an altered balance between kinase and phosphatase activities, kinases and phosphatases seem equally attractive therapeutic targets. Nevertheless, compared to kinases, phosphatase research, and phosphatase-directed therapies have lagged behind for a long time. This has in part been due to the fact that the first phosphatase was discovered 20 years after the first kinase, and that protein phosphatases were for a long time regarded as significantly less specific and less amenable to regulation by external stimuli, rendering them less attractive as therapeutic targets (44, 45). However, more and more attention has been brought to the phosphatases nowadays, hopefully resulting in more clinical applications in the near future (37, 46, 47).

The large majority of protein phosphorylation occurs on serine (Ser) and threonine (Thr) residues (48). The Ser/Thr phosphatase PP2A constitutes about 1% of the total cellular protein content and is together with Protein Phosphatase 1 (PP1) responsible for more than 90% of all Ser/Thr phosphatase activity in the cell (49, 50). PP1 and PP2A are both holoenzymes, consisting of different subunits. PP2A consists of a dimeric core enzyme composed of a catalytic C subunit and a scaffolding A subunit (**Figure 1**). In humans, each of these subunits have two isoforms, α and β, of which the α isoform is the most commonly expressed in most cell types (51, 52). However, in

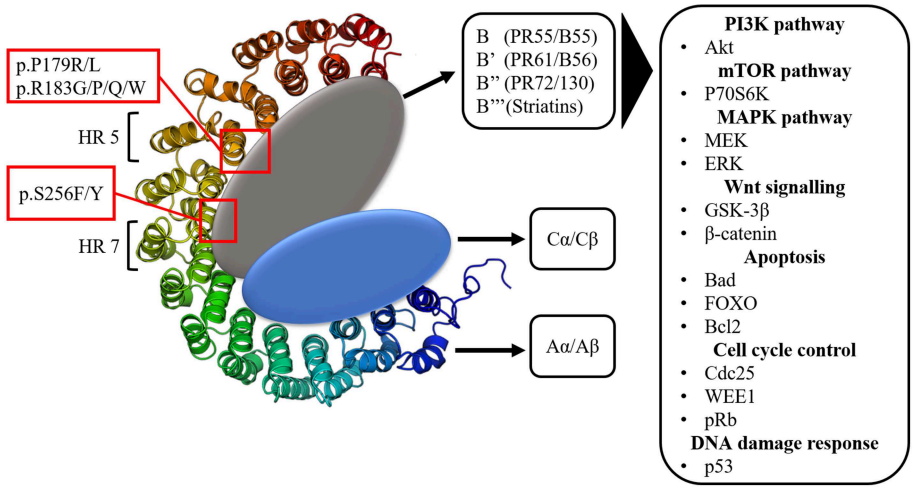


FIGURE 1 | The structure of the protein phosphatase PP2A. Three subunits can be distinguished: the scaffolding A subunit (Aα/Aβ) composed of 15 heat repeats (HR), the catalytic C subunit (Cα/Cβ) and the 4 classes of regulatory B subunits. *PPP2R1A* hotspot mutations occur in HR 5 (p.P179R/L, p.R183G/P/Q/W) and HR 7 (p.S256F/Y), which are involved in B subunit binding. The many regulatory B subunits allow for PP2A to target a vast array of components of important signaling pathways often involved in tumorigenesis.

order to target specific protein substrates, a third subunit needs to be associated with the AC core dimer, resulting in the formation of the trimeric PP2A holoenzyme (**Figure 1**). This third subunit is referred to as the regulatory B subunit and determines the subcellular localization and substrate specificity (53). The human genome encodes four different families of B subunits, which mutually exclusively bind the AC core dimer: PR55 (B/B55), PR61 (B'/B56), PR72/130 (B"), and B"' (Striatins). Furthermore, each family of B subunits consists of several isoforms (α up to ε) and splice variants, allowing for the formation of many different PP2A holoenzymes (53).

This huge structural diversity of PP2A holoenzymes forms the basis for its diverse functions in cellular signaling by allowing PP2A to act on various components within important signaling pathways (54). The main pathways affected by PP2A are the PI3K (Akt), mTOR (p70S6K) and MAPK (MEK/ERK) pathways (**Figure 1**). Additionally, PP2A also targets the oncoprotein cMyc as well as components involved in Wnt (GSK-3β, β-catenin) signaling, apoptosis (Bcl2, Bad, FOXO), cell cycle regulation (cdc25, WEE1, pRb) and DNA damage response (p53, ATM, Chk) (51, 55–60). All of these pathways are key regulators of processes imbalanced in tumorigenesis (e.g., protein synthesis, cell proliferation, cell survival, cell migration, and invasion). Since PP2A usually negatively affects these pathways, it was denoted as a potential tumor suppressor (61).

The tumor suppressive properties of PP2A were first demonstrated in *in vitro* experiments using the tumor promoting agent and selective PP2A inhibitor, okadaic acid (OA), as well as the simian virus 40 (SV40) small T antigen. These experiments showed that PP2A inactivation is an absolute requirement in order to achieve oncogene-induced transformation of immortalized human epithelial cells (e.g., by oncogenic H-*Ras*). OA is able to inhibit PP2A by acting on the catalytic C subunit, while SV40 small T antigen inhibits PP2A by binding the Aα subunit, thereby replacing specific B subunits (61–64). The tumor suppressive nature of PP2A was further corroborated by *in vivo* evidence in mice. For example, mice completely lacking the B56δ subunit, or showing ≥50% decreased expression of the phosphatase 2A phosphatase activator (PTPA), spontaneously developed tumors (65–67). Additionally, the general physiologic importance of PP2A function was demonstrated in several mouse models (68). For example, PP2A Aα or PP2A Cα knock-out mice are embryonically lethal, indicating the importance of PP2A already during development. Furthermore, a vast array of pathological phenotypes were observed in mice with different genetic PP2A dysfunctions, stressing the importance of functional PP2A in many crucial signaling pathways and in tissue homeostasis (68).

In line with these studies, many human cancers have shown to be associated with PP2A dysfunction (69–71). The main mechanism of PP2A inactivation in cancer is via the overexpression of the endogenous PP2A inhibitors SET (Suvar/Enhancer of zeste/Trithorax) and CIP2A (Cancerous Inhibitor of PP2A) (69, 72, 73). However, PP2A can also be inactivated via aberrant post-translational modifications, mostly via PP2A methylesterase (PME-1) upregulation, thereby stabilizing inactive PP2A complexes through binding and/or demethylation of the C subunit C-terminal tail (74–76). Another way of PP2A inactivation is via mutations in one of its subunits (77), or via mutations or heterozygous loss of the cellular PP2A activator PTPA (*PPP2R4*) (66). Interestingly, PP2A dysfunction is very common in endometrial cancer, as well as in other gynecologic malignancies, such as ovarian and cervical cancer. In the following part, we will give an overview of how PP2A is specifically inactivated in these gynecologic cancers.

THE PP2A STATUS IN ENDOMETRIAL AND OTHER COMMON GYNECOLOGIC CANCERS

PP2A Status in Endometrial Cancer

Inactivation of PP2A is observed in both type I and type II ECs. However, the way PP2A is inactivated seems to be quite different in both EC subtypes.

In type I endometrioid ECs, PP2A inactivation is likely indirect via an upregulation of the endogenous PP2A inhibitors CIP2A or PME-1. Immunohistochemical analysis of paraffin-embedded EC tissue revealed positive CIP2A staining in 79% of the cases. Additionally, increased CIP2A mRNA levels were observed in fresh human EC tissue compared to healthy endometrial tissue (78). CIP2A depletion in endometrioid cancer cells decreased cell proliferation and invasion, while apoptosis was increased, indicating the oncogenic role of CIP2A in type I EC and its potential as a therapeutic target (78). Detailed information on the mechanism of CIP2A overexpression in ECs is still lacking. However, in estrogen receptor (ER)-positive breast cancer cells, estradiol (E2) was able to increase CIP2A protein levels through the ERα (79). Therefore, it can be hypothesized that the same could be true for the estrogen-dependent type I ECs. Additionally, it could be one of the explanations why CIP2A overexpression is rare in the estrogen-independent type II ECs.

In addition, Wandzioch et al. demonstrated PME-1 overexpression in endometroid EC cell lines as well as patient samples (80). PME-1 expression was about 20 times higher in tumor tissue compared to healthy tissue, indicating PME-1 could also be a new potential biomarker for type I ECs. In case of PME-1 upregulation, PP2A activity was significantly reduced, resulting in an increased oncogenic phenotype via upregulation of the PP2A targets Akt and ERK (80).

In contrast to type I ECs, PP2A inactivation in type II ECs is, to our knowledge, not associated with CIP2A or PME-1 overexpression. Instead, up to 40% of type II EC tumors are associated with heterozygous missense mutations in *PPP2R1A* (34, 35, 81–95). *PPP2R1A* mutations also occur in type I ECs, albeit at very low frequencies (2.5–6.9%) (32).

PPP2R1A encodes the Aα subunit of PP2A and is an established tumor suppressor gene (96, 97). The structure of Aα is characterized by 15 Huntingtin-Elongation-A subunit-TOR (HEAT) repeats (98) (**Figure 1**). Each of these HEAT repeats consists of a pair of anti-parallel alpha helices connected via intra-repeat loops. These intra-repeat loops are responsible for the interaction with the C and B subunits. More precisely, HEAT repeats 1–10 are able to bind the regulatory B subunits while HEAT repeats 11–15 bind the catalytic C subunit. Remarkably, most of the *PPP2R1A* mutations cluster together in HEAT repeats 5 and 7, which are involved in B subunit binding. Another intriguing fact is that these *PPP2R1A* mutations almost always occur at the same residues across several cancer types, forming so called hotspot mutations. These hotspot mutations include p.P179R/L, p.R183G/P/Q/W (HEAT repeat 5), and p.S256F/Y (HEAT repeat 7) (93, 94, 99–101). Remarkably, in type II EC, p.P179L/R hotspot mutations are a lot more abundant than in most other cancer types (**Figure 2**). Biochemical studies in endometrial cancer cells demonstrated that C subunit binding for these hotspot mutations was significantly reduced. Strikingly, loss of C subunit binding was more severe for p.P179R (80% less C binding compared to wild type) than for p.R183W and p.S256F (50–60% less C binding compared to wild type). This

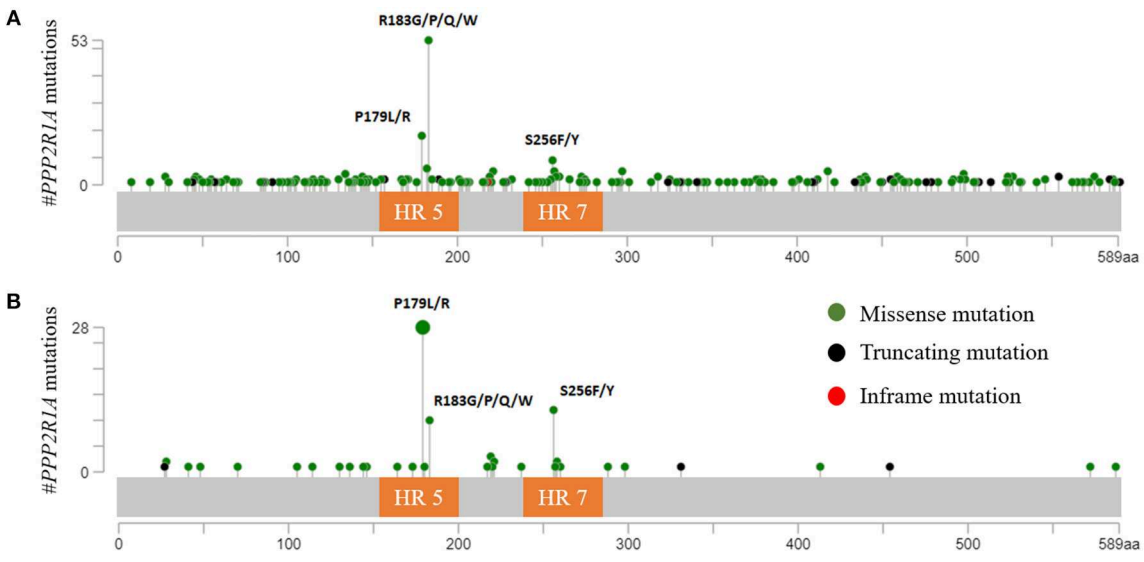


FIGURE 2 | (A) *PPP2R1A* mutations across all available cBioportal cancer studies except ECs. The three hotspot mutations (p.P179; p.R183; and p.S256) are clearly depicted. Most tumors (66.3%) had mutations in p.R183 while only 22.5% and 11.3% had mutations in p.P179 and p.S256, respectively. **(B)** *PPP2R1A* missense mutations reported in type II ECs (cBioportal). In contrast with other cancers, *PPP2R1A* mutations mostly occur at residue p.P179 (56%), while only 20% had mutations in residue p.R183. Also more EC tumors (24%) had mutations in p.S256 compared to other cancer types. HR, HEAT-repeat.

biochemical difference between the hotspot mutations could result in distinct functional consequences, although further research is needed to investigate this. Moreover, *PPP2R1A* hotspot mutations resulted in deficient binding to specific regulatory B subunits (B55α, B56α,β,ε, B"/PR72), while some B subunits (i.e., B56δ and B56γ, and the striatins) retained binding to the mutated Aα subunit (102, 103). Nevertheless, despite the retained binding of these B subunits, the phosphatase activity of the Aα mutated PP2A-B56δ, γ trimers was reduced. Therefore, it was suggested that *PPP2R1A* mutations are not simply loss-of-function (i.e., by their inability to bind specific B-type subunits), but may also lead to a dominant-negative inhibition of specific PP2A complexes (i.e., by their ability to retain binding to specific B-type subunits, but capturing them in trimeric complexes with decreased phosphatase activity). This hypothesis was further mechanistically underbuilt by mass spectrometry-based identification of the (mutant) Aα interactomes. These interactomes indeed revealed that the Aα mutants had an increased binding to the endogenous PP2A inhibitor TIPRL1, which could explain the decreased PP2A activities of retained mutant Aα-PP2A trimeric complexes (102). In accordance, ectopic expression of several Aα mutants, among which p.P179R and p.S256F, in the wild-type *PPP2R1A*-expressing HEC-1A EC cell line, resulted in increased anchorage-independent cell growth and increased xenografted tumor growth in nude mice, and correlated with increased Akt and mTOR/S6K oncogenic signaling (102).

Besides the *PPP2R1A* mutations, also mutations in *PPP2R1B,* encoding the PP2A Aβ isoform, have been reported for ECs in the cBioportal database, albeit with occurrences of <1% (mainly deletions) in both EC subtypes (93, 94).

PP2A Status in Ovarian and Cervical Cancer

Inactivation of PP2A has also been reported in other gynecologic cancers. In contrast to type II ECs, and similar to type I ECs, ovarian (0–7%), and cervical cancers (0–1%) hardly present any mutations in *PPP2R1A* (91, 93, 94, 104–112). Interestingly, however, the few number of *PPP2R1A* mutations that are present mainly occur at the hotspot residue p.R183, which is most frequently affected across all cancer types (**Figure 2A**).

Likewise, mutations in *PPP2R1B*, were also reported in ovarian and cervical cancers, albeit at very low frequencies, and without any hotspot mutations. Furthermore, the presence of *PPP2R1B* mutations or loss of heterozygosity was not relevant for ovarian and cervical tumorigenesis (113–116).

Instead, the main way of PP2A inactivation in both ovarian and cervical cancers is indirect. Indeed, a retrospective analysis of serous ovarian cancer demonstrated 40.4% of the specimens to have strong CIP2A immunoreactivity and another 42.4% had weak positive staining (117). Another retrospective study of 152 ovarian cancer specimens (serous, endometrioid, mucinous and clear cell) further corroborated this by showing CIP2A overexpression in 65.79% of samples tested (118). Likewise, recent studies have shown SET overexpression in ovarian cancers (119).

Increased CIP2A expression levels have also been reported in cervical cancer. For example, one study reported on the expression of CIP2A in 60.8% of samples from patients with squamous cervical cancer while this was only 5.7% in normal cervical epithelial tissue. Furthermore, five cervical cancer cell lines harbored elevated CIP2A levels (120). This was further corroborated by a study of Huang et al. in which CIP2A expression was observed in cervical cancer cell lines but not in normal epithelial cells. Additionally, cervical cancer tissue had higher CIP2A mRNA levels compared to healthy adjacent tissue (121). In cervical cancer, CIP2A is mainly upregulated via the E6 and E7 oncoproteins expressed by the human papilloma virus (HPV) type 16 (122–124), the most common type of HPV in cervical cancers (125). In addition to the indirect PP2A inhibition via CIP2A, Pim et al. also proposed a direct way of PP2A inhibition in cervical cancers. They observed that the E7 oncoprotein is able to bind the PP2A Aα and Cα subunits, thereby displacing the B subunit. This way E7 is probably acting in the same way as the SV40 small T antigen rendering PP2A unable to dephosphorylate and inhibit its oncogenic targets (126, 127). In contrast, however, White et al. were not able to demonstrate this interaction between the E7 oncoprotein and PP2A, despite some similarities between E7 and the SV40 small T antigen (128). Finally, another way of decreased PP2A activity during cervical carcinogenesis might be via reduced PP2A Cα expression, potentially by a microRNA-dependent mechanism (129).

Summarized, based on the mechanism of PP2A inactivation, two groups can be distinguished within gynecologic cancers. The first group comprises type I endometrioid EC, ovarian and cervical cancers and is characterized by PP2A inactivation mainly via CIP2A or PME-1, and perhaps also via SET. This group also has a very low frequency of *PPP2R1A* mutations. However, when present, these mutations recur mostly at hotspot residue p.R183. The second group comprises the type II ECs. This cancer type lacks CIP2A and PME-1 overexpression, but harbors frequent heterozygous missense mutations in *PPP2R1A*. Moreover, these missense mutations are different from the ones associated with the first group. More precisely, *PPP2R1A* mutations in type II ECs most frequently recurred at residues p.P179 and p.S256 (**Figure 2B**). Indeed, when looking at the cBioportal database, almost all hotspot mutations in p.P179 and p.S256 were associated with type II ECs, while hotspot mutations in p.R183 are more frequently observed across other cancer types (**Figure 2A**) (90, 93, 94, 130). The reasons for this distinct mutational pattern remain currently unclear. However, distinct *PPP2R1A* missense mutations might affect B subunit binding and PP2A activity in a slightly different way (102), thereby, in part, contributing to a different tumor biology in type I and II ECs as well as other cancers. However, further research is warranted in order to fully understand the involvement of specific PP2A holoenzymes in different cancers. Nevertheless, the distinct PP2A inactivating mechanisms between type I and type II ECs, as well as other gynecologic cancers, open up specific opportunities for direct or indirect, personalized therapeutic targeting of PP2A, in order to (re)-activate this phosphatase.

PP2A AS A POTENTIAL THERAPEUTIC TARGET IN ENDOMETRIAL CANCER

In the last few years, the notion that phosphatases as opposed to kinases could also be useful therapeutic targets has gained more and more attention (54, 131–134). In contrast to therapeutic inhibition of kinases, the focus with targeting phosphatases, and in particular the tumor suppressive phosphatase PP2A, is on the development of activating or reactivating compounds (133). These compounds are either able to relieve the inhibition by endogenous inhibitors, or directly bind and activate PP2A. In contrast, some studies also reported on the benefit of PP2A inhibition rather than activation. Despite the promising therapeutic potential of these compounds in multiple pre-clinical studies in several other human cancer types, pre-clinical studies, specifically in EC, are however, mostly lacking. In the following four subsections, we will provide an overview of the most promising PP2A-targeted therapies, which—pending additional dedicated studies—may become applicable to EC as well.

Compounds Indirectly Activating PP2A

CIP2A Targeting Compounds

Since CIP2A is commonly overexpressed in several cancers, it has become an interesting therapeutic target in order to re-activate PP2A. Specifically in type I EC, CIP2A depletion decreased proliferation and invasion, and increased apoptosis *in vitro* (78), indicating it could be a valuable therapeutic target. Furthermore, depletion of CIP2A also showed anti-tumorigenic potential in ovarian and cervical cancer cells (118, 120, 135).

Most of the currently described CIP2A targeting compounds are able to increase PP2A activity by reducing the CIP2A protein levels, either via downregulation of CIP2A expression or by promoting its degradation. In cervical and endometrial cancer cells, CIP2A expression is mainly regulated by two transcription factors, Elk1 and Ets1, which are both necessary for regulating CIP2A protein levels (136). Additionally, in cervical cancer, the transcription factor E2F1 has also been implicated in the regulation of CIP2A expression via the E7 oncoprotein (124, 137). Therefore, compounds targeting one of these factors could have potential therapeutic value. For example, several erlotinib derivatives were able to reduce CIP2A levels and increase PP2A activity in breast cancer and hepatocellular carcinoma cells via disrupting the interaction between the transcription factor Elk1 and the *CIP2A* promotor (138, 139). On the other hand, lapatinib downregulated CIP2A through regulation of protein stability in breast cancer cells (140). Also, bortezomib, a US Food and Drug Administration (FDA)-approved proteasome inhibitor, was able to reduce CIP2A expression levels in several cancer cell lines, although the mechanism of action is not elucidated yet (141–143). Furthermore, increased CIP2A degradation through autophagy was seen in breast cancer cells upon mTORC1 inhibition (e.g., using rapamycin) (144). Additionally, several natural compounds have demonstrated PP2A re-activating potential via downregulation of CIP2A. Despite the lack of studies testing these compounds in EC, positive results have already been obtained in other cancer studies. For example, rhabdocoetsin B, arctigenin and the red wine component ellagic acid were able to reduce CIP2A transcription levels in breast and lung cancer cells (145–147). On the other hand, celastrol and gambogenic acid promoted CIP2A degradation in lung and liver cancer cells (148, 149). Additionally, the compounds genistein and ethoxysanguinarine promoted both transcriptional suppression and proteasomal degradation of CIP2A (150, 151). Lastly, fusogenic-oligoarginine peptide-mediated delivery of siRNA targeting CIP2A has also appeared as a new therapeutic strategy, showing anti-tumorigenic potential *in vitro* and *in vivo* in oral cancer cells (152, 153). It would be extremely interesting to test whether any of these known CIP2A inhibiting compounds would have therapeutic benefits in CIP2A-overexpressing endometrioid EC models.

PME-1 Targeting Compounds

PME-1 has also emerged as a potential therapeutic target in endometrioid EC, especially since PME-1 depletion using RNA interference resulted in increased PP2A activity, thereby reducing the oncogenic phenotype of type I EC cells *in vitro* and in xenograft assays (80). Additionally, PME-1 depletion in HeLa cells, a cervical cancer cell line, also led to decreased proliferation and colony formation by increasing PP2A activity and thereby inhibiting MAPK pathway activity (154).

So far, two classes of pharmacologic PME-1 inhibitors have been discovered, the ABL (Aza-β-lactam) inhibitors and the sulfonyl acrylonitrile inhibitors, which both irreversibly bind to PME-1 and inhibit PME-1 esterase activity (155, 156). Pusey et al. tested two of these PME-1 inhibitors, ABL-127 and AMZ-30, of which ABL-127 was the most potent one in EC models. However, *in vivo* testing of this compound in xenograft assays could not corroborate the *in vitro* data, implying that inhibition of solely the PME-1 esterase activity may be insufficient to inhibit PME-1's oncogenic characteristics (157).

SET Targeting Compounds

Although the relevance of SET overexpression (if any) in EC is currently unclear, SET inhibitors can mainly be divided into three groups according to their origin. The first group comprises sphingolipid-based compounds, such as ceramide and FTY720 (also called Fingolimod), as well as their derivatives. The second group resembles the apolipoprotein E (ApoE) and these compounds are denoted as SET interfering peptides (e.g., COG112 and OP449). More recently, potent cytotoxic effects were reported for cell penetrating peptides, the third group of SET inhibitors, which constitute the precise SET-PP2A interaction interface (158). Although the mechanism of action of all these SET inhibitors is not always well understood, they most likely increase PP2A activity in the same way, i.e., via disruption of the interaction between SET and PP2A (53, 59, 133). Recently, the interaction between the sphingolipid-based compounds (i.e., ceramide and FTY720) and PP2A were investigated in more detail using NMR spectroscopy (159). In this study, they observed that the sphingolipid compounds probably work by disrupting the dimerization of SET, which is thought to be important for its PP2A binding and inhibiting activity.

Compounds Directly Activating PP2A

Recently, small molecule activators of PP2A (SMAPs) have been developed (160). These SMAPs are derived from the anti-psychotic phenothiazines and are predicted to directly activate PP2A. Although a direct binding to the PP2A Aα subunit has been demonstrated (161), the mechanism by which these SMAPs are able to activate PP2A remains unknown. Nevertheless, several pre-clinical studies have shown the promising anti-proliferative potential of SMAP treatment, for example in T-cell acute lymphoblastic leukemia, castration-resistant prostate cancer, KRAS mutant lung adenocarcinoma, and tyrosine kinase inhibitor (TKI)-resistant lung adenocarcinoma (161–164). Hence, it would be of amazing interest to test these promising compounds in pre-clinical EC models with intact as well as impaired PP2A functionality.

The Therapeutic Potential of Combination Therapies

Besides the therapeutic potential of single agent targeting of PP2A, also combination therapies of PP2A activators with other drugs have gained attention (47, 165). The combination of a PP2A activator with a kinase inhibitor seemed particularly beneficial in cases where oncogenic kinase activation simultaneously resulted in PP2A inhibition, and therapy resistance to a single agent kinase inhibitor occurred (166–168). For example, *KRAS*-mutant lung cancer and pancreatic ductal adenocarcinoma cells showed resistance to MEK inhibitors and mTOR inhibitors, respectively. This resistance occurred due to cross-talk with the PI3K/Akt/mTOR pathway and cMyc oncoprotein upregulation, probably via PP2A inhibition. Hence, they tested the combination of a direct PP2A activator (SMAP) with a MEK or mTOR inhibitor, which resulted in significantly increased anti-cancer effects *in vitro* as well as *in vivo* (169, 170). Such combinatorial benefit was further demonstrated in myeloid leukemia where the combination of a SET inhibitor (indirect PP2A activation) with a tyrosine kinase inhibitor resulted in synergistic anti-cancer effects (171, 172). In TKI-resistant lung adenocarcinoma cells, the synergistic effects of a SMAP and the TKI afatinib were in part also contributed to a downregulation of CIP2A (164).

The combination of PP2A activators with chemotherapy has also been investigated, although not yet in EC. Several studies tested the effect of combining SET inhibitors (FTY720, OP449) with different chemotherapy regimens (e.g., doxorubicin, cisplatin). These studies demonstrated synergistic anti-cancer effects in myeloid leukemia, breast cancer cells, colorectal cancer cells as well as in cisplatin-resistant melanoma and lung cancer cells (171, 173–175).

Overall, these successful precedents open up possibilities to test these PP2A activating compounds in EC models, possibly in combination with kinase inhibitors or chemotherapeutics. This could be specifically interesting for the type II serous ECs, in which therapeutic combinations with PP2A activators might sensitize these cancer cells toward the current, mainly ineffective, therapies.

Exploiting PP2A Inhibition for Therapeutic Purposes

In contrast to the therapeutic potential of PP2A activation, some studies also reported on the therapeutic relevance of PP2A inhibition, when applied together with a DNA damaging treatment, or when combined with immunotherapy (176, 177). The anti-cancer effect of PP2A inhibition in combination with DNA damaging agents can be explained by the enabling role of PP2A in DNA damage response and repair pathways as well as in cell cycle regulation. Hence, PP2A inactivation in this situation (i.e., combined with chemo-or radiation therapy) leads to aberrant cell cycle progression and checkpoint activation, resulting in mitotic catastrophe and, consequently, cell death (133, 178, 179). Likewise, PP2A is also involved in the immune response by negatively regulating the function of cytotoxic T-lymphocytes (176, 180). Therefore, PP2A inhibition combined with immunotherapy could enhance the immune-mediated anti-tumor response.

The small molecule LB-100 is one of the best studied PP2A inhibitors so far, without any apparent toxicities in animals and with promising results in a first human clinical trial (181, 182). Pre-clinical studies demonstrated LB-100 was able to sensitize many different solid tumor cells to DNA damaging agents. For example, LB-100 enhanced cisplatin-mediated cytotoxicity in ovarian carcinoma cells *in vitro* and *in vivo* in xenografts (178, 183, 184). Likewise, the combination of LB-100 with the immune checkpoint inhibitor aPD-1 in colon and melanoma cancer cells resulted in an enhanced and durable T-cell-dependent anti-tumor response, with more effector T-cell and less suppressive regulatory T-cell infiltration (176). On a critical note, it needs to be mentioned here though, that recent evidence has suggested that LB-100 is not entirely specific for PP2A, and also inhibits the catalytic activity of the related Ser/Thr phosphatase PP5 (182). As PP5 is considered as tumor promoting, PP5 inhibition could contribute to the anti-tumor activities of LB-100. This was further corroborated *in vitro* in ovarian cancer cells, where knockdown of PP5 resulted in decreased cell proliferation and colony formation.

To conclude, further research is warranted to fully understand how both PP2A activation and inhibition can be therapeutically viable as anti-cancer treatment for EC.

PP2A DYSFUNCTIONS AS PREDICTIVE BIOMARKERS FOR TARGETED THERAPIES IN EC

CIP2A-Mediated PP2A Inhibition

So far, a wealth of studies reported on the role of CIP2A overexpression, and thereby likely PP2A inactivation, as a potential predictive biomarker for diverse therapies (targeted and untargeted), in a large variety of solid cancers (54). For example, in lung and breast cancer, the overexpression of CIP2A resulted in resistance to the EGFR inhibitors lapatinib and erlotinib, while RNA interference-mediated CIP2A depletion sensitized the cells toward these compounds (140, 185). CIP2A overexpression also resulted in resistance toward Chk1 kinase

inhibitors in gastric adenocarcinoma and breast cancer cells (186). Furthermore, CIP2A overexpression conferred resistance to chemotherapy in several solid tumor types, including cervical and ovarian cancers (187–190). Liu et al. further demonstrated that in HeLa cells, treated with several chemotherapeutics such as paclitaxel, doxorubicin and cisplatin, CIP2A expression was significantly associated with drug insensitivity through increased expression of p-glycoprotein drug efflux pumps (190). Additionally, the use of siRNA, targeting CIP2A *in vitro,* resulted in sensitization of HeLa cells to different chemotherapeutics (190). Finally, the natural CIP2A inhibitors, ethoxysanguinarine and gambogenic acid, sensitized lung and hepatocellular cancer cells to chemotherapy (149, 151).

While there is a general lack of studies on the predictive potential of CIP2A expression in EC models, it is very likely that, based upon the evidence obtained in other solid (gynecologic) tumors, CIP2A expression could mediate therapeutic resistance in EC cells as well. This further implies that CIP2A status of the EC tumors should better be taken into account in clinical trial set-ups. Additionally, the data illustrate the potential advantages of combining PP2A activators with EC therapies that are mainly ineffective on their own.

Recurrent *PPP2R1A* Hotspot Mutations

Whether *PPP2R1A* mutations are present in EC tumors or not, could have consequences for the efficacy of targeted therapies. For example, kinase inhibitors targeting the PI3K/Akt/mTOR pathway or MEK/MAPK pathway, commonly affected in ECs, could be less effective when PP2A, counteracting the targeted kinase, is mutated. The rationale behind this is that a kinase inhibitor can only work to its full potential when the opposing phosphatase is not inactivated. In case the phosphatase is dysfunctional, the net phosphorylation would be largely unaffected and the pathway would remain activated. Simply put, the use of certain kinase inhibitors in case of PP2A dysfunction would be the equivalent of pouring water into a bucket with holes in it. This biochemical logic was nicely underscored by Kauko et al. who showed that PP2A inhibition achieved by siRNA-mediated knockdown of Aα, conferred resistance to a MEK inhibitor in *KRAS*-mutant lung cancer cells (169).

On the other hand, *PPP2R1A* mutations in type II ECs could also be predictors of positive outcome to certain kinase inhibitors. For example, Haesen et al. showed hyperactivation of the PI3K/Akt/mTOR pathway in *PPP2R1A* mutated EC cells, while the MAPK pathway was actually downregulated (102). This indicates single agent kinase inhibitors targeting the PI3K pathway might be effective in *PPP2R1A* mutated ECs, since cross-talk to the MAPK pathway would possibly be absent and *PPP2R1A* mutant EC cells might be dependent on PI3K/Akt/mTOR signaling for growth and survival (32). Patient stratification based on *PPP2R1A* status of the tumor could also be applied to other gynecologic cancer types, even when *PPP2R1A* mutations are rare. For example, Papp et al. demonstrated ovarian cancer cell lines harboring *PPP2R1A* mutations to be more sensitive to a PI3K/mTOR inhibitor (108). However, in this experiment they took cell lines with mutations in *PPP2R1A* and *PARP1* into account, which could bias the results.

Finally, the response of type II ECs to SMAPs could also be dependent on the *PPP2R1A* mutational status of the cancer cells, especially since these SMAPs bind to the Aα subunit in close proximity to the *PPP2R1A* hotspot mutations (161). Therefore, mutations in this subunit could disturb the interaction with the SMAPs and consequently render the compound ineffective. Targeted pre-clinical studies addressing these possibilities should provide further insights in these issues in the near future.

Others

Although no studies have yet addressed the predictive role of PME-1 overexpression in type I EC, overexpression of PME-1 in glioma drives resistance to various multikinase inhibitors. Consequently, PME-1 depletion resulted in enhanced sensitivity to these inhibitors *in vitro* and *in vivo* in xenografts (191).

As identified through a large siRNA screen, decreased expression of the PP2A activator PTPA conferred significantly increased resistance of cervical HeLa cells to several cytotoxic agents, including cisplatin, taxol, and etoposide (192), perhaps suggestive for a similar dismal predictive role for heterozygous loss or mutation of PTPA in type II endometrial carcinosarcoma (66).

Likewise, increased SET expression has been associated with resistance to TKI's, cisplatin, paclitaxel, oxaliplatin, and 5-fluoro-uracil in diverse cancer types (54, 193, 194). Whether this would be relevant for EC, remains, again, to be determined.

PP2A DYSFUNCTIONS AS PROGNOSTIC BIOMARKERS FOR EC

CIP2A Overexpression

In several cancers, PP2A inactivation is associated with significantly worse prognosis (54). For example, overexpression of the PP2A inhibitor CIP2A is correlated with worse prognosis in several solid tumors as well as in myeloma (195–197). Specifically, for gynecologic cancers, several studies reported on the prognostic potential of CIP2A in cervical and ovarian cancer. In serous ovarian cancers, strong CIP2A immunoreactivity correlated with worse prognosis (117), and the same was observed in a retrospective study on 152 ovarian cancer specimens, including serous, endometrioid, mucinous, and clear cell subtypes (118). In cervical cancer, CIP2A was found to associate with H-Ras to promote epithelial-mesenchymal transition, resulting in increased migration and invasion of cervical cancer cells *in vitro* and *in vivo* (120). Furthermore, cervical cancer tissue analysis revealed that CIP2A expression correlated with lymph node metastasis and high-grade and advanced stage cervical cancer (120). However, these results are in contrast with the human protein atlas database (www.proteinatlas.org) which does not put CIP2A forward as an unfavorable prognostic marker in both ovarian and cervical cancers (198).

Data concerning the prognostic potential of CIP2A in EC are scarce. One study of Yu et al. demonstrated that CIP2A expression in type I endometrioid EC correlated with increased FIGO stage and tumor grade (78). Furthermore, according to the human protein atlas, CIP2A expression correlated with worse

prognosis in patients with EC. Nevertheless, more studies are necessary to further demonstrate the prognostic potential of CIP2A in EC.

Recurrent *PPP2R1A* Hotspot Mutations

In the absence of any published work on the prognostic marker potential of *PPP2R1A* in type II EC so far, we used the cBioportal database to analyze the survival data of type II ECs. However, survival data were only available for 44 patients (UCEC-TCGA study) with type II serous EC, of which only 12 presented with mutations in *PPP2R1A* (93, 94). Analysis of this limited data set revealed no significant difference in overall survival between patients with and without *PPP2R1A* mutations ($P = 0.39$) (**Figure 3**). However, more patient data are definitely required in order to obtain more conclusive results. Longer patient follow-up and centralized data collection in multi-institutional centers could boost the data collection for patients with this rare endometrial cancer subtype.

Others

Similarly, PP2A inactivation via *PPP2R4* (PTPA) haploinsufficiency leads to a worse prognosis in many cancer types, including endometrial carcinosarcomas (66). Also, lower PP2A/C expression in cervical cancer was closely associated with the nodal status of cervical cancer patients (129).

Although there is a lack of studies on the role of SET in EC, the protein atlas database (www.proteinatlas.org) indicates that there is no correlation between SET expression and a worse prognosis in gynecologic cancers (198). On the other hand, a strong correlation was seen between SET expression levels and decreased survival of ovarian cancer patients (119).

FUTURE STEPS

In this review, we discussed the therapeutic potential of PP2A targeting as well as the biomarker potential of PP2A dysfunctions in EC, and other gynecologic cancers. Specifically, in type II EC, *PPP2R1A* mutations are remarkably common, while in type I EC, PP2A dysfunction rather occurs through non-genomic mechanisms, involving increased expression of PP2A inhibitors CIP2A and PME-1. However, in order for *PPP2R1A* to become a clinically relevant biomarker for type II ECs, reliable and fast detection of somatic mutations in this gene will be necessary. Therefore, future development of methods able to detect somatic mutations in tumor samples, or preferably in liquid biopsies, will be crucial.

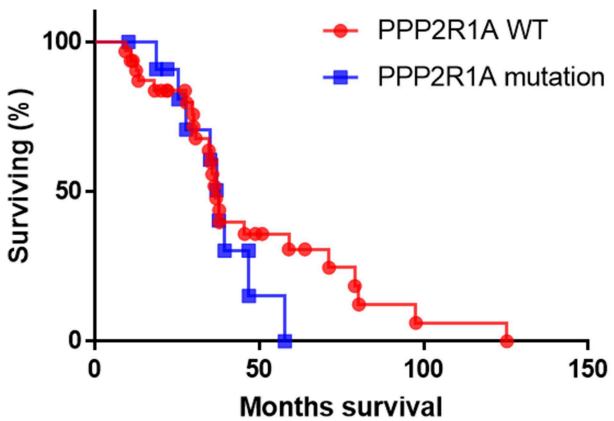


FIGURE 3 | The overall survival of patients with type II serous EC with (blue line) and without *PPP2R1A* mutations (red line). ($n = 44$), data were extracted from the UCEC-TCGA study available in the cBioportal database.

Over the last few years, several promising methods have been developed for the detection of mutations in oncogenes. For example, Spaans et al. designed a Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) based somatic mutation panel able to detect hotspot mutations in 13 genes frequently mutated in gynecologic cancers (199). This method is able to provide reproducible, high-throughput data based on low quality and quantity DNA from formalin-fixed paraffin-embedded (FFPE) samples. Additionally, next-generation sequencing (NGS) has come forward as a potential technique to detect mutations in the clinic. NGS is able to rapidly detect all mutations in the complete gene of interest. However, data analysis is still rather complex and differentiating between somatic passenger and driver mutations can be time consuming. However, optimizations of this technique could lead to a more user-friendly method for the detection of specific mutations. For example, Cottrell et al. validated a NGS assay (WuCaMP) which targets a specific panel of genes with known clinical importance (200). This highly specific and sensitive assay allowed for a fast analysis of the target genes, thereby reducing time and costs of NGS.

More recently, also non-or minimally-invasive liquid biopsies have been investigated as a way to detect somatic mutations in patients with EC (201). For example, circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) extracted from blood samples or uterine aspirates showed potential as a way to screen for somatic mutations. However, whether CTCs can be useful for patients with high-risk EC is still unclear due to its debatable prevalence in blood samples. Bogani et al. reported a low prevalence of CTCs in pre-operative blood samples of patients with high-risk EC and even an absence of CTCs in patients with type II EC (202). In contrast, Alonso-Alconada et al. demonstrated the presence of CTCs in patients with high-risk EC (203). Thus, further research is warranted in order to prove the potential usefulness of CTCs in type II ECs. On the other hand, also cell-free DNA can be used to detect mutations. NGS was able to detect specific endometrioid EC-associated mutations in the cell-free DNA derived from peripheral blood samples of patients with early and late stage endometrioid EC (204). These promising results indicate the potential of this technique for the detection of type II ECs, when implementing *PPP2R1A* in the targeted gene panel.

Furthermore, also DNA obtained during a routine Pap (Papanicolaou) test can be analyzed for the detection of oncogenic mutations. For example, Wang et al. designed a test called PapSEEK, which is able to detect mutations in 18 commonly mutated genes in endometrial cancer (205). This is particularly interesting since with this method, oncogenic

mutations could be detected already during a routine check-up, making early stage detection of (type II) EC possible. This is especially important since patients with type II EC show few symptoms until the disease is already in late stages and therefore less amenable to the current therapies.

In contrast, detection of CIP2A or PME-1 overexpression in (type I) EC cannot rely on genetic methods, but should rather focus on the mRNA or protein level, and is therefore, much more dependent on the availability of tumor biopsies or resected tumor material. In this respect, the use of reliable, validated antibodies, able to specifically detect these oncoproteins via immunohistochemistry techniques is crucial. However, the fact that CIP2A is always found expressed at high levels in the tumor tissue, while being nearly undetectable in the corresponding non-proliferating normal tissue, virtually eliminates the issues associated with the poorer suitability of immunohistochemistry as a technique to reliably quantify protein expression in tissues. In addition, the use of autoantibodies as serum biomarkers for CIP2A showed promising results in breast cancer patients (206). Therefore, it might also be interesting to investigate this in the setting of EC.

Despite the identification of the phosphatase PP2A as a promising molecular marker for ECs, few pre-clinical studies have investigated its potential as a direct therapeutic target, nor as a stratification marker for targeted kinase inhibitor treatments in this cancer type. Nevertheless, a plethora of studies in other solid tumor types suggest PP2A to have potential as a new therapeutic target for both type I and, more importantly, for the more aggressive type II ECs. We put several potential therapeutic compounds forward that could be tested in EC studies, potentially in combination with chemotherapy or targeted therapy.

So far, hormonal intervention and the immunotherapeutic pembrolizumab are the only two FDA-approved targeted therapies for hormone-dependent type I ECs, while there are none for the type II ECs (207). Nevertheless, molecular analyses of ECs have revealed that, in the large majority of ECs, the PI3K pathway is overactivated, which led to a number of (pre-)clinical studies investigating kinase inhibitors targeting this pathway (e.g., several mTOR and PI3K inhibitors) (32). The outcome of these studies was largely disappointing, not only due to the development of inherent resistance mechanisms (e.g., cross-talk to MEK/MAPK pathway), but also in a big part due to the complete lack of patient stratification in clinical studies (208, 209). The importance of the latter was further illustrated by the clear therapeutic benefit of the HER2 inhibitors trastuzumab (anti-HER2 antibody) and lapatinib (tyrosine kinase inhibitor, TKI), in type II serous EC cells stratified based on *HER2* amplification vs. normal *HER2*, or based on *HER2* amplification and functional PI3K vs. those with *HER2* amplification and mutant *PIK3CA* (32, 210, 211). Likewise, we hypothesized here that the PP2A status of the endometrial tumor, should be an important additional stratification marker for testing these targeted kinase inhibitors, given that PP2A mainly acts as a negative regulator of PI3K and HER2 downstream signaling, and hence its functional or dysfunctional state could co-determine kinase inhibitor therapy outcome. In case dysfunctional PP2A would mediate therapy resistance, the use of SMAP combination therapy could be a valuable solution. Also type I ECs could benefit from the combination of PP2A activators with standard therapies, since sensitization of the cells to chemo- or radiation therapy could result in a lower dose and duration of the therapy, required to treat type I ECs. This in turn, would reduce the physical and economic burden associated with chemotherapy.

Furthermore, since the mechanism of PP2A inactivation is different between both subtypes of EC, it will be important to stratify patients in those having type I and those having type II tumors within (pre)-clinical trials. This way, existing, or new therapeutic compounds will be tested on a more rational basis and no bias will occur toward the biggest group of indolent type I ECs. Therefore, the presence of certain PP2A dysfunctions in the EC tumor could indicate whether the patient is eligible for certain (targeted) therapies.

In conclusion, we highlighted the therapeutic potential of PP2A activating as well as inactivating compounds in several gynecologic cancers. However, it has to be noted that more studies should focus on these promising compounds in the specific context of type I and type II ECs. Furthermore, we demonstrated, based on studies in several other cancers, among which ovarian and cervical cancer, that PP2A dysfunction, due to mutations or cellular PP2A inhibitors, could be an indicator for worse prognosis as well as a predictor for therapeutic outcome in EC. Therefore, stratification of patients with type II EC based on their *PPP2R1A* mutational status, or of patients with type I EC based on their CIP2A or PME-1 status could help to establish more reliable testing of current and future targeted therapies in clinical trials. Furthermore, the presence of CIP2A or PME-1 expression could also broaden the therapeutic possibilities for the type I ECs. Dedicated pre-clinical studies in EC cells, with functional vs. dysfunctional PP2A status, should address these issues in the near future. Additionally, the presence of *PPP2R1A* mutations could also help to diagnose patients with type II EC in earlier stages (e.g., via liquid biopsies), thereby also contributing to a better patient outcome. However, further research is warranted in order to confirm this marker potential of *PPP2R1A* in type II ECs. In the end, only such dedicated studies, will help treatments for patients with type I and type II EC to catch-up with the emerging personalized medicine and targeted therapies already established in many other cancers.

AUTHOR CONTRIBUTIONS

MR: writing. VJ: review and Editing.

FUNDING

MR is a recipient of an F.W.O.-SB fellowship of the Research Foundation-Flanders. VJ is supported by grants from the Belgian Foundation against Cancer (FAC-F/2016/822), the Research Foundation-Flanders (F.W.O. G.0B01.16N and F.W.O. G.0B17.19N) and the KU Leuven Research Fund (C24/17/073).

REFERENCES

1. Arruebo M, Vilaboa N, Saez-Gutierrez B, Lambea J, Tres A, Valladares M, et al. Assessment of the evolution of cancer treatment therapies. *Cancers*. (2011) 3:3279–330. doi: 10.3390/cancers3033279
2. Hajdu SI, Vadmal M, Tang P. A note from history: landmarks in history of cancer, part 7. *Cancer*. (2015) 121:2480–513. doi: 10.1002/cncr.29365
3. Holloway RW, Ahmad S. Robotic-assisted surgery in the management of endometrial cancer. *J Obstet Gynaecol Res*. (2012) 38:1–8. doi: 10.1111/j.1447-0756.2011.01744.x
4. Kim M, Suh DH, Lee KH, Eom KY, Toftdahl NG, Mirza MR, et al. Major clinical research advances in gynecologic cancer in 2018. *J Gynecol Oncol*. (2019) 30:e18. doi: 10.3802/jgo.2019.30.e18
5. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, et al. Mutational landscape and significance across 12 major cancer types. *Nature*. (2013) 502:333–9. doi: 10.1038/nature12634
6. An X, Tiwari AK, Sun Y, Ding PR, Ashby CR Jr., Chen ZS. BCR-ABL tyrosine kinase inhibitors in the treatment of Philadelphia chromosome positive chronic myeloid leukemia: a review. *Leuk Res*. (2010) 34:1255–68. doi: 10.1016/j.leukres.2010.04.016
7. Paz-Ares L, Sanchez JM, Garcia-Velasco A, Massuti B, Lopez-Vivanco G, Provencio M, et al. A prospective phase II trial of erlotinib in advanced non-small cell lung cancer (NSCLC) patients (p) with mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR). *J Clin Oncol*. (2006) 24:369s. doi: 10.1200/jco.2006.24.18_suppl.7020
8. Paz-Ares L, Soulieres D, Melezinek I, Moecks J, Keil L, Mok T, et al. Clinical outcomes in non-small-cell lung cancer patients with EGFR mutations: pooled analysis. *J Cell Mol Med*. (2010) 14:51–69. doi: 10.1111/j.1582-4934.2009.00991.x
9. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med*. (2010) 363:1693–703. doi: 10.1056/NEJMoa1006448
10. Shaw AT, Engelman JA. Ceritinib in ALK-rearranged non-small-cell lung cancer. *N Engl J Med*. (2014) 370:2537–9. doi: 10.1056/NEJMoa1311107
11. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. (1987) 235:177–82. doi: 10.1126/science.3798106
12. Arteaga CL, Engelman JA. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. *Cancer Cell*. (2014) 25:282–303. doi: 10.1016/j.ccr.2014.02.025
13. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*. (2011) 364:2507–16. doi: 10.1056/NEJMoa1103782
14. Amant F, Mirza MR, Koskas M, Creutzberg CL. Cancer of the corpus uteri. *Int J Gynaecol Obstet*. (2015) 131(Suppl 2):S96–104. doi: 10.1016/j.ijgo.2015.06.005
15. Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol*. (1983) 15:10–7. doi: 10.1016/0090-8258(83)90111-7
16. Lax SF. Pathology of endometrial carcinoma. *Adv Exp Med Biol*. (2017) 943:75–96. doi: 10.1007/978-3-319-43139-0_3
17. Eritja N, Yeramian A, Chen BJ, Llobet-Navas D, Ortega E, Colas E, et al. Endometrial carcinoma: specific targeted pathways. *Adv Exp Med Biol*. (2017) 943:149–207. doi: 10.1007/978-3-319-43139-0_6
18. Levenback C, Burke TW, Silva E, Morris M, Gershenson DM, Kavanagh JJ, et al. Uterine papillary serous carcinoma (UPSC) treated with cisplatin, doxorubicin, and cyclophosphamide (PAC). *Gynecol Oncol*. (1992) 46:317–21. doi: 10.1016/0090-8258(92)90224-7
19. Smith MR, Peters WA. III, Drescher CW. Cisplatin, doxorubicin hydrochloride, and cyclophosphamide followed by radiotherapy in high-risk endometrial carcinoma. *Am J Obstet Gynecol*. (1994) 170:1677–81. discussion: 1681–2.
20. Buhtoiarova TN, Brenner CA, Singh M. Endometrial carcinoma: role of current and emerging biomarkers in resolving persistent clinical dilemmas. *Am J Clin Pathol*. (2016) 145:8–21. doi: 10.1093/ajcp/aqv014
21. Morgan J, Hoekstra AV, Chapman-Davis E, Hardt JL, Kim JJ, Buttin BM. Synuclein-gamma (SNCG) may be a novel prognostic biomarker in uterine papillary serous carcinoma. *Gynecol Oncol*. (2009) 114:293–8. doi: 10.1016/j.ygyno.2009.04.036
22. El-Sahwi KS, Schwartz PE, Santin AD. Development of targeted therapy in uterine serous carcinoma, a biologically aggressive variant of endometrial cancer. *Exp Rev Anticancer Ther*. (2012) 12:41–9. doi: 10.1586/era.11.192
23. Hogberg T, Signorelli M, de Oliveira CF, Fossati R, Lissoni AA, Sorbe B, et al. Sequential adjuvant chemotherapy and radiotherapy in endometrial cancer–results from two randomised studies. *Eur J Cancer*. (2010) 46:2422–31. doi: 10.1016/j.ejca.2010.06.002
24. Goff BA, Kato D, Schmidt RA, Ek M, Ferry JA, Muntz HG, et al. Uterine papillary serous carcinoma: patterns of metastatic spread. *Gynecol Oncol*. (1994) 54:264–8. doi: 10.1006/gyno.1994.1208
25. Kato DT, Ferry JA, Goodman A, Sullinger J, Scully RE, Goff BA, et al. Uterine papillary serous carcinoma (UPSC): a clinicopathologic study of 30 cases. *Gynecol Oncol*. (1995) 59:384–9. doi: 10.1006/gyno.1995.9957
26. Black JD, English DP, Roque DM, Santin A D. Targeted therapy in uterine serous carcinoma: an aggressive variant of endometrial cancer. *Womens Health*. (2014) 10:45–57. doi: 10.2217/WHE.13.72
27. Hamilton CA, Cheung MK, Osann K, Chen L, Teng NN, Longacre TA, et al. Uterine papillary serous and clear cell carcinomas predict for poorer survival compared to grade 3 endometrioid corpus cancers. *Br J Cancer*. (2006) 94:642–6. doi: 10.1038/sj.bjc.6603012
28. Cirisano FD Jr., Robboy SJ, Dodge RK, Bentley RC, Krigman HR, Synan IS, et al. The outcome of stage I-II clinically and surgically staged papillary serous and clear cell endometrial cancers when compared with endometrioid carcinoma. *Gynecol Oncol*. (2000) 77:55–65. doi: 10.1006/gyno.2000.5737
29. Acharya S, Hensley ML, Montag AC, Fleming GF. Rare uterine cancers. *Lancet Oncol*. (2005) 6:961–71. doi: 10.1016/S1470-2045(05)70463-0
30. Kamijo Y, Ichikawa M. Cost information of chemotherapy for cervical and endometrial cancer in Japan. *Jpn J Nurs Sci*. (2014) 11:190–9. doi: 10.1111/jjns.12020
31. Pennington M, Gentry-Maharaj A, Karpinskyj C, Miners A, Taylor J, Manchanda R, et al. Long-term secondary care costs of endometrial cancer: a prospective cohort study nested within the United Kingdom collaborative trial of ovarian cancer screening (UKCTOCS). *PLoS ONE*. (2016) 11:e0165539. doi: 10.1371/journal.pone.0165539
32. Remmerie M, Janssens V. Targeted therapies in type II endometrial cancers: too little, but not too late. *Int J Mol Sci*. (2018) 19:2380. doi: 10.3390/ijms19082380
33. Gibson WJ, Hoivik EA, Halle MK, Taylor-Weiner A, Cherniack AD, Berg A, et al. The genomic landscape and evolution of endometrial carcinoma progression and abdominopelvic metastasis. *Nat Genet*. (2016) 48:848–55. doi: 10.1038/ng.3602
34. Cherniack AD, Shen H, Walter V, Stewart C, Murray BA, Bowlby R, et al. Integrated molecular characterization of uterine carcinosarcoma. *Cancer Cell*. (2017) 31:411–23. doi: 10.1016/j.ccell.2017.02.010
35. Cancer Genome Atlas Research Network, Kandoth CN, Schultz C, Cherniack N, Akbani AD, Liu R, et al. Integrated genomic characterization of endometrial carcinoma. *Nature*. (2013) 497:67–73. doi: 10.1038/nature12113
36. Graves JD, Edwin Krebs G. Protein phosphorylation and signal transduction. *Pharmacol Therapeut*. (1999) 82:111–21. doi: 10.1016/S0163-7258(98)00056-4
37. Sacco F, Perfetto L, Castagnoli L, Cesareni G. The human phosphatase interactome: an intricate family portrait. *FEBS Lett*. (2012) 586:2732–9. doi: 10.1016/j.febslet.2012.05.008
38. Ardito F, Giuliani M, Perrone D, Troiano G, Lo Muzio L. The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (Review). *Int J Mol Med*. (2017) 40:271–80. doi: 10.3892/ijmm.2017.3036
39. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. (2000) 100:57–70. doi: 10.1016/S0092-8674(00)81683-9
40. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. *Nature*. (2001) 411:355–65. doi: 10.1038/35077225
41. Paul MK, AMukhopadhyay K. Tyrosine kinase – Role and significance in Cancer. *Int J Med Sci*.. (2004) 1:101–15. doi: 10.7150/ijms.1.101
42. Cutillas PR. Role of phosphoproteomics in the development of personalized cancer therapies. *Proteom Clin Appl*. (2015) 9:383–95. doi: 10.1002/prca.201400104

43. Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science*. (2002) 298:1912–34. doi: 10.1126/science.1075762
44. Brautigan DL. Protein Ser/Thr phosphatases–the ugly ducklings of cell signalling. *FEBS J*. (2013) 280:324–45. doi: 10.1111/j.1742-4658.2012.08609.x
45. Chen MJ, Dixon JE, Manning G. Genomics and evolution of protein phosphatases. *Sci Signal*. (2017) 10:eaag1796. doi: 10.1126/scisignal.aag1796
46. Westermarck J. Phosphatases catching up with the level of knowledge: finally druggable? *Int J Biochem Cell Biol*. (2018) 96:96–7. doi: 10.1016/j.biocel.2018.01.007
47. Westermarck J. Targeted therapies don't work for a reason; the neglected tumor suppressor phosphatase PP2A strikes back. *FEBS J*. (2018) 285:4139–45. doi: 10.1111/febs.14617
48. Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, et al. Global, *in vivo*, and site-specific phosphorylation dynamics in signaling networks. *Cell*. (2006) 127:635–48. doi: 10.1016/j.cell.2006.09.026
49. Cho US, Xu W. Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. *Nature*. (2007) 445:53–7. doi: 10.1038/nature05351
50. Shi Y. Serine/threonine phosphatases: mechanism through structure. *Cell*. (2009) 139:468–84. doi: 10.1016/j.cell.2009.10.006
51. Janssens V, Goris J. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J*. (2001) 353(Pt 3):417–39. doi: 10.1042/bj3530417
52. Khew-Goodall Y, Hemmings BA. Tissue-specific expression of mRNAs encoding alpha- and beta-catalytic subunits of protein phosphatase 2A. *FEBS Lett*. (1988) 238:265–8. doi: 10.1016/0014-5793(88)80493-9
53. Lambrecht C, Haesen D, Sents W, Ivanova E, Janssens V. Structure, regulation, and pharmacological modulation of PP2A phosphatases. *Methods Mol Biol*. (2013) 1053:283–305. doi: 10.1007/978-1-62703-562-0_17
54. Meeusen B, Janssens V. Tumor suppressive protein phosphatases in human cancer: emerging targets for therapeutic intervention and tumor stratification. *Int J Biochem Cell Biol*. (2018) 96:98–134. doi: 10.1016/j.biocel.2017.10.002
55. Wlodarchak N, Xing Y. PP2A as a master regulator of the cell cycle. *Crit Rev Biochem Mol Biol*. (2016) 51:162–84. doi: 10.3109/10409238.2016.1143913
56. Janssens V, Rebollo A. The role and therapeutic potential of Ser/Thr phosphatase PP2A in apoptotic signalling networks in human cancer cells. *Curr Mol Med*. (2012) 12:268–87. doi: 10.2174/156652412799218930
57. Thompson JJ, Williams CS. Protein phosphatase 2A in the regulation of Wnt signaling, stem cells, and cancer. *Genes*. (2018) 9:121. doi: 10.3390/genes9030121
58. Grech G, Baldacchino S, Saliba C, Grixti MP, Gauci R, Petroni V, et al. Deregulation of the protein phosphatase 2A, PP2A in cancer: complexity and therapeutic options. *Tumour Biol*. (2016) 37:11691–700. doi: 10.1007/s13277-016-5145-4
59. Perrotti D, Neviani P. Protein phosphatase 2A: a target for anticancer therapy. *Lancet Oncol*. (2013) 14:e229–38. doi: 10.1016/S1470-2045(12)70558-2
60. Kolupaeva V, Janssens V. PP1 and PP2A phosphatases–cooperating partners in modulating retinoblastoma protein activation. *FEBS J*. (2013) 280:627–43. doi: 10.1111/j.1742-4658.2012.08511.x
61. Janssens V, Goris J, Van Hoof C. PP2A: the expected tumor suppressor. *Curr Opin Genet Dev*. (2005) 15:34–41. doi: 10.1016/j.gde.2004.12.004
62. Hahn WC, Dessain SK, Brooks MW, King JE, Elenbaas B, Sabatini DM, et al. Enumeration of the simian virus 40 early region elements necessary for human cell transformation. *Mol Cell Biol*. (2002) 22:2111–23. doi: 10.1128/MCB.22.7.2111-2123.2002
63. Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC. Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell*. (2004) 5:127–36. doi: 10.1016/S1535-6108(04)00026-1
64. Sablina AA, Hector M, Colpaert N, Hahn WC. Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Res*. (2010) 70:10474–84. doi: 10.1158/0008-5472.CAN-10-2855
65. Lambrecht C, Libbrecht L, Sagaert X, Pauwels P, Hoorne Y, Crowther J, et al. Loss of protein phosphatase 2A regulatory subunit B56delta promotes spontaneous tumorigenesis in vivo. *Oncogene*. (2018) 37:544–52. doi: 10.1038/onc.2017.350
66. Sents W, Meeusen B, Kalev P, Radaelli E, Sagaert X, Miermans E, et al. PP2A Inactivation Mediated by PPP2R4 Haploinsufficiency promotes cancer development. *Cancer Res*. (2017) 77:6825–37. doi: 10.1158/0008-5472.CAN-16-2911
67. Meeusen B, Janssens V. *In vivo* pieces of the PP2A onco-puzzle fallen into place. *Oncoscience*. (2017) 4:154–5. doi: 10.18632/oncoscience.384
68. Reynhout S, Janssens V. Physiologic functions of PP2A: Lessons from genetically modified mice. *Biochim Biophys Acta Mol Cell Res*. (2019) 1866:31–50. doi: 10.1016/j.bbamcr.2018.07.010
69. Haesen D, Sents W, Ivanova E, Lambrecht C, Janssens V. Cellular inhibitors of protein phosphatase PP2A in cancer. *Biomed Res*. (2012) 23:197–211.
70. Haesen D, Sents W, Lemaire K, Hoorne Y, Janssens V. The basic biology of PP2A in hematologic cells and malignancies. *Front Oncol*. (2014) 4:347. doi: 10.3389/fonc.2014.00347
71. Seshacharyulu P, Pandey P, Datta K, Batra SK. Phosphatase: PP2A structural importance, regulation and its aberrant expression in cancer. *Cancer Lett*. (2013) 335:9–18. doi: 10.1016/j.canlet.2013.02.036
72. Kauko O, Westermarck J. Non-genomic mechanisms of protein phosphatase 2A (PP2A) regulation in cancer. *Int J Biochem Cell Biol*. (2018) 96:157–64. doi: 10.1016/j.biocel.2018.01.005
73. Khanna A, Pimanda JE. Clinical significance of cancerous inhibitor of protein phosphatase 2A in human cancers. *Int J Cancer*. (2016) 138:525–32. doi: 10.1002/ijc.29431
74. Longin S, Zwaenepoel K, Martens E, Louis JV, Rondelez E, Goris J, et al. Spatial control of protein phosphatase 2A (de)methylation. *Exp Cell Res*. (2008) 314:68–81. doi: 10.1016/j.yexcr.2007.07.030
75. Kaur A, Westermarck J. Regulation of protein phosphatase 2A (PP2A) tumor suppressor function by PME-1. *Biochem Soc Trans*. (2016) 44:1683–93. doi: 10.1042/BST20160161
76. Yabe R, Tsuji S, Mochida S, Ikehara T, Usui T, Ohama T, et al. A stable association with PME-1 may be dispensable for PP2A demethylation - implications for the detection of PP2A methylation and immunoprecipitation. *FEBS Open Bio*. (2018) 8:1486–96. doi: 10.1002/2211-5463.12485
77. Sangodkar J, Caroline Farrington C, Kimberly M., Matthew Galsky D, David Kastrinsky B, and Narla G. All roads lead to PP2A: exploiting the therapeutic potential of this phosphatase. *FEBS J*. (2016) 283:1004–24. doi: 10.1111/febs.13573
78. Yu N, Zhang T, Zhao D, Cao Z, Du J, Zhang Q. CIP2A is overexpressed in human endometrioid adenocarcinoma and regulates cell proliferation, invasion and apoptosis. *Pathol Res Pract*. (2018) 214:233–9. doi: 10.1016/j.prp.2017.11.011
79. Choi YA, Koo JS, Park JS, Park MY, Jeong AL, Oh KS, et al. Estradiol enhances CIP2A expression by the activation of p70 S6 kinase. *Endocr Relat Cancer*. (2014) 21:189–202. doi: 10.1530/ERC-13-0453
80. Wandzioch E, Pusey M, Werda A, Bail S, Bhaskar A, Nestor M, et al. PME-1 modulates protein phosphatase 2A activity to promote the malignant phenotype of endometrial cancer cells. *Cancer Res*. (2014) 74:4295–305. doi: 10.1158/0008-5472.CAN-13-3130
81. McConechy MK, Anglesio MS, Kalloger SE, Yang W, Senz J, Chow C, et al. Subtype-specific mutation of PPP2R1A in endometrial and ovarian carcinomas. *J Pathol*. (2011) 223:567–73. doi: 10.1002/path.2848
82. McConechy MK, Ding J, Cheang MC, Wiegand K, Senz J, Tone A, et al. Use of mutation profiles to refine the classification of endometrial carcinomas. *J Pathol*. (2012) 228:20–30. doi: 10.1002/path.4056
83. Le Gallo M, O'Hara AJ, Rudd ML, Urick ME, Hansen NF, O'Neil NJ, et al. Exome sequencing of serous endometrial tumors identifies recurrent somatic mutations in chromatin-remodeling and ubiquitin ligase complex genes. *Nat Genet*. (2012) 44:1310–5. doi: 10.1038/ng.2455
84. Kuhn E, Wu RC, Guan B, Wu G, Zhang J, Wang Y, et al. Identification of molecular pathway aberrations in uterine serous carcinoma by genome-wide analyses. *J Natl Cancer Inst*. (2012) 104:1503–13. doi: 10.1093/jnci/djs345
85. Zhao S, Choi M, Overton JD, Bellone S, Roque DM, Cocco E, et al. Landscape of somatic single-nucleotide and copy-number mutations in uterine serous carcinoma. *Proc Natl Acad Sci USA*. (2013) 110:2916–21. doi: 10.1073/pnas.1222577110

86. Kogan L, Octeau D, Amajoud Z, Abitbol J, Laskov I, Ferenczy A, et al. Impact of lower uterine segment involvement in type II endometrial cancer and the unique mutational profile of serous tumors. *Gynecol Oncol Rep*. (2018) 24:43–7. doi: 10.1016/j.gore.2018.03.004
87. Hoang LN, McConechy MK, Meng B, McIntyre JB, Ewanowich C, Gilks CB, et al. Targeted mutation analysis of endometrial clear cell carcinoma. *Histopathology*. (2015) 66:664–74. doi: 10.1111/his.12581
88. DeLair DF, Burke KA, Selenica P, Lim RS, Scott SN, Middha S, et al. The genetic landscape of endometrial clear cell carcinomas. *J Pathol*. (2017) 243:230–41. doi: 10.1002/path.4947
89. Le Gallo M, Rudd ML, Urick ME, Hansen NF, Zhang S, Nisc Comparative Sequencing Program, et al. Somatic mutation profiles of clear cell endometrial tumors revealed by whole exome and targeted gene sequencing. *Cancer*. (2017) 123:3261–8. doi: 10.1002/cncr.30745
90. Forbes SA, Beare D, Boutselakis H, Bamford S, Bindal N, Tate J, et al. COSMIC: somatic cancer genetics at high-resolution. *Nucleic Acids Res*. (2017) 45:D777–83. doi: 10.1093/nar/gkw1121
91. Shih Ie M, Panuganti PK, Kuo KT, Mao TL, Kuhn E, Jones S, et al. Somatic mutations of PPP2R1A in ovarian and uterine carcinomas. *Am J Pathol*. (2011) 178:1442–7. doi: 10.1016/j.ajpath.2011.01.009
92. Nagendra DC, Burke J III, Maxwell GL, Risinger JI. PPP2R1A mutations are common in the serous type of endometrial cancer. *Mol Carcinog*. (2012) 51:826–31. doi: 10.1002/mc.20850
93. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal*. (2013) 6:pl1. doi: 10.1126/scisignal.2004088
94. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov*. (2012) 2:401–4. doi: 10.1158/2159-8290.CD-12-0095
95. *COSMIC: Catalogue of Somatic Mutations in Cancer*. Available online at: http://cancer.sanger.ac.uk
96. Ruediger R, Ruiz J, Walter G. Human cancer-associated mutations in the Aalpha subunit of protein phosphatase 2A increase lung cancer incidence in Aalpha knock-in and knockout mice. *Mol Cell Biol*. (2011) 31:3832–44. doi: 10.1128/MCB.05744-11
97. Chen W, Arroyo JD, Timmons JC, Possemato R, Hahn WC. Cancer-associated PP2A Aalpha subunits induce functional haploinsufficiency and tumorigenicity. *Cancer Res*. (2005) 65:8183–92. doi: 10.1158/0008-5472.CAN-05-1103
98. Groves MR, Hanlon N, Turowski P, Hemmings BA, Barford D. The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. *Cell*. (1999) 96:99–110. doi: 10.1016/S0092-8674(00)80963-0
99. Gao J, Chang MT, Johnsen HC, Gao SP, Sylvester BE, Sumer SO, et al. 3D clusters of somatic mutations in cancer reveal numerous rare mutations as functional targets. *Genome Med*. (2017) 9:4. doi: 10.1186/s13073-016-0393-x
100. Kamburov A, Lawrence MS, Polak P, Leshchiner I, Lage K, Golub TR, et al. Comprehensive assessment of cancer missense mutation clustering in protein structures. *Proc Natl Acad Sci USA*. (2015) 112:E5486–95. doi: 10.1073/pnas.1516373112
101. Bailey MH, Tokheim C, Porta-Pardo E, Sengupta S, Bertrand D, Weerasinghe A, et al. Comprehensive characterization of cancer driver genes and mutations. *Cell*. (2018) 173:371–85. e18. doi: 10.1016/j.cell.2018.02.060
102. Haesen D, Abbasi Asbagh L, Derua R, Hubert A, Schrauwen S, Hoorne Y, et al. Recurrent PPP2R1A mutations in uterine cancer act through a dominant-negative mechanism to promote malignant cell growth. *Cancer Res*. (2016) 76:5719–31. doi: 10.1158/0008-5472.CAN-15-3342
103. Jeong AL, Han S, Lee S, Su Park J, Lu Y, Yu S, et al. Patient derived mutation W257G of PPP2R1A enhances cancer cell migration through SRC-JNK-c-Jun pathway. *Sci Rep*. (2016) 6:27391. doi: 10.1038/srep27391
104. The Cancer Genome Atlas Research Network, Bell D, Berchuck A, Birrer M, Chien J, Cramer DW, et al. Integrated genomic analyses of ovarian carcinoma. *Nature*. (2011) 474:609. doi: 10.1038/nature10166
105. The Cancer Genome Atlas Research Network, Robert Burk D, Zigui C, Charles S, Katherine T, Andre Carvalho L, et al. Integrated genomic and molecular characterization of cervical cancer. *Nature*. (2017) 543:378–84. doi: 10.1038/nature21386
106. Berger AC, Korkut A, Kanchi RS, Hegde AM, Lenoir W, Liu W, et al. A comprehensive pan-cancer molecular study of gynecologic and breast cancers. *Cancer Cell*. (2018) 33:690–705.e9. doi: 10.1016/j.ccell.2018.03.014
107. Jones S, Wang TL, Shih Ie M, Mao TL, Nakayama K, Roden R, et al. Frequent mutations of chromatin remodeling gene ARID1A in ovarian clear cell carcinoma. *Science*. (2010) 330:228–31. doi: 10.1126/science.1196333
108. Papp E, Hallberg D, Konecny GE, Bruhm DC, Adleff V, Noe M, et al. Integrated genomic, epigenomic, and expression analyses of ovarian cancer cell lines. *Cell Rep*. (2018) 25:2617–33. doi: 10.1016/j.celrep.2018.10.096
109. Spaans VM, Trietsch MD, Peters AA, Osse M, Ter Haar N, Fleuren GJ, et al. Precise classification of cervical carcinomas combined with somatic mutation profiling contributes to predicting disease outcome. *PLoS ONE*. (2015) 10:e0133670. doi: 10.1371/journal.pone.0133670
110. Ojesina AI, Lichtenstein L, Freeman SS, Pedamallu CS, Imaz-Rosshandler I, Pugh TJ, et al. Landscape of genomic alterations in cervical carcinomas. *Nature*. (2014) 506:371–5. doi: 10.1038/nature12881
111. Rahman M, Nakayama K, Rahman MT, Nakayama N, Katagiri H, Katagiri A, et al. PPP2R1A mutation is a rare event in ovarian carcinoma across histological subtypes. *Anticancer Res*. (2013) 33:113–8.
112. Jones S, Wang TL, Kurman RJ, Nakayama K, Velculescu VE, Vogelstein B, et al. Low-grade serous carcinomas of the ovary contain very few point mutations. *J Pathol*. (2012) 226:413–20. doi: 10.1002/path.3967
113. Campbell IG, Manolitsas T. Absence of PPP2R1B gene alterations in primary ovarian cancers. *Oncogene*. (1999) 18:6367–9. doi: 10.1038/sj.onc.1203070
114. Wang F, Zou Y, Liu FY, Yu XH, Huang H, Zhang N, et al. Infrequent mutations of the PPP2R1A and PPP2R1B genes in patients with ovarian cancer. *Mol Med Rep*. (2013) 7:1826–30. doi: 10.3892/mmr.2013.1416
115. Yeh LS, Hsieh YY, Chang JG, Chang WW, Chang CC, Tsai FJ. Mutation analysis of the tumor suppressor gene PPP2R1B in human cervical cancer. *Int J Gynecol Cancer*. (2007) 17:868–71. doi: 10.1111/j.1525-1438.2007.00880.x
116. Wu R, Connolly DC, Ren X, Fearon ER, Cho KR. Somatic mutations of the PPP2R1B candidate tumor suppressor gene at chromosome 11q23 are infrequent in ovarian carcinomas. *Neoplasia*. (1999) 1:311–4. doi: 10.1038/sj.neo.7900042
117. Bockelman C, Lassus H, Hemmes A, Leminen A, Westermarck J, Haglund C, et al. Prognostic role of CIP2A expression in serous ovarian cancer. *Br J Cancer*. (2011) 105:989–95. doi: 10.1038/bjc.2011.346
118. Fang Y, Li Z, Wang X, Zhang S. CIP2A is overexpressed in human ovarian cancer and regulates cell proliferation and apoptosis. *Tumour Biol*. (2012) 33:2299–306. doi: 10.1007/s13277-012-0492-2
119. Jiang SW, Xu S, Chen H, Liu J, Duan P. Oncogenic Role of SET/I2PP2A for gynecologic cancers. *Curr Drug Targets*. (2017) 18:1152–7. doi: 10.2174/1389450118666170328114506
120. Wu Y, Gu TT, Zheng PS. CIP2A cooperates with H-Ras to promote epithelial-mesenchymal transition in cervical-cancer progression. *Cancer Lett*. (2015) 356 (2 Pt B):646–55. doi: 10.1016/j.canlet.2014.10.013
121. Huang LP, Adelson ME, Mordechai E, Trama JP. CIP2A expression is elevated in cervical cancer. *Cancer Biomark*. (2010) 8:309–17. doi: 10.3233/CBM-2011-0220
122. Liu J, Xiao W, Gengyin Z, Hong W, Lei X, Yizhe C, et al. Cancerous inhibitor of protein phosphatase 2A is overexpressed in cervical cancer and upregulated by human papillomavirus 16 E7 oncoprotein. *Gynecol Oncol*. (2011) 122:430–6. doi: 10.1016/j.ygyno.2011.04.031
123. Tian Y, Chen H, Qiao L, Zhang W, Zheng J, Zhao W, et al. CIP2A facilitates the G1/S cell cycle transition via B-Myb in human papillomavirus 16 oncoprotein E6-expressing cells. *J Cell Mol Med*. (2018) 22:4150–60. doi: 10.1111/jcmm.13693
124. Wang X, Peng G, Meng W, Jing L, Jiaxiang L, Shule Z, et al. Feedback between E2F1 and CIP2A regulated by human papillomavirus E7 in cervical cancer: implications for prognosis. *Am J Transl Res*. (2017) 9:2327–39.
125. Bosch FX, Manos MM, Munoz N, Sherman M, Jansen AM, Peto J, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International biological study on cervical cancer (IBSCC) study group. *J Natl Cancer Inst*. (1995) 87:796–802. doi: 10.1093/jnci/87.11.796

126. Pim D, Massimi P, Dilworth SM, Banks L. Activation of the protein kinase B pathway by the HPV-16 E7 oncoprotein occurs through a mechanism involving interaction with PP2A. *Oncogene*. (2005) 24:7830–8. doi: 10.1038/sj.onc.1208935
127. Bello JO, Nieva LO, Paredes AC, Gonzalez AM, Zavaleta LR, Lizano M. Regulation of the Wnt/beta-catenin signaling pathway by human papillomavirus E6 and E7 oncoproteins. *Viruses*. (2015) 7:4734–55. doi: 10.3390/v7082842
128. White EA, Rebecca Kramer E, Justin Hwang H, Arun Pores Fernando T, Nana N, William Hahn C, et al. Papillomavirus E7 oncoproteins share functions with polyomavirus small T antigens. *J Virol*. (2015) 89:2857–65. doi: 10.1128/JVI.03282-14
129. Chang YW, Chen MW, Chiu CF, Hong CC, Cheng CC, Hsiao M, et al. Arsenic trioxide inhibits CXCR4-mediated metastasis by interfering miR-520h/PP2A/NF-kappaB signaling in cervical cancer. *Ann Surg Oncol.* (2014) 21 (Suppl 4):S687–95. doi: 10.1245/s10434-014-3812-5
130. Chang MT, Asthana S, Gao SP, Lee BH, Chapman JS, Kandoth C, et al. Identifying recurrent mutations in cancer reveals widespread lineage diversity and mutational specificity. *Nat Biotechnol*. (2016) 34:155–63. doi: 10.1038/nbt.3391
131. Lazo JS, McQueeney KE, Sharlow ER. New approaches to difficult drug targets: the phosphatase story. *SLAS Discov*. (2017) 22:2472555217721142. doi: 10.1177/2472555217721142
132. Frankson R, Yu ZH, Bai Y, Li Q, Zhang RY, Zhang ZY. Therapeutic targeting of oncogenic tyrosine phosphatases. *Cancer Res*. (2017) 77:5701–5. doi: 10.1158/0008-5472.CAN-17-1510
133. O'Connor CM, Perl A, Leonard D, Sangodkar J, Narla G. Therapeutic targeting of PP2A. *Int J Biochem Cell Biol*. (2018) 96:182–93. doi: 10.1016/j.biocel.2017.10.008
134. Nunes-Xavier CE, Martin-Perez J, Elson A, Pulido R. Protein tyrosine phosphatases as novel targets in breast cancer therapy. *Biochim Biophys Acta*. (2013) 1836:211–26. doi: 10.1016/j.bbcan.2013.06.001
135. De P, Carlson JH, Leyland-Jones B, Dey N. Role of oncogenic nexus of CIP2A in breast oncogenesis: how does it work? *Am J Cancer Res.* (2015) 5:2872–91. doi: 10.18632/oncotarget.2127
136. Pallai R, Bhaskar A, Sodi V, Rice L M. Ets1 and Elk1 transcription factors regulate cancerous inhibitor of protein phosphatase 2A expression in cervical and endometrial carcinoma cells. *Transcription*. (2012) 3:323–35. doi: 10.4161/trns.22518
137. Zhang W, Chen H, Chen Y, Liu J, Wang X, Yu X, et al. Cancerous inhibitor of protein phosphatase 2A contributes to human papillomavirus oncoprotein E7-induced cell proliferation via E2F1. *Oncotarget.* (2015) 6:5253–62. doi: 10.18632/oncotarget.2867
138. Liu CY, Huang TT, Huang CT, Hu MH, Wang DS, Wang WL, et al. EGFR-independent Elk1/CIP2A signalling mediates apoptotic effect of an erlotinib derivative TD52 in triple-negative breast cancer cells. *Eur J Cancer*. (2017) 72:112–23. doi: 10.1016/j.ejca.2016.11.012
139. Yu HC, Hung MH, Chen YL, Chu PY, Wang CY, Chao TT, et al. Erlotinib derivative inhibits hepatocellular carcinoma by targeting CIP2A to reactivate protein phosphatase 2A. *Cell Death Dis.* (2014) 5:e1359. doi: 10.1038/cddis.2014.325
140. Zhao M, Howard EW, Parris AB, Guo Z, Zhao Q, Ma Z, et al. Activation of cancerous inhibitor of PP2A (CIP2A) contributes to lapatinib resistance through induction of CIP2A-Akt feedback loop in ErbB2-positive breast cancer cells. *Oncotarget*. (2017) 8:58847–64. doi: 10.18632/oncotarget.19375
141. Chen KF, Liu CY, Lin YC, Yu HC, Liu TH, Hou DR, et al. CIP2A mediates effects of bortezomib on phospho-Akt and apoptosis in hepatocellular carcinoma cells. *Oncogene*. (2010) 29:6257–66. doi: 10.1038/onc.2010.357
142. Lin YC, Chen KC, Chen CC, Cheng AL, Chen KF. CIP2A-mediated Akt activation plays a role in bortezomib-induced apoptosis in head and neck squamous cell carcinoma cells. *Oral Oncol*. (2012) 48:585–93. doi: 10.1016/j.oraloncology.2012.01.012
143. Tseng LM, Liu CY, Chang KC, Chu PY, Shiau CW, Chen KF. CIP2A is a target of bortezomib in human triple negative breast cancer cells. *Breast Cancer Res.* (2012) 14:R68. doi: 10.1186/bcr3175
144. Puustinen P, Rytter A, Mortensen M, Kohonen P, Moreira JM, Jaattela M. CIP2A oncoprotein controls cell growth and autophagy through mTORC1 activation. *J Cell Biol.* (2014) 204:713–27. doi: 10.1083/jcb.201304012
145. Ma L, Wen ZS, Liu Z, Hu Z, Ma J, Chen XQ, et al. Overexpression and small molecule-triggered downregulation of CIP2A in lung cancer. *PLoS ONE*. (2011) 6:e20159. doi: 10.1371/journal.pone.0020159
146. Huang Q, Qin S, Yuan X, Zhang L, Ji J, Liu X, et al. Arctigenin inhibits triple-negative breast cancers by targeting CIP2A to reactivate protein phosphatase 2A. *Oncol Rep*. (2017) 38:598–606. doi: 10.3892/or.2017.5667
147. Duan J, Ji-Cheng Z, Gui-Zhen W, Xin-Chun Z, Wei-Dong H, Guang-Biao Z. The red wine component ellagic acid induces autophagy and exhibits anti-lung cancer activity *in vitro* and *in vivo*. *J Cell Molecul Med*. (2019) 23:143–54. doi: 10.1111/jcmm.13899
148. Liu Z, Ma L, Wen ZS, Hu Z, Wu FQ, Li W, et al. Cancerous inhibitor of PP2A is targeted by natural compound celastrol for degradation in non-small-cell lung cancer. *Carcinogenesis.* (2014) 35:905–14. doi: 10.1093/carcin/bgt395
149. Yu XJ, Zhao Q, Wang XB, Zhang JX, Wang XB. Gambogenic acid induces proteasomal degradation of CIP2A and sensitizes hepatocellular carcinoma to anticancer agents. *Oncol Rep*. (2016) 36:3611–8. doi: 10.3892/or.2016.5188
150. Zhao Q, Zhao M, Parris AB, Xing Y, Yang X. Genistein targets the cancerous inhibitor of PP2A to induce growth inhibition and apoptosis in breast cancer cells. *Int J Oncol*. (2016) 49:1203–10. doi: 10.3892/ijo.2016.3588
151. Liu Z, Ma L, Wen ZS, Cheng YX, Zhou GB. Ethoxysanguinarine induces inhibitory effects and downregulates CIP2A in lung cancer cells. *ACS Med Chem Lett.* (2014) 5:113–8. doi: 10.1021/ml400341k
152. Alexander-Bryant AA, Dumitriu A, Attaway CC, Yu H, Jakymiw A. Fusogenic-oligoarginine peptide-mediated silencing of the CIP2A oncogene suppresses oral cancer tumor growth in vivo. *J Control Release*. (2015) 218:72–81. doi: 10.1016/j.jconrel.2015.09.026
153. Cantini L, Attaway CC, Butler B, Andino LM, Sokolosky ML, Jakymiw A. Fusogenic-oligoarginine peptide-mediated delivery of siRNAs targeting the CIP2A oncogene into oral cancer cells. *PLoS ONE*. (2013) 8:e73348. doi: 10.1371/journal.pone.0073348
154. Puustinen P, Junttila MR, Vanhatupa S, Sablina AA, Hector ME, Teittinen K, et al. PME-1 protects extracellular signal-regulated kinase pathway activity from protein phosphatase 2A-mediated inactivation in human malignant glioma. *Cancer Res*. (2009) 69:2870–7. doi: 10.1158/0008-5472.CAN-08-2760
155. Bachovchin DA, Mohr JT, Speers AE, Wang C, Berlin JM, Spicer TP, et al. Academic cross-fertilization by public screening yields a remarkable class of protein phosphatase methylesterase-1 inhibitors. *Proc Natl Acad Sci USA*. (2011) 108:6811–6. doi: 10.1073/pnas.1015248108
156. Bachovchin DA, Zuhl AM, Speers AE, Wolfe MR, Weerapana E, Brown SJ, et al. Discovery and optimization of sulfonyl acrylonitriles as selective, covalent inhibitors of protein phosphatase methylesterase-1. *J Med Chem*. (2011) 54:5229–36. doi: 10.1021/jm200502u
157. Pusey M, Bail S, Xu Y, Buiakova O, Nestor M, Yang JJ, et al. Inhibition of protein methylesterase 1 decreased cancerous phenotypes in endometrial adenocarcinoma cell lines and xenograft tumor models. *Tumour Biol*. (2016) 37:11835–42. doi: 10.1007/s13277-016-5036-8
158. Tian L, Zhang XG, Haesen D, Bravo J, Fominaya J, Choquet S, et al. Identification of PP2A/set binding sites and design of interacting peptides with potential clinical applications. *Int J Peptide Res Therap*. (2018) 24:479–88. doi: 10.1007/s10989-017-9633-1
159. De Palma RM, Parnham SR, Li Y, Oaks JJ, Peterson YK, Szulc ZM, et al. The NMR-based characterization of the FTY720-SET complex reveals an alternative mechanism for the attenuation of the inhibitory SET-PP2A interaction. *FASEB J*. (2019) 33:7647–66. doi: 10.1096/fj.201802264R
160. Kastrinsky DB, Sangodkar J, Zaware N, Izadmehr S, Dhawan NS, Narla G, et al. Reengineered tricyclic anti-cancer agents. *Bioorg Med Chem*. (2015) 23:6528–34. doi: 10.1016/j.bmc.2015.07.007
161. Sangodkar J, Perl A, Tohme R, Kiselar J, Kastrinsky DB, Zaware N, et al. Activation of tumor suppressor protein PP2A inhibits KRAS-driven tumor growth. *J Clin Invest*. (2017) 127:2081–90. doi: 10.1172/JCI89548
162. Gutierrez A, Pan L, Groen RW, Baleydier F, Kentsis A, Marineau J, et al. Phenothiazines induce PP2A-mediated apoptosis in T cell acute lymphoblastic leukemia. *J Clin Invest*. (2014) 124:644–55. doi: 10.1172/JCI65093
163. McClinch K, Avelar RA, Callejas D, Izadmehr S, Wiredja D, Perl A, et al. Small-molecule activators of protein phosphatase 2A for the treatment

of castration-resistant prostate cancer. *Cancer Res*. (2018) 78:2065–80. doi: 10.1158/0008-5472.CAN-17-0123
164. Tohme R, Izadmehr S, Gandhe S, Tabaro G, Vallabhaneni S, Thomas A, et al. Direct activation of PP2A for the treatment of tyrosine kinase inhibitor-resistant lung adenocarcinoma. *JCI Insight*. (2019) 4:e125693. doi: 10.1172/jci.insight.125693
165. Mazhar S, Taylor SE, Sangodkar J, Narla G. Targeting PP2A in cancer: combination therapies. *Biochim Biophys Acta Mol Cell Res*. (2018) 1866:51–63. doi: 10.1016/j.bbamcr.2018.08.020
166. Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci*. (2011) 36:320–8. doi: 10.1016/j.tibs.2011.03.006
167. Conciatori F, Ciuffreda L, Bazzichetto C, Falcone I, Pilotto S, Bria E, et al. mTOR cross-talk in cancer and potential for combination therapy. *Cancers*. (2018) 10:23. doi: 10.3390/cancers10010023
168. Murray BW, Miller N. Durability of kinase-directed therapies–a network perspective on response and resistance. *Mol Cancer Ther*. (2015) 14:1975–84. doi: 10.1158/1535-7163.MCT-15-0088
169. Kauko O, O'Connor CM, Kulesskiy E, Sangodkar J, Aakula A, Izadmehr S, et al. PP2A inhibition is a druggable MEK inhibitor resistance mechanism in KRAS-mutant lung cancer cells. *Sci Transl Med*. (2018) 10:eaaq1093. doi: 10.1126/scitranslmed.aaq1093
170. Allen-Petersen BL, Risom T, Feng Z, Wang Z, Jenny ZP, Thoma MC, et al. Activation of PP2A and inhibition of mTOR synergistically reduce MYC signaling and decrease tumor growth in pancreatic ductal adenocarcinoma. *Cancer Res*. (2019) 79:209–19. doi: 10.1158/0008-5472.CAN-18-0717
171. Agarwal A, MacKenzie RJ, Pippa R, Eide CA, Oddo J, Tyner JW, et al. Antagonism of SET using OP449 enhances the efficacy of tyrosine kinase inhibitors and overcomes drug resistance in myeloid leukemia. *Clin Cancer Res*. (2014) 20:2092–103. doi: 10.1158/1078-0432.CCR-13-2575
172. Smith AM, Dun MD, Lee EM, Harrison C, Kahl R, Flanagan H, et al. Activation of protein phosphatase 2A in FLT3+ acute myeloid leukemia cells enhances the cytotoxicity of FLT3 tyrosine kinase inhibitors. *Oncotarget*. (2016) 7:47465–78. doi: 10.18632/oncotarget.10167
173. Cristobal I, Rincon R, Manso R, Carames C, Zazo S, Madoz-Gurpide J, et al. Deregulation of the PP2A inhibitor SET shows promising therapeutic implications and determines poor clinical outcome in patients with metastatic colorectal cancer. *Clin Cancer Res*. (2015) 21:347–56. doi: 10.1158/1078-0432.CCR-14-0724
174. Rincon R, Cristobal I, Zazo S, Arpi O, Menendez S, Manso R, et al. PP2A inhibition determines poor outcome and doxorubicin resistance in early breast cancer and its activation shows promising therapeutic effects. *Oncotarget*. (2015) 6:4299–314. doi: 10.18632/oncotarget.3012
175. Ishitsuka A, Fujine E, Mizutani Y, Tawada C, Kanoh H, Banno Y, et al. FTY720 and cisplatin synergistically induce the death of cisplatin-resistant melanoma cells through the downregulation of the PI3K pathway and the decrease in epidermal growth factor receptor expression. *Int J Mol Med*. (2014) 34:1169–74. doi: 10.3892/ijmm.2014.1882
176. Ho WS, Wang H, Maggio D, Kovach JS, Zhang Q, Song Q, et al. Pharmacologic inhibition of protein phosphatase-2A achieves durable immune-mediated antitumor activity when combined with PD-1 blockade. *Nat Commun*. (2018) 9:2126. doi: 10.1038/s41467-018-04425-z
177. Lu J, Kovach JS, Johnson F, Chiang J, Hodes R, Lonser R, et al. Inhibition of serine/threonine phosphatase PP2A enhances cancer chemotherapy by blocking DNA damage induced defense mechanisms. *Proc Natl Acad Sci USA*. (2009) 106:11697–702. doi: 10.1073/pnas.0905930106
178. Hong CS, Ho W, Zhang C, Yang C, Elder JB, Zhuang Z. LB100, a small molecule inhibitor of PP2A with potent chemo- and radio-sensitizing potential. *Cancer Biol Ther*. (2015) 16:821–33. doi: 10.1080/15384047.2015.1040961
179. Gong FR, Wu MY, Shen M, Zhi Q, Xu ZK, Wang R, et al. PP2A inhibitors arrest G2/M transition through JNK/Sp1- dependent down-regulation of CDK1 and autophagy-dependent up-regulation of p21. *Oncotarget*. (2015) 6:18469–83. doi: 10.18632/oncotarget.4063
180. Taffs RE, Redegeld FA, Sitkovsky MV. Modulation of cytolytic T lymphocyte functions by an inhibitor of serine/threonine phosphatase, okadaic acid Enhancement of cytolytic T lymphocyte-mediated cytotoxicity. *J Immunol*. (1991) 147:722–8.
181. Chung V, Mansfield AS, Braiteh F, Richards D, Durivage H, Ungerleider RS, et al. Safety, tolerability, and preliminary activity of LB-100, an inhibitor of protein phosphatase 2A, in patients with relapsed solid tumors: an open-label, dose escalation, first-in-human, phase I trial. *Clin Cancer Res*. (2017) 23:3277–84. doi: 10.1158/1078-0432.CCR-16-2299
182. D'Arcy BM, Swingle MR, Papke CM, Abney KA, Bouska ES, Prakash A, et al. The antitumor drug LB-100 is a catalytic inhibitor of protein phosphatase 2A (PPP2CA) and 5 (PPP5C) coordinating with the active-site catalytic metals in PPP5C. *Mol Cancer Ther*. (2019) 18:556–66. doi: 10.1158/1535-7163.MCT-17-1143
183. Chang KE, Wei BR, Madigan JP, Hall MD, Simpson RM, Zhuang Z, et al. The protein phosphatase 2A inhibitor LB100 sensitizes ovarian carcinoma cells to cisplatin-mediated cytotoxicity. *Mol Cancer Ther*. (2015) 14:90–100. doi: 10.1158/1535-7163.MCT-14-0496
184. Liu L, Wang H, Cui J, Zhang Q, Zhang W, Xu W, et al. Inhibition of protein phosphatase 2A sensitizes mucoepidermoid carcinoma to chemotherapy via the PI3K-AKT pathway in response to insulin stimulus. *Cell Physiol Biochem*. (2018) 50:317–31. doi: 10.1159/000494008
185. Wang CY, Chao TT, Chang FY, Chen YL, Tsai YT, Lin HI, et al. CIP2A mediates erlotinib-induced apoptosis in non-small cell lung cancer cells without EGFR mutation. *Lung Cancer*. (2014) 85:152–60. doi: 10.1016/j.lungcan.2014.05.024
186. Khanna A, Kauko O, Bockelman C, Laine A, Schreck I, Partanen JI, et al. Chk1 targeting reactivates PP2A tumor suppressor activity in cancer cells. *Cancer Res*. (2013) 73:6757–69. doi: 10.1158/0008-5472.CAN-13-1002
187. Li W, Zhang H, Yang L, Wang Y. Cancerous inhibitor of protein phosphatase 2A regulates cisplatin resistance in ovarian cancer. *Oncol Lett*. (2019) 17:1211–6. doi: 10.3892/ol.2018.9653
188. Choi YA, Park JS, Park MY, Oh KS, Lee MS, Lim JS, et al. Increase in CIP2A expression is associated with doxorubicin resistance. *FEBS Lett*. (2011) 585:755–60. doi: 10.1016/j.febslet.2011.01.018
189. Zhang X, Xu B, Sun C, Wang L, Miao X. Knockdown of CIP2A sensitizes ovarian cancer cells to cisplatin: an *in vitro* study. *Int J Clin Exp Med*. (2015) 8:16941–7.
190. Liu J, Wang M, Zhang X, Wang Q, Qi M, Hu J, et al. CIP2A is associated with multidrug resistance in cervical adenocarcinoma by a P-glycoprotein pathway. *Tumour Biol*. (2016) 37:2673–82. doi: 10.1007/s13277-015-4032-8
191. Kaur A, Denisova OV, Qiao X, Jumppanen M, Peuhu E, Ahmed SU, et al. PP2A inhibitor PME-1 drives kinase inhibitor resistance in glioma cells. *Cancer Res*. (2016) 76:7001–11. doi: 10.1158/0008-5472.CAN-16-1134
192. MacKeigan JP, Murphy LO, Blenis J. Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nat Cell Biol*. (2005) 7:591–600. doi: 10.1038/ncb1258
193. Sobral LM, Sousa LO, Coletta RD, Cabral H, Greene LJ, Tajara EH, et al. Stable SET knockdown in head and neck squamous cell carcinoma promotes cell invasion and the mesenchymal-like phenotype in vitro, as well as necrosis, cisplatin sensitivity and lymph node metastasis in xenograft tumor models. *Mol Cancer*. (2014) 13:32. doi: 10.1186/1476-4598-13-32
194. Hung MH, Wang CY, Chen YL, Chu PY, Hsiao YJ, Tai WT, et al. SET antagonist enhances the chemosensitivity of non-small cell lung cancer cells by reactivating protein phosphatase 2A. *Oncotarget*. (2016) 7:638–55. doi: 10.18632/oncotarget.6313
195. Tang M, Shen JF, Li P, Zhou LN, Zeng P, Cui XX, et al. Prognostic significance of CIP2A expression in solid tumors: a meta-analysis. *PLoS ONE*. (2018) 13:e0199675. doi: 10.1371/journal.pone.0199675
196. Come C, Laine A, Chanrion M, Edgren H, Mattila E, Liu X, et al. CIP2A is associated with human breast cancer aggressivity. *Clin Cancer Res*. (2009) 15:5092–100. doi: 10.1158/1078-0432.CCR-08-3283
197. Liu X, Cao W, Qin S, Zhang T, Zheng J, Dong Y, et al. Overexpression of CIP2A is associated with poor prognosis in multiple myeloma. *Signal Transduct Target Ther*. (2017) 2:17013. doi: 10.1038/sigtrans.2017.13
198. Uhlen M, Zhang C, Lee S, Sjostedt E, Fagerberg L, Bidkhori G, et al. A pathology atlas of the human cancer transcriptome. *Science*. (2017) 357:eaan2507. doi: 10.1126/science.aan2507
199. Spaans VM, Trietsch MD, Crobach S, Stelloo E, Kremer D, Osse EM, et al. Designing a high-throughput somatic mutation profiling panel specifically for gynaecological cancers. *PLoS ONE*. (2014) 9:e93451. doi: 10.1371/journal.pone.0093451

200. Cottrell CE, Al-Kateb H, Bredemeyer AJ, Duncavage EJ, Spencer DH, Abel HJ, et al. Validation of a next-generation sequencing assay for clinical molecular oncology. *J Mol Diagn*. (2014) 16:89–105. doi: 10.1016/j.jmoldx.2013.10.002
201. Muinelo-Romay L, Casas-Arozamena C, Abal M. Liquid biopsy in endometrial cancer: new opportunities for personalized oncology. *Int J Mol Sci*. (2018) 19:E2311. doi: 10.3390/ijms19082311
202. Bogani G, Liu MC, Dowdy SC, Cliby WA, Kerr SE, Kalli KR, et al. Detection of circulating tumor cells in high-risk endometrial cancer. *Anticancer Res*. (2015) 35:683–7.
203. Alonso-Alconada L, Muinelo-Romay L, Madissoo K, Diaz-Lopez A, Krakstad C, Trovik J, et al. Molecular profiling of circulating tumor cells links plasticity to the metastatic process in endometrial cancer. *Mol Cancer*. (2014) 13:223. doi: 10.1186/1476-4598-13-223
204. Bolivar AM, Luthra R, Mehrotra M, Chen W, Barkoh BA, Hu P, et al. Targeted next-generation sequencing of endometrial cancer and matched circulating tumor DNA: identification of plasma-based, tumor-associated mutations in early stage patients. *Mod Pathol*. (2019) 32:405–14. doi: 10.1038/s41379-018-0158-8
205. Wang Y, Li L, Douville C, Cohen JD, Yen TT, Kinde I, et al. Evaluation of liquid from the Papanicolaou test and other liquid biopsies for the detection of endometrial and ovarian cancers. *Sci Transl Med*. (2018) 10:eaap8793. doi: 10.1126/scitranslmed.aap8793
206. Liu X, Chai Y, Li J, Ren P, Liu M, Dai L, et al. Autoantibody response to a novel tumor-associated antigen p90/CIP2A in breast cancer immunodiagnosis. *Tumour Biol*. (2014) 35:2661–7. doi: 10.1007/s13277-013-1350-6
207. Lheureux S, McCourt C, Rimel BJ, Duska L, Fleming G, Mackay H, et al. Moving forward with actionable therapeutic targets and opportunities in endometrial cancer: a NCI clinical trials planning meeting report. *Gynecol Oncol*. (2018). In press. doi: 10.1016/j.ygyno.2018.02.005
208. Fleming GF, Sill MW, Darcy KM, McMeekin DS, Thigpen JT, Adler LM, et al. Phase II trial of trastuzumab in women with advanced or recurrent, HER2-positive endometrial carcinoma: a Gynecologic Oncology Group study. *Gynecol Oncol*. (2010) 116:15–20. doi: 10.1016/j.ygyno.2009.09.025
209. Leslie KK, Sill MW, Lankes HA, Fischer EG, Godwin AK, Gray H, et al. Lapatinib and potential prognostic value of EGFR mutations in a Gynecologic Oncology Group phase II trial of persistent or recurrent endometrial cancer. *Gynecol Oncol*. (2012) 127:345–50. doi: 10.1016/j.ygyno.2012.07.127
210. Black JD, Lopez S, Cocco E, Bellone S, Altwerger G, Schwab CL, et al. PIK3CA oncogenic mutations represent a major mechanism of resistance to trastuzumab in HER2/neu overexpressing uterine serous carcinomas. *Br J Cancer*. (2015) 113:1020–6. doi: 10.1038/bjc.2015.306
211. Lopez S, Cocco E, Black J, Bellone S, Bonazzoli E, Predolini F, et al. Dual HER2/PIK3CA targeting overcomes single-agent acquired resistance in HER2-amplified uterine serous carcinoma cell lines *in vitro* and *in vivo*. *Mol Cancer Ther*. (2015) 14:2519–26. doi: 10.1158/1535-7163.MCT-15-0383

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

ORIGINAL RESEARCH
published: 11 April 2019
doi: 10.3389/fonc.2019.00211

Hepatoma-Derived Growth Factor and DDX5 Promote Carcinogenesis and Progression of Endometrial Cancer by Activating β-Catenin

Chunhua Liu[1†], Lijing Wang[1†], Qingping Jiang[2†], Junyi Zhang[3†], Litong Zhu[1], Li Lin[1], Huiping Jiang[1], Dan Lin[1], Yanyi Xiao[1], Weiyi Fang[1,3] and Suiqun Guo[1]*

[1] Department of Obstetrics and Gynecology, The Third Affiliated Hospital, Southern Medical University, Guangzhou, China, [2] Department of Pathology, Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, [3] Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, Guangzhou, China

OPEN ACCESS

Edited by:
Massimo Nabissi,
University of Camerino, Italy

Reviewed by:
Saori Furuta,
University of Toledo, United States
Farhad Jadidi-Niaragh,
Tabriz University of Medical Sciences, Iran
Guang-Rong Yan,
Third Affiliated Hospital of Guangzhou Medical University, China

***Correspondence:**
Suiqun Guo
guosq2005@126.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: 14 November 2018
Accepted: 11 March 2019
Published: 11 April 2019

Citation:
Liu C, Wang L, Jiang Q, Zhang J, Zhu L, Lin L, Jiang H, Lin D, Xiao Y, Fang W and Guo S (2019) Hepatoma-Derived Growth Factor and DDX5 Promote Carcinogenesis and Progression of Endometrial Cancer by Activating β-Catenin. Front. Oncol. 9:211. doi: 10.3389/fonc.2019.00211

Background: Our previous work determined the correlation between high nuclear expression of hepatoma-derived growth factor (HDGF) and clinicopathological data of endometrial cancer (EC); however, the modulatory mechanisms and biological role of HDGF in EC have not been reported.

Methods: Lentiviral particles carrying human HDGF short hairpin RNA (shHDGF-1, -2, and -3) vector and plasmids for HDGF, DDX5, and β-catenin expression were, respectively introduced into EC cells to evaluate the effects and molecular mechanisms underlying EC cell proliferation, migration, invasion, and metastasis. Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) and western blotting were used to determine HDGF and DDX5 expression. Co-immunoprecipitation (co-IP), mass spectrometry, and an immunofluorescence co-localization study were conducted to explore the relationship between HDGF, DDX5, and β-catenin. Immunohistochemistry was used to analyze the clinical associations between HDGF and DDX5 in EC.

Results: Knocking down HDGF expression significantly decreased EC cellular proliferation, migration, invasion *in vitro*, as well as tumorigenesis and metastasis *in vivo*. Conversely, HDGF overexpression reversed these effects. Stable knockdown-based HDGF suppression activated the PI3K/AKT signaling pathway, along with downstream β-catenin-mediated cell cycle and epithelial-mesenchymal transition signaling. Furthermore, co-IP combined with mass spectrometry and an immunofluorescence co-localization study indicated that HDGF interacts with DDX5, whereas β-catenin was associated with DDX5 but not HDGF. Overexpression of DDX5 reversed the suppression of shHDGF. Immunohistochemistry analysis showed that high expression of DDX5 constituted an unfavorable factor with respect to the clinicopathological characteristics of EC tissues and that HDGF and DDX5 high expression (HDGF+/DDX5+) led to a worse prognosis for patients with EC ($P < 0.001$). In addition, we found that the

expression of HDGF and DDX5 was positively correlated in EC tissues ($r = 0.475$, $P < 0.001$).

Conclusion: Our results provide novel evidence that HDGF interacts with DDX5 and promotes the progression of EC through the induction of β-catenin.

Keywords: EC, HDGF, DDX5, β-catenin, PI3K/AKT

INTRODUCTION

Endometrial cancer (EC) comprises the most common malignancy involving the female genital tract and the fourth most common malignancy in women after breast, lung, and colorectal cancers (1). In 2012, approximately 320,000 new cases of EC were diagnosed worldwide and the incidence is increasing (2). Currently, endometrial carcinogenesis is thought to be a multi-step process involving the coordinated interaction of hormonal regulation, gene mutation, adhesion molecules, and apoptosis; however, the molecular mechanisms underlying the pathogenesis of EC have not been fully elucidated (3). Therefore, a better understanding of the molecular mechanism underlying the progression of EC will likely lead to new insights regarding novel therapeutic targets.

Hepatoma-derived growth factor (HDGF), the gene for which is located on chromosome 1q21–23, is a heparin-binding growth factor that was originally purified from media conditioned with the human hepatoma cell line, HuH7 (4). HDGF is ubiquitously expressed in normal tissues and tumor cell lines that exhibit growth factor properties. The most recent study reported that HDGF acts as a coactivator of SREBP1-mediated transcription of lipogenic genes (5). HDGF is characterized as a mitogen for many cell types and localizes to the nucleus, which is necessary for its mitogenic activity. Characteristics such as promoting growth, suppressing differentiation, and exhibiting angiogenic properties, suggest a role for HDGF in cancer induction and tumor progression (6–8). Accordingly, a number of studies have focused on the significance of HDGF as a prognostic marker and have demonstrated its clinical value for oral cancer (9), esophageal cancer (10), gastrointestinal stromal tumors (11, 12), meningiomas (13), hepatocellular cancer (14), and non-small cell lung carcinoma (15). Consistent with these findings, in our previous study (16), we determined a correlation between high nuclear expression of HDGF and clinicopathological data of EC; however, the functional significance of HDGF in EC remains unknown.

The DEAD-box RNA helicase, DDX5 (p68), constitutes a multi-functional protein with an important role in regulating transcription in conjunction with multiple, sequence-specific transcription factors (17). Recently, DDX5 has been demonstrated to act as a potent transcriptional co-activator of the estrogen receptor (18, 19), androgen receptor (20), tumor suppressor p53 (21), MyoD (22), and β-catenin (23). DDX5 has been implicated in cancer development and progression by functioning in several key cancer cell activities, such as proliferation, migration, cytoskeletal reorganization, and the epithelial-mesenchymal transition (EMT) (24–28). Recently, a small molecule inhibitor for DDX5, Supinoxin^{TM} (RX-5902), has been developed for cancer therapy, and is currently in a clinical trial in patients with metastatic triple negative breast cancer (ClinicalTrials.gov identifier: NCT02003092) (29), which suggested the significance of DDX5 in the pathogenesis of tumor.

In the current study, we examined and characterized the interaction between HDGF and DDX5, and determined its effect on the activation of PI3K/AKT and downstream β-catenin-mediated cell cycle and EMT signaling to promote the proliferation, invasion, and metastasis of EC.

MATERIALS AND METHODS

Cell Culture

The EC cell lines Ishikawa and RL95-2 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China). All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (ExCell, Shanghai, China). Ishikawa and RL95-2 cell lines used in this study were incubated in a humidified chamber with 5% CO_2 at 37°C.

Immunohistochemistry and Evaluation of Staining

One hundred and twenty two endometrial carcinoma (EC) paraffin sections (3 mm) samples from 2002 to 2008 were obtained in the Third Affiliated Hospital of Guangzhou Medical University, Guangzhou City, China. Detailed information and the IHC of HDGF about the 122 EC tissue specimens was performed in our previous study (16). Immunohistochemistry was performed according to standard procedures. The staining intensity of DDX5 (1:100, Abcam, Cambridge, MA, USA) was scored as previously described (20).

Establishment of EC Cell Lines With Stable Knockdown of HDGF

Lentiviral particles carrying human HDGF short hairpin RNA (shHDGF-1, -2, and -3; **Supplementary Table 1**) vector and empty vector controls (PLV-Ctr) were constructed by GeneChem (Shanghai, China). Ishikawa and RL95-2 cells were infected with lentiviral vectors, and cells with green fluorescent protein signals (**Supplementary Figure 1**) were selected for further experiments using qRT-PCR and western blotting analyses.

Abbreviations: co-IP, co-immunoprecipitation; DDX5, DEAD-box RNA helicase; EC, endometrial cancer; EMT, epithelial-mesenchymal transition; HDGF, hepatoma-derived growth factor; MTT, 3-(4 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; PCNA, proliferating cell nuclear antigen; PI3K, phosphoinositide 3-kinase; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction.

Transient Transfection Using Plasmids or Small Interfering RNAs or PI3K Inhibitor Ly294002

HDGF, DDX5, and β-catenin plasmids were generated by Biosense Technologies (Guangzhou, China). Small interfering RNA (siRNA) for DDX5 and β-catenin (named as siDDX5 and siβ-catenin, respectively) were designed and synthesized by Guangzhou RiboBio (RiboBio Inc., China). At 24 h before transfection, EC cells were plated onto a 6- or 96-well plate (Nest Biotech, China) at 30%–50% confluence. Plasmids were then transfected into cells using Lipofectamine™ 2,000 (Invitrogen Biotechnology, Shanghai, China), according to the manufacturer's protocol. EC cells overexpressing HDGF were treated with or without Ly294002 according to a previous description (30). Cells were collected after 48–72 h for further experiments.

RNA Isolation, Reverse Transcription, and qRT-PCR

RNA was extracted from EC cell lines and the empty vector controls using TRIzol (TaKaRa, Shiga, Japan). RNA was transcribed into cDNA (TaKaRa) and amplified with specific primers. The targeted HDGF sequences were: sense 5′-GCT TCC GGC TAT CAG TCC TC-3′; antisense: 5′-CTG CCT CCT TCT CCT CTC CT-3′; The targeted DDX5 sense: 5′-GGC CTG ATC ACA GAA CCA TT-3′; antisense: 5′-ACC ACC CTT ATT CCC AAA CC-3′; and ARF5 (used as an internal control) sense: 5′-ATC TGT TTC ACA GTC TGG GAC G-3′; antisense: 5′-CCT GCT TGT TGG CAA ATA CC-3′. The assays were performed in accordance with the manufacturer's instructions (TaKaRa) and according to a previous description (31). The PCR reaction for each gene was repeated three times.

Western Blotting Analysis

Western blotting was performed as previously described (32). The antibodies included anti-HDGF, DDX5, pRb, E2F1, CCND1, CDK4, c-Myc, P27, PI3K, p-PI3K, AKT, p-AKT, β-catenin, E-cadherin, N-cadherin, vimentin, Snail, and β-actin (**Supplementary Table 2**). β-actin was used as a loading control for all blots. The images were captured using a ChemiDoc™ CRS+ Molecular Imager (Bio-Rad, Berkeley, CA, USA).

MTT Assay

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the rate of *in vitro* cell proliferation. For MTT assay, cells were processed as described earlier (31). Briefly, after transfected with shHDGF or PLV-Ct, cells were incubated, dissolve and measured the absorbance value (OD) at 490 nm.

EdU Incorporation Assays

Proliferating EC cells were examined using the Cell-Light EdU Apollo 567 *in vitro* Imaging Kit (RiboBio, Guangzhou, China), according to the manufacturer's protocol. Briefly, after incubation with 10 mM EdU for 2 h, EC cells were fixed with 4% paraformaldehyde, permeabilized in 0.3% Triton X-100, and stained with Apollo fluorescent dyes. A total of 5 mg/mL of DAPI was used to stain cell nuclei for 10 min. The number of EdU-positive cells was counted under a fluorescence microscope in five random fields. All assays were independently performed three times.

Colony Formation Assay

Cells were plated in 6-well culture plates at 200 cells/well (3 wells/cell group). After incubation at 37°C in a 5% CO_2 incubator for 15 days, cells were washed twice with phosphate buffered saline (PBS) and stained with hematoxylin solution. The visible colony numbers were counted. All experiments were repeated at least three times.

Cell Cycle Analysis

A total of 5×10^6 EC cells were harvested after a 48-h incubation, and then washed with cold PBS. The cells were further fixed with 70% ice-cold ethanol at 4°C overnight. Fixed cells were washed three times with cold PBS. After incubation with PBS containing 10 mg/mL of propidium iodide and 0.5 mg/mL of RNase A for 15 min at 37°C. FACS caliber flow cytometry (BD Biosciences, San Jose, CA, USA) was used to obtain the DNA content of the labeled cells.

In vivo Tumorigenesis in Nude Mice

The animal studies were approved by the Animal Ethics Committee of the Southern Medical University. A total of 5×10^6 logarithmically growing EC cells transfected with shHDGF or PLV-Ctr ($n = 5$ per group) in 0.1 mL of RPMI-1640 medium were subcutaneously injected into the left-right symmetric flank of 4–5-week-old male BALB/c-nu mice. The mice were maintained in a barrier facility on HEPA-filtered racks. The animals were fed an autoclaved laboratory rodent diet. After 21 days, the mice were sacrificed and tumor tissues were excised and weighed.

Wound Healing Assay

EC cells were plated in 6-well plates and incubated overnight until 90% confluent. An injury line was made using a 10-μL plastic filter tip to create a wound approximately 10 μm in diameter. Then we removed the culture medium and used PBS to eliminate dislodged cells. Subsequently, the wells were covered with serum-free medium to incubate for 48 h. "Wound closure" was observed at 0, 12, 24, 48 h under an inverted microscope.

Transwell Migration and Invasion Assays

For cell migration assays, 1×10^5 cells in 100 μL of RPMI-1640 medium without serum were seeded on a fibronectin-coated polycarbonate membrane insert in a Transwell apparatus (Corning, Armonk, NY, USA). In the lower chamber, 500 μL of RPMI-1640 with 10% serum was added as a chemoattractant. After the cells were incubated for 10 h at 37°C in a 5% CO2 atmosphere, the insert was washed with PBS and cells on the top surface of the insert were removed with a cotton swab. Cells adhering to the lower surface were fixed with methanol, stained with Giemsa solution, and counted under a microscope in 5 pre-determined fields (200×). For the cell invasion assay, the procedure was similar to the Transwell migration assay, except that the Transwell membranes were pre-coated with 24 μg/mL

of Matrigel (R&D Systems, Minneapolis, MN, USA) for 4 h. All assays were independently repeated three times.

In vivo Metastasis Assays

In vivo metastasis assays were performed according to a previous study (30). A total of 5×10^6 EC-shHDGF and -PLV-Ctr cells were injected into nude mice ($n = 5$ for each group) through the liver membrane. Whole-body optical images were visualized to monitor primary tumor growth and formation of metastatic lesions. After 2 months, all mice were sacrificed, individual organs were removed, and metastatic tissues were analyzed by hematoxylin and eosin staining.

Co-immunoprecipitation (Co-IP)

Co-IP was carried out using a Pierce Co-Immunoprecipitation kit (Thermo Scientific, Waltham, MA, USA), according to the

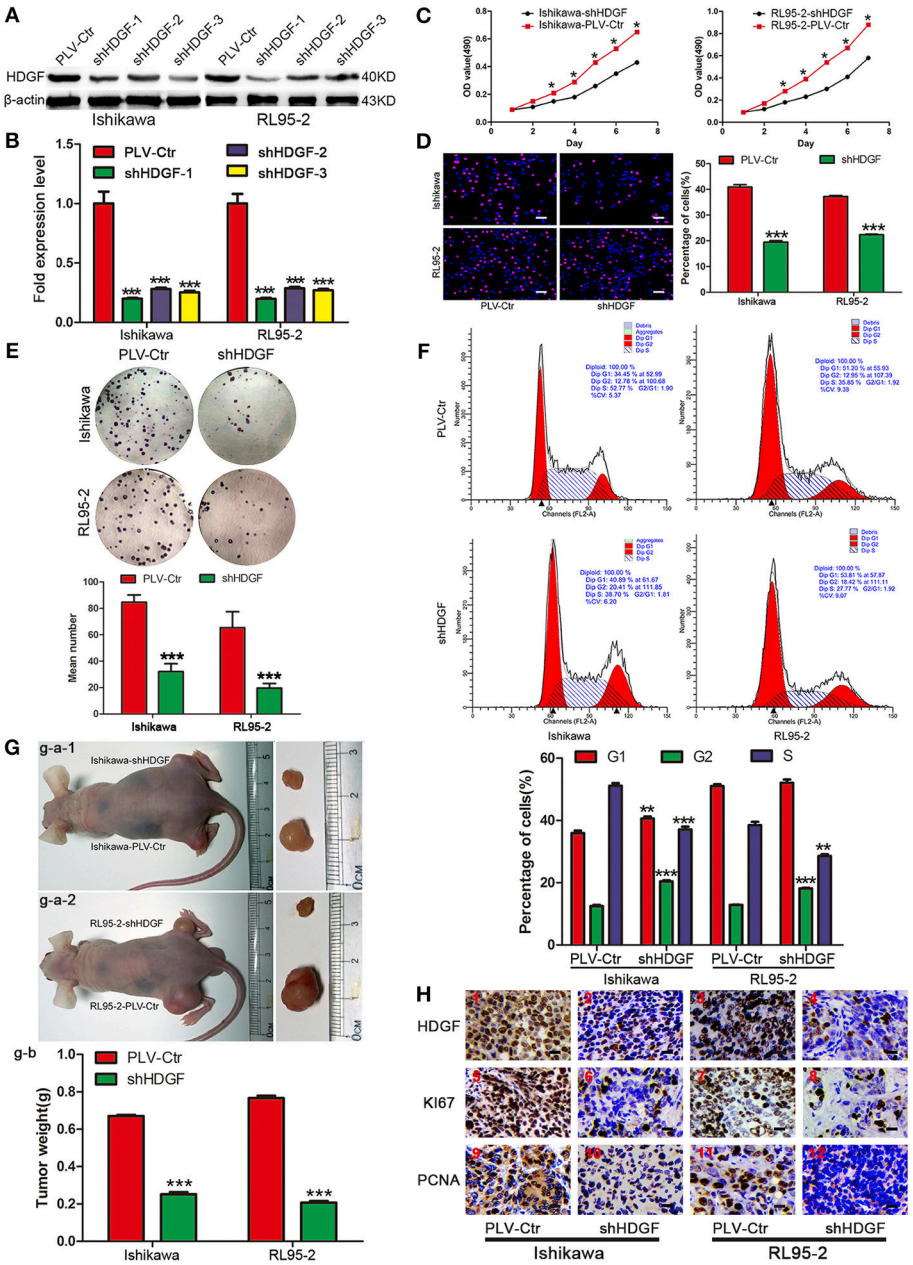


FIGURE 1 | Stable knockdown HDGF inhibits cell growth in EC. HDGF knockdown confirmed by western blot **(A)** and qRT-PCR **(B)**. Student's *t*-test, $^{***}P < 0.001$. MTT assay **(C)**, EdU incorporation assays **(D)**, clone formation **(E)**, and cell cycle assay **(F)** after HDGF knockdown. One-way ANOVA and Dunnett's multiple comparison test, $^{*}P < 0.05$. **(G)** g–a: When compared with PLV-Ctr, *in vivo* tumorigenicity of shHDGF-EC cells was markedly reduced ($^{***}P < 0.001$). g–b: Tumor weight statistics for each mouse group ($n = 5$ per group) **(H)**. HDGF, Ki67, and PCNA was evaluated by immunohistochemical. Compared with shHDGF-EC cells (2,4,6,8,10,12), the PLV-Ctr cell tumor tissues(1,3,5,7,9,11) were high expression.

manufacturer's instructions. The cells were lysed and the protein concentrations were measured. Then, 2000 μg of protein in 400 μL of supernatant was incubated with 10 μg anti-HDGF, anti-DDX5, and anti-β-catenin or anti-IgG antibodies coated on beads on a rotator overnight at 4°C. The beads were washed, eluted in sample buffer, and boiled for 8 min at 95°C. Immune complexes were subjected to Coomassie brilliant blue staining, mass spectrometry (Geneseed Biotech Co., Ltd, Guangzhou, China) and western blotting analysis. Anti-IgG was used as a negative control.

Immunofluorescence

Immunofluorescent staining was performed according to standard procedures as a previous study (33). Briefly, cells were cultured overnight, then fixed with 3.5% paraformaldehyde and permeabilization by 0.2% Triton X-100 at room temperature. Cells were incubated with mouse anti-β-catenin and rabbit anti-DDX5 antibody overnight at 4°C. After three washes in PBS, the coverslips were incubated for 1 h with secondary antibody. Then, coverslips were mounted onto slides with mounting solution containing 0.2 mg/mL of DAPI and sealed with nail polish. Slides were stored in a dark box and observed using a scanning confocal microscope (Zeiss LSM 800, Oberkochen, Germany).

Statistical Analysis

SPSS 21.0 (Chicago, IL, USA) and Graph Pad Prism 5.0 software (LaJolla, CA, USA) were used to analyze all data for statistical significance. Comparisons between two groups were performed using Student's *t*-test, one-way analysis of variance (ANOVA) for multiple groups, and a parametric generalized linear model with random effects for tumor growth and the MTT assay. Data are expressed as the mean ± SD from at least three independent experiments. The chi-squared test was applied to determine the relationship between the level of DDX5 expression and clinicopathological characteristics. Analysis of HDGF and DDX5 expression in 122 EC tissues was performed using paired-samples *t*-tests. The relationships were analyzed using Spearman's correlation analysis. Survival analysis was performed using the Kaplan-Meier method. The multivariate Cox proportional hazards method was used for analyzing the relationship between variables and patient survival time. A prognostic value of < 0.05 ($P < 0.05$) was considered significant, and all tests were two-sided. Statistical significance was denoted as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

Stable Knockdown of HDGF Expression Inhibits Cell Proliferation *in vitro* and *in vivo*

To gain insight into the role of HDGF in EC, we first used a lentiviral vector to specifically and stably knockdown the expression of HDGF in the Ishikawa and RL95-2 cells. The levels of HDGF were assessed by western blotting (**Figure 1A**) and qRT-PCR (**Figure 1B**). The most efficient knockdowns of HDGF expression were shown in shHDGF-3-Ishikawa and shHDGF-1-RL95-2 cells compared to the PLV-Ctr ($P < 0.001$) (**Supplementary Figure 2**).

Next, we assessed the effect of decreased HDGF expression on EC cell growth *in vitro*. The growth curves determined by MTT assays showed that growth of the shHDGF-Ishikawa and shHDGF-RL95-2 cells was significantly slower than that of PLV-Ctr cells ($P < 0.05$; **Figure 1C**). Conversely, overexpression of

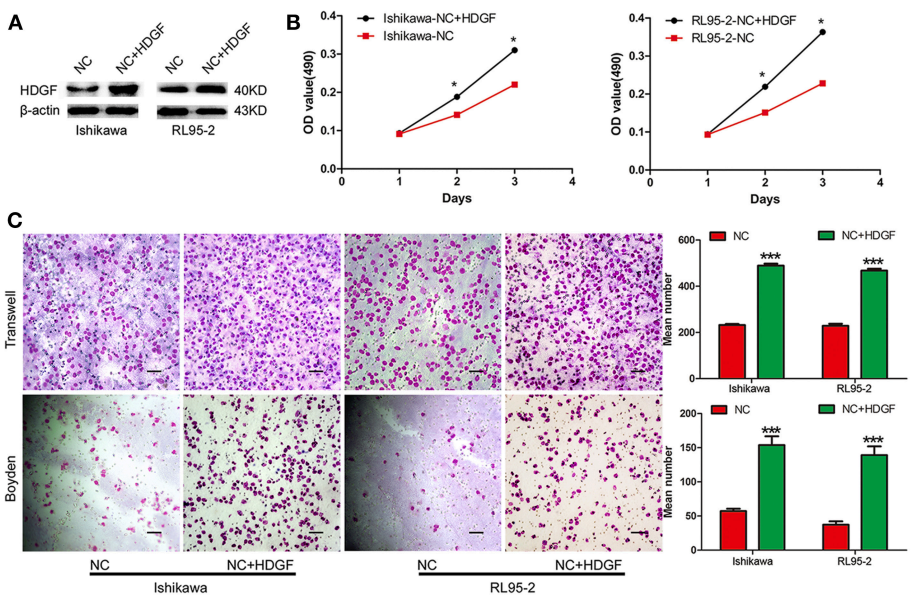


FIGURE 2 | Transient overexpression of HDGF by plasmid-promoted cell proliferation, migration, and invasion. Efficiency of plasmid overexpression of HDGF in EC cell lines **(A)**. Transient increase in expression of HDGF by plasmid promoted cell proliferation in EC cells **(B)**. Transient upregulation of HDGF dramatically increased the migration and invasion ability of EC cells *in vitro* **(C)**. Data are presented as the mean ± SD for three independent experiments ($^{*}P < 0.05$, $^{***}P < 0.001$).

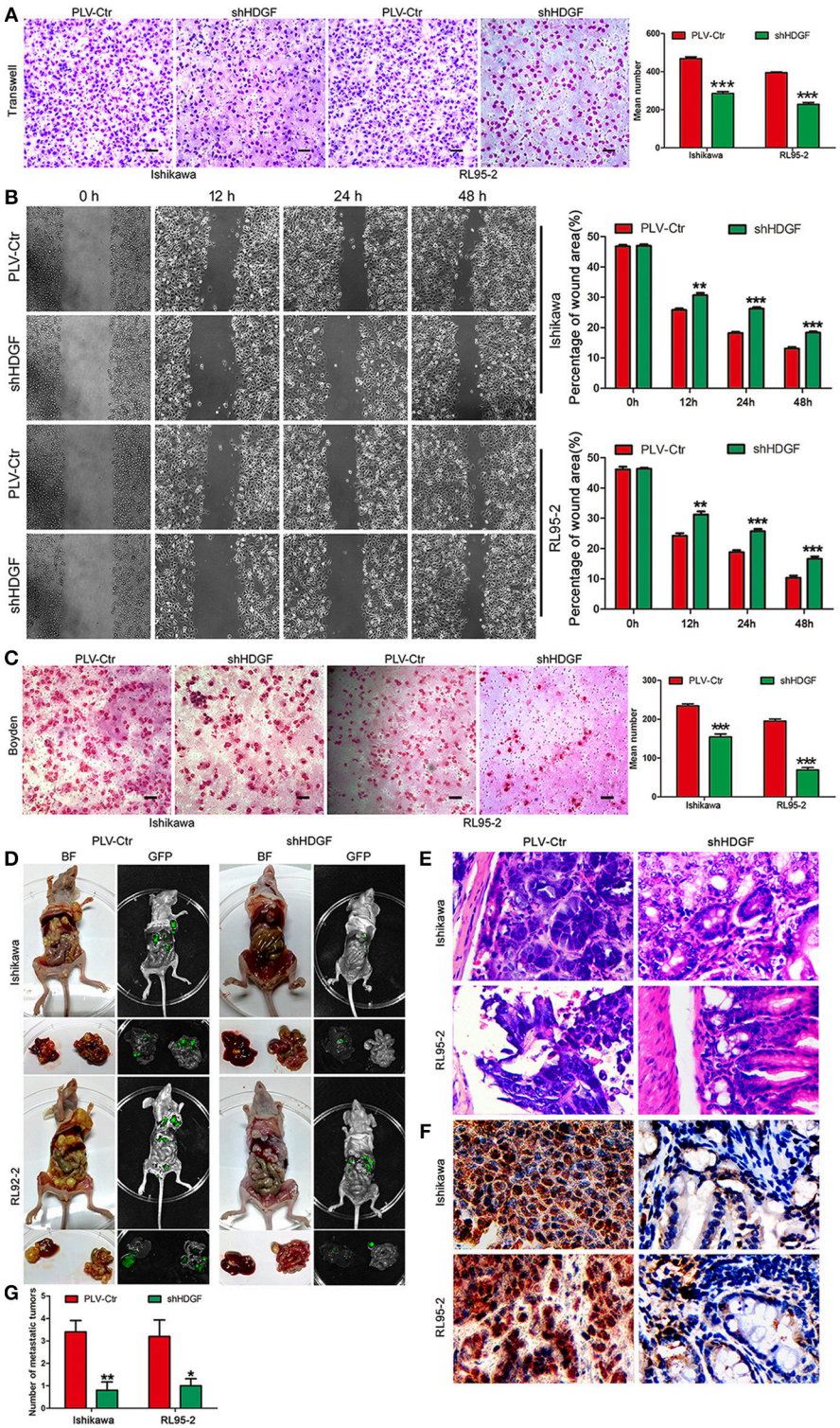


FIGURE 3 | HDGF expression knockdown decreases cell migration, invasion, and metastasis. Stable downregulation of HDGF reduced the *in vitro* migration ability of Ishikawa and RL95-2 cells **(A)**. The wound healing assay indicated that shHDGF transfection into Ishikawa and RL95-2 cells for 48 h impaired migration capacity compared with the negative control group **(B)**. Stable suppression of HDGF reduced the *in vitro* invasiveness of shHDGF-Ishikawa and shHDGF-RL95-2 cells **(C)**. *In vivo* intrahepatic metastasis assay results after shHDGF-Ishikawa and PLV-Ctr-Ishikawa injection: PLV-Ctr-EC cells were more easily transferred to intestinal tissue **(D)**. The HE **(E)** and IHC **(F)** of the metastatic intestinal tissues. Original magnification 200×. The numbers of metastatic tumors **(G)**. Data are presented as the mean ± SD for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, statistically significant difference.

HDGF (**Figure 2A**) reversed these effects ($P < 0.05$; **Figure 2B**). The EdU incorporation assay revealed that the percentage of cells in S phase decreased following the downregulation of HDGF expression ($P < 0.001$; **Figure 1D**). Colony formation assays showed that knockdown of HDGF significantly decreased cell proliferation ($P < 0.001$; **Figure 1E**). Furthermore, cell

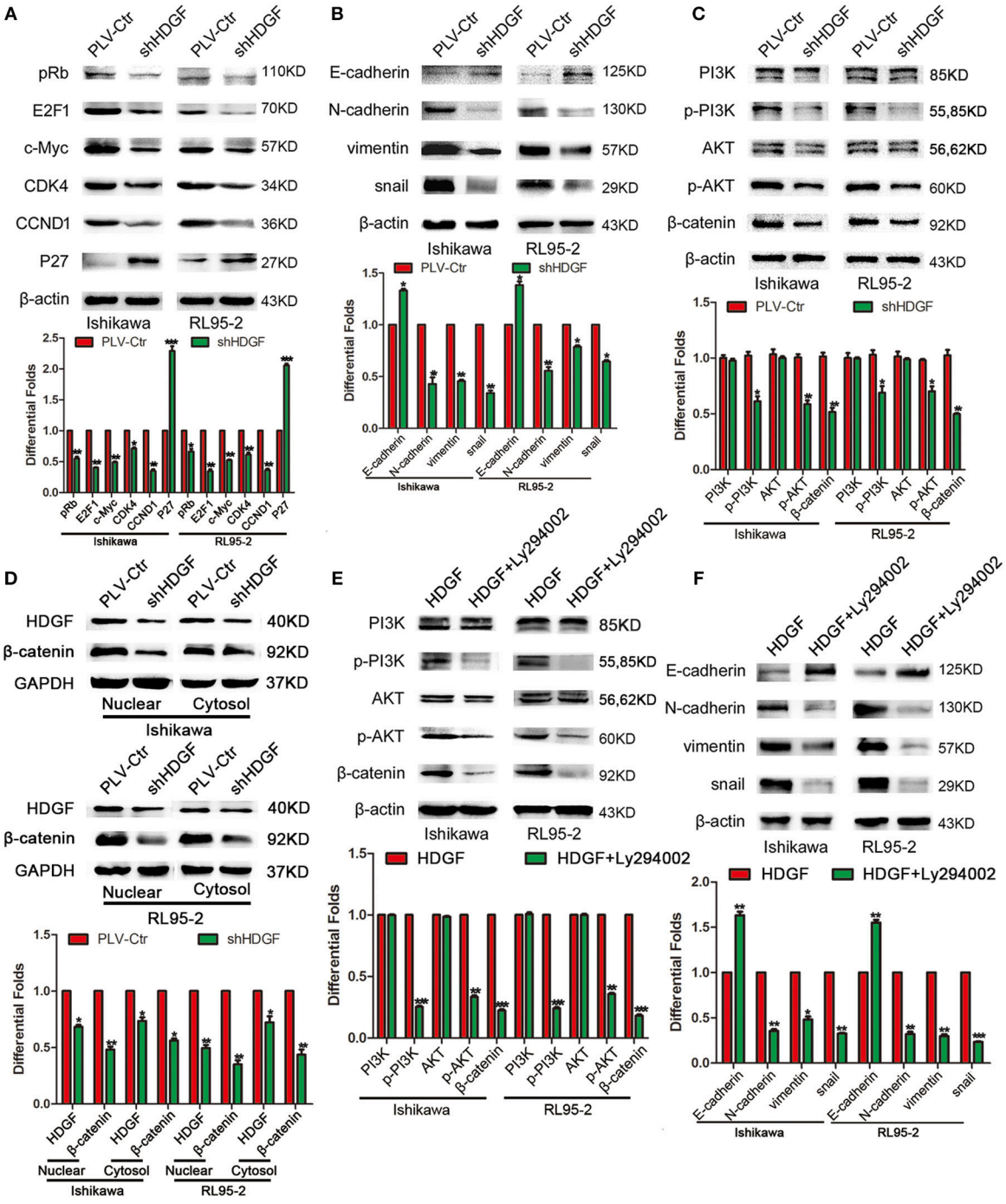


FIGURE 4 | HDGF controls cell cycle and EMT-associated gene expression in EC via the PI3K/AKT and β-catenin pathways. Knocking down endogenous HDGF expression reduced the expressions of pRb, E2F1, c-Myc, CDK4, and CCND1, and enhanced P27 in EC cells **(A)**. Suppressing HDGF expression decreased the expression of EMT marker genes (N-cadherin, Vimentin, and Snail) and enhanced E-cadherin expression **(B)**. Reduced HDGF expression decreased the expressions of p-PI3K, p-AKT, and β-catenin **(C)**. Knocking down of HDGF suppressed both nuclear and cytosol protein expression of β-catenin **(D)**. Suppressing the expression of p-PI3K by its specific inhibitor Ly294002 (50 nM) reduced p-AKT, β-catenin, Snail, N-cadherin, Vimentin, and upregulated E-cadherin in overexpressing HDGF EC cells **(E,F)**. β-actin served as the internal control. Each experiment was repeated three times with similar results, and error bars represent the mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cycle analysis demonstrated that HDGF suppression dramatically reduced cell cycle progression from the G1 to S phase ($P < 0.05$; **Figure 1F**).

Subsequently, to confirm the growth effect of HDGF *in vivo*, we performed an *in vivo* tumorigenesis study by inoculating EC cells into nude mice. Mice in the shHDGF-EC and PLV-Ctr groups were sacrificed 21 days after inoculation, with average tumor weights of 0.187 g and 0.793 g, respectively ($P < 0.001$; **Figure 1G**). The mice injected with shHDGF-Ishikawa and shHDGF-RL95-2 cells had smaller tumor burdens (**Figure 1G**) and displayed lower expression of HDGF, Ki67 and proliferating cell nuclear antigen (PCNA) in tumor tissues relative to the controls (**Figure 1H**). These results suggested that HDGF significantly promotes tumorigenesis *in vivo*.

HDGF Downregulation Suppresses Cell Migration, Invasion, and Intrahepatic Metastasis of EC Cells *in vitro* and *in vivo*

To examine the effect of HDGF on cell migration and invasion, a Transwell apparatus, wound healing assay, and Boyden chamber coated with Matrigel were used. After incubation

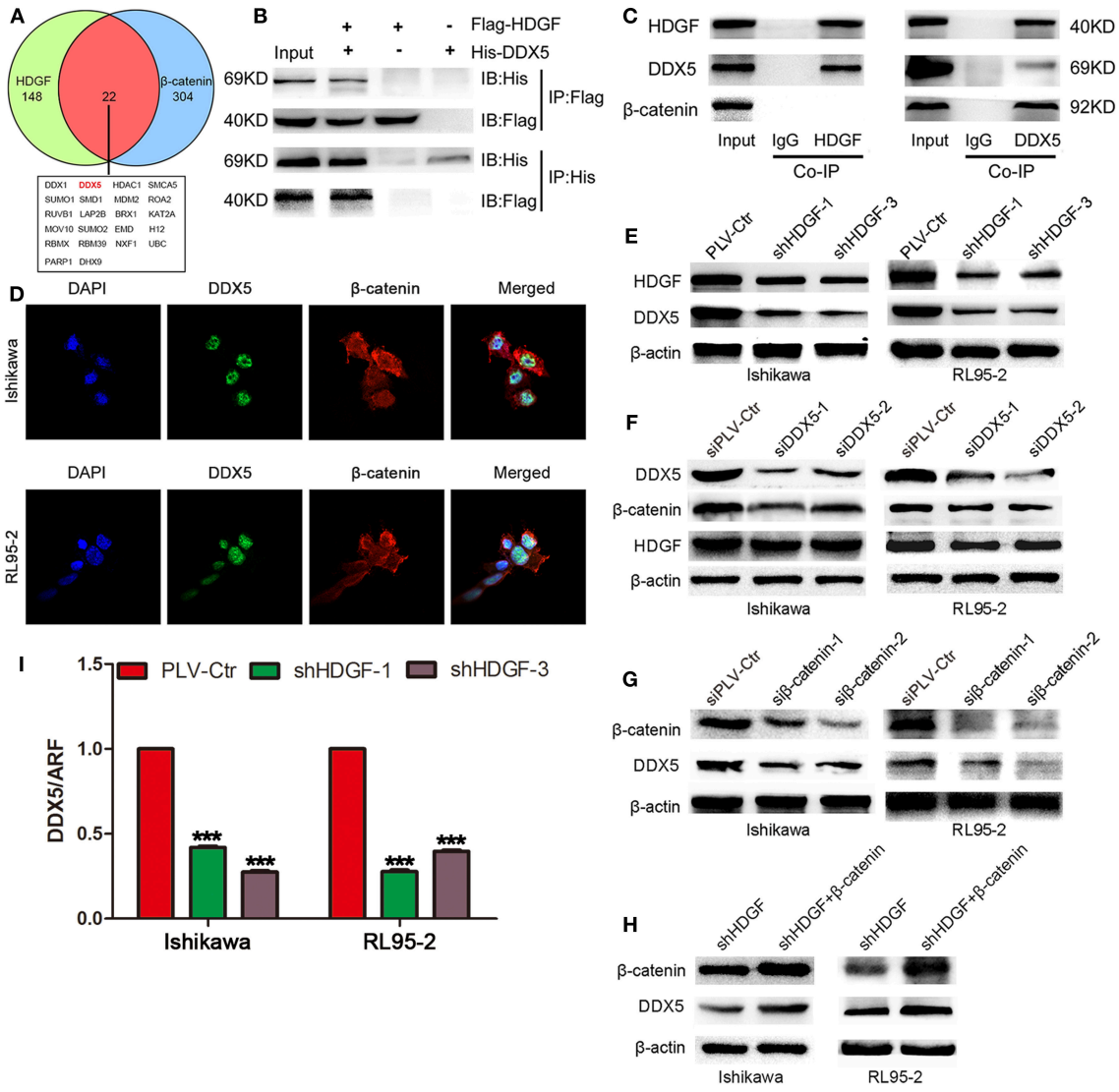


FIGURE 5 | DDX5 interact with both HDGF and β-catenin in EC cells. Venn diagram depicting overlaps between HDGF and β-catenin proteins: the 22 common proteins are listed **(A)**. Co-immunoprecipitation (Co-IP) detected the interaction of exogenous HDGF and DDX5 in RL95-2 cells **(B)**. Co-IP detected the interaction of endogenous HDGF and DDX5 in RL95-2 cells **(C)**. Nuclear co-localization of HDGF and DDX5, as well as β-catenin and DDX5 proteins, in EC cells by immunofluorescence under a scanning confocal microscope **(D)**. DDX5 levels after HDGF knockdown, as assessed by western blotting **(E)**. HDGF and β-catenin levels after DDX5 knockdown **(F)**. Overexpression of β-catenin plasmid in shHDGF-EC cells increased DDX5 levels **(G)**. DDX5 expression after β-catenin knockdown **(H)**. DDX5 mRNA expression after HDGF knockdown, normalized to the expression of ARF5. One-way ANOVA and Dunnett's multiple comparison test ***$P < 0.001$ **(I)**.

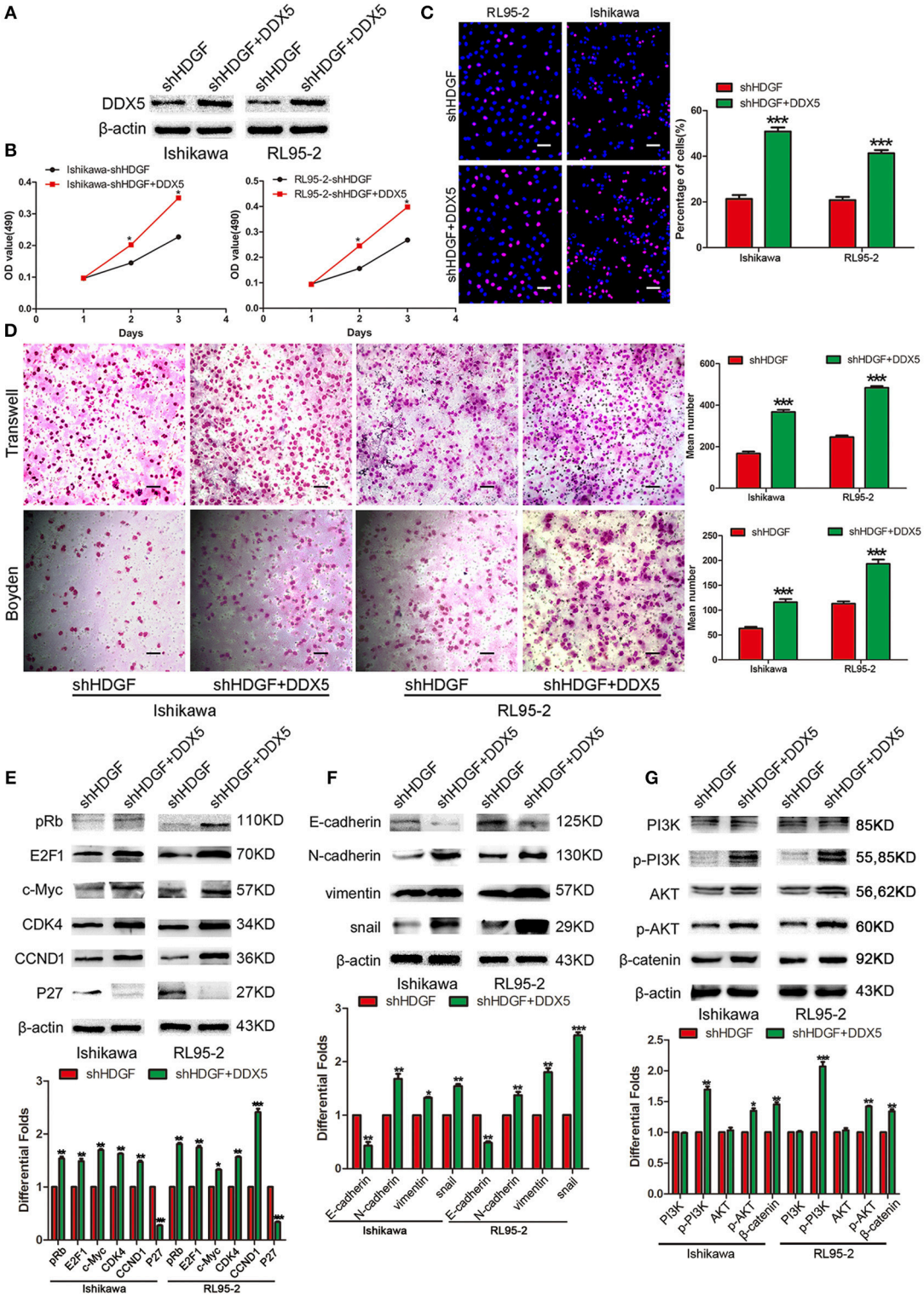


FIGURE 6 | Transient overexpression of DDX5 from a plasmid reverses the suppression of shHDGF. Efficiency of plasmid overexpression of DDX5 in EC cell lines **(A)**. Transient increase in the expression of DDX5 by plasmid promotes cell proliferation in EC cells, as assessed by the MTT **(B)** and EdU incorporation assays **(C)**. Transient upregulation of DDX5 dramatically increases the migration and invasion ability of EC cells *in vitro* **(D)**. Western blotting analysis of the protein levels of p-PI3K, p-AKT, β-catenin, pRb, E2F1, c-Myc, CDK4, CCND1, and P27, as well as invasion and migration according to the relevant protein levels of E-cadherin, N-cadherin, Vimentin, and Snail after transient transfection of DDX5 plasmid into EC cells **(E–G)**. β-actin served as the internal control. Data are presented as the mean ± SD for three independent experiments (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

for 10 h, a reduced number of migrated cells were observed for shHDGF-Ishikawa and shHDGF-RL9-2 cells compared with PLV-Ctr cells ($P < 0.001$; **Figure 3A**). In addition, the wound healing assay demonstrated that shHDGF-Ishikawa and shHDGF-RL95-2 cells inhibited the migration capacity ($P < 0.05$; **Figure 3B**). As shown in **Figure 3C**, the results of the Boyden chamber coated with Matrigel assays were similar to those of the Transwell assays ($P < 0.01$ for each); however, overexpressing HDGF reversed these effects ($P < 0.05$; **Figure 2C**).

To further assess the effect of HDGF on EC intrahepatic metastasis, shHDGF-Ishikawa and control cells were independently injected into the liver capsules of nude mice. Fluorescence imaging was used to identify scattered metastatic

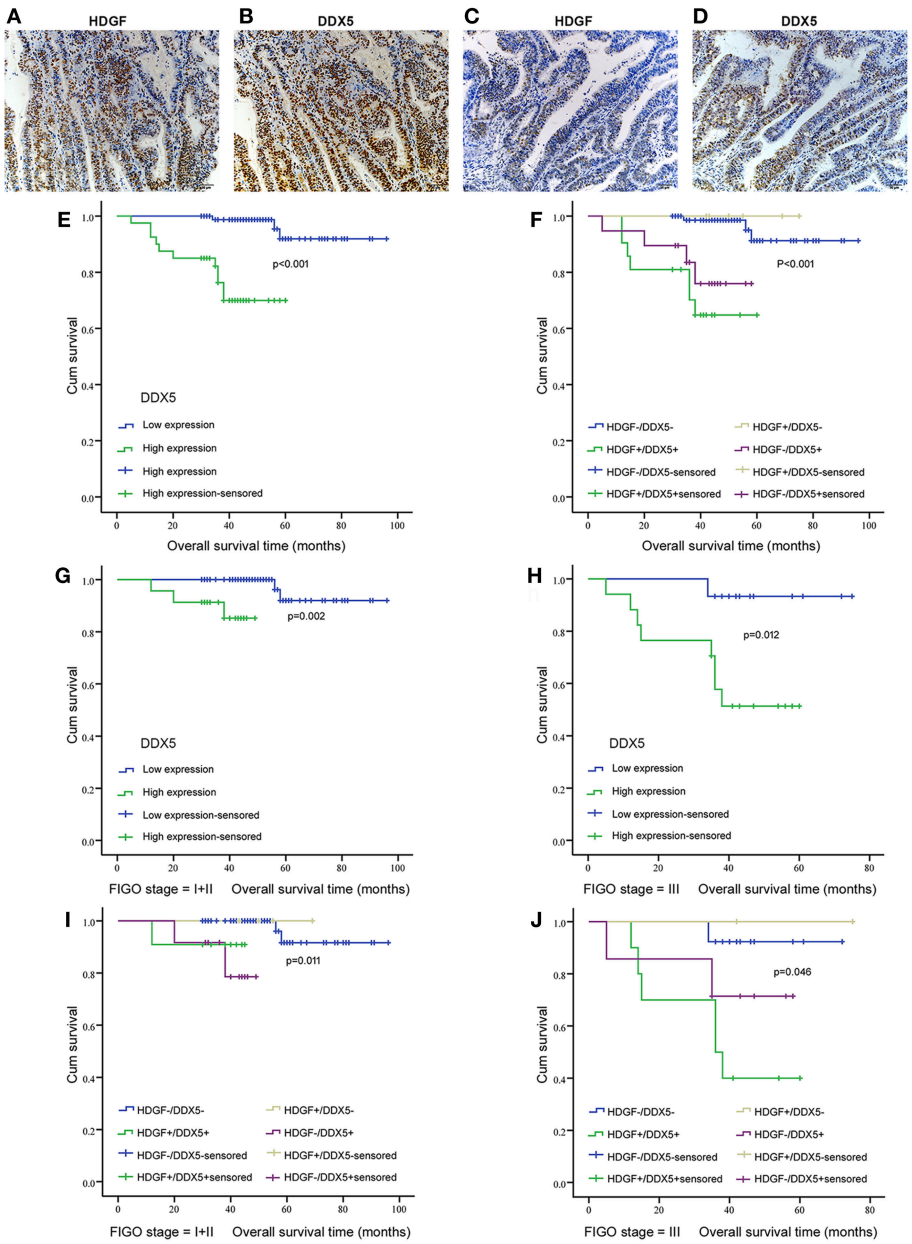


FIGURE 7 | Correlation between HDGF and DDX5 in EC tissues and the clinical significance. Immunohistochemistry results showing the level of HDGF and DDX5 expression at the same locations in EC tissues. **(A,B)** represent high expression of HDGF and DDX5, respectively. **(C,D)** represent low expression of HDGF and DDX5, respectively. Original magnification 200×. We analyzed DDX5 expression in the primary tumors of patients with EC **(E)** and the correlation between HDGF high expression and prognosis for patients with EC by strata analysis against FIGO stage **(G,H)**. Kaplan-Meier survival curve comparing subgroups based on HDGF and DDX5 expression in the primary tumors of patients with EC. Low HDGF expression cases with high or low DDX5 expression, as well as high HDGF expression cases with high or low DDX5 expression **(F–J)**. The *P*-value is based on a log-rank test.

nodules in the livers and intestines of nude mice that formed in the mice after 2 months. Only a few scattered metastatic cells were observed following injection of shHDGF-Ishikawa cells, whereas a variety of large clusters were observed in the PLV-Ctr group (**Figure 3D**). As can be seen from **Figure 3D**, control PLV-Ctr cells were more easily transferred to intestinal tissue, while shHDGF was less or not metastasized. The HE and IHC of the metastatic intestinal tissues and numbers of metastatic foci were shown in **Figures 3E–G**. Taken together, these results suggest that HDGF effectively promotes cell migration, invasion, and intrahepatic metastasis of EC cells *in vitro* and *in vivo*.

HDGF Regulates the Expression of Cell Cycle- and EMT-Associated Genes via the PI3K/AKT and β-Catenin Signaling Pathways in EC Cells

To further study the mechanism by which HDGF regulates cell proliferation, migration, invasion, and metastasis, the protein levels of cell cycle- and EMT-associated genes were examined in Ishikawa and RL95-2 cells with stably suppressed HDGF. Knockdown of HDGF inhibited the activation of oncogenic cell cycle regulators, including pRb, E2F1, c-Myc, CCND1, and CDK4, and increased the level of P27 (**Figure 4A**). Further, we found that EMT markers (N-cadherin, Vimentin, and Snail) were suppressed, whereas the E-cadherin level increased (**Figure 4B**). Simultaneously, the levels of p-PI3K, p-AKT, and β-catenin were significantly decreased (**Figure 4C**). Further, we observed that knockdown of HDGF significantly suppressed both nuclear and cytosol protein expressions of β-catenin in EC cells (**Figure 4D**). In subsequent study, we used specific inhibitor (Ly294002) of PI3K to suppress the expression of p-PI3K and observed that the protein expression of p-PI3k, p-AKT, β-catenin, Snail, N-cadherin, Vimentin was decreased, and E-cadherin was upregulated in overexpressing HDGF EC cells (**Figures 4E,F**). These results suggest that HDGF regulates the expression of cell cycle- and EMT-associated genes via the PI3K/AKT and β-catenin signaling pathways in EC cells.

DDX5 Interacts With HDGF and β-Catenin

In previous study, our team had shown the interaction of β-catenin and HDGF or DDX5 in Lung Adenocarcinoma (33). To explore the precise molecular mechanisms of HDGF in EC, co-IP, combined with mass spectrometry, was used in Ishikawa cells. This analysis yielded 242 potential HDGF-interacting proteins (**Supplementary Table 3**), including DDX5 (69 kDa band) and β-catenin (92 kDa band). In addition, we used data sets from public domain data to draw a Venn diagram to show the proteins that interact with HDGF (34) and β-catenin (35) proteins, and observed that there were 22 overlapping proteins (**Figure 5A**). Exogenous and endogenous co-IP demonstrated that HDGF and DDX5 interact in Ishikawa cells, whereas β-catenin was associated with DDX5, but not HDGF (**Figures 5B,C**). Moreover, nuclear co-localization of DDX5 and β-catenin proteins, as well as that of HDGF and DDX5 proteins (33), was observed by immunofluorescence using a scanning confocal microscope (**Figure 5D**). Taken together, these results suggest that HDGF is associated with DDX5 in EC, as is the case with β-catenin and DDX5.

Subsequently, western blotting indicated that stable knockdown of HDGF expression resulted in DDX5 protein downregulation (**Figure 5E**); however, knockdown of DDX5 did not significantly affect HDGF expression, but resulted in β-catenin protein downregulation (**Figure 5F**). In addition,

TABLE 1 | Correlation between the clinicopathological factors and the expression of HDGF and DDX5 in endometrial cancer.

	HDGF (%)				DDX5 (%)			
Characteristics	***N***	**High**	**Low**	***P***	***N***	**High**	**Low**	***P***
Age								
< 50	42	10(23.8)	32(76.2)	0.830	42	13(31.0)	29(69.0)	0.754
≧50	80	21(26.3)	59(73.7)		80	27(33.8)	53(66.2)	
Menopausal status								
Premenopausal	65	15(23.1)	50(76.9)	0.537	65	18(27.7)	47(72.3)	0.201
Postmenopausal	57	16(28.1)	41(71.9)		57	22(38.6)	35(61.4)	
FIGO stage								
I+II	90	18(20.0)	72(80.0)	0.032	90	20(22.2)	70(77.8)	< 0.001
III	32	13(40.6)	19(59.4)		32	20(62.5)	12(37.5)	
Histological grading								
G1	44	15(34.1)	29(65.9)	0.140	44	15(34.1)	29(65.9)	0.067
G2	62	11(17.7)	51(82.3)		62	16(25.8)	46(74.2)	
G3	16	5(31.3)	11(68.7)		16	9(56.3)	7(43.7)	
Depthof myometrial invasion								
< 50%	85	19(22.4)	66(77.6)	0.263	85	27(31.8)	58(68.2)	0.834
≧50%	37	12(32.4)	25(67.6)		37	13(35.1)	24(64.9)	
Lymph node status								
Negative	105	25(23.8)	80(76.2)	0.369	105	32(30.5)	73(69.5)	0.264
Positive	17	6(35.3)	11(64.7)		17	8(47.1)	9(52.9)	

we observed that β-catenin suppression decreased the level of the DDX5 protein (**Figure 5G**). Furthermore, overexpression of β-catenin from a plasmid in shHDGF-EC cells increased DDX5 expression (**Figure 5H**). qRT-PCR showed that the expression of DDX5 was reduced after knockdown of HDGF (**Figure 5I**), which indicated that HDGF affects DDX5 at the level of transcription.

DDX5 Overexpression Reverses the Suppression of shHDGF

Transiently transfecting DDX5 into shHDGF EC cells (**Figure 6A**) enhanced cell proliferation, as assessed by the MTT (**Figure 6B**) and EdU incorporation assays (**Figure 6C**). Transwell and Boyden chamber assays showed that EC cell migration and invasive ability were enhanced (**Figure 6D**). Furthermore, we found that DDX5 overexpression induced the expression of pRb, E2F1, c-Myc, CCND1, CDK4, N-cadherin, Vimentin, Snail, p-PI3K, p-AKT, and β-catenin, but reduced the expression of P27 and E-cadherin (**Figures 6E–G**). These results indicated that DDX5 overexpression can overcome the EC cell growth suppression induced by shHDGF.

Association of HDGF and DDX5 Expression With the Clinicopathological Characteristics of EC Tissues

Combined with data from our previous work (16), **Figures 7A–D** displays the expression of HDGF and DDX5. Immunohistochemical staining showed that HDGF and DDX5 positive signals were mostly located in the nuclei of EC cells, with minor cytoplasmic distribution. Our previous results indicated that 25.5% (31/122) and 74.5% (91/122) of cases exhibited high and low nuclear expression of HDGF, respectively (16). Here, we found that DDX5 protein was positively expressed in 32.8% (40/122) and not expressed in 67.2% of tumors (82/122; **Table 1**). DDX5 expression was significantly related to FIGO stage (I+II vs. III; $P < 0.001$; **Table 1**); however, no significant correlation existed with respect to age, menopausal status, histologic grading, depth of myometrial invasion, or lymph node status in patients with EC ($P > 0.05$; **Table 1**). The results were similar to those obtained for the HDGF protein.

Correlation Analysis of HDGF and DDX5 With Overall Survival of Patients With EC

We stratified the data from patients with EC into four groups, according to the combination of different levels of HDGF and DDX5 expression: low HDGF/low DDX5 (HDGF–/DDX5–) expression; high HDGF/high DDX5 (HDGF+/DDX5+) expression; low HDGF/high DDX5 (HDGF–/DDX5+) expression; and high HDGF/high DDX5 (HDGF+/DDX5+) expression. Our previous work showed that 25.5% (31/122) and 74.5% (91/122) of cases exhibited high and low nuclear expression of HDGF, respectively (16). Here, we found that 22 EC tissues were HDGF+/DDX5+, whereas 73 cases were HDGF–/DDX5– (**Table 2**). The expression of HDGF+/DDX5+ correlated with the FIGO stage ($P = 0.003$; **Table 2**).

Patients with high expression of DDX5 had worse prognoses than those with low expression of DDX5 ($P < 0.001$; **Figure 7E**), and HDGF+/DDX5+ also correlated with a short survival time for patients with EC ($P < 0.001$; **Figure 7F**). We further

TABLE 2 | Co-expression of HDGF and DDX5 in endometrial cancer.

Characteristics	N	HDGF and DDX5(%)				*P*
		HDGF+/DDX5+	HDGF+/DDX5-	HDGF–/DDX5+	HDGF–/DDX5–	
Age						0.988
< 50	42	7(16.7)	3(7.1)	6(14.3)	26(61.9)	
≧50	80	15(18.8)	6(7.5)	12(15.0)	47(58.8)	
Menopausal status						0.647
Premenopausal	65	10(15.4)	5(7.7)	8(12.3)	42(64.6)	
Postmenopausal	57	12(21.1)	4(7.0)	10(17.5)	31(54.4)	
FIGO stage						0.003
I+II	90	10(11.1)	8(8.9)	10(11.1)	62(68.9)	
III	32	12(37.5)	1(3.1)	8(25.0)	11(34.4)	
Histological grading						0.130
G1	44	11(25.0)	4(9.1)	4(9.1)	25(56.8)	
G2	62	6(9.7)	5(8.1)	10(16.1)	41(66.1)	
G3	16	5(31.3)	0(0)	4(25.0)	7(43.8)	
Depth of myometrial invasion						0.605
< 50%	85	13(15.3)	6(7.1)	14(16.5)	52(61.2)	
≧50%	37	9(24.3)	3(8.1)	4(10.8)	21(56.8)	
Lymph node status						0.546
Negative	105	17(16.2)	8(7.6)	15(14.3)	65(61.9)	
Positive	17	5(29.4)	1(5.9)	3(17.6)	8(47.1)	

conducted survival analysis by strata analysis against FIGO stage. These results indicated that high DDX5 protein levels and HDGF+/DDX5+ are significantly associated with the survival time for patients with EC based on FIGO stage = I+II ($P = 0.002$, $P = 0.011$, respectively; **Figures 7G,I**) and FIGO stage = III ($P = 0.012$, $P = 0.046$, respectively; **Figures 7H,J**).

Univariate analyses showed that FIGO stage, histological grading, lymph node status, depth of myometrial invasion, high DDX5 expression, and post-operative hormone therapy significantly correlated with patient survival ($P = 0.002$, $P = 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$, and $P = 0.044$, respectively; **Table 3**). Multivariate analysis showed that the level of DDX5 expression, FIGO stage, and depth of myometrial invasion were independent prognostic factors for EC ($P = 0.038$, $P = 0.018$, and $P = 0.014$, respectively; **Table 3**). However, HDGF expression, histological grading, lymph node status, and post-operative chemotherapy were not independent prognostic factors for EC ($P = 0.984$, $P = 0.160$, $P = 0.702$, and $P = 0.631$, respectively; **Table 3**).

Correlation Between HDGF and DDX5 Expression in EC

There was a significant positive correlation between HDGF and DDX5 protein levels in EC tissues ($r = 0.475$, $P < 0.001$; **Table 4**).

DISCUSSION

In previous studies, HDGF has been shown to promote the progression of tumors by activating the AKT–MAPK (36), Akt and TGF-β (37), and VEGF signaling pathways (38), and interacting with β-catenin as a positive feedback loop (39); however, the molecular mechanism involved in HDGF-associated EC cell proliferation, invasion, and metastasis has not been elucidated.

In the present study, we observed that HDGF knockdown markedly decreased cell proliferation, migration, invasion, and metastasis *in vitro*. Furthermore, subcutaneous tumor

TABLE 3 | Summary of univariate and multivariate Cox regression analysis of overall survival duration.

Parameter	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age						
< 50 vs. ≥50	0.093	0.401	0.138–1.165			
Family history of tumor						
Negative vs. positive	0.279	0.325	0.043–2.487			
Education						
< Graduation vs. ≥graduation	0.298	26.921	0.05–13271.753			
Health insurance						
No vs. yes	0.089	0.020	0.000–1.811			
Career						
≦Worker vs. >worker	0.272	27.978	0.07–10713.674			
Menopausal status						
Premenopausal vs. postmenopausal	0.559	0.721	0.240–2.160			
Complications						
With vs. without	0.125	0.309	0.069–1.384			
FIGO stags						
I +II vs. III	0.002	5.652	1.892–16.883	0.018	4.385	1.283–14.994
Histological grading						
G1 vs. G2 vs. G3	0.001	4.514	1.896–10.745	0.160	2.178	0.735–6.453
Lymph node status						
Negative vs. positive	< 0.001	12.232	4.196–35.659	0.702	1.477	0.200–10.905
Depth of myometrial invasion						
< 50% vs. ≥50%	< 0.001	9.745	2.713–34.999	0.014	7.867	1.522–40.678
HDGF expression						
Low vs. high	0.004	4.951	1.686–14.544	0.984	0.985	0.245–3.964
DDX5 expression						
Low vs. high	< 0.001	12.126	3.209–45.821	0.038	5.677	1.100–29.281
Postoperative irradiation						
Yes vs. no	0.512	1.652	0.368–7.409			
Postoperative chemotherapy						
Yes vs. no	0.175	2.081	0.721–6.005			
Postoperative hormone therapy						
Yes vs. no	0.044	0.267	0.074–0.963	0.631	1.491	0.293–7.597

experiments in nude mice demonstrated that knockdown of HDGF inhibited tumorigenesis, invasion, and metastasis *in vivo*. These findings are consistent with an earlier report by Zhou et al. (40) in which downregulation of HDGF inhibited the proliferation and invasiveness of hepatocellular carcinoma cells.

Multiple studies have shown that PI3K/AKT constitutes a key signal mediator during carcinogenesis (41, 42) and that activation of PI3K/AKT may regulate β-catenin signaling through Akt phosphorylation and inactivation of GSK3-β (43). In the current study, we observed that decreased HDGF expression suppressed p-PI3K and p-AKT levels, and the downstream β-catenin-mediated cell cycle and EMT signal molecules, such as pRb, E2F1, c-Myc, CCND1, CDK4, Snail, Vimentin, and N-cadherin, while elevating the expression of P27 and E-cadherin in EC cells. In addition, using Ly294002 to treat overexpressing HDGF EC cells induced a decrease of p-PI3K, p-AKT, β-catenin and metastatic effect related proteins such as Snail, Vimentin, and N-cadherin. Therefore, we hypothesized that HDGF knockdown significantly suppresses EC cell proliferation, migration, invasion, and metastasis through the PI3K/AKT and β-catenin pathways in EC.

To better understand the molecular mechanisms underlying HDGF promotion of EC proliferation and metastasis, we searched public domain data and screened DDX5 as a candidate interaction protein of HDGF. DDX5 is a member of DEAD box family proteins that plays an important role in the progression of many tumors (44–48). Subsequently, we observed that HDGF not only combined with DDX5 but also induced the expression of DDX5 in EC. Furthermore, DDX5 could reverse the inhibitive effects on cell growth, migration, and invasion in shHDGF EC cells. Previously, HDGF was found to bind the promoter of β-catenin, resulting in β-catenin transcriptional activation (39). Phosphorylated p68 (DDX5) can enter the cytoplasm, which leads to its interaction with β-catenin and displacement of Axin (49). DDX5 (P68) forms a complex with β-catenin and facilitates its transcription activation to regulate both cell adhesion and gene expression (23). Notably, as β-catenin can, in turn, function as a transcription factor to stimulate DDX5 expression by binding to its promoter (50), we thus speculated that HDGF upregulated the expression of DDX5 by inducing β-catenin expression. Consistent with this speculation, we observed that DDX5 was significantly increased in shHDGF-EC cells after transfection of the β-catenin cDNA.

In previous reports, DDX5 had been documented to promote cell proliferation and EMT by interacting with Wnt-β-catenin signaling and inducing the nuclear translocation of β-catenin (23, 26, 28, 51). Consistent with these previous reports, we observed that DDX5 not only directly combined with β-catenin, but also induced the expression of β-catenin via activating PI3K/AKT signaling and stimulated its nuclear translocation, thus inducing cell cycle transition and EMT signaling and the promotion of cell growth and metastasis in EC. These data demonstrated that HDGF interacts with DDX5 to further induce β-catenin to participate in cell growth and metastasis in EC.

In our previous study of HDGF expression in 122 samples of EC, we found that high nuclear expression of HDGF was positively correlated with FIGO stage ($P = 0.032$) and that patients with high expression of HDGF had poorer overall survival rates compared to those with low expression ($P = 0.001$) (16). In the current study, we further showed that DDX5 protein was expressed in 32.8% (40/122) of EC samples. Similar to HDGF, DDX5 expression was also significantly associated with FIGO stage ($P < 0.001$), and patients with high expression of DDX5 had poorer overall survival rates than those with low expression ($P < 0.001$). Multivariate analyses showed that high expression of DDX5 was an independent predictor of prognosis for patients with EC ($P = 0.038$). Notably, our data showed a significant positive correlation between HDGF and DDX5 in EC tissues ($r = 0.475$, $P < 0.001$). Finally, we observed that high nuclear levels of HDGF and DDX5 led to the worst prognosis for patients with EC.

CONCLUSIONS

In summary, this study provides compelling evidence that HDGF in combination with DDX5 induces β-catenin to form a complex, which significantly promotes EC cell proliferation, migration,

TABLE 4 | Correlation between HDGF and DDX5 expression in endometrial cancer tissues.

	HDGF		*r*	*p*
	High	Low		
DDX5			0.475	< 0.001
High	22	18		
Low	9	73		

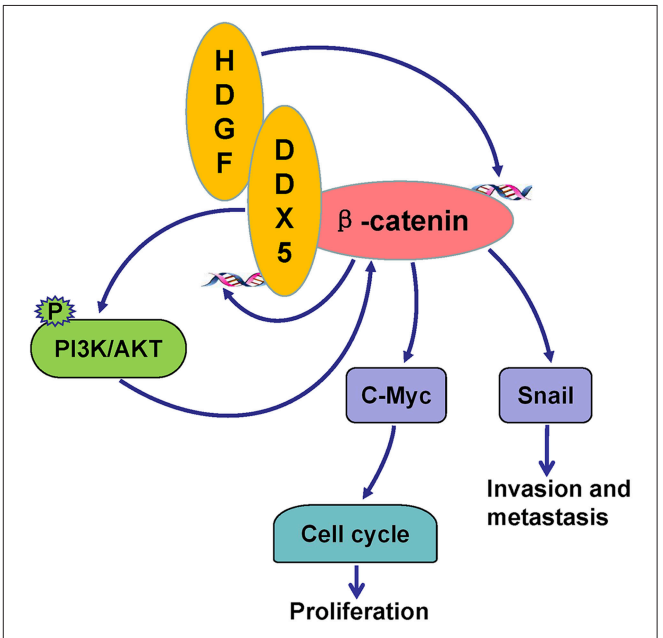


FIGURE 8 | Potential signaling pathways utilized by HDGF/DDX5/β-catenin to promote proliferation, invasion, and metastasis in EC.

invasion, and metastasis *in vitro* and *in vivo*. The underlying mechanism likely involves the activation of PI3K/AKT signaling and downstream β-catenin-mediated cell cycle and EMT signaling proteins (**Figure 8**). Our results suggest that HDGF–DDX5 and β-catenin work together to play important roles in EC carcinogenesis and progression.

ETHICS STATEMENT

For the use of the human tissue specimens for research purposes, prior consent from the patients and approval from the Ethics Committees of the Third Affiliated Hospital of Guangzhou Medical University were obtained. All animal studies were conducted in accordance with the principles and procedures outlined in the Southern Medical University Guide for the Care and Use of Animals.

AUTHOR CONTRIBUTIONS

CL, LW, QJ, and JZ conducted the research. SG and WF designed the research study. CL, LZ, LL, HJ, DL, and YX performed the statistical analysis. CL, LW, LZ, and WF wrote the manuscript. All authors have read and approved the final manuscript.

FUNDING

This project was funded by the Nature Science Fund of Guangdong Province (2015A030313240) and the Medical Research Fund of Guangdong Province (A2015467).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00211/full#supplementary-material

REFERENCES

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer.* (2015) 136:E359–86. doi: 10.1002/ijc.29210
2. Morice P, Leary A, Creutzberg C, Abu-Rustum N, Darai E. Endometrial cancer. *Lancet.* (2016) 387:1094–108. doi: 10.1016/S0140-6736(15)00130-0
3. McAlpine JN, Temkin SM, Mackay HJ. Endometrial cancer: not your grandmother's cancer. *Cancer.* (2016) 122:2787–98. doi: 10.1002/cncr.30094
4. Huang JS, Chao CC, Su TL, Yeh SH, Chen DS, Chen CT, et al. Diverse cellular transformation capability of overexpressed genes inhuman hepatocellular carcinoma. *Biochem Biophys Res Commun.* (2004) 315:950–8. doi: 10.1016/j.bbrc.2004.01.151
5. Min X, Wen J, Zhao L, Wang K, Li Q, Huang G, et al. Role of hepatoma-derived growth factor in promoting de novo lipogenesis and tumorigenesis in hepatocellular carcinoma. *Mol Oncol.* (2018) 12:1480–97. doi: 10.1002/1878-0261.12357
6. Everett AD, Stoops T, McNamara CA. Nuclear targeting isrequired for hepatoma-derived growth factor-stimulated mitogenesisin vascular smooth muscle cells. *J Biol Chem.* (2001) 276:37564–8. doi: 10.1074/jbc.M105109200
7. Everett AD, Bushweller J. Hepatoma derived growth factor is anuclear targeted mitogen. *Curr Drug Targets.* (2003) 4:367–71. doi: 10.2174/1389450033490975
8. Kishima Y, Yamamoto H, Izumoto Y, Yoshida K, Enomoto H, Yamamoto M, et al. Hepatoma-derived growth factor stimulatescell growth after translocation to the nucleus by nuclear localization signals. *J Biol Chem.* (2002) 277:10315–22. doi: 10.1074/jbc.M111122200
9. Lin YW, Li CF, Chen HY, Yen CY, Lin LC, Huang CC, et al. The expression and prognostic significance of hepatoma-derived growth factor in oral cancer. *Oral Oncol.* (2012) 48:629–35. doi: 10.1016/j.oraloncology.2012.01.014
10. Yamamoto S, Tomita Y, Hoshida Y, Morii E, Yasuda T, Doki Y, et al. Expression level of hepatoma-derived growth factor correlates with tumor recurrence of esophageal carcinoma. *Ann Surg Oncol.* (2007) 14:2141–9. doi: 10.1245/s10434-007-9369-9
11. Chang KC, Tai MH, Lin JW, Wang CC, Huang CC, Hung CH, et al. Hepatoma-derived growth factor is a novel prognosticfactor for gastrointestinal stromal tumors. *Int J Cancer.* (2007) 121:1059–65. doi: 10.1002/ijc.22803
12. Yamamoto S, Tomita Y, Hoshida Y, Takiguchi S, Fujiwara Y, Yasuda T, et al. Expression of hepatoma-derived growth factor is correlated with lymph node metastasis and prognosis of gastric carcinoma. *Clin Cancer Res.* (2006) 12:117–22. doi: 10.1158/1078-0432.CCR-05-1347
13. Dunn J, Ferluga S, Sharma V, Futschik M, Hilton DA, Adams CL, et al. Proteomic analysis discovers the differential expression of novel proteins and phosphoproteins in meningioma including NEK9, HK2 and SET and deregulation of RNA metabolism. *EBioMedicine.* (2019) 40:77–91. doi: 10.1016/j.ebiom.2018.12.048
14. Yoshida K, Tomita Y, Okuda Y, Yamamoto S, Enomoto H, Uyama H, et al. Hepatoma-derived growth factor is a novel prognostic factor for hepatocellular carcinoma. *Ann Surg Oncol.* (2006) 13:159–67. doi: 10.1245/ASO.2006.11.035
15. Ren H, Tang X, Lee JJ, Feng L, Everett AD, Hong WK, et al. Expression of hepatoma-derived growth factor is a strong prognostic predictor for patients with early-stage non–small-cell lung cancer. *J Clin Oncol.* (2004) 22:3230–7. doi: 10.1200/JCO.2004.02.080
16. Wang L, Jiang Q, Hua S, Zhao M, Wu Q, Fu Q, et al. High nuclear expression of HDGF correlates with disease progression and poor prognosis in human endometrial carcinoma. *Dis Markers.* (2014) 2014:298795. doi: 10.1155/2014/298795
17. Fuller-Pace FV. The DEAD box proteins DDX5 (p68) and DDX17 (p72): multi-tasking transcriptional regulators. *Biochim Biophys Acta.* (2013) 1829:756–63. doi: 10.1016/j.bbagrm.2013.03.004
18. Métivier R, Penot G, Hübner MR, Reid G, Brand H, Kos M, et al. Estrogen receptor-α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell.* (2003) 115:751–63. doi: 10.1016/S0092-8674(03)00934-6
19. Watanabe M, Yanagisawa J, Kitagawa H, Takeyama K, Ogawa S, Arao Y, et al. A subfamily of RNA-binding DEAD-box proteins acts as an estrogen receptor α coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator, SRA. *EMBO J.* (2001) 20:1341–52. doi: 10.1093/emboj/20.6.1341
20. Clark EL, Coulson A, Dalgliesh C, Rajan P, Nicol SM, Fleming S, et al. The RNA helicase p68 is a novel androgen receptor coactivator involved in splicing and is overexpressed in prostate cancer. *Cancer Res.* (2008) 68:7938–46. doi: 10.1158/0008-5472.CAN-08-0932
21. Bates GJ, Nicol SM, Wilson BJ, Jacobs AM, Bourdon JC, Wardrop J, et al. The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor. *EMBO J.* (2005) 24:543–53. doi: 10.1038/sj.emboj.7600550
22. Caretti G, Schiltz RL, Dilworth FJ, Padova MD, Zhao P, Ogryzko V, et al. The RNA helicases p68/p72 and the non-coding RNA SRA are coregulators of MyoD and skeletal muscle differentiation. *Dev Cell.* (2006) 11:547–60. doi: 10.1016/j.devcel.2006.08.003

23. Shin S, Rossow KL, Grande JP, Janknecht R. Involvement of RNA Helicases p68 and p72 in Colon Cancer. *Cancer Res.* (2007) 67:7572–8. doi: 10.1158/0008-5472.CAN-06-4652
24. Yang L, Lin C, Zhao S, Wang H, Liu ZR. Phosphorylation of p68 RNA helicase plays a role in platelet-derived growth factor-induced cell proliferation by upregulating cyclin D1 and c-Myc expression. *J Biol Chem.* (2007) 282:16811–9. doi: 10.1074/jbc.M610488200
25. Wang H, Gao X, Yang JJ, Liu ZR. Interaction between p68 RNA helicase and Ca2+-calmodulin promotes cell migration and metastasis. *Nat Commun.* (2013) 4:1354. doi: 10.1038/ncomms2345
26. Yang L, Lin C, Liu ZR. P68 RNA helicase mediates PDGF-induced epithelial mesenchymal transition by displacing Axin from beta-catenin. *Cell.* (2006) 127:139–55. doi: 10.1016/j.cell.2006.08.036
27. Wang D, Huang J, Hu Z. RNA helicase DDX5 regulates microRNA expression and contributes to cytoskeletal reorganization in basal breast cancer cells. *Mol Cell Proteomics*. (2012) 11:M111.011932. doi: 10.1074/mcp.M111.011932
28. Mazurek A, Luo W, Krasnitz A, Hicks J, Powers RS, Stillman B. DDX5 regulates DNA replication and is required for cell proliferation in a subset of breast cancer cells. *Cancer Discov.* (2012) 2:812–25. doi: 10.1158/2159-8290.CD-12-0116
29. Xing Z, Ma WK, Tran EJ. The DDX5/Dbp2 subfamily of DEAD-box RNA helicases. *Wiley Interdiscip Rev RNA*. (2019) 10:e1519. doi: 10.1002/wrna.1519
30. Zhao M, Fang W, Wang Y, Guo S, Shu L, Wang L, et al. Enolase-1 is a therapeutic target in endometrial carcinoma. *Oncotarget.* (2015) 6:15610–27. doi: 10.18632/oncotarget.3639
31. ZhaoM, LuoR, LiuY, GaoL, FuZ, FuQ, et al. miR-3188 regulates nasopharyngeal carcinoma proliferation and chemosensitivity through a FOXO1-modulated positive feedback loop with mTOR-p-PI3K/AKT-c-JUN. *Nat Commun.* (2016) 7:11309. doi: 10.1038/ncomms11309
32. Zhen Y, LiuZ, Yang H, Yu X, Wu Q, Hua S, et al. Tumor suppressor PDCD4 modulates miR-184-mediated direct suppression of C-MYC and BCL2 blocking cell growth and survival in nasopharyngeal carcinoma. *Cell Death Dis.* (2013) 4:e872. doi: 10.1038/cddis.2013.376
33. Fu Q, Song X, Liu Z, Deng X, Luo R, Ge C, et al. miRomics and proteomics reveal a miR-296–3p/PRKCA/FAK/Ras/c-Myc feedback loop modulated by HDGF/DDX5/beta-catenin complex in lung adenocarcinoma. *Clin Cancer Res.* (2017) 23:6336–50. doi: 10.1158/1078-0432.CCR-16-2813
34. Physical protein-protein interactions with reliability scores. Available online at: http://hintdb.hgc.jp/htp/proteins/P51858.html (accessed 20 January, 2018).
35. Physical protein-protein interactions with reliability scores. Available online at: http://hintdb.hgc.jp/htp/proteins/P35222.html (accessed 20 January, 2018).
36. Thakar K, Kröcher T, Savant S, Gollnast D, Kelm S, Dietz F. Secretion of hepatoma-derived growth factor is regulated by n-terminal processing. *Biol Chem.* (2010) 391:1401–10. doi: 10.1515/BC.2010.147
37. Song Y, Hu Z, Long H, Peng Y, Zhang X, Que T, et al. A complex mechanism for HDGF-mediated cell growth, migration, invasion, and TMZ chemosensitivity in glioma. *J Neurooncol.* (2014) 119:285–95. doi: 10.1007/s11060-014-1512-4
38. Liu YF, Zhao R, Guo S, Wang XQ, Lian PL, Chen YG, et al. Expression and clinical significance of hepatoma-derived growth factor as a prognostic factor in human hilar cholangiocarcinoma. *Ann Surg Oncol.* (2011) 18:872–9. doi: 10.1245/s10434-010-1303-x
39. Lian J, Tang J, Shi H, Li H, Zhen T, Xie W, et al. Positive feedback loop of hepatoma-derived growth factor and beta-catenin promotes carcinogenesis of colorectal cancer. *Oncotarget*. (2015) 6:29357–74. doi: 10.18632/oncotarget.4982
40. Xiong Y, Deng Y, Wang K, Zhou H, Zheng X, Si L, et al. Profiles of alternative splicing in colorectal cancer and their clinical significance: A study based on large-scale sequencing data. *EBioMedicine.* (2018) 36:183–95. doi: 10.1016/j.ebiom.2018.09.021
41. Han K, Xu X, Chen G, Zeng Y, Zhu J, Du X, et al. Identification of a promising PI3K inhibitor for the treatment of multiple myeloma through the structural optimization. *J Hematol Oncol.* (2014) 7:9. doi: 10.1186/1756-8722-7-9
42. Akinleye A, Avvaru P, Furqan M, Song Y, Liu D. Phosphatidylinositol 3-kinase (PI3K) inhibitors as cancer therapeutics. *J Hematol Oncol.* (2013) 6:88. doi: 10.1186/1756-8722-6-88
43. Pap M, Cooper GM. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J Biol Chem.* (1998) 273:19929–32. doi: 10.1074/jbc.273.32.19929
44. Causevic M, Hislop RG, Kernohan NM, Carey FA, Kay RA, Steele RJ, et al. Overexpression and poly-ubiquitylation of the DEAD-box RNA helicase p68 in colorectal tumours. *Oncogene.* (2001) 20:7734–43. doi: 10.1038/sj.onc.1204976
45. Wortham NC, Ahamed E, Nicol SM, Thomas RS, Periyasamy M, Jiang J, et al. The DEAD-box protein p72 regulates ERalpha-/oestrogen-dependent transcription and cell growth, and is associated with improved survival in ERalpha-positive breast cancer. *Oncogene.* (2009) 28:4053–64. doi: 10.1038/onc.2009.261
46. Beier UH, Maune S, Meyer JE, Gorogh T. Overexpression of p68 mRNA in head and neck squamous cell carcinoma cells. *Anticancer Res.* (2006) 26:1941–6.
47. Ma Z, Kong R, Feng J, Ma Y, Sun L, Yang X, et al. Knockdown of DDX5 inhibits the proliferation and tumorigenesis in esophageal cancer. *Oncol Res.* (2017) 25:887–95. doi: 10.3727/096504016X14817158982636
48. Taniguchi T, Iizumi Y, Watanabe M, Masuda M, Morita M, Aono Y, et al. Resveratrol directly targets DDX5 resulting in suppression of the mTORC1 pathway in prostate cancer. *Cell Death Dis.* (2016) 7:e2211. doi: 10.1038/cddis.2016.114
49. Wang H, Gao X, Huang Y, Yang J, Liu ZR. P68 RNA helicase is a nucleocytoplasmic shuttling protein. *Cell Res.* (2009) 19:1388–400. doi: 10.1038/cr.2009.113
50. Guturi KK, Sarkar M, Bhowmik A, Das N, Ghosh MK. DEAD-box protein p68 is regulated by beta-catenin/transcription factor 4 to maintain a positive feedback loop in control of breast cancer progression. *Breast Cancer Res.* (2014) 16:496. doi: 10.1186/s13058-014-0496-5
51. Wang Z, Luo Z, Zhou L, Li X, Jiang T, Fu E. DDX5 promotes proliferation and tumorigenesis of non-small-cell lung cancer cells by activating beta-catenin signaling pathway. *Cancer Sci.* (2015) 106:1303–12. doi: 10.1111/cas.12755

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Oncology

SYSTEMATIC REVIEW
published: 07 May 2019
doi: 10.3389/fonc.2019.00359

Anti-estrogen Treatment in Endometrial Cancer: A Systematic Review

Willem Jan van Weelden[1]*, *Leon F. A. G. Massuger*[1], *ENITEC*[†], *Johanna M. A. Pijnenborg*[1‡] *and Andrea Romano*[2‡]

[1] Department of Obstetrics and Gynecology, Radboud Institute for Health Sciences, Radboud University Medical Center, Nijmegen, Netherlands, [2] Department of Obstetrics and Gynecology, GROW–School for Oncology and Developmental Biology, Maastricht University Medical Centre, Maastricht, Netherlands

Introduction: Hormonal therapy in endometrial cancer (EC) is used for patients who wish to preserve fertility and for patients with advanced or recurrent disease in a palliative setting. First line hormonal therapy consists of treatment with progestins, which has a response rate of 25% in an unselected population. Treatment with anti-estrogens is an alternative hormonal therapy option, but there is limited data on the effect and side-effects of anti-estrogens in EC. Therefore, we performed a systematic review to investigate the response rate and toxicity of anti-estrogenic therapy in patients with endometrial cancer.

Methods: A systematic search in electronic databases was performed to identify studies on selective estrogen receptor modulators (SERM) and down-regulators (SERD) and aromatase inhibitors that reported on response rates (RR) among EC patients. Outcome in estrogen receptor (ER) positive and negative disease was assessed independently.

Results: Sixteen studies on advanced stage and recurrent EC were included. Ten studies investigated anti-estrogen monotherapy and seven investigated a combination of anti-estrogenic drugs with either progestin or targeted treatment. Due to heterogeneity in patient population, no meta-analysis was performed. The median age of the patients in the included studies ranged from 61 to 71 years and the proportion of low grade tumors ranged from 38 to 80%. The RR for tamoxifen ranged from 10 to 53%, for other SERMs and SERDs 9–31%, for aromatase inhibitors from 8 to 9%, for combined tamoxifen/progestin treatment 19–58%, for combined chemo- and hormonal therapy 43% and for combination of anti-estrogenic treatment with mammalian target of rapamycin (mTOR) inhibitors 14–31%. Toxicity consisted mainly of nausea and thrombotic events and was higher in combination therapy of chemotherapy and hormonal therapy and hormonal therapy and mTOR inhibitors compared to other therapies.

Conclusion: Tamoxifen or a combination of tamoxifen and progestin should be the preferred choice when selecting second line hormonal treatment because the RRs are similar to first line progestin treatment and the toxicity is low. The response can be optimized by selecting patients with endometrioid tumors and positive estrogen receptor status, which should be based on a pretreatment biopsy.

Keywords: endometrial cancer, anti-estrogen, tamoxifen, fulvestrant, aromatase inhibitor, review

OPEN ACCESS

Edited by:
Paola Gehrig,
University of North Carolina at Chapel Hill, United States

Reviewed by:
Emma Rossi,
University of North Carolina at Chapel Hill, United States
Kevin Schuler,
TriHealth, United States

***Correspondence:**
Willem Jan van Weelden
willemjan.vanweelden@radboudumc.nl

[†]*ENITEC: European Network for Individualised Treatment of Endometrial Cancer (within the European Society of Gynaecological Oncology: www.esgo.org; www.esgo.org/network/enitec/)*

[‡]*These authors share last authorship*

Specialty section:
This article was submitted to Women's Cancer, a section of the journal Frontiers in Oncology

Received: *14 January 2019*
Accepted: *18 April 2019*
Published: *07 May 2019*

Citation:
van Weelden WJ, Massuger LFAG, ENITEC, Pijnenborg JMA and Romano A (2019) Anti-estrogen Treatment in Endometrial Cancer: A Systematic Review. Front. Oncol. 9:359. doi: 10.3389/fonc.2019.00359

INTRODUCTION

Endometrial cancer (EC) is the most common gynecologic malignancy in the Western world (1). The incidence of EC is increasing and is expected to rise further in the coming years (2). The most important risk factors for the development of EC are related to exogenous or endogenous estrogen exposure, including: estrogen medication, nulliparity, early menarche, late menopause, and obesity, which contributes to estrogen exposure by aromatase dependent conversion of androgen into estrogen (3–7). In general, two types of EC are identified based on tumor histology and presumed carcinogenesis. Endometrioid EC (EEC) represents 80% of EC cases and most EECs are caused by an excess estrogen exposure that, in the absence of counteractive effects of progesterone, induces endometrial proliferation and subsequent endometrial hyperplasia and cancer (8). Non-endometrioid EC (NEEC) is responsible for 20% of EC incidence and is assumed to develop independent of estrogen (8, 9). Standard therapy for EC consists of surgery followed by adjuvant radio- and/or chemotherapy depending on final tumor characteristics (10, 11). Hormonal therapy is an alternative treatment for patients who wish to preserve their fertility, and for those with metastatic or recurrent disease without curative options (12). Historically, progestin therapy has been the most widely applied hormonal treatment and it is still the preferred choice as first line hormonal therapy (10, 13). In addition to progestins, inhibition of estrogen-induced proliferation by anti-estrogens is used as an alternative to progestin treatment in EC (14). Currently used anti-estrogenic drugs are selective estrogen receptor modulators (SERM) or down-regulators (SERD) and aromatase inhibitors. SERMs and SERDs such as tamoxifen and fulvestrant have an anti-proliferative effect by blocking the estrogen receptor (ER) through which estrogen effects are mediated. Within the group of SERMs, tamoxifen has both stimulatory and blocking effects on ER in the endometrium, while other SERMs like raloxifene and arzoxifene only block ER (15–17). Fulvestrant, the main SERD, only has antagonistic effects through down regulation of ER (18). Aromatase inhibitors like anastrozole, letrozole, and exemestane, limit the estrogen tumor exposure by aromatase in fat tissue, especially in postmenopausal women (12). The use of anti-estrogens is well established in breast cancer, but up till now, there is limited data on the response rates in EC. In one systematic review and meta-analysis, first and second line hormonal therapy in recurrent EC was evaluated, but the different types of hormonal therapy were not evaluated separately (19). Two separate reviews presented an overview of available (pre)clinical evidence on, respectively, fulvestrant and aromatase inhibitors. Unfortunately, no complete overview of anti-estrogenic treatment was given (20, 21). As a consequence, choice for anti-estrogenic drugs as second line hormonal therapy is based on experience of the treating physician, rather than on refined and up-to-date clinical data. Therefore, we performed this systematic review to determine the response rates and toxicity of anti-estrogenic therapy in patients with endometrial cancer and to relate it to the response rate of progestin therapy.

METHODS

Search Strategy

This review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (22). An electronic search was performed in the following databases from inception until 3rd of October 2018: Pubmed, Embase, clinicaltrials.gov and Cochrane database of Systematic Review. The search string included *"endometrial cancer,"* outcome measures like *"response rate," "disease progression,"* or *"survival"* and drug terms like *"estrogen antagonists," "aromatase inhibitors," "estrogen receptor modulators," "estrogen receptor down-regulator,"* and individual drug names. The full search string is shown in **Supplementary Table 1**. Citations of relevant articles and reviews were manually screened to ensure that no study was missed and that the search was complete.

Study Selection

Studies were included if they reported on (1) women with endometrial cancer, who used anti-estrogenic therapy for fertility preservation or for advanced or recurrent disease. Studies investigating (2) estrogen receptor modulators, estrogen receptor down-regulators or aromatase inhibitors were eligible if (3) clinical outcome was reported. Studies reporting findings on patients with sarcomas or endometrial stroma sarcomas were excluded as well as conference papers, reviews and letters to the editor. Case reports and case series with <10 patients were excluded. Studies that combined anti-estrogen treatment with other therapy, i.e., progestins, chemotherapy, or targeted therapy were included and reviewed separately.

Data Extraction and Quality Assessment

Data from included articles was extracted using data collection forms with information regarding study design, in- and exclusion criteria, number of included patients, age, tumor stage and grade, estrogen (ER) and progesterone receptor status, previous treatment(s) and complete response (CR), partial response (PR), stable disease (SD), progressive disease, progression free survival (PFS), and overall survival (OS) was noted. Additional information was requested from study authors if necessary.

The quality of each individual study was assessed in five domains based of the National Institute of Health Quality Assessment Tool for Case Series Studies (23). Each full-text article was evaluated independently by three authors (WvW, JP, and AR) and risk of bias was subsequently discussed in a consensus meeting.

Outcome Assessment and Statistical Analyses

The primary outcome was the response rate (RR) to hormonal therapy and was defined as the proportion of patients with CR and PR. Other outcomes were the clinical benefit rate (CBR), which is defined as the proportion of patients with either CR, PR, or SD and toxicity which is defined as any adverse event occurring during treatment. Toxicity was ideally evaluated with a standardized measuring scale including grading of severity.

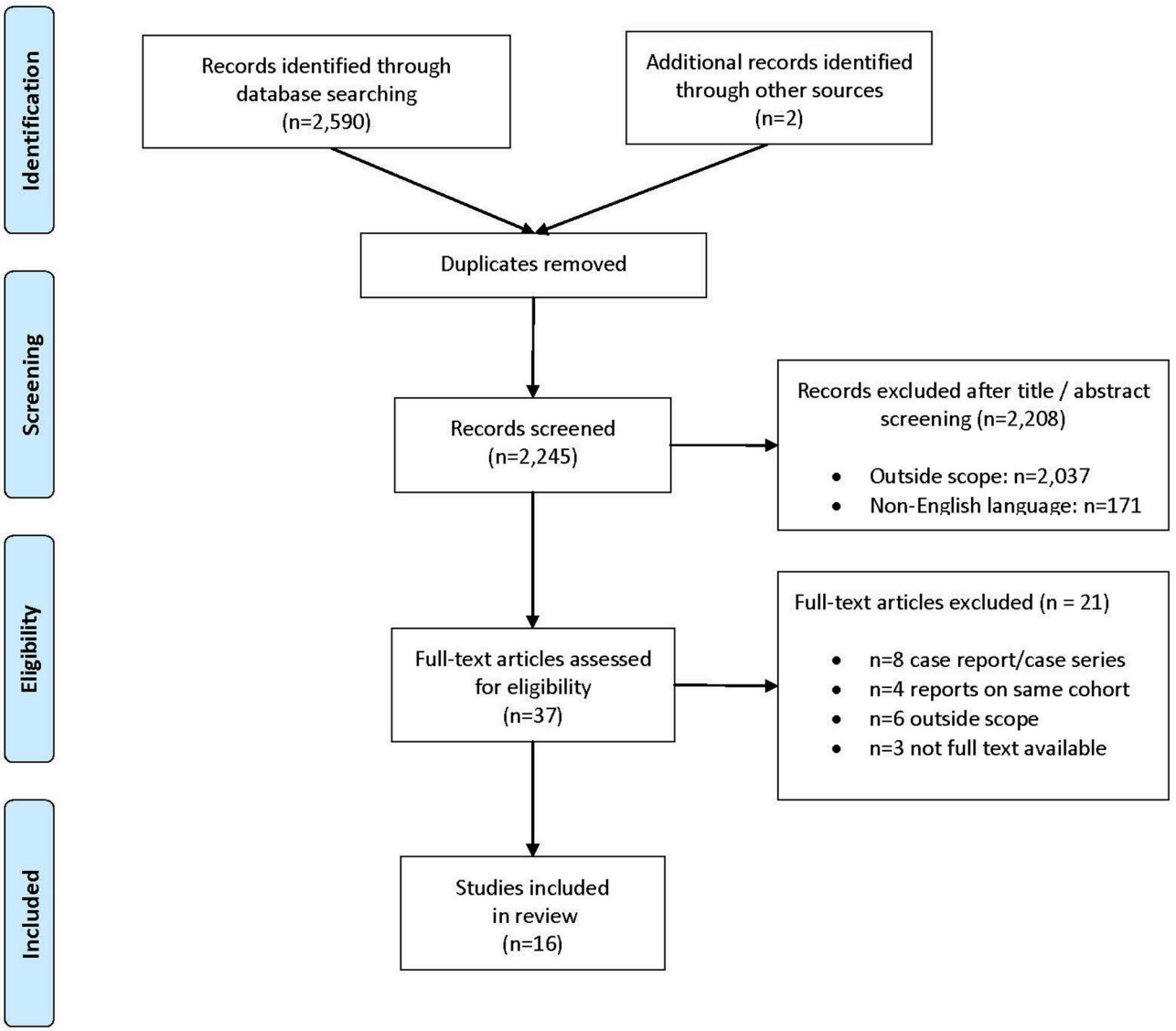


FIGURE 1 | Selection of studies for systematic review.

Individual treatment arms of randomized studies were analyzed separately. RR and CBR are reported for tamoxifen, other SERMs/SERDs, aromatase inhibitors, combination regimens and for ER positive and negative tumors separately. The specific expression of the two ER isoforms (ERα and ERβ) was not considered. Due to the large heterogeneity in the included studies, meta-analysis could not be performed. In case it was not reported in the study, the 95% confidence interval for RR and CBR was calculated using the normal approximation method of the binomial confidence interval (24).

RESULTS

The search resulted in identification of 2,592 records. After removal of duplicates, 2,245 unique records were screened on title and abstract. For the systematic review, 2,208 records were excluded, leaving 37 articles for full text evaluation (**Figure 1**). A total of 21 articles were excluded from the final analysis due to: case reports or case series with <10 patients ($n = 8$), reports on the same patient cohort ($n = 4$) or studies that were outside the scope of the review ($n = 6$), including studies on endometrial stroma sarcoma and studies on chemotherapy and radiotherapy (25–30). Three other studies published between 1983 and 1990 could not be evaluated because the full text articles were not available (31–33). In addition, nine ongoing studies were identified (34–42).

Included Studies

Sixteen studies were included in the final systematic review. All included studies investigated patients with advanced stage and

TABLE 1 | Bias assessment.

References	Blinded treatment	Robust outcome assessment	Incomplete outcome data	Selective outcome reporting?	Other problems that introduced bias	Any disclosure reported by the authors	Total
Bonte et al. (43)	☹	☹	☺	☺	☹	Not specified	☹
Rendina et al. (44)	☹	☺	☺	☺	☺	Not specified	☺
Quinn and Campbell (46)	☹	☺	☺	☺	☺	Not specified	☺
Thigpen et al. (49)	☹	☺	☺	☺	☺	Not specified	☺
McMeekin et al. (50)	☹	☺	☺	☹	☺	Support and co-author from Lilly	☺
Covens et al. (54)	☹	☺	☺	☺	☺	Not specified	☺
Emons et al. (55)	☹	☺	☺	☺	☺	Support from Astra Zeneca	☺
Rose et al. (47)	☹	☺	☺	☺	☺	Nothing to disclose	☺
Ma et al. (52)	☹	☺	☺	☺	☺	Support from Novartis	☺
Lindemann et al. (57)	☹	☺	☺	☺	☺	Nothing to disclose	☺
Pandya et al. (48)	☹	☺	☺	☹	☹	Not specified	☹
Fiorica et al. (51)	☹	☺	☺	☺	☺	Nothing to disclose	☺
Whitney et al. (53)	☹	☺	☺	☺	☺	Nothing to disclose	☺
Ayoub et al. (45)	☹	☺	☺	☺	☺	Support from ICI Americas Inc.	☺
Fleming et al. (56)	☹	☺	☺	☺	☹	Nothing to disclose	☺
Slomovitz et al. (58)	☹	☺	☺	☺	☺	Support from Novartis	☺

☺, *Low risk of bias;* ☹, *High risk of bias.*

recurrent EC. Ten studies described the use of monotherapy of which one reported outcomes on mono- and combined therapy, resulting in a total of seven studies on combined therapy (43–58). There were two case series and 14 prospective studies. Bias was assessed as recommended by the National Institute of Health on five criteria (blinded treatment arms, robustness of outcome assessment, completeness of the data, selective outcome reporting and other biases) (23). Results of bias assessment and conflict of interest disclosures are shown in **Table 1**. Blinded treatment was not performed in any study included in the systematic review and was therefore regarded as high risk in all studies. Outcome assessment was performed with objective and reproducible criteria in all but one study (43). Two studies had a high risk of bias in three domains and were considered low quality studies (43, 48). All included studies investigated the effect of hormonal therapy among patients with advanced or recurrent EC.

Anti-estrogens as Monotherapy

An overview of the included studies that evaluated anti-estrogens as monotherapy in advanced and recurrent EC is shown in **Table 2**. Four studies investigated the use of tamoxifen, three studies investigated other SERMs or SERDs and three other studies reported on the use of aromatase inhibitors. Among all studies, the median age of included patients ranged from 61 to 71 years, and the proportion of patients with NEEC histology varied between 8 and 48%.

The overall RR of anti-estrogen monotherapy ranged from 8% (95% CI: 1–15) to 53% (95% CI: 29–78) among included studies (**Figure 2**). For tamoxifen the RR ranged from 10% (95% CI: 6–18) to 53% (95% CI: 29–78), for the other SERMs and SERDs the RR varied between 9% (95% CI: 2–17) and 31% (95% CI: 15–51) and for aromatase inhibitors the RR ranged from 8% (95% CI: 1–15) to 9% (95% CI: 2–25). Results of the RR and CBR of all individual studies are illustrated in **Figure 2**.

Toxicity was scored according to a standardized scale in 6 out of 10 eligible studies. The remaining four studies did not report toxicity at all [$n = 1$, (43)] or did not report severity of complaints [$n = 3$, (44, 46, 49)] (**Table 2**). Nausea and thromboses were the most common side-effects. Thrombotic events were not reported in studies investigating tamoxifen or arzoxifene. The use of fulvestrant resulted in thrombosis in 6% of patients. Aromatase inhibitors resulted in thrombosis in 3–5% of patients.

Anti-estrogens in Combined Therapy

As shown in **Table 3**, the seven studies included in our analysis investigated either a combination of progestin and tamoxifen (four studies), a combination of chemotherapy with progestin and tamoxifen (one study), or a combination of anti-estrogen treatment with mammalian target of rapamycin (mTOR) inhibitors (two studies). Two studies on progestin/tamoxifen combined daily progestin with tamoxifen while the other two studies alternated between progestin and tamoxifen or added progestin to daily tamoxifen only in even weeks.

Among the seven studies, median age ranged from 61 to 70 years, and the proportion of low grade EEC tumors ranged from 38 to 80%. The overall RR of combined therapy ranged from 14% (95% CI: 3–36) to 43% (95% CI: 23–64). For combined progestin/tamoxifen treatment the RR varied between 19% (95%

TABLE 2 | Study characteristics in monotherapy.

References (bias risk)	N°	Drug and dose	Study type	Population	Previous treatment	Age (median)	Histology/ grade	Response	PFS (months)	OS (months)	Toxicity
TAMOXIFEN											
Bonte et al. (43) (high)	17	40 mg/day	Case series	Stage III–IV or recurrent EC	Unresponsive to progestin	Range 26–72	Not reported	12% CR 41% PR	Not reported	Not reported	Not reported
Rendina et al. (44)* (low)	45	40 mg/day	Prospective	Stage III–IV or recurrent EC	Not reported	61	80% grade 1–2	13% CR 22% PR 18% SD	11.5	16	No treatment interruption
Quinn and Campbell (46) (low)	49	40 mg/day	Case series	Stage III–IV or recurrent EC	Unresponsive to progestin	66	84% EEC 8% NEEC 37% grade 1–2	12% CR 8% PR 0% SD	Not reported	6–34 depending on response	Nausea (16%)
Thigpen et al. (49) (low)	68	40 mg/day	Prospective	Stage III–IV or recurrent EC	No prior therapy	87% >60 y	56% EEC 44% NEEC	4% CR 6% PR	1.9	8.8	Nausea (6%)
OTHER SERM/SERD											
McMeekin et al. (50) (low)	29	Arzoxifene 20 mg/day	Prospective	Stage III–IV or recurrent EC ER or PR+ or gr1/2 (if ER/PR unknown)	Progestin stopped >3 weeks No earlier chemotherapy	66	100% EEC 74% grade 1–2	3% CR 28% PR 7% SD	3.7	Not reported	No grade 3–4 toxicity
Covens et al. (54) (low)	53	Fulvestrant 250 mg IM/4 week	Prospective	Stage III–IV or recurrent EC	No prior hormonal therapy	70% >60 y	66% EEC 23% NEEC 49% grade 1–2	2% CR 8% PR 25% SD	2	ER+: 26 ER–: 9	Grade 3–4: Thrombosis (8%)
Emons et al. (55) (low)	35	Fulvestrant 250 mg IM/4 week	Prospective	Stage IVB or recurrent EC, ER or PR+ or unknown	No prior hormonal therapy	70	71% EEC 26% NEEC 69% grade 1–2	0% CR 11% PR 23% SD	2.3	13.2	Grade 3–4: Pulmonary embolism (3%) Nausea (6%)
AROMATASE INHIBITOR											
Rose et al. (47) (low)	23	Anastrozole 1 mg/day	Prospective	Stage III–IV or recurrent EC	Maximum 1 prior hormonal therapy No prior chemotherapy	83% >60	52% EEC 48% NEEC 39% grade 1–2	9% PR 9% SD	1	6	Grade 3–4: Pulmonary embolism (4%)
Ma et al. (52) (low)	32	Letrozole 2.5 mg/day	Prospective	Stage IV or recurrent EC	Progestin therapy allowed No earlier chemo.	71	Not reported	3% CR 6% PR 34% SD	Not reported	NR	Grade 3 de-pression (3%); thrombosis (3%)
Lindemann et al. (57) (low)	51	Examestane 25 mg/day	Prospective	Stage III–IV or recurrent EC,	No hormonal or chemotherapy	69	61% grade 1–2	5% CR 5% PR 20% SD	3.1	10.9	Grade 3–4: Anorexia (4%) Thrombosis (6%) Anemia (55%)

**Consecutive primary and combined hormonal therapy. IM, intramuscular administration; EC, endometrial cancer; EEC, endometrioid endometrial cancer; NEEC, non endometrioid endometrial cancer; CR, complete response; PR, partial response; SD, stable disease.*

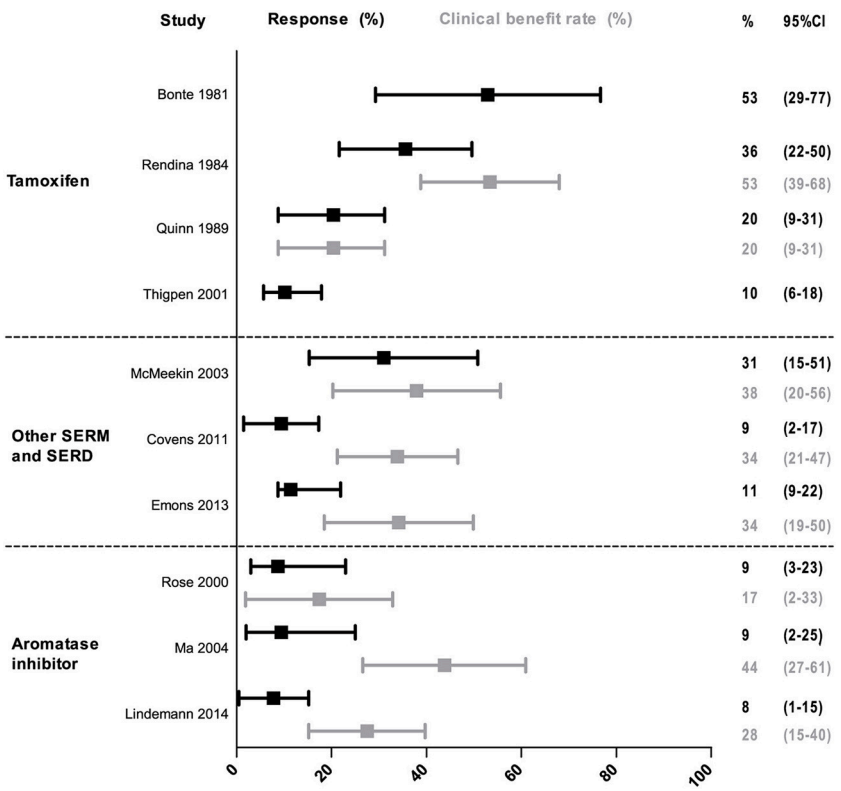


FIGURE 2 | Response and clinical benefit rate of monotherapy. The response and clinical benefit rate are shown with 95% confidence intervals between the error bars. Response rate is defined as the proportion of patients with complete and partial response. The clinical benefit rate is defined as the proportion of patients with either complete response partial response or stable disease.

CI: 7–31) and 37% (95% CI: 27–47), for chemotherapy with progestin/tamoxifen the RR was 43% (95% CI: 23–64) and for combination therapy of hormonal treatment and mTOR inhibitor the RR ranged from 14% (95% CI: 3–36) to 31% (95% CI: 17–49) (**Figure 3**).

Toxicity was scored according to a standardized scale in five out of seven studies. Thrombosis occurred in 2% of patients with daily tamoxifen and progestin in even weeks only and in 9% of patients who alternated tamoxifen with progestins (51, 53). Chemotherapy and progestin/tamoxifen resulted in moderate to severe hematologic or gastro-intestinal toxicity in 14 and 12% of the patients (45). Seventy-five percent of patients received the optimal treatment dose. The combination of the mTOR inhibitor temsirolimus with progestin and tamoxifen resulted in serious thrombotic events in 43% of the patients, causing a premature stop to accrual in this study (56). The study that combined everolimus and letrozole reported grade 3–4 fatigue in 11% and nausea or vomiting in 6% of the patients (58). No thrombosis was reported. Thirty-two percent of patients required a dose reduction because of side effects, but no patient had to stop treatment due to toxicity.

Effect According to ER Status

Among all included studies, six investigated RR and/or CBR for patients with ER positive and ER negative tumors separately (**Table 4**). Tumor tissue used for ER analysis was taken either before start of hormonal therapy, from the primary tumor or from the recurrence. Immunohistochemical analysis for ER was performed using a staining-intensity index in most studies with different cutoff values, although two studies dichotomized ER status based on percentage of positive tumor cells. RR in ER positive patients ranged from 10% (95% CI: 1–19) to 47% (95% CI: 25–70) and RR in ER negative patients was 0% in all but one study. The highest RRs were found in studies that based ER positivity on tumor samples taken from the metastatic site before start of hormonal therapy. CBR ranged from 35% (95% CI: 20–50) to 59% (95% CI: 39–80) in ER positive to 0 to 18% (95% CI: 2–34) in ER negative disease.

DISCUSSION

In this systematic review, we have outlined the effect of selective estrogen receptor modulators (SERM), down-regulators (SERD) and aromatase inhibitors in patients with advanced stage and recurrent endometrial cancer (EC). None of the included studies investigated patients with a wish to preserve fertility indicating that there is a lack of evidence for anti-estrogenic treatment in this population. Among studies on advanced stage and recurrent EC, comparison between different types of anti-estrogenic drugs

TABLE 3 | Study characteristics in combined therapy.

References (bias risk)	N°	Drug and dose	Study type	Population	Previous treatment	Age (median)	Histology/ grade	Response	PFS (months)	OS (months)	Toxicity
PROGESTIN/TAMOXIFEN											
Rendina et al. (44)* (low)	89	TMX 40 mg/day and MPA 1 g/week IM	Prospective	Stage III–IV or recurrent EC	Unresponsive to TMX or MPA	61	80% grade 1–2	8% CR 29% PR 21% SD	8–12	10–16	Not reported
Pandya et al. (48) (high)	42	TMX 20 mg/day and MA 160 mg/day	Prospective	Stage III–IV or recurrent EC	No prior hormonal therapy	68	55% grade 1–2	2% CR 17% PR 2% SD	Not reported	8.6	Grade 3–4: Pulmonary embolism (2%) Other sever (2%)
Fiorica et al. (51) (low)	56	Alternating TMX 40 mg and MA 160 mg	Prospective	Stage III–IV or recurrent EC	No prior hormonal and chemotherapy	70	79% EEC 21% NEEC 59% grade 1–2	21% CR 5% PR	2.7	14	Grade 3–4: Thrombosis (9%)
Whitney et al. (53) (low)	58	TMX40 mg/day + MPA 200 mg/day in even weeks	Prospective	Stage III–IV or recurrent EC	No prior hormonal and chemotherapy	75%>60	71% EEC 28% NEEC 53% grade 1–2	10% CR 22% PR	3	13	Grade 3–4: Thrombosis (2%) Anemia (3%) weight gain (3%)
HORMONAL THERAPY COMBINED WITH CHEMOTHERAPY											
Ayoub et al. (45) (high)	23	CAF+alternating MPA 200 mg/day and TMX20 mg	Prospective	Stage IV or recurrent EC	Palliative radiotherapy in 60%.	63	Not reported	26% CR 17% PR	Not reported	14	Moderate-severe: 14% hematologic 12% nausea 7% cystitis 22% flebitis
HORMONAL THERAPY COMBINED WITH mTOR inhibitor											
Fleming et al. (56) (low)	21	Temsirolimus 25 mg weekly and alternating TMX 40 mg/day OR MA160 mg	Prospective	Stage III–IV or recurrent EC	No prior hormonal therapy	72%>60	67% EEC 33% NEEC 38% grade 1–2	0% CR 14% PR	4.2	9.6	Grade 3–4: Thrombosis (24%) Pulmonary embolism (10%) Myocardial infarction (5%) Sudden death (5%)
Slomovitz et al. (58) (low)	35	Everolimus 10 g/d and letrozole 2.5 mg/day	Prospective	Stage III–IV or recurrent EC	Chemotherapy	62	71% EEC 29% NEEC	26% CR 6% PR 9% SD	3	14	Grade 3–4: Fatigue (11%) Nausea/vomit (6%)

**Consecutive primary and combined hormonal therapy. TMX, tamoxifen; MPA, medroxyprogsterone acetate; MA, megestrol acetate; CAF, cyclophosphamide, adriamycin, 5-fluorouracil; EC, endometrial cancer; CR, complete response; PR, partial response; SD, stable disease; EEC, endometrioid endometrial cancer; NEEC, non endometrioid endometrial cancer.*

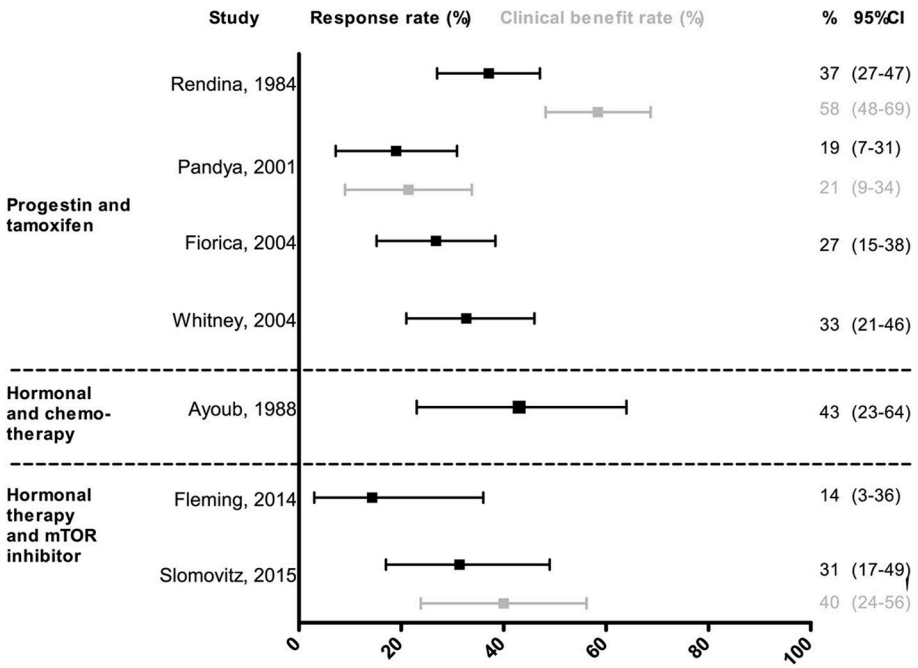


FIGURE 3 | Response and clinical benefit rate of combined treatment. The response and clinical benefit rate are shown with 95% confidence intervals between the error bars. Response rate is defined as the proportion of patients with complete and partial response. The clinical benefit rate is defined as the proportion of patients with either complete response partial response or stable disease.

was challenging because of the lack of randomized studies and differences in patient and tumor characteristics. The investigated treatments reported similar response rates for all treatments except for aromatase inhibitors, which had a limited effect in the investigated populations. Serious side-effects were rare for anti-estrogens, but occurred more frequently when anti-estrogenic drugs were combined with chemotherapy or mTOR inhibitors. The expression of estrogen receptor in the tumor taken prior to start of treatment was associated with improved response to anti-estrogens.

The observed RR and CBR differed according to the selected population, with higher response rates in endometrioid tumors with positive ER status. Among studies investigating tamoxifen, Rendina et al reported a RR as high as 36% (95% CI: 22–50) in patients with predominantly grade 1–2 tumors, whereas Thigpen found a RR of 10% (95% CI: 6–18) in patients with NEEC histology in 44% of cases (44, 49). The limited therapeutic response in NEEC reflects the low impact of estrogen in the carcinogenesis of these tumors (60). The reported tamoxifen related toxicity was limited to nausea.

As expected, the therapeutic response to anti-estrogens was higher among EECs, as illustrated by the study of McMeekin in which a RR of 31% (95% CI: 15–51) to arzoxifene was reported in a cohort that included only EEC. Despite these data, arzoxifene was never introduced into clinical practice. Two studies explored the use of fulvestrant, reporting limited responses ranging from 9 to 11%. Furthermore, fulvestrant can only be administered through intramuscular injection because of low oral bioavailability, which complicates the clinical implementation of this drug in a palliative setting. Aromatase inhibitors were shown to have only limited response rate in the investigated populations. Thus, aromatase inhibitors should not be a first choice when selecting anti-estrogenic therapy for EC. As aromatization of androgens into estrogen occurs predominantly in fat tissue, patients with obesity might represent a subgroup of EC patients in which aromatase inhibitors can be more effective. However, this hypothesis has not been tested in EC patients and studies in breast cancer do not show superior results of aromatase inhibitors compared to other hormonal treatments in obese patients (61).

Out of the four studies investigating combined treatment of tamoxifen and progestin, three studies enrolled a comparable patient population and reported a RR ranging from 19 to 27%. Also considering that serious toxicity occurred in just 2–5% of the included patients, the use of tamoxifen combined with progestins is an attractive treatment regimen. The addition of progestin and tamoxifen to chemotherapy was evaluated by one study, which reported a higher RR for the combination compared to chemotherapy alone. However, the applied chemotherapy regimen in this study is no longer in use in EC and no studies that combined anti-estrogenic therapy with currently used chemotherapeutic drugs have been performed (62). The combination of hormonal therapy with an mTOR inhibitor did not result in superior RRs compared with other anti-estrogenic treatments. Toxicity remains an important concern, especially for the combination of temsirolimus with alternating treatment with progestin and tamoxifen. Interestingly, the combination of letrozole with

TABLE 4 | Overall response and clinical benefit rate according to estrogen receptor status.

References	N°	Type	Tumor used for ER analysis	Type of immunohistochemical analysis	Response rate [% (95% CI)]		Clinical benefit [% (95% CI)]	
					ER+	ER–	ER+	ER–
Singh et al. (59); Whitney et al. (53)	46	TMX daily and MPA in alternating weeks	Before start of hormonal therapy	Staining intensity index with range 0–500 Cutoff 75	47 (25–70)	26 (9–42)	Not reported	
Covens et al. (54)	53	Fulvestrant	Recurrence/metastasis	% of positive nuclei Cutoff 10%	16 (3–29)	0	45 (28–63)	18 (2–34)
Emons et al. (55)	27	Fulvestrant	Primary tumor	NR	11 (0–23)	0	Not reported	
Lindemann et al. (57)	51	Examestane	Primary tumor or recurrence	Staining intensity index Cutoff: high intensity 10% of nuclei	10 (1–19)	0	35 (20–50)	0
Fleming et al. (56)	20	Temsirolimus and alternating MA or TMX	Primary tumor	Any level of staining	13 (0–31)	0	Not reported	
Slomovitz et al. (58)	30	Everolimus and letrozole	Primary tumor or recurrence	Staining intensity index range 0–8 Cutoff: 3	Not reported		59 (39–80)	13 (0–35)

MPA, medroxyprogesterone-acetate; TMX, tamoxifen; MA, megestrol acetate.

everolimus was less toxic. A recent GOG study presented at the SGO meeting 2018 showed similar RRs and adverse events for letrozole/everolimus and progestin/tamoxifen (63). Upon validation, this regimen could be an alternative to progestin/tamoxifen. Further investigation into molecular alterations that lead to resistance to hormonal therapy might also provide us with improved individualized combination treatment for these patients (64).

In summary, treatment with tamoxifen or combined treatment of tamoxifen and progestin are currently the best options in anti-estrogen therapy, because of similar or higher RR when compared to other treatments and limited toxicity. Preferably, patients with ER positive tumor and endometrioid histology should be selected for anti-estrogen therapy in order to optimize the chance of response.

Whether combined tamoxifen/progestin results in improved response when compared to progestins, has unfortunately not been studied in a randomized trial. The only study that randomized between progestin and progestin with tamoxifen was a low quality study that stopped the progestin arm prematurely due to poor accrual (48). However, several good quality studies reported an average response rate of 25% to progestin in an unselected population, which is similar to the responses to tamoxifen and progestin/tamoxifen found in this review (65, 66). The rationale for adding tamoxifen to progestin is to counteract the down regulation of the progesterone receptor that is induced by progestin treatment in order to prolong the duration of response (67, 68). Different combinations of progestin and tamoxifen have been explored. One option is to start progestin monotherapy and add or replace progestin by tamoxifen upon progression, as shown by two studies among progestin unresponsive patients (43, 44). Alternatively, combined treatment of tamoxifen with progestin or alternating treatment can be applied. From the reported RR in our study, it is not possible to define which regimen is superior.

Immunohistochemical expression of ER was evaluated by the studies included in this review using different methods and cutoffs for positivity. One study defined an optimal cutoff based on a staining intensity index, but even among ER negative patients, a high response rate of 26% was observed suggesting that differentiation between ER positive and ER negative can still be optimized (59). Future studies on this topic would ideally result in a test that can be used for all types of stored and fresh EC tissues and will be adopted worldwide. Most studies used primary tumor tissue for ER analysis. Yet, primary tumor and metastases are not comparable due to changes in the tumor caused by intercurrent therapy and the metastatic process itself (69–71). Therefore, it is essential that tumor tissue is obtained directly before start of hormonal therapy to relate receptor status to response. In case tumor tissue cannot be procured, non-invasive visualization of estrogen receptor status on a PET scan with an estrogen tracer might be an alternative approach (72, 73).

While the strengths of this review include the systematic approach and the quality assessment for eligible studies, there are some limitations to be addressed. First, systematic reviews are based on published data, and may therefore be biased toward selective reporting of positive results. Although we have tried to improve the quality by excluding case series with <10 patients, still this limitation should be taken into account. Second, criteria for response duration were not consistently used among all studies hampering proper comparison of outcome. Finally, most of the included studies evaluated patients with advanced and recurrent EC. However, both patient groups might differ in patient and tumor characteristics. Unfortunately, we could not discriminate in this review between advanced stage and recurrent EC,

since most studies did not report outcome separately for both groups.

The effect of anti-estrogens in advanced and recurrent EC needs further improvement. In our review, the average response or clinical benefit rates were (far) below 50% and the effect of anti-estrogen therapy on progression free survival and overall survival was limited. Therefore, there is a need for additional biomarkers to improve selection of patients that benefit most from anti-estrogen hormonal therapy. Currently, selection for hormonal treatment is mainly based on estrogen and progesterone receptor status. However, several studies observe a benefit for patients even in ER negative disease, highlighting the need for in depth analysis of the intracellular pathway that is activated upon binding of estrogen to the estrogen receptor (54, 58, 59). An initial study on this topic has reported promising results in breast cancer, but so far no research on this topic has been performed in endometrial cancer (74). Furthermore, recent studies have shown that proteins involved in intracellular conversion of inactive estrogens to active estrogens have a prognostic role in EC (75, 76). These proteins can theoretically also oppose the effects of hormonal therapy warranting further research on this topic. Also, combining hormonal therapy with targeted therapies is an attractive strategy to overcome resistance to hormonal treatment and is the subject of many of the ongoing studies (34–36, 38, 39, 64). Finally, new studies should focus on patients with stable disease instead of complete or partial response only. Stable disease can be considered of clinical benefit for patients in a palliative setting especially if the disease remains stable for several months. Ideally, future studies would incorporate a predefined period of stable disease as outcome measure and would report on clinical benefit as primary outcome, as some of the included studies already have (57, 58).

CONCLUSION

Treatment with tamoxifen or the combination of tamoxifen and progestin should be first choice in anti-estrogen therapy for patients with advanced and recurrent endometrial cancer because response rates are comparable to first line hormonal treatment with progestins and toxicity is limited. Therefore, these therapies are a good second-line hormonal treatment option in endometrial cancer. Responses to anti-estrogen therapy can be improved by selecting patients with endometrioid tumors and positive estrogen receptor status, which should be based on a pretreatment biopsy.

AUTHOR CONTRIBUTIONS

AR and JP came up with the concept. WvW and AR performed the search and selected relevant articles. WvW, AR, and JP performed bias assessment. WvW, LM, JP, and AR interpreted the data. WvW, AR, and JP wrote the first draft. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

The study was partly supported by the Dutch Cancer Society (KWF Kankerbestrijding, www.kwf.nl), contract number UM-13-5782 granted to AR.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2019.00359/full#supplementary-material

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin.* (2018) 68:394–424. doi: 10.3322/caac.21492
2. Shaw E, Farris M, McNeil J, Friedenreich C. Obesity and endometrial cancer. *Recent Results Cancer Res.* (2016) 208:107–36. doi: 10.1007/978-3-319-42542-9_7
3. Bergstrom A, Pisani P, Tenet V, Wolk A, Adami HO. Overweight as an avoidable cause of cancer in Europe. *Int J Cancer.* (2001) 91:421–30. doi: 10.1002/1097-0215(200002)9999:9999<::AID-IJC1053>3.0.CO;2-T
4. Key TJ, Allen NE, Verkasalo PK, Banks E. Energy balance and cancer: the role of sex hormones. *Proc Nutr Soc.* (2001) 60:81–9. doi: 10.1079/PNS200068
5. Kaaks R, Lukanova A, Kurzer MS. Obesity, endogenous hormones, and endometrial cancer risk: a synthetic review. *Cancer Epidemiol Biomarkers Prev.* (2002) 11:1531–43.
6. Allen NE, Tsilidis KK, Key TJ, Dossus L, Kaaks R, Lund E, et al. Menopausal hormone therapy and risk of endometrial carcinoma among postmenopausal women in the European prospective investigation into cancer and nutrition. *Am J Epidemiol.* (2010) 172:1394–403. doi: 10.1093/aje/kwq300
7. Zhao H, Zhou L, Shangguan AJ, Bulun SE. Aromatase expression and regulation in breast and endometrial cancer. *J Mol Endocrinol.* (2016) 57:R19–33. doi: 10.1530/JME-15-0310
8. Ellenson HL, Ronnett BM, Kurman RJ. Precursor lesions of endometrial carcinoma. In: Kurman RJ, editor. *Blaustein's Pathology of the Female Genital Tract*. New York, NY: Springer (2011). p. 359–91. doi: 10.1007/978-1-4419-0489-8_8
9. Bokhman JV. Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol.* (1983) 15:10–7. doi: 10.1016/0090-8258(83)90111-7
10. Colombo N, Creutzberg C, Amant F, Bosse T, Gonzalez-Martin A, Ledermann J, et al. ESMO-ESGO-ESTRO consensus conference on endometrial cancer: diagnosis, treatment and follow-up. *Int J Gynecol Cancer.* (2016) 26:2–30. doi: 10.1097/IGC.0000000000000609
11. Amant F, Mirza MR, Koskas M, Creutzberg CL. Cancer of the corpus uteri. *Int J Gynaecol Obstet.* (2018) 143(Suppl 2):37–50. doi: 10.1002/ijgo.12612
12. Carlson MJ, Thiel KW, Leslie KK. Past, present, and future of hormonal therapy in recurrent endometrial cancer. *Int J Women's Health.* (2014) 6:429–35. doi: 10.2147/IJWH.S40942
13. Kelley RM, Baker WH. Progestational agents in the treatment of carcinoma of the endometrium. *N Engl J Med.* (1961) 264:216–22. doi: 10.1056/NEJM196102022640503
14. Davies C, Godwin J, Gray R, Clarke M, Cutter D, Darby S, et al. Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. *Lancet.* (2011) 378:771–84. doi: 10.1016/S0140-6736(11)60993-8
15. Delmas PD, Bjarnason NH, Mitlak BH, Ravoux AC, Shah AS, Huster WJ, et al. Effects of raloxifene on bone mineral density, serum cholesterol

concentrations, and uterine endometrium in postmenopausal women. *N Engl J Med.* (1997) 337:1641–7. doi: 10.1056/NEJM199712043372301
16. Cosman F, Lindsay R. Selective estrogen receptor modulators: clinical spectrum. *Endocr Rev.* (1999) 20:418–34. doi: 10.1210/edrv.20.3.0371
17. Goldstein SR, Bhattoa HP, Neven P, Cox DA, Dowsett SA, Alam J, et al. Gynecologic effects of arzoxifene in postmenopausal women with osteoporosis or low bone mass. *Menopause.* (2012) 19:41–7. doi: 10.1097/gme.0b013e318223bbf4
18. Bross PF, Baird A, Chen G, Jee JM, Lostritto RT, Morse DE, et al. Fulvestrant in postmenopausal women with advanced breast cancer. *Clin Cancer Res.* (2003) 9:4309–17.
19. Ethier JL, Desautels DN, Amir E, MacKay H. Is hormonal therapy effective in advanced endometrial cancer? A systematic review and meta-analysis. *Gynecol Oncol.* (2017) 147:158–66. doi: 10.1016/j.ygyno.2017.07.002
20. Bogliolo S, Gardella B, Dominoni M, Musacchi V, Cassani C, Zanellini F, et al. Effectiveness of aromatase inhibitors in the treatment of advanced endometrial adenocarcinoma. *Arch Gynecol Obstet.* (2016) 293:701–8. doi: 10.1007/s00404-015-3974-9
21. Bogliolo S, Cassani C, Dominoni M, Orlandini A, Ferrero S, Iacobone AD, et al. The role of fulvestrant in endometrial cancer. *Expert Opin Drug Metab Toxicol.* (2017) 13:537–44. doi: 10.1080/17425255.2016.1244264
22. Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
23. NIH National Heart Lung and Blood Institute. *Study Quality Assessment Tools*. Available online at: https://www.nhlbi.nih.gov/health-topics/study-quality-assessment-tools (accessed October 1, 2018).
24. Wallis S. Binomial confidence intervals and contingency tests: mathematical fundamentals and the evaluation of alternative methods. *J Quant Linguist.* (2013) 20:178–208. doi: 10.1080/09296174.2013.799918
25. Rose PG, Cha SD, Tak WK, Fitzgerald T, Reale F, Hunter RE. Radiation therapy for surgically proven para-aortic node metastasis in endometrial carcinoma. *Int J Rad Oncol Biol Phys.* (1992) 24:229–33. doi: 10.1016/0360-3016(92)90676-9
26. Pinelli DM, Fiorica JV, Roberts WS, Hoffman MS, Nicosia SV, Cavanagh D. Chemotherapy plus sequential hormonal therapy for advanced and recurrent endometrial carcinoma: a phase II study. *Gynecol Oncol.* (1996) 60:462–7. doi: 10.1006/gyno.1996.0074
27. Covens A, Brunetto VL, Markman M, Orr JW, Lentz SS, Benda J. Phase II trial of danazol in advanced, recurrent, or persistent endometrial cancer: a gynecologic oncology group study. *Gynecol Oncol.* (2003) 89:470–4. doi: 10.1016/S0090-8258(03)00149-5
28. Dahhan T, Fons G, Buist MR, ten Kate FJW, van der Velden J. The efficacy of hormonal treatment for residual or recurrent low-grade endometrial stromal sarcoma. A retrospective study. *Eur J Obstet Gynecol Reprod Biol.* (2009) 144:80–4. doi: 10.1016/j.ejogrb.2009.02.005
29. George S, Feng Y, Manola J, Nucci MR, Butrynski JE, Morgan JA, et al. Phase 2 trial of aromatase inhibition with letrozole in patients with uterine leiomyosarcomas expressing estrogen and/or progesterone receptors. *Cancer.* (2014) 120:738–43. doi: 10.1002/cncr.28476
30. Beck TL, Giannakopoulos NV, Rendi MH, Isacson C, Goff BA. Impact of oophorectomy and hormone suppression in low grade endometrial stromal sarcoma: a multicenter review. *Gynecol Oncol.* (2008) 149:297–300. doi: 10.1016/j.ygyno.2018.03.008
31. Hald I, Salimtschik M, Mouridsen HT. Tamoxifen treatment of advanced endometrial carcinoma. A phase II study. *Eur J Gynaecol Oncol.* (1983) 4:83–7.
32. Kline RC, Freedman RS, Jones LA, Atkinson EN. Treatment of recurrent or metastatic poorly differentiated adenocarcinoma of the endometrium with tamoxifen and medroxyprogesterone acetate. *Cancer Treat. Rep.* (1987) 71:327–8.
33. Horvath G, Stendahl U, Kalling M, Ferno M, Himmelmann A, Hajba A. Antiestrogenic treatment of advanced and recurrent carcinoma corporis uteri–a phase II study of toremifene. *Anticancer Res.* (1990) 10:323–5.
34. Sinai Hospital of Baltimore (2016). *Single Arm Trial With Combination of Everolimus and Letrozole in Treatment of Platinum Resistant Relapse or Refractory or Persistent Ovarian Cancer/Endometrial Cancer*.
35. Gynecologic Oncology Group, Novartis Pharmaceuticals. (2018). *Everolimus and Letrozole or Hormonal Therapy to Treat Endometrial Cancer*.
36. Centre Leon Berard (2019). *Hormone Receptor Positive Endometrial Carcinoma Treated by Dual mTORC1/mTORC2 Inhibitor and Anastrozole (VICTORIA)*.
37. National Cancer Institute and National Institutes of Health Clinical Center (2019). *Endoxifen in Adults With Hormone Receptor Positive Solid Tumors*.
38. ArQule (2020). *Open-Label PHASE 1b Study of ARQ 092 in Combination With Anastrozole*.
39. Dana-Farber Cancer Institute, Eli Lilly and Company (2020). *A Phase 2 Study of LY3023414 and Abemaciclib With or Without Letrozole in Endometrial Cancer*.
40. National Cancer Institute (2020). *Exemestane in Treating Patients With Complex Atypical Hyperplasia of the Endometrium/Endometrial Intraepithelial Neoplasia or Low Grade Endometrial Cancer*.
41. Mayo Clinic and National Cancer Institute (2021). *Ribociclib and Letrozole in Treating Patients With Relapsed ER Positive Ovarian, Fallopian Tube, Primary Peritoneal, or Endometrial Cancer*.
42. Memorial Sloan Kettering Cancer Center, Eli Lilly and Company (2021). *Evaluating Cancer Response to Treatment With Abemaciclib and Fulvestrant in Women With Recurrent Endometrial Cancer*.
43. Bonte J, Ide P, Billiet G, Wynants P. Tamoxifen as a possible chemotherapeutic agent in endometrial adenocarcinoma. *Gynecol Oncol.* (1981) 11:140–61. doi: 10.1016/S0090-8258(81)80003-0
44. Rendina GM, Donadio C, Fabri M, Mazzoni P, Nazzicone P. Tamoxifen and medroxyprogesterone therapy for advanced endometrial carcinoma. *Eur J Obstet Gynecol Reprod Biol.* (1984) 17:285–91. doi: 10.1016/0028-2243(84)90071-6
45. Ayoub J, Audet-Lapointe P, Methot Y, Hanley J, Beaulieu R, Chemaly R, et al. Efficacy of sequential cyclical hormonal therapy in endometrial cancer and its correlation with steroid hormone receptor status. *Gynecol Oncol.* (1988) 31:327–37. doi: 10.1016/S0090-8258(88)80012-X
46. Quinn MA, Campbell JJ. Tamoxifen therapy in advanced/recurrent endometrial carcinoma. *Gynecol Oncol.* (1989) 32:1–3. doi: 10.1016/0090-8258(89)90839-1
47. Rose PG, Brunetto VL, VanLe L, Bell J, Walker JL, Lee RB. A Phase II trial of anastrozole in advanced recurrent or persistent endometrial carcinoma: a gynecologic oncology group study. *Gynecol Oncol.* (2000) 78:212–6. doi: 10.1006/gyno.2000.5865
48. Pandya KJ, Yeap BY, Weiner LM, Krook JE, Erban JK, Schinella RA, et al. Megestrol and tamoxifen in patients with advanced endometrial cancer: an Eastern Cooperative Oncology Group study (E4882). *Am J Clin Oncol Cancer Clin Trials.* (2001) 24:43–6. doi: 10.1097/00000421-200102000-00007
49. Thigpen T, Brady MF, Homesley HD, Soper JT, Bell J. Tamoxifen in the treatment of advanced or recurrent endometrial carcinoma: a Gynecologic Oncology Group study. *J Clin Oncol.* (2001) 19:364–7. doi: 10.1200/JCO.2001.19.2.364
50. McMeekin DS, Gordon A, Fowler J, Melemed A, Buller R, Burke T, et al. A phase II trial of arzoxifene, a selective estrogen response modulator, in patients with recurrent or advanced endometrial cancer. *Gynecol Oncol.* (2003) 90:64–9. doi: 10.1016/S0090-8258(03)00203-8
51. Fiorica JV, Brunetto VL, Hanjani P, Lentz SS, Mannel R, Andersen W. Phase II trial of alternating courses of megestrol acetate and tamoxifen in advanced endometrial carcinoma: a Gynecologic Oncology Group study. *Gynecol Oncol.* (2004) 92:10–4. doi: 10.1016/j.ygyno.2003.11.008
52. Ma BBY, Oza A, Eisenhauer E, Stanimir G, Carey M, Chapman W, et al. The activity of letrozole in patients with advanced or recurrent endometrial cancer and correlation with biological markers - A study of the National Cancer Institute of Canada Clinical Trials Group. *Int J Gynecol Cancer.* (2004) 14:650–8. doi: 10.1111/j.1048-891X.2004.14419.x
53. Whitney CW, Brunetto VL, Zaino RJ, Lentz SS, Sorosky J, Armstrong DK, et al. Phase II study of medroxyprogesterone acetate plus tamoxifen in advanced endometrial carcinoma: a Gynecologic Oncology Group study. *Gynecol Oncol.* (2004) 92:4–9. doi: 10.1016/j.ygyno.2003.09.018
54. Covens AL, Filiaci V, Gersell D, Lutman CV, Bonebrake A, Lee YC. Phase II study of fulvestrant in recurrent/metastatic endometrial carcinoma: a Gynecologic Oncology Group study. *Gynecol Oncol.* (2011) 120:185–8. doi: 10.1016/j.ygyno.2010.10.015

55. Emons G, Gunthert A, Thiel FC, Camara O, Strauss HG, Breitbach GP, et al. Phase II study of fulvestrant 250 mg/month in patients with recurrent or metastatic endometrial cancer: a study of the Arbeitsgemeinschaft Gynakologische Onkologie. *Gynecol Oncol.* (2013) 129:495–9. doi: 10.1016/j.ygyno.2013.02.039
56. Fleming GF, Filiaci VL, Marzullo B, Zaino RJ, Davidson SA, Pearl M, et al. Temsirolimus with or without megestrol acetate and tamoxifen for endometrial cancer: a gynecologic oncology group study. *Gynecol Oncol.* (2014) 132:585–92. doi: 10.1016/j.ygyno.2014.01.015
57. Lindemann K, Malander S, Christensen RD, Mirza MR, Kristensen GB, Aavall-Lundqvist E, et al. Examestane in advanced or recurrent endometrial carcinoma: a prospective phase II study by the Nordic Society of Gynecologic Oncology (NSGO). *BMC Cancer*. (2014) 14:68. doi: 10.1186/1471-2407-14-68
58. Slomovitz BM, Jiang Y, Yates MS, Soliman PT, Johnston T, Nowakowski M, et al. Phase II study of everolimus and letrozole in patients with recurrent endometrial carcinoma. *J Clin Oncol.* (2015) 33:930–6. doi: 10.1200/JCO.2014.58.3401
59. Singh M, Zaino RJ, Filiaci VJ, Leslie KK. (2007). Relationship of estrogen and progesterone receptors to clinical outcome in metastatic endometrial carcinoma: a Gynecologic Oncology Group study. *Gynecol Oncol.* 106:325–33. doi: 10.1016/j.ygyno.2007.03.042
60. Lax SF. Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. *Virchows Arch.* (2004) 444:213–23. doi: 10.1007/s00428-003-0947-3
61. Slowik A, Fraczek PA, Krzemieniecki K. Body mass index and aromatase inhibitors: a step forward in individualizing therapy for breast cancer patients? *Expert Rev Anticancer Ther.* (2016) 16:759–66. doi: 10.1080/14737140.2016.1191949
62. Bestvina CM, Fleming GF. Chemotherapy for endometrial cancer in adjuvant and advanced disease settings. *Oncologist.* (2016) 21:1250–9. doi: 10.1634/theoncologist.2016-0062
63. Slomovitz BM, Filiaci VL, Coleman RL, Walker JL, Fleury AC, Holman LL, et al. (2018). GOG 3007, a randomized phase II (RP2) trial of everolimus and letrozole (EL) or hormonal therapy (medroxyprogesterone acetate/tamoxifen, PT) in women with advanced, persistent or recurrence endometrial carcinoma (EC): a GOG Foundation study. In: *SGO 49th Annual Meeting on Women's Cancer*. New Orleans, LA. doi: 10.1016/j.ygyno.2018.04.012
64. MacKay HJ, Levine DA, Bae-Jump VL, Bell DW, McAlpine JN, Santin A, et al. Moving forward with actionable therapeutic targets and opportunities in endometrial cancer: NCI clinical trials planning meeting report on identifying key genes and molecular pathways for targeted endometrial cancer trials. *Oncotarget.* (2017) 8:84579–94. doi: 10.18632/oncotarget.19961
65. Lentz SS, Brady MF, Major FJ, Reid GC, Soper JT. High-dose megestrol acetate in advanced or recurrent endometrial carcinoma: a Gynecologic Oncology Group study. *J Clin Oncol.* (1996) 14:357–61. doi: 10.1200/JCO.1996.14.2.357
66. Thigpen JT, Brady MF, Alvarez RD, Adelson MD, Homesley HD, Manetta A, et al. Oral medroxyprogesterone acetate in the treatment of advanced or recurrent endometrial carcinoma: a dose-response study by the Gynecologic Oncology Group. *J Clin Oncol.* (1999) 17:1736–44. doi: 10.1200/JCO.1999.17.6.1736
67. Mortel R, Levy C, Wolff JP, Nicolas JC, Robel P, Baulieu EE. Female sex steroid receptors in postmenopausal endometrial carcinoma and biochemical response to an antiestrogen. *Cancer Res.* (1981) 41:1140–7.
68. Carlson JA Jr., Allegra JC, Day TG Jr., Wittliff JL. Tamoxifen and endometrial carcinoma: alterations in estrogen and progesterone receptors in untreated patients and combination hormonal therapy in advanced neoplasia. *Am J Obstet Gynecol.* (1984) 149:149–53. doi: 10.1016/0002-9378(84)90187-X
69. Tacca O, Penault-Llorca F, Abrial C, Mouret-Reynier MA, Raoelfils I, Durando X, et al. Changes in and prognostic value of hormone receptor status in a series of operable breast cancer patients treated with neoadjuvant chemotherapy. *Oncologist.* (2007) 12:636–43. doi: 10.1634/theoncologist.12-6-636
70. Vandenput I, Trovik J, Leunen K, Wik E, Stefansson I, Akslen L, et al. Evolution in endometrial cancer: evidence from an immunohistochemical study. *Int J Gynecol Cancer.* (2011) 21:316–22. doi: 10.1097/IGC.0b013e31820575f5
71. Bartosch C, Monteiro-Reis S, Vieira R, Pereira A, Rodrigues M, Jeronimo C, et al. Endometrial endometrioid carcinoma metastases show decreased ER-Alpha and PR-A expression compared to matched primary tumors. *PLoS ONE.* (2015) 10:e0134969. doi: 10.1371/journal.pone.0134969
72. Tsujikawa T, Yoshida Y, Kiyono Y, Kurokawa T, Kudo T, Fujibayashi Y, et al. Functional oestrogen receptor alpha imaging in endometrial carcinoma using 16alpha-[(1)(8)F]fluoro-17beta-oestradiol PET. *Eur J Nucl Med Mol Imaging.* (2011) 38:37–45. doi: 10.1007/s00259-010-1589-8
73. van Kruchten M, de Vries EG, Brown M, de Vries EF, Glaudemans AW, Dierckx RA, et al. PET imaging of oestrogen receptors in patients with breast cancer. *Lancet Oncol.* (2013) 14:e465–75. doi: 10.1016/S1470-2045(13)70292-4
74. Verhaegh W, van Ooijen H, Inda MA, Hatzis P, Versteeg R, Smid M, et al. Selection of personalized patient therapy through the use of knowledge-based computational models that identify tumor-driving signal transduction pathways. *Cancer Res.* (2014) 74:2936–45. doi: 10.1158/0008-5472.CAN-13-2515
75. Cornel KM, Kruitwagen RF, Delvoux B, Visconti L, Van de Vijver KK, Day JM, et al. Overexpression of 17beta-hydroxysteroid dehydrogenase type 1 increases the exposure of endometrial cancer to 17beta-estradiol. *J Clin Endocrinol Metab.* (2012) 97:E591–601. doi: 10.1210/jc.2011-2994
76. Cornel KM, Krakstad C, Delvoux B, Xanthoulea S, Jori B, Bongers MY, et al. High mRNA levels of 17beta-hydroxysteroid dehydrogenase type 1 correlate with poor prognosis in endometrial cancer. *Mol Cell Endocrinol.* (2017) 442:51–7. doi: 10.1016/j.mce.2016.11.030

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: info@frontiersin.org | +41 21 510 17 00

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership