ROLE OF SEX STEROIDS AND THEIR RECEPTORS IN CANCERS

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ROLE OF SEX STEROIDS AND THEIR RECEPTORS IN CANCERS

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EDITORIAL
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Editorial: Role of Sex Steroids and Their Receptor in Cancers

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Editorial on the Research Topic

Role of Sex Steroids and Their Receptors in Cancers

The way we view steroid hormones has changed overtime: from simple transcription factors targeting male and female sexual organs, such as epididymis and testes or breast, ovary and uterus, respectively, to complex signalling proteins able to regulate a plethora of processes in a wide range of cell and tissues. Sex steroid receptors were classically considered transcription factors controlling a variety of responses in reproductive tissues both at physiological and at pathological level. Principally represented by oestrogen, progesterone, androgen, and glucocorticoid receptors (ER, PR, AR, and GR), upon binding their hormone, they translocate to the nucleus where recognize specific hormone responsive elements (HREs) located by the promoter of different genes and regulate their transcription (1). In more recent times, numerous studies have demonstrated that steroid receptors also can work in a non-transcriptional manner (2). In a few seconds or minutes after ligand binding, sex steroid receptors activate transduction pathways (such as PI3K/AKT or MAPKs) and alter a multitude of physiological and pathological processes not only in organs recognized as steroid-dependent but also in distinct anatomical sites. By both "genomic" and "non-genomic" mechanisms, steroid receptors influence the regulation of key genes, important for organ development and function but also promote the development and the progression of cancers by influencing tumour growth and invasiveness, epithelial-mesenchymal transition (EMT; 2–4).

In addition to the classical hormone-related cancers of the breast, prostate, ovary, and testis, an increasing number of scientists is studying the role of sex steroid receptors in different kind of cancers (5–14), trying to understand how and when steroid hormones and their receptors influence their incidence in men or women (5, 15, 16).

This Research Topic focuses the attention on the role of steroid receptors in all types of cancers and highlights the importance of updating detection methods to include all isoforms and variants that are continuously discovered. To date, at least 20 different variants of the androgen receptor in prostate (17), 5 variants for the oestrogen receptor β (named from ERβ1 to ERβ5) and 3 variants for the oestrogen receptor a (the full ERα, and two truncated forms ERα36 and ERα46) have been characterized. Pagano et al. illustrate the importance of the newly discovered ERα variant, ERα36, in different human cancers. This variant, with a molecular mass of 36kDa, is involved in tumour progression, metastatic potential, drug-resistance and is expressed in a wide range of human cancers such as neuronal tumours, gastric cancer, hepatocarcinoma, laryngeal, endometrial, renal cell, and

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papillary thyroid carcinomas. Its expression is also revealed in ER-positive and ER-negative breast cancers where it could be responsible for the drug-resistance.

It's equally important to choose the best model and use the right technique to study the role of steroid receptors in cancer, as demonstrated by Lacouture et al. By using a FACS-free method, they isolate ERα-positive mammary mouse epithelial cells that, in 3D cultures completely recapitulate the mammary gland's morphology. In their study, the authors highlight the role of estrogen or ERα in controlling mammary gland metabolism during carcinogenesis. The expression of steroid receptors in classically hormone-dependent cancers has long been used to select the more efficient therapy, but upcoming studies have tried to analyse their involvement in predicting other clinical and biological features of cancers such as overall and disease-free survival, therapy responsiveness, and prognosis. For example, in metastatic breast cancer patients, the prognosis of single hormone receptor (ERα or PR) positive tumours, with or without the HER2 overexpression, was similar as that of double-positive or double-negative (ERα and PR) tumours, indicating that other characteristics, such as age and race of patients, tumour grade, TNM stage, and surgery, have a major weight (Mao et al.). Another important marker for breast cancer is the AR. Its expression is, in most cases, a good prognostic factor in ERα-positive breast cancer and a poor prognostic factor in ERα-negative breast cancer (18, 19). In post-menopausal women, the AR expression is associated to a better survival outcome, while high levels of circulating androgens and an high AR/ER ratio are associated with poor outcomes in ERα-positive breast cancer (18, Rajarajan et al.). Rajarajan et al. evaluated the AR/ER ratio in pre–menopausal breast cancer patients and observed that, also in women younger than 50 years old, a high AR/ER ratio was a poor prognostic factor. They concluded that is not exclusively the AR expression, but the ER activity and the hormonal milieu that determine the clinical outcome. In addition to steroid receptors, Ki67, a proliferation marker, can be used to indicate the responsiveness to neoadjuvant endocrine therapy in ERα–positive breast cancer (Zhang et al.).

The major novelty of this Research Topic lies in the assembled data covering the role of steroid receptors in cancers not viewed as hormone responsive. Different research groups enabled this issue by submitting review and original articles. Bernardo et al. described that, in bladder cancer, besides to the GATA3 expression, higher in low grade and low stage tumours, the ERα expression is lower in low grade tumours, but the reduced number of cases makes it difficult to define the prognostic role of ERα or ERβ in these cancers. Wang et al. demonstrated that in oesophageal cancer, oestradiol inhibits cell viability and migration, thereby providing a novel insight for cancer development, treatment, and prevention. These data justify the sex difference observed in the occurrence of this group of cancer. In glioblastoma, PR and the cytoplasmic kinase src work together to regulate the activity of proteins, such as the focal adhesion kinase (FAK) and paxillin, involved in migration and invasion. Furthermore, the c–src activation could be responsible for the putative PR phosphorylation on Y87 residue, thus connecting genomic and non–genomic action triggered by progesterone, as studied by Bello–Alvarez et al. Indukuri et al. underlined that in the colon, the ERβ influences the inflammatory signalling through NFκB possibly reducing the incidence of colorectal cancers. In particular, by comparing two different colon cancer–derived cell lines, and adding expression of ERβ, they observed that the steroid receptor hinders p65 chromatin binding to genes controlling cell adhesion, migration, and circadian clock, while enabling binding by genes modulating cell proliferation and Notch signalling.

All the collected manuscripts indicate that a deepened knowledge of steroid hormone receptors could help the precision medicine to predict the impact of gender on tumours' incidence and help developing personalized therapies to efficaciously cure a wide group of cancers.

AUTHOR CONTRIBUTIONS

All authors listed have equally, substantially, and intellectually contributed to this editorial and approved it for publication.

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Interaction of Estradiol and Endoplasmic Reticulum Stress in the Development of Esophageal Carcinoma

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Gender differences in esophageal cancer patients indicate that estradiol may have antitumor effects on esophageal cancer. The initiation of endoplasmic reticulum stress (ERS) can induce apoptosis in esophageal cancer cells. However, it is still unknown whether estradiol inhibits the development of esophageal cancer by activating ERS pathway. In this study, the gender difference in the development of esophageal cancer was observed by analyzing clinical data and the experimental tumor xenografts in mice. Meanwhile, we investigated the mechanism of ERS in estradiol-mediated inhibition of esophageal cancer using esophageal squamous cell carcinoma cell line EC109. The proportion of male patients with esophageal cancer was significantly higher than female patients. Meanwhile, male patients were prone to have adventitial invasion. The weight of transplanted tumors in female mice was significantly smaller than that in male mice. *In vitro* experiments showed estradiol inhibits the viability and migration of EC109 cells by increasing the expression of ERS-related proteins, whereas ERS inhibitor 4-PBA abolished the effects of estradiol. In conclusion, our data demonstrate that sex difference exists in the occurrence of esophageal cancer. Estradiol can inhibit the viability and migration of esophageal cancer cells through the activation of ERS, providing a novel insight for esophageal cancer development, treatment, and prevention.

Keywords: esophageal cancer, endoplasmic reticulum stress, gender difference, estradiol, EC109 cell

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INTRODUCTION

Esophageal cancer is one of the eight most common cancers in the world (1), with poor prognosis and low long-term survival (2). Esophageal cancers were mainly classified by the tumor–node–metastasis (TNM) grading standard, and surgery is a conventional treatment for most types of esophageal cancer. There are also auxiliary treatment methods such as radiotherapy and chemotherapy (3). However, surgery requires high physical conditions of the patients, and the recovery is slow after operation (4). Radiotherapy and chemotherapy often cause adverse reactions, which affects the function of various tissues and organs of the patients and reduces the repair ability of esophageal mucosa (5). Therefore, it is urgent to explore new therapy methods.

Decades of research found that there are significant gender differences in esophageal cancer among all races and across the world (6). The incidence of esophageal cancer is three to four times

more common among male than female individuals, but the exact mechanism is unclear (7). The identified risk factors for esophageal cancer also cannot fully explain this gender difference and may be related to sex chromosome mechanism. However, the epidemiological studies (8–10) and some preclinical studies indicate that sex hormones might play an important role in esophageal cancer. Sex steroids such as estrogens contribute to the physiological maturation and cell proliferation of estrogen-dependent tissues, such as breast, ovary, and endometrium (11, 12). Canceration of these tissues is associated with abnormal changes in sex steroid levels. However, the functions of steroid hormones in esophageal cancer are often ignored, although there are significant gender differences in esophageal cancer patients. Steroids can affect cell behaviors through non-genomic and genomic actions (11). Thus, studying the functions and mechanisms of steroids and steroid antagonists holds great promise for esophageal cancer treatment and prevention. With the latest research, it has been found that overexpressions of estrogen receptor α and β in esophageal malignant tumors are associated with prognosis (6, 13). *In vitro* studies also demonstrated that estrogens have remarkable inhibitory effect on the occurrence of esophageal cancer (14, 15). Although the antitumor effect of estrogens on esophageal cancer has been reported, its molecular mechanism is still unknown.

Endoplasmic reticulum stress (ERS) is a reaction induced by the disorder of Ca^{2+} balance and overload accumulation of protein in endoplasmic reticulum when cells are injured. ERS-induced apoptosis is the third apoptosis pathway in addition to the death receptor- and mitochondrial-mediated apoptosis pathways. Recent studies indicate that ERS plays a key role in tumor progression. The initiation of ERS signaling can induce apoptosis in esophageal cancer cells (16, 17), which may represent a novel insight for the therapeutic intervention of esophageal cancer. Several studies have demonstrated the role of E2 treatment in enhancing ERS in a few tumors (18–20). E2-treated MCF-7 cells showed increased ERS, inflammatory stress response, and apoptosis (21). ERS is the key biological event that determines the fate of cells after E2 treatment. However, whether estrogens inhibit the occurrence of esophageal cancer by interaction with ERS has not been investigated.

Therefore, in this study, we analyzed the age and gender data of patients with esophageal cancer and used the murine xenograft model in both sexes to confirm the gender difference in esophageal cancer. Furthermore, the inhibitory effects of estradiol and ERS in the viability and migration of esophageal cancer cells were verified using cell experiments.

MATERIALS AND METHODS

Clinical Data

The data of 372 patients with esophageal cancer treated in the First Affiliated Hospital of Hebei North University from June 2012 to March 2020 were collected. The diagnosis was confirmed by pathological section analysis after operation, and the classification of esophageal cancer was determined at the same time. The age, sex, and the relationship between gender difference and lymphatic metastasis or adventitial invasion were analyzed.

Cell Culture

Human esophageal squamous cell carcinoma cell lines EC109 were generously provided by Life Science Research Center of Hebei North University. The cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/ml), and streptomycin (100 U/ml). All cells were maintained in the presence of 5% CO_2 at 37°C in a humidified atmosphere.

Xenograft Model Establishment

EC109 cells in exponential stage were collected and centrifuged at 1,000 rpm for 5 min. After two washes with phosphate-buffered saline (PBS), and the cell concentration was adjusted to 5×10^7 cell/ml with RPMI 1640 medium without fetal bovine serum. EC109 cell tumor xenografts were established by subcutaneously injecting 1×10^7 cells into the right flanks of 4- to 6-week-old mice. The tumor-bearing mice were divided into male and female group; each group included eight mice. All procedures were performed under sodium pentobarbital anesthesia. The animal experiment was approved by the Animal Ethics Committee of Hebei North University. After 4 weeks of rearing, mice were sacrificed by cervical dislocation. Tumor tissues were harvested, photographed, and weighed. The tumor inhibition rate of the female group was calculated with the formula as follows: tumor inhibition rate = (average tumor weight in male group – average tumor weight in female group)/average tumor weight in male group × 100%.

Analysis of Cell Viability

EC109 cells were assigned into the control group (vehicle), E2 group (10 nM), E2 + ICI group (10 nM E2 and 1 μM ICI 182, 780, an estrogen receptor antagonist), E2 + 4-PBA (ERS inhibitor) group (10 nM E2 and 5 mM 4-PBA), and ERS agonist tunicamycin (TM) group (10 μg/ml). Cell Counting Kit-8 (CCK-8, Applygen Technologies Inc.) was used to measure cell viability according to the manufacturer's protocol. In brief, cells growing at the exponential stage were seeded into 96-well plates at a density of 5,000 cells/well in a final volume of 100 μl and exposed to various treatments for 24 h. Ten microliters of CCK-8 solution was added to each well for a 4-h incubation. Cell viability was calculated by measuring the absorbance at 450 nm. All experiments were repeated three times, and the data are expressed as the mean ± SEM of three wells per treatment.

Analyses of Cell Migration

A cell culture wound-healing assay was performed to analyze cell migration. Cells growing at the exponential stage were seeded into six-well plates at a density of 1×10^5/ml in a final volume of 2 ml. Cells were grown to confluence, and a linear wound was created in the confluent monolayer using a 200-μl micropipette tip. The cells were then washed with PBS to eliminate detached cells. Pictures were taken under the microscope to record the scratches on each well. In order to reduce the effect of DNA

replication and proliferation on the cell migration rate, the serum-free medium was used in the current experimentation according to the previous reports (22, 23). After exposure to various treatments for 24 h, the movement of the wound edge was monitored under a microscope (200 ×). The area between the two sides of the scratch is measured using ImageJ software. Cellular migration rate is calculated by the relative area between the two sides of the scratch. The formula of calculation is as follows: cell migration rate = (scratch area before treatment – scratch area after treatment)/(scratch area before treatment) × 100%.

Analyses of Immunofluorescence

After counting the cells, cells growing at the exponential stage were seeded into confocal dish at a density of 1×10^5/ml in a final volume of 200 μl and cultured for 16–18 h. After cultured in serum-free RPMI 1640 medium for 24 h, the cells were exposed to various treatments for 24 h. Then, cells were washed with PBS and fixed with 4% paraformaldehyde/PBS (30 min) for confocal microscopic analysis. After permeabilization with Triton X-100, cells were blocked with 5% normal bovine serum albumin for 30 min and incubated with antiglucose regulated protein 78 (GRP78) (1:500, ab21685, Abcam), antiestrogen receptor α (ERα) (1:100, ab32063, Abcam), and anti-ERβ (1:100, ab212351, Abcam), respectively, at 4°C overnight. After rinsing with PBS, the dishes were incubated with corresponding fluorescence secondary antibodies for 90 min. The dishes were mounted after staining with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min and analyzed under an Olympus laser confocal microscope (Olympus, Japan).

Analyses of Western Blotting

EC109 cells were seeded into six-well plates and were treated with different agents for 24 h when the confluence reached 80%. The cells were washed with PBS and lysed in 100 μl radioimmunoprecipitation assay (RIPA). After brief sonication and centrifugation, the supernatants were collected for protein concentration measurement by bicinchoninic acid (BCA) kit. The proteins in each group were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% milk, the membranes were incubated with primary antibodies (1:1,000), including anti-GRP78 (ab21685, Abcam), antiactivating transcription factor 6 (ATF6) (ab203119, Abcam), anti-inositol-requiring enzyme 1α (IRE1α) (ab37117, Abcam), antiprotein kinase RNA-like endoplasmic reticulum kinase (PERK) (70R-17036, Fitzgerald), and anti-β-actin (E2317, Cell Signaling Technology). After incubation overnight at 4°C, the membranes were washed and incubated with secondary antibodies (diluted 1: 2000) at room temperature for 1 h. Enhanced chemiluminescence (ECL) was used for signal development. BioRad imaging system was used to capture the chemiluminescence. Analysis was conducted using Quantity One software, and the relative protein levels were expressed as the intensity ratios of target protein to β-actin.

Statistical Analyses

Statistical analysis was performed using SPSS version 22. All experiments were independently performed at least three times. All values are expressed as the mean ± SEM. Differences among groups were analyzed using one-way analysis of variance (ANOVA) by a least significant difference, and *post hoc* test was performed for testing for all data. The related results of xenograft were tested by two independent samples *t*-test. A difference of $P < 0.05$ was considered to be statistically significant.

RESULTS

Gender Differences in Esophageal Cancer

Among the 372 esophageal cancer patients, 339 were male (91.13%) and 33 were female (8.87%), with a ratio of male to female of 10.27:1. The proportion of male patients with esophageal cancer was significantly higher than that of female patients ($P < 0.05$). Among the 372 patients, the youngest was 36 years old and the oldest was 80 years old, and the average age of onset in male patients (61.38 ± 8.34) was slightly lower than that in female patients (65.97 ± 7.51, $P < 0.05$). The sex and age information of patients with esophageal cancer is shown in **Table 1**. The type of esophageal cancer is mainly squamous carcinoma with a percentage of 88.98% (331/372) in total patients, 88.79% (301/339) in male patients, and 90.91% (30/33) in female patients (**Table 2**). There was no statistic difference in the percentage of squamous carcinoma between the male and female patients ($P > 0.05$) (**Table 2**). In addition, there was no relationship between gender and lymphatic metastasis ($P > 0.05$) (**Figure 1A**), but difference was significant in correlation between gender difference and adventitial invasion ($P < 0.05$) (**Figure 1B**).

TABLE 1 | Sex and age distributions in patients with esophageal cancer derived from the First Affiliated Hospital of Hebei North University from June 2012 to March 2020.

Sex	Number of cases (%)	Age distribution	Average age
Male	339 (91.13%)	36–80	61.38 ± 8.34
Female	33 (8.87%)	48–79	65.97 ± 7.52
P	< 0.05		< 0.05

% represents a percentage of the total cases.

TABLE 2 | Type distribution in esophageal cancer patients derived from the First Affiliated Hospital of Hebei North University from June 2012 to March 2020.

Type of esophageal cancer	Male (%)	Female (%)
Squamous carcinoma	301 (88.79%)	30 (90.91%)
Adenocarcinoma	32 (9.44%)	3 (9.09%)
neuroendocrine neoplasm	6 (1.77%)	0 (0.00%)
Total	339	33

% represents a percentage of the same sex cases.

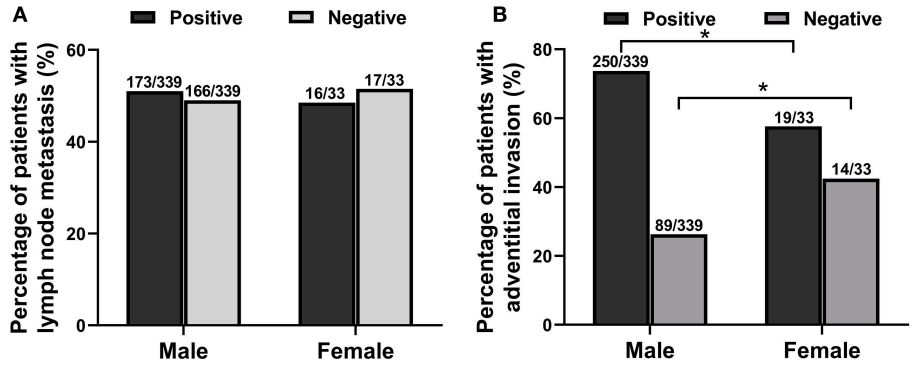

FIGURE 1 | The relationship between gender difference and lymphatic metastasis or adventitial invasion in patients with esophageal cancer from June 2012 to March 2020. **(A)** The relationship between gender and lymphatic metastasis. **(B)** The correlation between gender and adventitial invasion. *$P < 0.05$, compared with the male group.

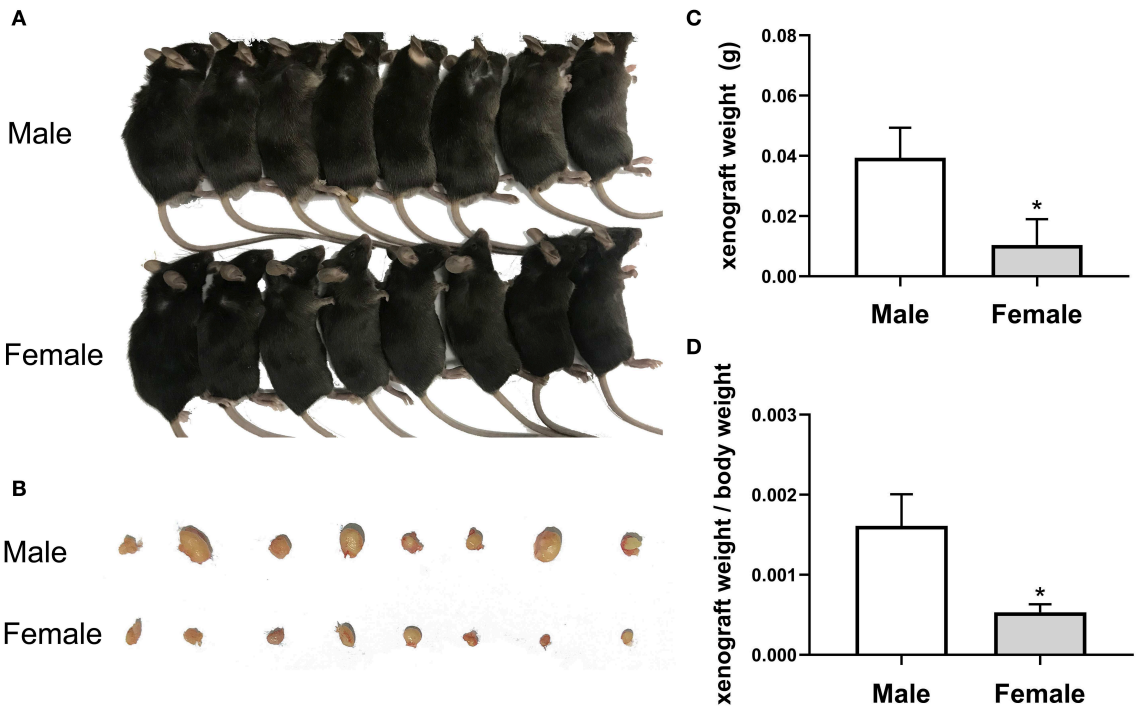

FIGURE 2 | The growth of esophageal cancer xenograft in male and female mice. **(A)** The establishment of xenograft model in mice. **(B)** The appearance of tumor xenograft from the male and female mice. **(C)** The tumor weight analysis. The results are expressed as the means ± SEM, $n = 8$. *$P < 0.05$, compared with the male group. **(D)** The ratio of tumor weight to body weight. The results are expressed as the means ± SEM, $n = 8$. *$P < 0.05$, compared with the male group.

Effect of Gender Differences on Xenograft Tumor in Mice

The current study investigated the anticancer potential of female patients by establishing a model of EC109 cell xenograft. The results showed that the xenograft weight and the ratio of xenograft weight to body weight in the female mice (0.010375 ± 0.001908 g, 0.00053 ± 0.000101) were significantly lower than that of the male mice (0.039375 ± 0.009952 g, 0.00161 ± 0.000395, $P < 0.05$) (**Figures 2A–D**). After calculation, the tumor inhibition rate of the female group was as high as 73.65% compared with the male group. These data revealed that tumor growth was lower in female patients ($P < 0.05$), indicating that estradiol may inhibited tumor growth.

The Expressions of ERs in EC109 Cells

To determine the roles of estradiol in esophageal carcinoma, we detected the protein expressions of ERs in EC109, including ERα

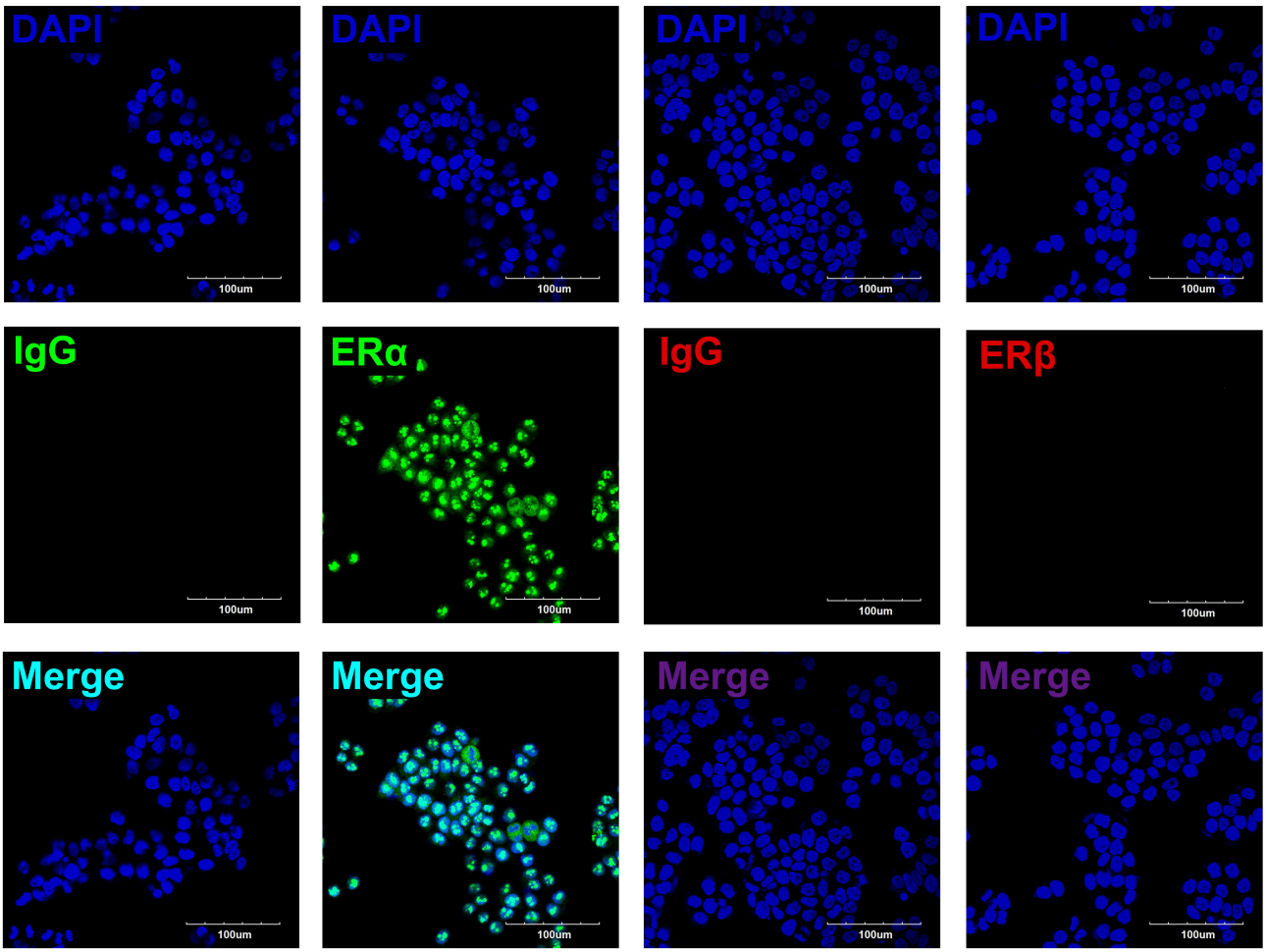

FIGURE 3 | The expressions of estrogen receptors (ERs) in EC109 cells. The representative pictures showed that the positive expression of ERα was located in the nuclei, whereas ERβ was undetectable.

and ERβ. Immunofluorescence showed that ERα was expressed in the nuclei of EC109, whereas ERβ was undetectable. These results indicate that estradiol acts mainly through ERα in EC109 (**Figure 3**).

Effects of Estradiol on the Migration and Viability of EC109 Cells

To investigate whether estradiol has antitumor effects in esophageal cancer, we attempted to determine whether estradiol affects EC109 cell migration and viability *in vitro*. Estradiol or TM treatment for 24 h significantly decreased cell migration ability ($P < 0.05$), when compared with the control group. On the contrary, estrogen receptor antagonist ICI and ERS inhibitor 4-PBA counteracted the effect of estradiol on the migration ability of EC109 cells (**Figures 4A,B**). Furthermore, the data showed that both estradiol and TM treatment significantly reduced the viability of EC109 cells compared with the control group, respectively ($P < 0.05$). Meanwhile, ICI and 4-PBA enhanced the viability of EC109 cells after treatment with estradiol (**Figure 4C**). These results suggested that estradiol may inhibit EC109 cell migration and viability, and this inhibition is mediated by ERS.

Effect of Estradiol on the Expressions of ERS-Related Proteins in EC109 Cells

In order to further identify the molecular mechanism by which estradiol inhibits the viability of EC109 cells, we analyzed the protein expressions of GRP78, ATF6, IRE1α, and PERK, which were involved in ERS. Immunofluorescence was used to detect the expression of GRP78 in EC109 cells after different treatments. Compared with the control group, GRP78 was greatly increased in cells treated with estradiol and TM. Meanwhile, ICI and 4-PBA treatments significantly decreased GRP78 expression in the E2-treated cells, respectively (**Figure 5A**). In order to confirm the response of ERS in EC109 cells treated with estradiol, the expressions of ERS-related proteins were detected by Western blotting. Estradiol and TM treatments resulted in the increase in protein

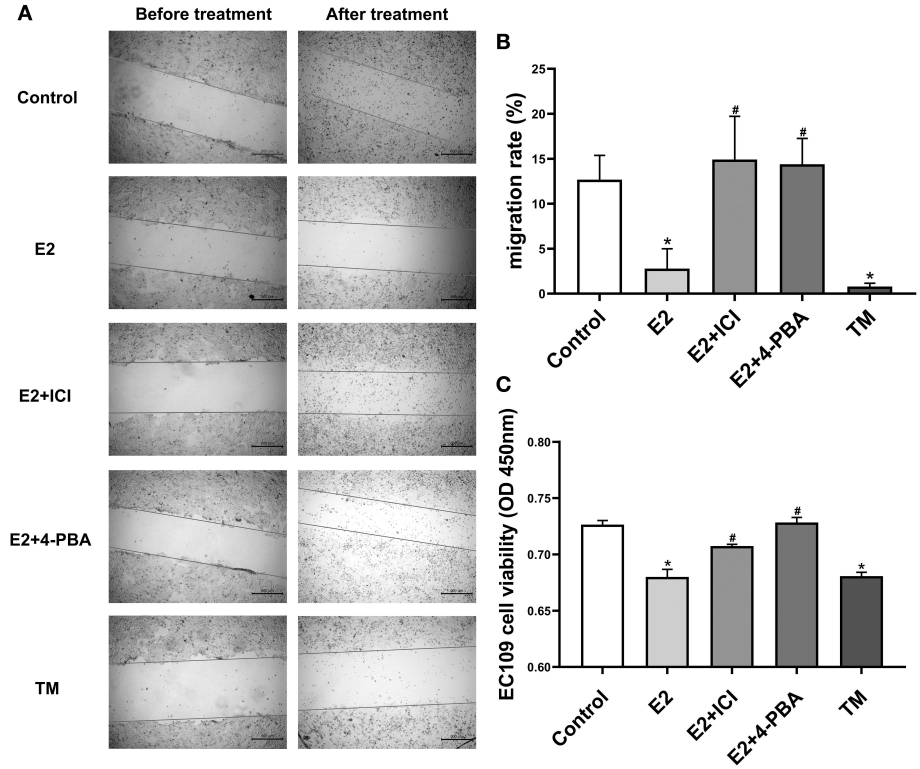

FIGURE 4 | Estradiol treatment reduces the viability and migration rate of esophageal cancer cell line EC109. The cells were cultured in serum-free Roswell Park Memorial Institute (RPMI) 1640 medium for 24 h, and treated with 17-estradiol (E2), E2 and ICI 182,780 (E2 + ICI), E2 and 4-phenylbutyric acid (4-PBA), and tunicamycin (TM) for 24 h, respectively. Then, the cellular migration and proliferation were detected with the cell wound scratch assay and Cell Counting Kit-8 (CCK8) method, respectively. **(A)** Representative wound healing images. The migration rate is calculated by the relative area between the two sides of the scratch. **(B)** The migration rate analysis of EC109 cell. The results are expressed as the means ± SEM, $n = 3$. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the E2 group. **(C)** The EC109 cell viability analysis. The results are expressed as the means ± SEM, $n = 3$. $^{*}P < 0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the E2 group.

expressions of GRP78, ATF6, IRE1α, and PERK in EC109 cells when compared with the control group ($P < 0.05$). Meanwhile, ICI and 4-PBA treatment abolished the estradiol-induced ERS protein expression ($P < 0.05$) (**Figures 5B–E**). These results suggested that estradiol upregulated ERS in EC109 cells.

DISCUSSION

There are sex differences in the development of esophageal cancer, and women who underwent resection has a higher overall survival rate than men (9). The known causes (such as smoking, obesity, etc.) cannot well-explain this gender difference. First of all, this study collected and analyzed the clinical data of 372 patients with esophageal cancer treated by surgery in the First Affiliated Hospital of Hebei North University in the past 9 years. The proportion of male patients with esophageal cancer was significantly higher than that of female patients. Next, in this study, we established the xenograft model to verify whether the sex of mice affected the development of esophageal cancer. The results showed that the xenograft weight and xenograft weight/body weight in the female mice were significantly lower than that in the male mice, with a tumor inhibition rate of 73.65% in the female than in the male mice. The results confirm the relation between sex and the development of esophageal cancer.

In light of epidemiological and preclinical studies, more scholars believe that sex hormones may play an important role in the incidence of esophageal cancer (24, 25). Previous report showed that the increased risk of esophageal cancer is related to the decrease in estrogens level (6). Early menopause increases the risk factors for esophageal squamous cell carcinoma (9). It has been shown that premenopausal female patients have a prolonged survival than postmenopausal patients and that female ESCC patients with higher serum estradiol level have a favorable survival rate (10). An epidemiological study of menopausal hormone therapy (MHT) confirmed that ever-users of MHT were at a decreased risk of esophageal adenocarcinoma, gastric adenocarcinoma, and esophageal squamous cell carcinoma. The estrogens-only MHT users had a decreased risk of esophageal and gastric adenocarcinoma in particular (8). The previous study also supported the protective effects of female hormones on the risk of esophageal squamous cell carcinoma (26) and esophageal adenocarcinoma (27). Through several epidemiological studies,

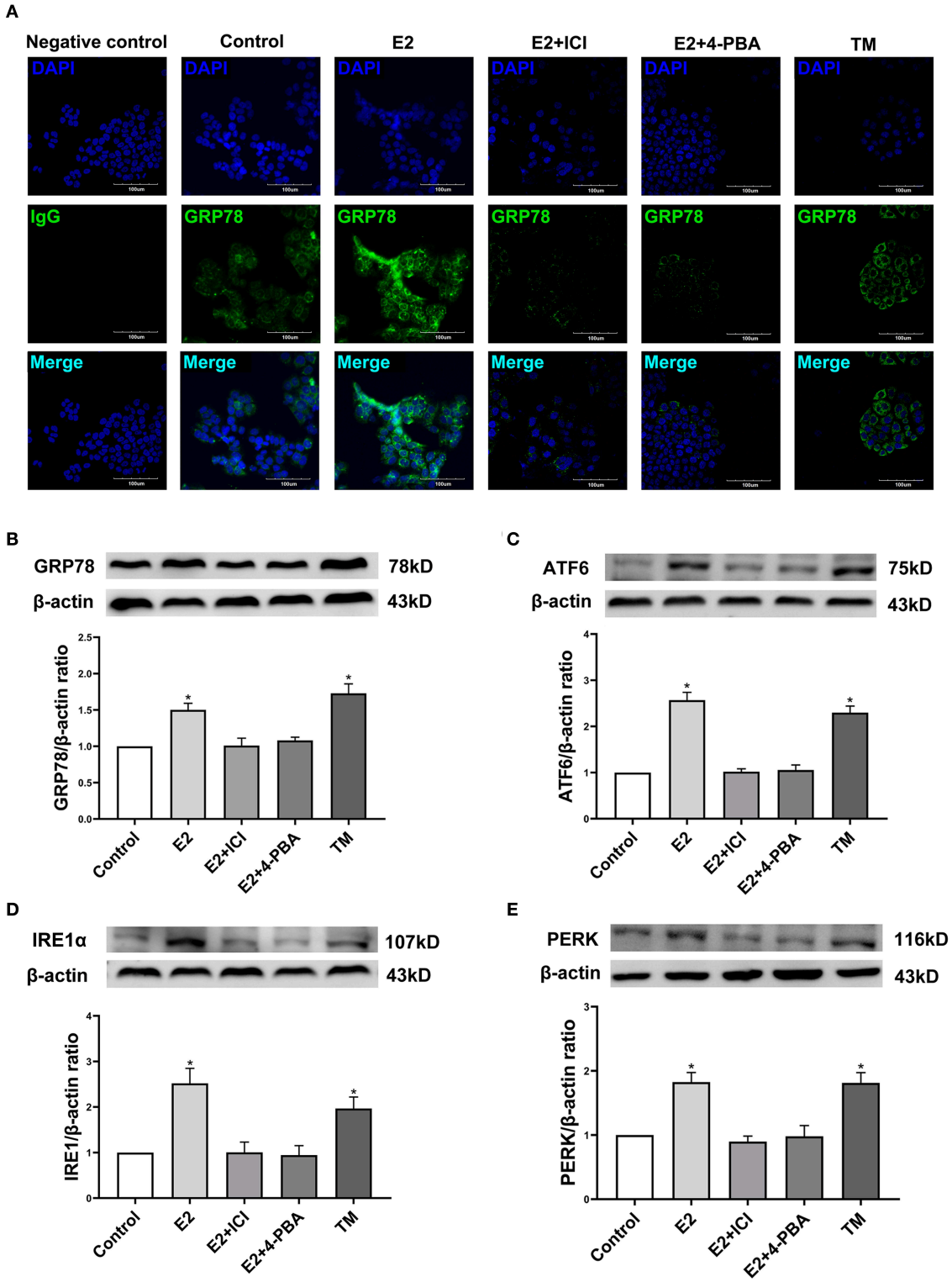

FIGURE 5 | Estradiol treatment upregulates the expression of endoplasmic reticulum stress (ERS) related proteins in esophageal cancer cell line EC109. The cells were cultured in serum-free Roswell Park Memorial Institute (RPMI) 1640 medium for 24 h, and treated with 17-estradiol (E2), E2 and ICI 182,780 (E2 + ICI), E2 and 4-phenylbutyric acid (4-PBA), and tunicamycin (TM) for 24 h, respectively. **(A)** GRP78 proteins in EC109 cells under an Olympus laser confocal microscope. **(B–E)** The expressions of GRP78, ATF6, IRE1α, and PERK in EC109 cells after various treatments. Densitometric values were normalized to β-actin. The results are expressed as the means ± SEM, $n = 3$. $^{*}P<0.05$, compared with the control group; $^{\#}P < 0.05$, compared with the E2 group. The cells were cultured in serum-free RPMI 1640 medium for 24 h.

the hypothesis of estrogen protection in esophageal cancer has been proposed.

The previous studies found that esophageal squamous cell carcinoma and adenocarcinoma tissues express ERs, including ERα and ERβ (10, 28, 29). Estrogen plays a protective role through ERs, and the patients with esophageal cancer who express ERβ in the nucleus have a better prognosis (10, 28). Some studies have also suggested that ERα plays a protective role in esophageal cancer (12, 30), which is consistent with our data, showing that ERα is highly expressed in EC109 but not ERβ. Esophageal cancer cells also express other sex hormone receptors, such as androgen receptor, progesterone receptor, and so on (30, 31). *In vitro* studies have shown that estrogens have a certain inhibitory effect on cell growth and promote the apoptosis of esophageal cancer cells, which is mediated by the interaction with ERs (32). Only esophageal cancer cells with ERs are inhibited by estrogen, whereas cells without ERs are not (33–35). The Cell Counting Kit-8 (CCK8) results of this study showed that estradiol treatment for 24 h significantly decreased the viability of EC109 cells, and the cell scratch assay demonstrated that the estradiol treatment for 24 h significantly decreased the migration ability of EC109 cells. ICI, ER antagonist, improved the viability and migration ability of EC109 cells treated with estradiol. The results indicate that estradiol can reduce the viability and cell migration ability of esophageal cancer EC109 cells, which is consistent with the previous studies that estradiol has a certain inhibitory effect on the growth of esophageal cancer cells.

Endoplasmic reticulum is an important organelle in cells. Under a variety of physiological or pathological conditions, various stimuli can cause unfolded or misfolded proteins to gather in the endoplasmic reticulum, which is named ERS. ERS is divided into two stages: the early unfolded protein response (UPR) (36) and the late induction of apoptosis. In recent years, studies showed that the initiation of ERS induces apoptosis in esophageal cancer cells (16), and manipulation of ERS signaling has been identified as a therapeutic target for the esophageal cancer. However, whether estrogens interact with ERS to inhibit the occurrence of esophageal cancer has not been reported. GRP78, as immunoglobulin heavy chain binding protein (Bip), belongs to the heat shock proteins family (37), which localized on the ER membrane of all eukaryotic cells. Under ERS, the expression of GRP78 is increased and plays an important role in cell survival and apoptosis by regulating transmembrane ERS sensor (38). Together, ERS is mainly mediated by endoplasmic reticulum molecular chaperone GRP78 protein. In this study, the results of immunofluorescence and Western blotting showed that estradiol treatment for 24 h significantly increased the expression of GRP78 in EC109 cells. Therefore, ERS response was evident in EC109 cells after estradiol treatment. In addition, our results showed that the viability and migration ability of EC109 cells were significantly decreased after treatment with ERS agonist TM. The results suggest that ERS can inhibit the growth of esophageal cancer cells. Moreover, 4-PBA, an ERS inhibitor, could improve the viability and migration ability of E2-treated EC109 cells. In summary, the inhibitory effect of estradiol on the growth of EC109 cells is partly due to the interaction with ERS.

Studies indicate that ATF6, IRE1α, and PERK were the main signaling molecules of ERS. The activation of these signaling molecules were demonstrated by various stress and lead to ERS and UPR (39). These protein levels can directly or indirectly sense to misfolded proteins in the endoplasmic reticulum (40). In order to further identify the molecular

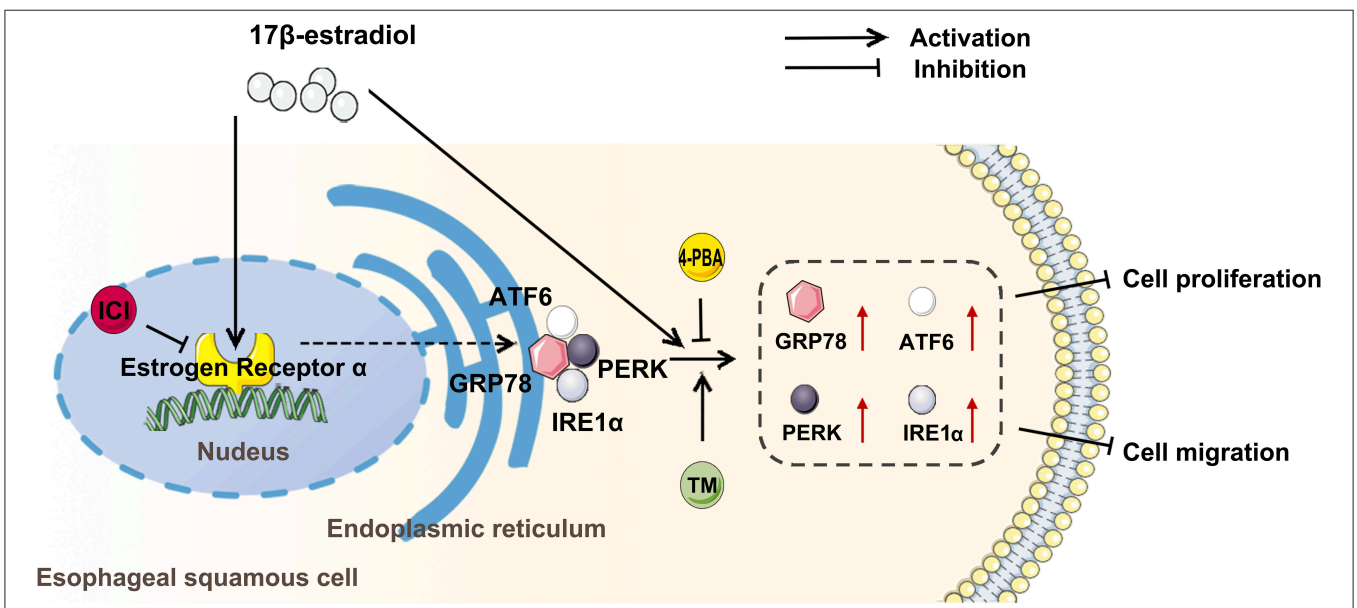

FIGURE 6 | Estradiol treatment inhibits the viability and migration of esophageal cancer cells through the activation of endoplasmic reticulum stress (ERS). ICI, an inhibitor of estrogen nuclear receptors; 4-PBA, an inhibitor of ERS; tunicamycin (TM), an agonist of ERS; GRP78, ATF6, PERK, and IRE1 were ERS-related proteins.

mechanism that estradiol inhibits the viability and migration of esophageal cancer cells by regulating ERS, we detected the expression of ERS-related signaling molecules. Our results showed that both estrogen and TM upregulated the expression of ATF6, IRE1α, and PERK proteins in EC109 cells. In contrast, ICI and 4-PBA treatments eliminated the increase in these proteins induced by estradiol. Recent study (41) showed that the activation of UPR starts from dissociation of GRP78 from ATF6, IRE1, and PERK. If the stress is temporary, the activation of ATF6, IRE1, and PERK can enhance the degradation of unfolded and misfolded proteins through proteasomes. However, if the cells suffer from prolonged or severe stress, additional responses are initiated, involving IRE1/Ask1/JNK, caspase-12/caspase-9/caspase-3, ERK/ATF-4/CCAAT/enhancer-binding protein homologous protein (CHOP) pathways, and these pathways can promote apoptosis (42). This is consistent with our findings that estradiol inhibits the viability and migration of EC109 cells by excessive activation of UPR.

In summary, the present study indicates that the gender difference is involved in the development of esophageal cancer, and estradiol treatment increases the expression of GRP78, ATF6, IRE1, and PERK through estrogen receptor, and upregulates ERS to inhibit the viability and migration of esophageal cancer cells (**Figure 6**). However, the specific molecular mechanism by which EC109 cell apoptosis is induced by estradiol through upregulating ERS-related pathways still needs to be further studied. It should be pointed out that there were several limitations in the xenograft experiments. The roles of androgens or estradiol, ICI or bicalutamide, and 4-PBA or TM treatments in affecting tumor growth were not investigated, and the sera levels of steroid hormones were not detected in these mice. Therefore, the various treatments and castrated mice should be used to verify the roles of androgens and estradiol in xenograft model in the future study. In addition, an analysis of steroid hormones present in the serum of esophageal cancer patients or mice should be performed in the future. Clarifying the role of steroid hormones in the development of esophageal cancer will make the detection of serum steroid hormone level to be a simple and important tool in the screening and personalized therapy of esophageal cancer.

CONCLUSION

There are sex differences in the occurrence of esophageal cancer. Estradiol inhibits the proliferation and migration of esophageal cancer cells by interaction with ERS.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of Reproductive Hebei North University. In this retrospective study, we collected and analyzed the age distribution and average age from the 372 patients with esophageal cancer in the first affiliated Hospital of Hebei North University. However, surgery and treatment options for these patients were not involved in current study, and there was no harm or risk to these patients. Therefore, written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CW, PW, Z-AZ, RG, and YL performed the majority of the animal experiment and laboratory work. CW acquired and analyzed the data. J-CL and Y-SL collected the clinical data. Z-GZ and S-GL involved in the conception and design of the study, data interpretation, and critically revised the manuscript. All authors revised the manuscript critically, approved the version to be published and agreed to be accountable for all aspects of the work.

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MINI REVIEW
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A Role for Estrogen Receptor alpha36 in Cancer Progression

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Estrogen receptor α (ERα) functions as a ligand dependent transcription factor that directly binds specific estrogen responsive elements, thus regulating the transcription of estrogen sensitive genes. ERα has also been shown to be associated with the plasma membrane (membrane associated ERα, mERα), concentrated in lipid rafts, plasma membrane microdomains with a distinct lipid composition, where it transduces membrane-initiated estrogen-dependent activation of the mitogen-activated protein (MAP) kinase signaling pathway. Two isoforms of ERα have been described: the "traditional" ERα66 (66 kDa) and a lower molecular weight variant: the ERα46 (46 kDa). More recently, a novel ERα variant with a molecular mass of 36 kDa (ERα36) has been discovered. Notably, ERα36 has been found expressed in different human tumor cells, including both ER- positive and ER- negative breast cancer cells. Estrogen signaling at the cell membrane via ERα36 appears as capable of activating multiple pathways of importance for cancer aggressiveness and metastatic potential. The presence of serum autoantibodies reacting with mERα (anti-ERα Abs) in a large percentage of patients with breast cancer has recently been reported by our group. These anti-ERα Abs seem to act as estrogen agonists rapidly triggering MAP kinase pathway activation thus inducing tumor cell proliferation and overcoming cell resistance to anti-estrogen drug tamoxifen. In this review, we describe the involvement of ERα36 in different tumors. We also report the potential pathogenetic activity of anti-ERα Abs and their implication in drug resistance.

Keywords: estrogen, estrogen receptors, estrogen receptor 36, signaling, cancer, breast cancer, proliferation, autoantibodies

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INTRODUCTION

The biological effects of estrogen are mediated by specific receptors designated as estrogen receptors (ERs) (1). In humans, ERs play a key role in reproductive processes and are involved in the regulation of many physiological processes in several tissues, organs and systems such as central nervous system, cardiovascular and immune system. ERs belong to the steroid hormone superfamily of nuclear receptors, i.e., mainly detectable in the cell nucleus. However, ERs have also been found in the cytoplasm and even at mitochondrial level. Two different types of ERs have been identified: the estrogen receptor alpha (ERα) (2, 3), and the estrogen receptors beta (ERβ) (4, 5). Both ERα and ERβ are composed of several functional domains that serve specific roles (6, 7). Starting from NH_2- to COO-terminus, the principal domains are (i) the N-terminal A/B domain (NTD); (ii) the DNA-binding domain (DBD); (iii) the ligand-binding domain (LBD). Two activation function (AF) domains, AF1 and AF2, located within the NTD and LBD, respectively, appear as responsible for regulating the transcriptional activity of ER. The regulatory mechanisms exerted by estrogens are mainly carried out *via* the control of gene transcription. This can

occur after activation and dimerization of ERs, by binding an estrogen response element (ERE, AGGTCAnnnTGACCT). However, estrogen can also directly bind DNA through the involvement of cytoplasmic signaling proteins (8) and growth factor receptors (9, 10). Moreover, further estrogen signaling pathways have been observed. In fact, ERs can also be detected at the plasma membrane (membrane associated ER, mER), where they are embedded in lipid rafts, cholesterol enriched plasma membrane microdomains with a distinct lipid composition. These ERs can ignite non genomic pathways such as the activation of the mitogen-activated protein (MAP) kinase signaling pathway (11, 12). In the same vein, more recently, a seven-transmembrane receptor G protein-coupled receptor 30 (GPR30) structurally unrelated to the other ERs but able to mediate rapid non-genomic signals has also been identified (13).

In humans, the ERβ is 530 amino acids in length, with a molecular weight of 59 kDa and it is encoded by the gene estrogen receptor 2 (ESR2), located on chromosome 14, locus 14q23.2 (14). To date, three other truncated shorter isoforms at 54, 49, and 44 kDa and one elongated isoform at 61 kDa are known. The ERα is encoded by the ESR1 gene located on chromosome 6, locus 6q25.1 (15). The full-length size of ERα is 595 amino acids with a molecular weight of 66 kDa (ERα66). In the last few years, two further shorter isoforms (at 46 and 36 kDa) have been characterized. ERα46, the 46 kDa isoform of ERα, lacking of the N-terminal A/B or of the transcriptional activation domain 1 (AF-1), is expressed in various cell types, as macrophages (16), vascular endothelial cells (17), osteoblasts (18) and also in cancer cells. The other isoform, ERα36, the 36 kDa isoform of ERα, differs from the classic ERα66 in the lack of the AF-1 and AF-2 transcriptional activation domains but it retains the DNA-binding domain as well as the partial dimerization and ligand-binding domains (19). ERα36 shares a common overall structure with ERα46 but it is characterized by a unique 27 amino acids domain that replaces the last 138 amino acids encoded by both ERα46 and ERα66 gene. This unique amino acid sequence in ER36 may alter the ligand binding domain, which explains why ER36 has a different binding affinity. This receptor is mainly located in cytoplasm and at the plasma membrane of several different cancer cell types (19–21) and even in healthy tissues, among which ovarian, breast, kidney, lung, heart and bone (22).

ESTROGEN RECEPTOR ALPHA 36 MOLECULAR MECHANISMS

ERα36 is mainly involved in the initiation of non-genomic signaling pathways to activate the phosphatidylinositol-3-kinase/AKT (PI3K/AKT) and the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) (19, 23). The interaction of ERα36 with 17β-estradiol (E2) causes Src activation inducing downstream cascades: MEK activation, phosphorylation of ERK and paxillin (PXN), which induces a third messenger expression, cyclin D1 (24). ERα36 also activates ERK1/2 through the protein kinase C (PKC) delta signaling pathway, and phospholipase D (PLD), leading to an increase in the expression of cyclin D1/cyclin-dependent kinase 4, which regulates cell cycle progression, leading to an increase in the proliferative rate and to an enhancement of metastatic potential (25). Through ERα36, E2 and tamoxifen induce the activation of MAPK/ERK and PI3K/AKT pathways that, in turn, regulate c-Myc protein expression, contributing to the cancer metastatic potential (26, 27). More in general, several studies suggest that ERα36 could act as a negative dominant regulator of estrogen genomic signaling promoted by ERα66 and ERβ (28). However, there is a positive feedback mechanism between ERα66 and ERα36. In fact, ERα66 appears as able to suppress the ERα36 activity. The loss of ERα66 expression related to an increase in ERα36 expression represents one of the mechanisms leading to the acquisition of resistance to antiestrogen therapy, e.g., by tamoxifen (28). This review highlights the effects of the ERα36 on several different cancer cells types.

ERα36 IN DIFFERENT TYPES OF TUMORS

It is now known that estrogens, through their receptors, play an important role in the pathogenesis of many types of tumors. In particular, ERα36, has been demonstrated to be involved in tumor progression and growth, metastatic potential, resistance to treatment and poor prognosis (29). A high expression of this isoform has been described in some types of tumors, such as renal cell carcinoma, papillary thyroid carcinoma, laryngeal carcinoma, endometrial carcinoma, hepatocarcinoma, gastric cancer, neuronal tumors (neuroblastoma and glioblastoma), and breast cancer. However, whether ERα36 could play a role in other human cancers is still scarcely investigated and cannot be ruled out.

Renal Cell Carcinoma

Dysregulated estrogen signaling contributes to the initiation and progression of renal cell carcinomas (30), but the mechanism has not been well established. Wang et al., in a retrospective study, hypothesized that ERα36 may be involved in tumor progression (31). In this study the authors described a different expression level and cell localization of ERα36 in malignant and benign renal tumor cells. In particular, a greater expression of ERα36 in malignant tumor cells compared to benign ones with a predominantly cytoplasmic localization in the latter has been shown. Furthermore, the high expression levels of ERα36 have been found related (i) to renal cell carcinoma necrosis, and (ii) to increased metastastatic aggressiveness. Further studies appear as mandatory in order to evaluate the potential role of ERα36 expression levels as prognostic markers in renal cell carcinoma and to differentiate benign from malignant tumors (31).

Papillary Thyroid Carcinoma

Papillary thyroid cancer (PTC) represents about 80% of malignant thyroid tumors and is three times more common in women than in men suggesting a critical role of estrogen in its occurrence and development (32–34). Dai et al., analyzed the expression levels of ERα36, in association with the epidermal growth factor receptor (EGFR) and HER2, in PTCs, nodular

hyperplasias and normal thyroid tissues (35). The results obtained highlight the existence of a significant correlation between the high levels of expression of ERα36 in PTC tissues and the progression and increase of tumor metastases. In particular, by correlating the expression levels of ERα36, EGFR and HER2 with the clinicopathological characteristics of PTC, high levels of these receptors significantly correlate with extrathyroidal extension and lymph node metastasis have been found. Conversely, there is no correlation between ERα36 expression and the histologic subtype, age, gender and tumor size of PTC patients. Hence, ERα36, in association with the expression of EGFR and HER2, could represent, if further validated, a possible marker of the tumor stage of PTC (35).

Laryngeal Carcinoma

Significant differences between sexes have been described as concerns this carcinoma, with a male to female ratio of 11: 1 (36–38). This, suggests that sex hormones may be involved in the tumorigenesis of this form of cancer. Even though it is not uniformly accepted as a hormone-dependent tumor, laryngeal cancer expresses ERα36. By *in vitro* studies using laryngeal carcinoma epithelial cells (Hep2), the binding of ERα36 with E2 has been shown to induce a rapid activation of PKC and PLD, with an increase in the proliferative rate and in resistance to chemotherapy-induced apoptosis. In Hep2 cancer cell line, ERα36 is located in caveolae (sphingolipid and cholesterol rich plasma membrane microdomains) in association with caveolin-1 and, after E2 treatment, induces an upregulation of angiogenic and metastatic factors (39). By immunohistochemical analysis of human laryngeal tumors, an association between the amount of ERα36 and vascular endothelial-derived growth factor (VEGF) has been observed, supporting a role of ERα36 in vascularization and metastasis (39).

Endometrial Carcinoma

Endometrial carcinoma is one of the most frequent gynecological malignancies in women (40, 41). Among the most common risk factors are polycystic ovary syndrome, obesity (42) and prolonged exposure to endogenous estrogens (43). Estrogens, in addition to increasing the risk of tumor onset, play a key role in the development and progression of endometrial carcinoma (40). Comparing endometrial cancer cells to normal cells, an increase in aromatase activity, the enzyme that converts androgens into estrogens, has been observed (44).

Interestingly, endometrial carcinoma cells (Hec1A) express ERα36 at plasma membrane and cytoplasm level (45). A positive correlation between ERα36 and EGFR expression levels has been observed in Hec1A cultured cells suggesting the involvement of ERα36 in the activation of the extracellular signaling linked to EGFR in endometrial carcinoma (45). Moreover, as observed in other tumors, ERα36 promotes the agonist activity of tamoxifen in endometrial cancer cells (23). In fact, both estrogen and tamoxifen are able to promote the activation of the MAPK/ERK and PI3K/AKT pathways through ERα36 (23). Furthermore, treatments with estrogens or tamoxifen induce the expression of the proto-oncogene c-Myc in Hec1A cells (23, 25). Therefore, ERα36 could be considered as a potential prognostic biomarker of endometrial carcinoma (46).

Hepatocarcinoma

Hepatocellular carcinoma (HCC) is one of the most common malignancies and is the third cause of cancer-related death worldwide. The risk is increased in presence of chronic hepatitis and cirrhosis. The incidence of this carcinoma is 3 times higher in men than in women suggesting that sex hormones, estrogen in particular, could play a critical role in its development (47). In fact, some studies suggest that estrogens could exert a protective role in the development of HCC (48). Accordingly, the incidence of this carcinoma can be significantly lowered by estrogen treatment in post-menopausal women (49).

As concerns ERs, Miceli et al. (50) suggest that a switch from the expression of ERα66 to the expression of ERα36 could be associated with development and progression of human HCC. ERα36, poorly expressed in normal hepatocytes, is instead well expressed by hepatocarcinoma cells and is localized at the plasma membrane as well as in the cytoplasm, supporting the idea that it could be involved in HCC development and/or progression (50). Other studies highlight more complex role of ERα36. Its expression seems in fact to be higher in primary HCC in comparison with secondary HCC and it appears as inversely correlated to ERα66 expression (21). Hence, these authors hypothesize that the expression level of ERα36 might be considered a useful tool to differentiate the primary and secondary HCC. Further insights also derive from studies carried out with epigallocatechin-3-gallate (EGCG), a natural product that exerts its anti-cancer in HCC by inhibiting ERα36 (51). Hence, ERα36 appears to play a role in the pathogenesis of HCC, but further studies are needed to better understand the exact role of the different ER isoforms in this cancer.

Gastric Cancer

To date, the molecular and cellular mechanisms involved in the development of gastric cancer are still to be elucidated. Some studies suggest a protective role for estrogen in the development of gastric cancer (52–54). In fact, the incidence of this cancer is higher in men than in women before menopause and tends to increase in women after menopause (53). Other data highlight an involvement of estrogen in the tumorigenesis of gastric cancer (55). In this regard, ERα36 appears to be highly expressed in gastric cancer cell lines with a mainly cytoplasmic and/or surface localization. In addition, ERα36 expression is significantly associated with lymph node metastasis but not with the other clinicopathological features of gastric adenocarcinoma. Therefore, the increase of ERα36 expression in gastric adenocarcinoma and its association with metastasis could suggest that the evaluation of ERα36 level could represent a prognostic biomarker for gastric cancer progression (56). However, opposite results have been reported by another study conducted by Wang et al. These authors reported a lower expression of ERα36 in tumor tissues than in normal tissues and an expression level of ERα36 negatively correlated with the tumor size and the number of metastases (57). On these bases, the need of further studies aimed at clarifying the effective

role of ERα36 in gastric cancer onset and progression appears well evident.

Neuroblastoma

Neuroblastoma is a very aggressive solid tumor that occurs most frequently in children after leukemia and brain cancer. It is an embryonic tumor that originates from the sympathetic nervous system (58). In recent years, several lines of evidence have demonstrated that ERα can contribute to neuroblastoma tumorigenesis. In particular, the expression of ERα appears to be related to neuronal differentiation and to the survival rate of patients with neuroblastoma (59, 60). *In vitro* studies in human neuroblastoma SH-SY5Y cells, the knocking down of the ERα36 gene with the specific siRNA lead to a reduction of cell proliferation and an increase in apoptotic susceptibility. In particular, the silencing of ERα36 seems to be associated to a reduction in protein phosphatase 2A (PP2A) activity (of importance in cellular homeostasis and tumor suppression) and an increase in phosphorylation of the tau protein (of importance in cytoskeletal integrity and function). In addition, ERα36 gene silencing has been shown to reduce the expression of Cyclin Dl, the proliferating cell nuclear antigen (PCNA) and B cell lymphoma-2 (Bcl-2) antiapoptotic protein while increasing the expression of proapoptotic protein Bax. Furthermore, the regulation of some pathways such as MAPK/ERK and PI3K/AKT has been shown to be dependent on the interaction between Caveolin-1 and ERα36, i.e., on lipid raft function (61).

Glioblastoma

Glioblastoma (GBM) is a highly aggressive and highly invasive primary brain tumor. Patients with GBM have a poor prognosis with an average survival of approximately 1 year (62). Although several studies have shown that adjuvant treatment with tamoxifen could be capable of sensitizing glioblastoma cells to radiation therapy also inhibiting their proliferation, this approach cannot be used for all types of glioblastoma. In addition, long-term use of tamoxifen can lead to the induction of tamoxifen resistance (63, 64). Therefore, several studies have been conducted to understand the molecular mechanism of tamoxifen resistance and to improve the quality of life of patients with glioblastoma (65). In this regard, an involvement of ERα36, that is highly expressed in GBM, has been suggested as pivotal in the induction of resistance to tamoxifen treatment. In fact, Qu et al. observed that high levels of ERα36 could block the tamoxifen-mediated cell growth inhibition and induce autophagy by hindering the AKT/mTOR signaling pathway. However, the effect of autophagy on tumor cell viability is still to be elucidated. Indeed, it seems that in conditions of nutrient and oxygen deficiency, when the tumor size increased, autophagy could promote the survival of cancer cells. Accordingly, *in vitro* studies on glioblastoma cells treated with tamoxifen showed a significant increase in ERα36 expression level accompanied by an increased cytoprotection by autophagy. These results provide new insights into the mechanism underlying the antiproliferative, cytostatic, properties of tamoxifen and the involvement of ERα36 in resistance to tamoxifen treatment (65) also suggesting the contribution of autophagy pathway to the development and progression of glioblastoma (62).

Breast Cancer

This is the most common form of cancer among women. About 70% of breast cancers express ERα66, which is involved in the transcriptional regulation of estrogen-sensitive genes (66–68). Among the main therapeutic treatments used, there are anti-estrogens, which tend to block the molecular pathogenic pathways mediated by ERα66. Unfortunately, many patients develop *de novo* or acquired resistance to these therapeutic agents, which is associated with the onset of metastasis and poor prognosis (69–71). Recently, ERα36 has been found expressed in ER-positive and ER-negative breast cancer cells (20, 24, 72). ERα36 expression levels have been associated with some clinicopathological features of breast cancer (tumor size, clinical stage, histological grade, lymph node metastasis) (28). Triple negative breast cancers (TNBC), i.e., lacking of ERα66, progesterone receptor (PR) and epidermal growth factor receptor (EGFR), are very aggressive tumors with high recurrence, elevated mortality rates and limited therapeutic options. Maczis et al., observed that ERα36 is expressed in TNBC and is involved in E2-induced activation of sphingosine kinase 1 (SphK1) and for the production of shingosine-1-phosphate (S1P), which has a role in tumor growth, progression, transformation and metastasis (73). Interestingly, tamoxifen could act as an agonist of ERα36 and induce proliferation, invasion and metastasis in breast cancer cells. In support of these data, Wang et al. observed that ERα36, through the tamoxifen agonist activity, can be able to increase the expression of aldehyde dehydrogenase 1A1 (ALDH1A1), a molecule involved in cancer stem cell maintenance and metastasis. In particular, treatment with tamoxifen induces the nuclear translocation of the ERα36 receptor, which directly regulates the transcription of ALDH1A1, suggesting a genomic-type mechanism of action in stem/progenitor cells of ER-positive breast cancers (29, 74).

Previous studies have shown that there are several molecular mechanisms and/or pathways involved in treatment resistance, including EGFR and HER2 (69, 75). Recently, the existence of a positive correlation between the levels of expression of ERα36 and EGFR/HER2 has been suggested to be involved in the mechanism of resistance to tamoxifen and in the increased proliferative capacity of breast cancer (20, 27, 69, 75–77). The activation of the SphK1/S1P/S1PR axis mediated by E2 through ERα36 could be one of the mechanisms that lead to the development of *de novo* and acquired resistance to anti-estrogenic therapy of breast cancer (73). In addition, a positive correlation between I-kappa-B-kinase-epsilon IKKε (an oncogene, member of the IKK family) and ERα36 has been observed (78). In particular, IKKε interacts with ERα-36 and increases its expression. IKKε seems to promote the mitogenic signaling of estrogens through ERα36 with the consequent induction of proliferation in ER-negative breast cancer cells (78). To note, in ER-negative cancer cells, ERα36 can inhibit and/or activate the EGFR/signal transducers and activators of transcription 5 (STAT5) pathway through the regulatory function of Src. *In vitro* studies on ER-negative cells show how low

concentrations the tamoxifen are able to induce the activation of MAPK/ERK pathway whereas at higher concentrations the signal is turned off. This could be explained by the fact that different concentrations of anti-estrogens could lead to different conformations and/or functions of ERα36 (79). Overall, even though several molecular mechanisms are involved in the occurrence of anti-estrogenic resistance, ERα36 seems to contribute to these mechanisms also playing a role in the maintenance of stem/progenitor cells of breast cancer.

Recently, the presence of serum autoantibodies reacting with ERα (anti-ERα Abs) in a large percentage of patients with breast cancer has been shown (80, 81). *In vitro* studies with anti-ERα Abs purified from sera of patients by affinity with the recombinant ERα66, allowed us to observe that they bind to and activate ERα expressed at membrane level, located within the lipid rafts triggering rapid MAP kinase activation and inducing tumor cell proliferation (80). Moreover, anti-ERα Abs are also able to interfere with the efficacy of tamoxifen treatment suggesting that they can react and activate also the ERα36 isoform (81). However, in contrast, anti-ERα Abs showed no reactivity with the ERα66-negative, ERα36 positive MDA-MB-231 cells, indicating that ERα36 epitopes could be not accessible to antibodies, perhaps for conformational modification in these tumor cells. To note, treatment with simvastatin, removing or lowering cellular cholesterol, an integral component of lipid rafts (82, 83), inhibits anti-ERα Abs effect on proliferation and cell cycle progression (81).

Finally, even though it has been observed that cell surface ER could activate intracellular pathways, the precise mechanisms are still unknown. An interaction with receptor tyrosine kinases and/or with G protein-coupled receptors could be involved in downstream signaling pathways (e.g., phosphatidylinositol-3-kinase, Akt/protein kinase B and the mitogen-activated protein kinase cascade). Moreover, we also cannot exclude a cross-reaction of these autoantibodies with GPR30 leading to a rapid non-genomic signals. In all cases, the identification of autoantibodies reacting with mERα could be considered as a potential prognostic and predictive tool for breast cancer.

CONCLUSIONS

Summarazing, it can be hypothesized that ERα36 could play an important role in estrogen signaling during the development and progression of several forms of cancer. However, to date, some open questions remain unanswered.

(i) The first one regards the potential role of ERα36 in further forms of human cancers, in particular, those presenting significant sex/gender differences in terms of incidence or progression such as melanoma and colon cancer.
(ii) The second open question concerns the potential therapeutic usefulness of statins. These drugs, impairing ERα36 function in patients with estrogen-dependent cancers, could exert some beneficial effect.

Further studies are thus mandatory in order to clarify both these aspects but, also, to better evaluate the role of ERα36 in the clinical practice as prognostic biomarker and/or as therapeutic target leading to the reduction of tumor growth and progression and/or reducing the occurrence of anti-estrogenic therapy resistance.

AUTHOR CONTRIBUTIONS

MP study conception and design, manuscript drafting. EO study conception and design, critical revision. MD study conception and design, manuscript drafting, critical revision. All authors contributed to the article and approved the submitted version.

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Intracellular Progesterone Receptor and cSrc Protein Working Together to Regulate the Activity of Proteins Involved in Migration and Invasion of Human Glioblastoma Cells

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Glioblastomas are the most common and aggressive primary brain tumors in adults, and patients with glioblastoma have a median survival of 15 months. Some alternative therapies, such as Src family kinase inhibitors, have failed presumably because other signaling pathways compensate for their effects. In the last ten years, it has been proven that sex hormones such as progesterone (P4) can induce growth, migration, and invasion of glioblastoma cells through its intracellular progesterone receptor (PR), which is mostly known for its role as a transcription factor, but it can also induce non-genomic actions. These non-classic actions are, in part, a consequence of its interaction with cSrc, which plays a significant role in the progression of glioblastomas. We studied the relation between PR and cSrc, and its effects in human glioblastoma cells. Our results showed that P4 and R5020 (specific PR agonist) activated cSrc protein since both progestins increased the p-cSrc (Y416)/cSrc ratio in U251 and U87 human glioblastoma derived cell lines. When siRNA against the PR gene was used, the activation of cSrc by P4 was abolished. The co-immunoprecipitation assay showed that cSrc and PR interact in U251 cells. P4 treatment also promoted the increase in the p-Fak (Y397) (Y576/577)/Fak and the decrease in p-Paxillin (Y118)/Paxillin ratio, which are significant components of the focal adhesion complex and essential for migration and invasion processes. A siRNA against cSrc gene blocked the increase in the p-Fak (Y576/Y577)/Fak ratio and the migration induced by P4, but not the decrease in p-Paxillin (Y118)/Paxillin ratio. We analyzed the potential role of cSrc over PR phosphorylation in three databases, and one putative tyrosine residue in the amino acid 87 of PR was found. Our results showed that P4 induces the activation of cSrc protein through its PR. The latter and cSrc could interact in a bidirectional mode for regulating the activity of proteins involved in migration and invasion of glioblastomas.

Keywords: glioblastoma, progesterone receptor, cSrc, non-genomic actions, focal adhesion kinase, paxillin

INTRODUCTION

Astrocytomas are the most common primary brain tumors in the central nervous system (CNS). The WHO classifies these tumors according to the degree of malignancy in a range from I to IV. Grade IV represents the most malignant astrocytoma, also known as glioblastoma (1). Patients with glioblastoma have an overall survival of 15 months, even when receiving the standard therapy consisting of the maximum bearable surgical removal followed by radiotherapy and chemotherapy with temozolomide (2). The current standard treatment for glioblastoma has remained unchanged for more than ten years (3). Some alternatives, such as the use of angiogenesis blockers or Src family kinase inhibitors, have been tested in clinical assays, but none of them with successful outcomes (4, 5) The poor prognosis of patients with glioblastoma is a consequence of the high rate of recurrence of these tumors promoted by the inherently radio-resistance and chemo-resistance and the high rate of migration and tumor invasion cells (6). Glioblastoma cells can spread to the surrounding brain parenchyma, which makes extremely difficult the complete resection of the tumor, and finally provokes the recurrence of glioblastoma (7).

Migration and invasion of tumor cells to the normal brain are complex processes that involve multiple steps and molecular signaling. In this context, the focal adhesion complex has a significant role. Some of their structural and regulator components, including non-receptor cytoplasmic tyrosine kinase cSrc, Focal adhesion kinase (Fak), Paxillin (Pax), Tyrosine-protein phosphatase non-receptor type 12 (PTP-PEST), and integrins have been associated with the spread of glioblastoma cells (8–11). The tyrosine kinase Fak acts as a regulator and scaffold protein since it can recruit cSrc and Pax to the specific sites in the focal adhesion complex. In turn, cSrc can phosphorylate other proteins, including Fak and Pax to form an active complex able to mediate the cell-extracellular matrix (ECM) adhesion, protrusion of cytoplasm to form the leading edge, cell contraction, recruitment of proteases, and detachment of the trailing edge (12). The role of PTP-PEST in glioblastomas has been associated with the stability of focal adhesion substrates (Fak, Pax, among others) by the regulation of their phosphorylation-dependent ubiquitination (11).

Lewis-Tuffin and colleagues demonstrated that some Src Family Kinase members, such as cSrc, Fyn, Yes, and Lyn, have an essential role in the motility of glioblastoma cells since the knockdown of these kinases reduces the rate of migration in three different cell lines (8). Some stem cell markers, such as Oct-3/4 have been related to increased migration and invasion of glioma stem cells through cSrc and Fak upregulation (13).

As a result of the higher prevalence of glioblastoma in men than women (14), sex hormones and their receptors have gained particular attention. Several studies have demonstrated a central role of progesterone (P4) in the promotion of proliferation (15, 16), migration, and invasion (17) of glioblastoma cells. One of the proteins with a great affinity for P4 is the progesterone receptor (PR), which belongs to the nuclear receptor family, and acts as a ligand-inducible transcription factor (18). When oligonucleotide antisense against PR or RU486, an antagonist of PR, was administered, the effect of P4 over migration and invasion on human glioblastoma cells was significantly diminished (17). These results suggest that PR has a significant role in promoting the progression of glioblastomas. In some breast cancer cell lines, it has been proven that P4 activates cSrc through PR, and in turn, increases migration and invasion rate (19). However, the role of PR in cSrc activation and their participation in the migration and invasion of glioblastoma cells is unknown. In this work, we studied the interplay between PR and cSrc, and its effects on the activity of proteins involved in migration and invasion of glioblastoma cells. To study the potential relationship between these proteins, glioblastoma-derived cell lines were treated with P4 or R5020 (PR agonist), and the phosphorylated/non-phosphorylated ratio of cSrc was measured by western blot. P4 and R5020 increased cSrc phosphorylation. To confirm the participation of PR in the cSrc phosphorylation, cells were transfected with a commercial siRNA against PR. Cells transfected with the PR siRNA were unable to increase cSrc phosphorylation. To investigate the physical interaction between PR and cSrc, we performed a co-immunoprecipitation assay, and interaction between PR and cSrc was observed. *In silico* analysis showed that cSrc could participate in the phosphorylation of PR in the amino acid 87. The role of cSrc activation by P4 in the switch Fak-phosphofak and Pax-phosphopax ratios and the migratory capacity of glioblastoma cells was determined by western blot and wound-healing assay in cells transfected with a commercial siRNA against cSrc. Fak phosphorylation and migration decreased in cells transfected with siRNA against cSrc compared to cells treated with control siRNA. Findings of this work suggest for the first time that cSrc and PR interact in glioblastoma cells. P4 through PR induces cSrc activation, which in turn participates in regulating the activity of proteins involved in the migration and invasion of glioblastomas.

MATERIALS AND METHODS

Cell Culture and Treatments

U251 and U87 (ATCC, USA) human glioblastoma derived cell lines were plated in 10 cm dishes and sustained in DMEM medium (*In vitro*, S.A., D.F., MEX), supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 2 mM glutamine, 0.1 mM non-essential amino acids (GIBCO, NY, USA) at 37°C, 5% CO2. The culture medium mentioned above was replaced by DMEM medium (*In vitro*, S.A., CDMX., MEX) without phenol red and free of hormones, supplemented with charcoal-stripped serum FBS (sFBS) (Hyclone, Utah), 24 h before the treatments. Cells were treated with P4 (10, 50 and 250 nM), 10 nM of R5020 (progestin with high affinity for PR (Kd ≈ 2 nM)) (20) or vehicle (DMSO 0.001%). Cell treatments lasted 10 and 20 min to assess cSrc, Fak, and Pax phosphorylation.

Abbreviations: CNS, central nervous system; Fak, Focal adhesion kinase; Pax, paxillin; ECM, extracellular matrix; PTP-PEST, (tyrosine-protein phosphatase non-receptor type 12); TCGA, The Cancer Genome Atlas; P4, progesterone; PR, progesterone receptor; SFK, Src Family Kinase; ER, estrogen receptor, mPRs, membrane progesterone receptors; PAQR, Progestin and AdipoQ Receptor.

Protein Extraction and Western Blotting

Activation of cSrc, Fak, and Pax was determined by measuring protein phosphorylation. Cells were treated with P4 (10, 50, and 250 nM), R5020 (10 nM), or vehicle (DMSO 0.001%), and western blot was used to determine the content of p-cSrc, p-Fak, and p-Pax. After treatments, cells were homogenized in RIPA buffer with a cocktail of protease inhibitors (Sigma Aldrich, St Louis, MO USA, # P8340) and a group of phosphatase inhibitors (NaF, $Na_4P_2O_7$, and Na_3VO_4). Proteins were obtained by centrifugation at 12,500 rpm for 5 min and quantified using the NanoDrop-2000 spectrophotometer (Thermo Scientific, MA, USA). For protein separation, 30 µg were loaded on a polyacrylamide gel at a concentration of 8.5% for cSrc and Pax, and 7.5% for Fak, under denaturing conditions. Proteins were transferred to a nitrocellulose membrane under semi-dry conditions in a transfer (BIO-RAD) for 30 min at 25 V in the case of the 60 kDa (cSrc) and 68 kDa (Pax) proteins and 1 h at 25 V in the case of the 125 kDa protein (Fak). Blocking was performed with 5% bovine serum albumin at 37°C for 2 h. Membranes were incubated with the primary antibodies against the phosphorylated and total forms of the cSrc, Pax, and Fak proteins (phospho Src Tyr-416 Cell Signaling, MA, USA, Ref. 2101; Src Cell Signaling, MA, USA. USA, Ref. 2108; phospho Pax Tyr-118 Cell Signaling, MA, USA, Ref. 2541; Pax Cell Signaling, MA, USA, Ref. 2542; phospho Fak Tyr-397 Cell Signaling, MA, USA, Ref. 3283; Fak Cell Signaling, MA, USA, Ref. 3285). Antibodies against the total and phosphorylated forms were used in a 1/500 dilution. As a loading control, the alpha-tubulin protein was detected at a 1/1,000 dilution (Santa Cruz Biotechnology, St. Louis, TX, USA, Ref. sc-398103). All the antibodies were incubated for 48 h except that against alpha-tubulin, which was incubated for 24 h. Subsequently, the membranes were incubated with the secondary antibody against rabbit (Thermo Scientific, USA, Ref. 1858415) or mouse (Santa Cruz Biotechnology, TX, USA, Ref. sc-516102) (1/10,000) with shaking and at room temperature for 45 min. The primary and secondary antibodies were removed from the membranes with a solution containing Tris–HCl pH 6.8 at 0.06 M, SDS at 2%, and β-mercaptoethanol at 0.7% for 30 min at 50°C at stirring. The chemiluminescent signal was detected by exposing the membranes to the SuperSignal West Fento substrate (Thermo Scientific # 34096) with Kodak Biomax Light Film plates (Sigma-Aldrich, MO, USA).

siRNA Transfection

Commercial siRNA against PR was used to test if P4 induced the cSrc activation through its PR. Briefly, 2.5×10^5 U251 cells were plated in 6-well dishes in DMEM medium supplemented with 10% FBS, and 24 h later, the medium was replaced with DMEM phenol red-free medium without FBS and antibiotics. Cells were transfected with a PR siRNA (100 nM) or with control siRNA that does not induce specific mRNA degradation using Lipofectamine RNAiMAX (Thermo Scientific, USA). The medium was refreshed 12 h after the addition of a PR siRNA or control siRNA, and 48 h after siRNAs addition, the cells were harvested for total RNA extraction to determine the efficiency of the transfection. The same protocol was used with Commercial siRNA against cSrc to test the interplay between P4, cSrc, and Fak and Pax activation. In this case, the efficiency of transfection was determined by western blot, and 48 h after transfection, the cells were harvested for protein extraction as previously described.

RNA Extraction and RT-PCR

RNA extraction was performed using TRIzol reagent (Invitrogen, USA) and following the manufacturer's instructions. One µg of total RNA was used to synthesize the first-strand cDNA in a reaction carried out by M-MLV reverse transcriptase (Thermo Scientific, USA) following the manufacturer's protocol. The efficiency of transfection was determined by RT-PCR from 2 µl of synthesized cDNA. PCR conditions were: 5 min incubation at 94°C followed by 28 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 68°C, and a final incubation for 60 s at 68°C. The 18S ribosomal RNA gene was used as an internal expression control. The primers used were PR forward 5′-CCCGCCCTATCTCAACTACC-3′ and reverse 5′-GTTGTGCTGCCCTTCCATTTG-3'). 18S forward 5′-AGTGAAACTGCAATGGCTC-3′ and reverse 5′-TGACCGGGTTGGTTTTGAT-3'.

Migration Assay

The wound-healing assay was performed to determine the cell migration of U251 cells. 2.5×10^5 cells were plated in 6-well slides with DMEM high glucose supplemented until reaching 70% confluence. Then, cells were transfected as was described in the previous section. About 48 h after transfection and in 90% of confluence, a scratch was made using a 200 µl pipette tip. Floating cells were removed with PBS and DMEM medium (*In vitro*, S.A., CDMX., MEX) without phenol red and free of hormones, supplemented with 10% SFB also free of hormones were added again. Cytosine β-D-arabinofuranoside hydrochloride (10 µM, Ara-C, C1768, Sigma-Aldrich, St. Louis, MO, USA) was used to inhibit cell proliferation 1 h before adding the treatments. Cells were treated with P4 (50 nM), or vehicle (DMSO 0.001%). Four random fields were chosen per treatment to determine cell migration after 0, 6, and 12 h of treatment. Photographs were taken with an Infinity 1-2C camera (Lumenera, CA) connected to the inverted microscope Olympus CKX41 (Olympus, JPN).

Co-Immunoprecipitation

Cell cultures were lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and a cocktail of protease inhibitors (Sigma Aldrich, St. Louis, MO USA, # P8340) at 4°C overnight. Cell lysates were centrifuged at 12,500 rpm for 15 min. One mg of total protein present in the supernatant was incubated with 2 µg of antibody anti-PR (Santa Cruz Biotechnology Dallas, Texas, USA Ref B-30 sc-811) and 50 µL of sepharose-coupled protein A/G plus-agarose (sc-2003; Santa Cruz Biotechnology) under permanent agitation at 4°C overnight. The next day, samples were centrifuged, and the pellets washed three times with buffer (20 mM Tris HCl; 150 mM NaCl; 1mM EDTA, 0.1 Triton X-100,

and a cocktail of protease inhibitors (Sigma Aldrich, St. Louis, MO USA, # P8340 pH 7.5). Finally, the samples were denatured by boiling in loading buffer (120 mM Tris, pH 6.8; 4% SDS; 0.2% glycerol; 5% β-mercaptoethanol; and 10 mg/ml bromophenol blue) and separated in SDS-PAGE. Western blot for cSrc was done as previously described in the Protein extraction and Western blotting section.

TCGA Data Analysis

RNA-Seq counts from 196 grade II, 223 grade III, and 139 grade IV gliomas were obtained from Glioblastoma and Low-Grade Glioma projects of The Cancer Genome Atlas (TCGA) repository (https://portal.gdc.cancer.gov/). The data were downloaded and processed using TCGAbiolinks package version 2.12.6 for R.17 Additionally, expression profiles of 249 healthy brain cortex samples were obtained from the GTEx database (https://gtexportal.org/home/). Data were normalized by DESeq2 version 1.22.2 and plotted. Gene expression correlation in glioblastoma, from the TCGAbiolinks package for R.

Statistical Analysis

Data were and analyzed using Graph Pad Prism 5 program (GraphPad Software, Inc., USA). A one-way ANOVA with Bonferroni *post hoc* test (**Figures 1A, E, F**, **2C–E**, **3B**) or t-student test were used to establish the statistical differences between comparable groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Activation of cSrc by P4 Is Mediated by PR in Glioblastoma Cells

The role of PR and cSrc in breast cancer has been broadly studied. The stimulation of breast cancer cells with P4 activated cSrc through PR, and induced various signaling pathways that conducted to cancer progression (21–23). To test the potential role of P4 in cSrc activation in glioblastoma cells, at the beginning of the study, a time-dependence assay (0–60 min) using P4 (10 nM) was performed in U251 cells (**Supplementary Figure 1**), however, a significant effect on p-cSrc/cSrc ratio was not observed, and we decided to test higher P4 concentrations, at 10 (**Figures 1A, B)** and 15 min (**Supplementary Figure 2**). U251, and U87 cells were treated with three different concentrations of P4 (10, 50 and, 250 nM) for 10 min, and the phosphorylation of cSrc (Y416) was determined by western blot. P4 induced cSrc activation at 50 nM in U251 and U87 cells (**Figures 1A, B)**. P4 has affinity for other receptors besides PR (24, 25); Nevertheless, because of the high affinity of R5020 for PR (Kd ≈ 2 nM) (20) over other receptors (AR 1% binding affinity) (26–28), cells were also treated with 10 nM of R5020. As in the case of P4, R5020 increased the p-cSrc (Y416)/cSrc ratio in U251 and U87 cells (**Figures 1C, D)**. To finally demonstrate that cSrc activation by P4 was mediated by its intracellular PR, U251 cells were transfected with a commercial siRNA against PR or control siRNA (scramble sequence) and treated with P4 for 10 min at 50 nM. The efficiency of transfection was higher than 50% (**Figure 1E**). P4 induced the activation of cSrc in cells with control siRNA as in the previous experiments. In contrast, the siRNA against PR blocked the increase in p-cSrc (Y416)/cSrc ratio induced by P4 (**Figure 1F**). This result demonstrates the participation of PR in the cSrc activation by P4 (**Figure 4**). Considering the short time (10 min) for the activation of cSrc, this result suggests that PR exerts this effect through nongenomic actions.

cSrc and PR Interact in U251 Cells

Non-genomic actions of PR are associated with the polyproline domain which can interact with SH3 domains of a variety of proteins including cSrc. In breast cancer cell lines, a physical interaction between cSrc and PR has been demonstrated (22). Co-immunoprecipitation assay was performed to evaluate the interaction between PR and cSrc. U251 cells were immunoprecipitated with antibodies against PR, and a western blot was carried out. In both vehicle and P4 treated cells, a band corresponding to cSrc was detected, indicating that PR (isoforms A and B) and cSrc directly or indirectly interact in glioblastoma cells. The treatment with P4 increased the interaction between PR and cSrc (**Figure 1G**). This result suggests that activation of cSrc is possible because of the physical interaction between cSrc and PR in glioblastoma cells (**Figure 4**).

P4 Induces a Switch in Fak-Phosphofak and Pax-Phosphopax Ratios Through cSrc in Glioblastoma Cells

Fak and Pax are two of the most critical components of the focal adhesion complex, fundamental to regulating cell migration and invasion. To test if P4 was able to induce Fak and Pax activation, U251 and U87 cells were treated with P4 at 50 nM for 20 min, and their phosphorylation was determined by western blot. P4 (50 nM) increased the p-Fak/Fak ratio (Y397 and Y576/577) in U251 cells and the p-Fak/Fak ratio (Y576/577) in U87 cells, while in the case of Pax, P4 decreased the p-Pax/Pax ratio (Y118) in U251 cells at 20 min (**Figures 2A, B**). cSrc is one of the major kinases implicated in the phosphorylation of focal adhesion complex components, especially Fak (29). To test the role of cSrc in the phosphorylation of Fak and Pax, U251 cells were transfected with a commercial siRNA against cSrc or control siRNA (scramble sequence) and treated with P4 (50 nM) for 20 min. The efficiency of transfection was 50% (**Figure 2C**). The siRNA against cSrc blocked the increase in the p-Fak/Fak (Y576/577) ratio induced by P4 (**Figure 2D**), but not the decrease in p-Pax/Pax ratio (**Figure 2E**). This result indicates that P4 is involved in regulating focal adhesion complex through PR and cSrc in glioblastoma cells (**Figure 4**).

Silencing of cSrc Reduces the Migration Induced by P4

Previous results suggest that P4 and cSrc have a fundamental role in the migration of glioblastoma cells, which in turn, participate in the recurrence of this tumor (17, 30). To determine whether

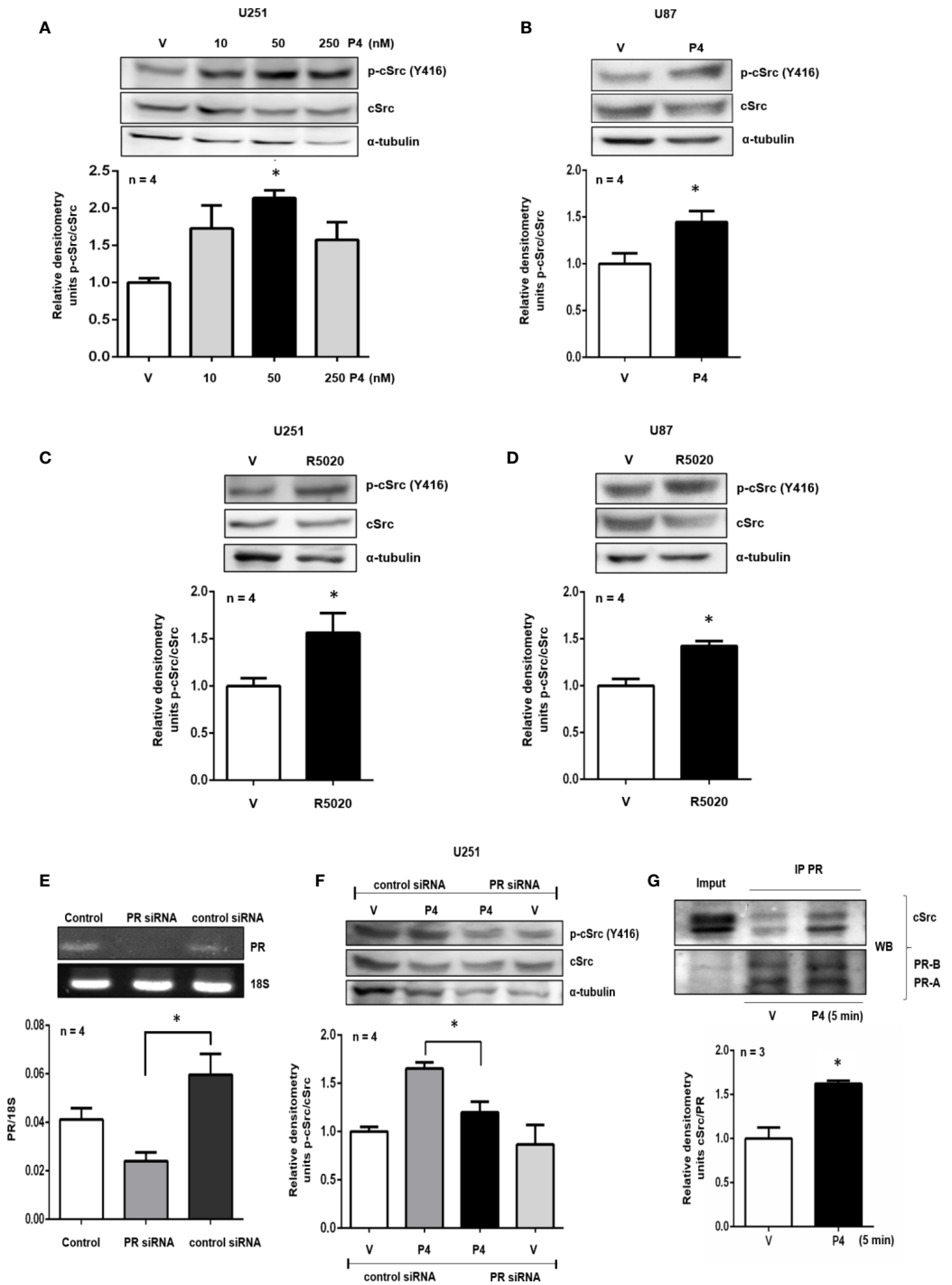

FIGURE 1 | P4 induces the activation of cSrc through PR. **(A, B)** U251 and U87 cells were treated with P4 (10, 50 and 250 nM) and P4 (50 nM) respectively or vehicle (V, DMSO 0.01%) for 10 min. **(C, D)** U251 and U87 cells were treated with R5020 (10 nM) or vehicle (V, DMSO 0.01%) for 10 min. **(E)** U251 cells were transfected with PR siRNA and a control siRNA (an aleatory RNA sequence) (100 nM) or were only treated with lipofectamine (Control). **(F)** Transfected cells with PR siRNA or control siRNA were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 10 min. Upper panels show the representative western blots for p-cSrc, cSrc, and α-tubulin or representative RT-PCR bands for PR and 18S mRNA. Lower panels show the densitometric analysis. **(G)** U251 cells were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 5 min and co-immunoprecipitated with PR. Data were normalized respect to the vehicle or control. Results are expressed as the mean ± S.E.M. **(A–F)** n = 4 **(G)** n = 3; *p < 0.05.

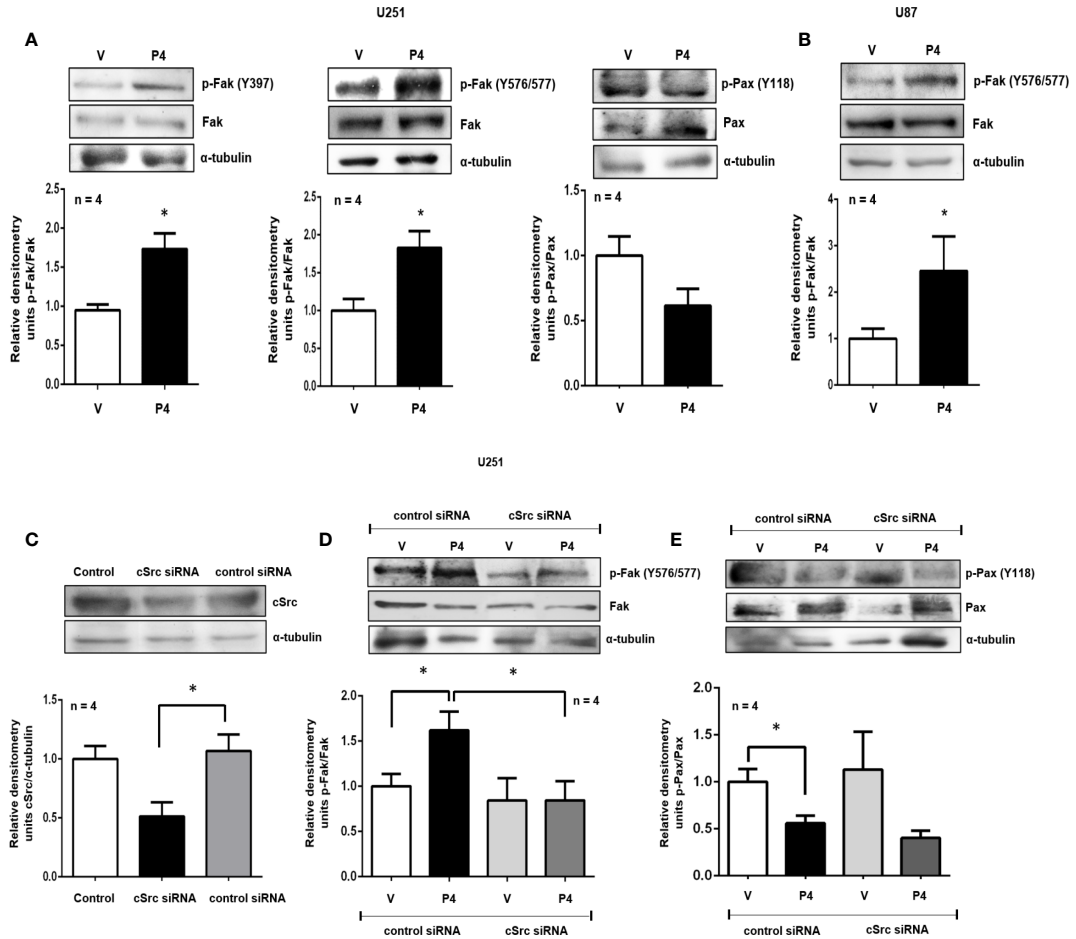

FIGURE 2 | P4 induces the activation of Fak and Pax through cSrc in glioblastoma cells. **(A, B)** U251 and U87 cells were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 20 min. **(C)** U251 cells were transfected with cSrc siRNA and a control siRNA (an aleatory RNA sequence) (100 nM) or were only treated with lipofectamine (Control). **(D, E)** Transfected cells with cSrc siRNA or control siRNA were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) for 20 min. Upper panels show the representative western blots for, cSrc, p-Fak, Fak, p-Pax, Pax, and α-tubulin. Lower panels show the densitometric analysis. Data were normalized respect to the vehicle or control. Results are expressed as the mean ± S.E.M. n = 4; *p < 0.05.

silencing of cSrc modify the migration induced by P4 in U251 cells, a scratch-wound assay was performed. In cells transfected with control siRNA and treated with P4, a slight increase in migration was observed as compared to vehicle at 6 and 12 h after treatment. This increase was inhibited in cells transfected with cSrc siRNA (**Figures 3A, B**). This result demonstrates that the migration of glioblastoma cells induced by P4 is related to the activation of cSrc and reinforces the previous molecular findings.

cSrc Has Several Putative Phosphorylation Sites Over PR

Phosphorylation of nuclear receptors, including PR, has great relevance in functions executed by these proteins. PR phosphorylation has been broadly studied in serine residues; however, there is scarce information about tyrosine residues. The potential role of active cSrc over PR phosphorylation was determined using three different databases (NetPhos 3.1, KinasePhos, and GPS 5.0). We found the same putative tyrosine residue in the amino acid 87 of PR in all of them. In the GPS 5.0 database, this residue presented the highest score, which means that it also has the highest potential for phosphorylation (**Table 1**). Even when this result must be confirmed in experimental assays, the information obtained by the databases opens the possibility of future investigation of functions and regulation of PR by cSrc phosphorylation (**Figure 4**).

PXN (Pax) and PTPN12 (PTP-PEST) Expression Depends on Tumor Grade and Glioblastoma Subtype

PXN and PTPN12 expression data from 196 grade II, 223 grade III, and 139 grade IV (glioblastoma) astrocytomas were obtained from TCGA and compared to 249 healthy human brain cortex samples from the GTEx database. The expression of these genes was also compared among the four subtypes of glioblastomas defined by Verhaak and colleagues (31). The PXN and PTPN12 mRNA expression increased in glioblastomas compared to normal brain,

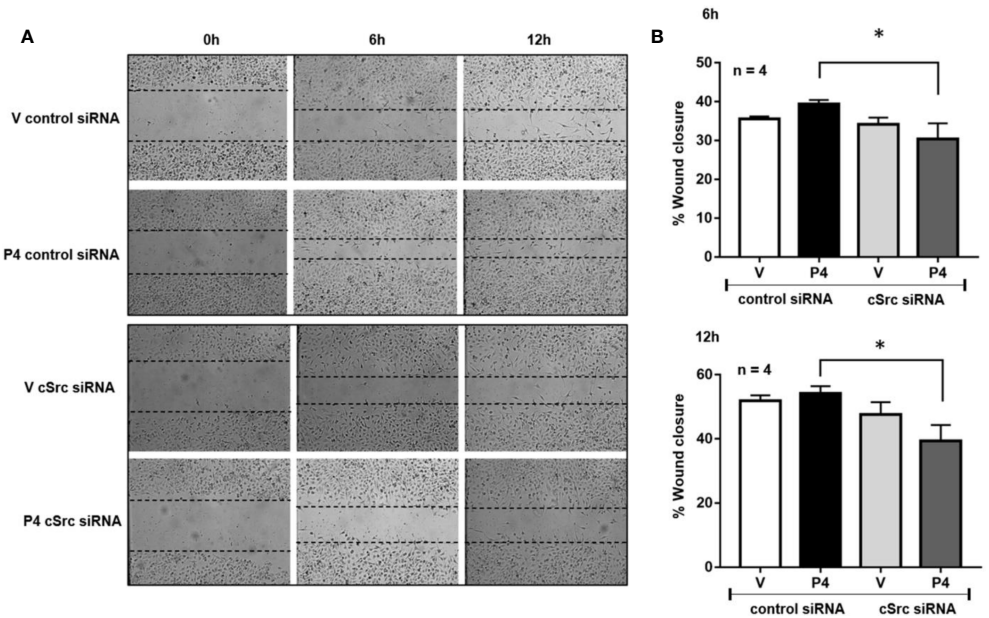

FIGURE 3 | cSrc participates in cell migration induced by P4. U251 cells transfected with cSrc siRNA and a control siRNA (an aleatory RNA sequence) (100 nM) were treated with P4 (50 nM) or vehicle (V, DMSO 0.01%) in cells. Photographs from the scratch area were taken at 0, 6 and 12 h and were captured with 400× magnification. **(A)** Representative image of the scratch area. **(B)** Graph of the wound closure (%), determined in the scratch area. Results are expressed as the mean ± S.E.M. n = 4; *p < 0.05.

and in the case of PTPN12, the expression was higher in glioblastomas compared to astrocytomas grades II and III (**Supplementary Figure 3A**). The analysis of expression among the four subtypes of glioblastomas showed that PXN and PTPN12 have the highest levels of expression in the mesenchymal subtype (the most aggressive glioblastoma subtype) (32) (**Supplementary Figure 3A**). The analysis of gene expression correlation between PXN and PTPN12 revealed a value of 0.61 (significant positive correlation) (**Supplementary Figure 3B**).

DISCUSSION

Glioblastoma is the most malignant brain tumor. Patients with glioblastoma have an overall survival of 14 months (1). One of the main influencing factors in the poor prognosis of these patients is the high capacity of glioblastoma cells to migrate and invade the brain parenchyma surrounding the tumor, which in turn makes extremely difficult a complete surgical resection (33). Several molecular signals are implicated in the processes of migration and invasion in glioblastoma; some are activated by cSrc kinase protein that belongs to the Src Family Kinase (SFK) (8, 34). Of all of the other family members (FYN, YES, BLK, YRK, FGR, HCK, LCK, and LYN) cSrc is the most often associated with cancer progression (35). This kinase has been associated with migration and invasion of multiple malignancies through the regulation of actomyosin contraction, actin polymerization (36), and ECM proteolysis (37). In glioblastomas, SFKs play an essential role in events related to motility and disruption of ECM.

It has been demonstrated that PR activated by P4 promotes the migration and invasion of glioblastoma cells (17). However, there is no information about the possible interplay between PR and cSrc in glioblastoma cells. In this work, we first investigated the capacity of P4 to activate cSrc through its PR and how this activation regulates the phosphorylation/dephosphorylation of kinases related to migration and invasion of glioblastoma cells. U251 and U87 cells were treated with P4 for 10 min, and the activation of cSrc was evaluated by western blot. The most effective concentration of P4 was 50 nM. The increase in the p-cSrc (Y416)/cSrc ratio in U251 cells was evident. Y416 is the amino acid residue localized in the domain SH1, which contains the autophosphorylation site required for the full cSrc activation (38). When U87 cells were treated with P4, a significant increase in the p-cSrc (Y416)/cSrc ratio was observed. Therefore, P4 induces the activation of cSrc in human glioblastoma derived cell lines.

In colorectal cancer, the increasing activity of cSrc rather than its overexpression is associated with metastasis (39, 40). cSrc is one of the first and most studied proto-oncogenes (41); however, its role in cancer progression is not entirely understood. The central role attributed to this kinase was increasing cellular proliferation (42). However, most recent investigations have found that cSrc regulates processes such as adhesion, invasion, and motility (38, 43). For example, the overexpression of cSrc in colon cancer does not induce the proliferation rate increase, but it facilitates the spread of cells (37). Some colleagues consider that cSrc induces cellular proliferation at the first stage of cancer development but regulates migration and invasion processes at the later stages (29). It has been demonstrated an essential role of

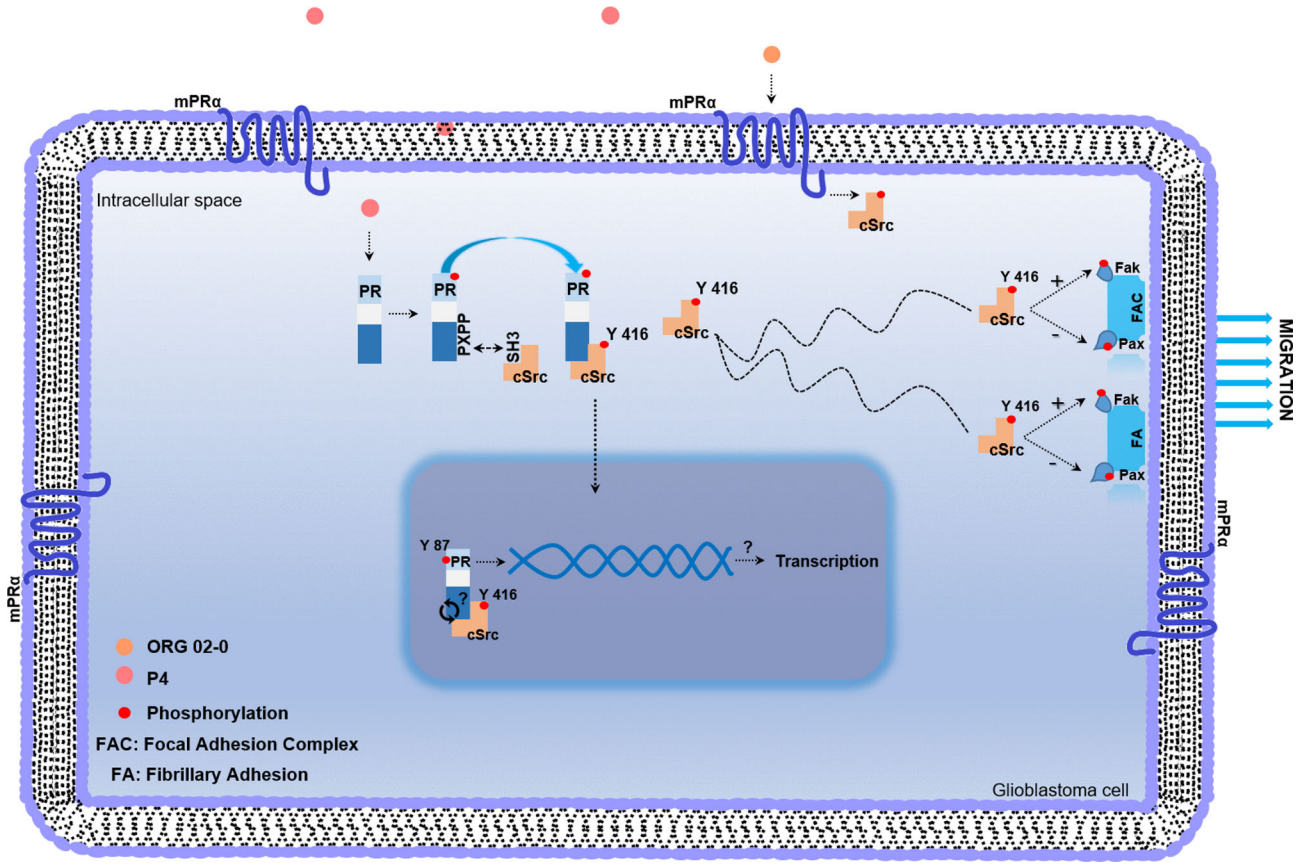

FIGURE 4 | Working model of non-genomic PR mechanism of action in glioblastoma cell. Once P4 enters the cell, it induces phosphorylation of PR in serine and threonine residues. After activation, PR recruits cSrc through the interaction between the polyproline (PXPP) and the SH3 domains of PR and cSrc, respectively. This interaction induces a conformational change in cSrc, which exposes its catalytic domain, and promotes the autophosphorylation of cSrc. Once activated, cSrc participates in regulating the phosphorylation of focal adhesion and fibrillary adhesion components such as Fak and Pax, which finally lead to the migration process. mPRs are expressed in glioblastoma cells and the agonist of mPRα (ORG 02-0) induces the activation of cSrc. The putative role of cSrc in the tyrosine 87 phosphorylation of PR could be involved in the transcriptional activity of PR.

TABLE 1 | *In silico* analysis of putative phosphorylation sites of cSrc over PR.

	ID	Position	AA	Kinase	Score	E-value	Peptide
Netphos 3.1	Progesterone Receptor Homo sapiens AAA60081.1	87	Y	SRC	0.516		VEGAYSRAE
				EGFR	0.444		
KinasePhos		87	Y	SRC		13	VEGAYSRAE
GPS 5.0		87	Y	TK/SRC/SRCA/YES1	27.217		LSDVEGAYSRAEATR

Three different databases to predict phosphorylation sites were used to analyze the potential role of cSrc in PR phosphorylation. One putative tyrosine residue in the amino acid 87of PR was found in three databases: NetPhos 3.1, KinasePhos, and GPS 5.0.

SFKs in the motility of glioblastoma cells (30, 44). The activation of cSrc by P4 may be involved in the regulation of events associated with the migration and invasion of glioblastoma cells.

One of the first identified substrates of cSrc was Fak, a non-receptor tyrosine kinase closely related to regulating a variety of cellular processes, including cell migration (45). At the focal adhesion complexes, cSrc induces Fak's phosphorylation and facilitates the turnover of these junctions, an essential step to cell migration. The complex Fak-cSrc can also phosphorylate Pax, which recruits other components to focal adhesion sites (38). It has been reported elevated levels of Fak expression in anaplastic astrocytoma and glioblastoma tumor biopsy samples compared to normal brain (46). In this work, we evaluated the capacity of P4 to activate Fak and Pax. P4 promoted the increase in the p-Fak(Y397)/Fak and p-Fak(Y576/577)/Fak ratio that corresponds to the autophosphorylation site and to another site directly phosphorylated by cSrc, respectively. Thus, P4 induces Fak's phosphorylation, including in the tyrosine residues directly related to cSrc and with the turnover of focal adhesions (38). To determine if P4 induces the phosphorylation of Fak through cSrc, we

transfected U251 cells with a commercial siRNA against cSrc or with control siRNA and treated them with P4 in the same conditions of the previous experiments. In this case, P4 failed to induce Fak activation in cells transfected with siRNA against cSrc.

Pax is a multifunctional protein that plays a scaffolding role at focal adhesions. Overexpression of this protein has been associated with high-grade astrocytomas as well with a poor survival (10). Upon integrin activation, Pax is mainly phosphorylated at two different tyrosine residues, namely Y31 and Y118, but this phosphorylation state is not permanent. Zaidel-Bar and colleagues found that tyrosine-phosphorylated Pax is associated with focal complex and focal adhesions, while non-phosphorylated Pax is associated with fibrillary adhesions. These colleagues proposed a hypothetical model in which Pax is initially phosphorylated and recruited to integrin adhesions. The rate of this recruitment is regulated by the presence of both phosphopax and Pax. Finally, phosphopax is dephosphorylated at a high rate under mechanical force, and the phosphorylation is reestablished at a low rate (47). In this work, we observed that P4 decreased the p-Pax(Y118)/Pax ratio in U251 cells 20 min after the treatment. This result suggests that P4 should induce Pax recruitment towards the integrins at fibrillary adhesions and contributes to the presence of both phosphorylated and unphosphorylated state, which is necessary to migration processes. When U251 cells were transfected with the siRNA against cSrc, the reduction in p-Pax(Y118)/Pax was more evident, which is in line with the role of cSrc in Pax phosphorylation. One of the proteins closely related to the dephosphorylation of Pax is the tyrosine phosphatase PTP-PEST. Shen and colleagues found that PTP-PEST coimmunoprecipitates with Fak and Pax in chicken embryo cells (48). These colleagues also demonstrated that the expression of PTP-PEST decreases the phosphotyrosine on Pax (49). In glioblastoma, PTP-PEST regulates the invasion events by phosphorylation-dependent ubiquitination of essential focal proteins such as Cas, Fak, Pax, and Src (11). Bioinformatic analysis revealed that PXN and PTPN12 mRNA expression was higher in astrocytomas (Grades II, III, and IV) compared to normal brain and showed the highest expression in the mesenchymal subtype (the most aggressive glioblastoma subtype, associated with bad prognostic) (32). The analysis of gene expression correlation revealed a value of 0.61 (significant positive correlation). These results suggest that these proteins together are implicated in the progression of glioblastomas. In the same line, the scratch-wound assay analysis showed that silencing of cSrc in U251 cells abolished the increase in cell migration induced by P4. Interestingly in 2013, Matias-Sanchez and colleagues found that PR, stimulated by P4 and the synthetic progestin medroxyprogesterone acetate, have an essential role in the actin polymerization, branching, and focal adhesion complex formation in cortical neurons. The molecular mechanism proposed by these colleagues involucrate the activation of Fak, and other proteins related to migration, such as WAVE and moesin. Phosphorylation of the latter was promoted by PR through the Ras homolog gene family, member A and Rho-associated kinase-2. Therefore, we should not underestimate the role of these last proteins in P4 effects (50).

The observed effects induced by P4 could also be mediated by membrane progesterone receptors (mPRs), G protein-coupled receptors that belong to the Progestin and AdipoQ Receptor Family (PAQR). Five subtypes of mPRs (mPRα, mPRβ, mPRδ, mPRϵ, and mPRγ) have been identified, and they are expressed in human glioblastoma cells (51, 52). Importantly, the activation of mPRα by ORG 02-0, a specific mPRα agonist, induces proliferation, migration, and invasion through the activation of cSrc and Akt in human derived glioblastoma cells (24).

P4 can exert its effects through various receptors in glioblastoma cells (24, 25). Therefore, an agonist of PR (R5020) was used to treat the U251 and U87 cells, and in both cases, an increase in p-cSrc (Y416)/cSrc ratio was observed. Considering the high affinity of R5020 for the PR and that this progestin is unable to be transformed into the active metabolites of P4 (53), it makes sense to think that results previously described are a consequence of the action of P4 through its PR and not by another receptor or metabolite. To determine if cSrc activation by P4 was induced through PR, a more specific assay was conducted. U251 cells were transfected with a commercial siRNA against PR or with control siRNA and treated with P4 in the same conditions of the previous experiments. As is shown in **Figure 1F**, P4 failed to induce cSrc activation in cells transfected with siRNA against PR. Thereby, the effect of P4 over cSrc activation is mediated by PR in these human glioblastoma cells.

Even though PR is widely known for its role as a transcription factor (53, 54), in the last two decades the attention has been focused on the actions that it can exert out of the nucleus (55). Non-genomics actions of PR are due to a polyproline domain (amino acids 396–456) that can interact with the SH3 domain of several proteins, including cSrc. Once this interaction occurs, the intramolecular interactions that hold cSrc in a closed configuration are disrupted, and the autophosphorylation site is exposed (22). This mechanism has been broadly studied in breast cancer. It has been demonstrated that in breast cancer cells, P4 can promote the interaction between PR and cSrc and, in turn, inducing proliferation (56), migration, and invasion (19). To test the interaction between PR and cSrc we performed a co-immunoprecipitation assay. This assay shows that PR (isoforms A and B) and cSrc interact in glioblastoma cells and that P4 enhances this interaction. This result suggests that activation of cSrc by P4 is due to a conformational change in cSrc that enables the autocatalytic domain to be exposed. However, this result must be interpreted with care since some colleagues have found that in breast cancer cell lines, the activation of cSrc through the PR is dependent on the estrogen receptor (ER). ERα plays an essential role in breast cancer cells by activating the Src/Erk pathway and increasing cell proliferation. Estrogens or progestins can induce this effect; however, according to studies conducted by Migliaccio and colleagues and Ballaré and colleagues, it is necessary to form a complex including ERα, PR, and Src (21, 23). Boonyaratanakornkit and colleagues, on the contrary, support the idea of PR self-sufficiency to induce cSrc activation without ER. They found that in breast cancer cells no expressing ER, P4 induced the activation of cSrc through PR (22). In glioblastomas, estradiol increased cell growth, migration, invasion, and the epithelial–mesenchymal transition (EMT) through activation of ERα (57, 58); therefore, we cannot dismiss the idea of the role of ERα in the PR-cSrc interaction.

Phosphorylation of PR is a post-translational modification broadly studied. Among other functions, it is directly related to regulating the transcriptional activity of this receptor and the degradation by the proteasome (59, 60). Serine and threonine phosphorylation of PR has been widely investigated (60–62); however, there are no reports about PR tyrosine phosphorylation. Therefore, we performed an *in silico* analysis in three databases to search putative phosphorylation sites of cSrc over PR. NetPhos 3.1, KinasePhos, and GPS 5.0. predicted the putative tyrosine residue in the amino acid 87 with the highest score. Even when *in vitro* and *in vivo* experiments are mandatory to demonstrate this prediction, we can speculate about this post-translational modification's possible role. The phosphorylation of the estrogen receptor by SFKs proteins has been studied in breast cancer cell lines. Its inhibition reduces its stability and transcriptional activity and alters the ligand binding (63).

Then, the interaction of PR and cSrc in glioblastoma cells could be bilateral and involve the genomic and non-genomic actions of P4. In conclusion, this work is the first report in demonstrating the interaction between cSrc and PR in human glioblastoma cells. This interaction induces cSrc activation, which in turn participates in the regulation of the activity of proteins involved in the migration and invasion of glioblastomas. The results presented here open new perspectives for the treatment of glioblastomas.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://portal.gdc.cancer.gov/

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by CB-A and AD-M. The first draft of the manuscript was written by CB-A and AD-M and reviewed by IC-A. AG-A participated in the analysis of results, and all authors commented the versions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.640298/full#supplementary-material

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Estrogen Receptor Beta Influences the Inflammatory p65 Cistrome in Colon Cancer Cells

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Inflammation is a primary component of both initiation and promotion of colorectal cancer (CRC). Cytokines secreted by macrophages, including tumor necrosis factor alpha (TNFα), activates the pro-survival transcription factor complex NFκB. The precise mechanism of NFκB in CRC is not well studied, but we recently reported the genome-wide transcriptional impact of TNFα in two CRC cell lines. Further, estrogen signaling influences inflammation in a complex manner and suppresses CRC development. CRC protective effects of estrogen have been shown to be mediated by estrogen receptor beta (ERβ, *ESR2*), which also impacts inflammatory signaling of the colon. However, whether ERβ impacts the chromatin interaction (cistrome) of the main NFκB subunit p65 (RELA) is not known. We used p65 chromatin immunoprecipitation followed by sequencing (ChIP-Seq) in two different CRC cell lines, HT29 and SW480, with and without expression of ERβ. We here present the p65 colon cistrome of these two CRC cell lines. We identify that RELA and AP1 motifs are predominant in both cell lines, and additionally describe both common and cell line-specific p65 binding sites and correlate these to transcriptional changes related to inflammation, migration, apoptosis and circadian rhythm. Further, we determine that ERβ opposes a major fraction of p65 chromatin binding in HT29 cells, but enhances p65 binding in SW480 cells, thereby impacting the p65 cistrome differently in the two cell lines. However, the biological functions of the regulated genes appear to have similar roles in both cell lines. To our knowledge, this is the first time the p65 CRC cistrome is compared between different cell lines and the first time an influence by ERβ on the p65 cistrome is investigated. Our work provides a mechanistic foundation for a better understanding of how estrogen influences inflammatory signaling through NFκB in CRC cells.

Keywords: p65, ERβ, ChIP, colon cancer, colorectal cancer (CRC), estrogen receptor

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INTRODUCTION

Colorectal cancer (CRC) accounts for the third highest number of cancer deaths among both women and men in the Western world (1). Benign adenomatous polyps evolve into carcinomas over 10-15 years, and screening is performed in many countries. While COX-2 inhibitors (e.g. celecoxib) or aspirin reduce inflammation and effectively prevent adenomatous polyp formation and CRC,

adverse effects exclude their general usage (2–4). Better preventive treatments are required, and a deeper understanding of exactly how inflammation impacts CRC is needed.

During inflammation, cytokines such as tumor necrosis factor alpha (TNFα) are released by macrophages. TNFα activates the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factor complex, which in turn induces several oncogenes and signaling pathways involved in tumor initiation and progression (5–7). Constitutive activation of NFκB has been observed in nearly 70% of CRC cell lines and 40% of clinical CRC specimens (8–11). NFκB is a homo- or heterodimer comprised of two to five of subunits p65 (RelA/RELA), RelB (RELB), cRel (RELC), p105/p50 (NFκB1), or p100/p52 (NFκB2), that appears in multiple forms (12). The N-terminal Rel domain is present in all subunits and binds to a specific DNA sequence known as the κB site. Together with the recruitment of cofactors, this induces or suppresses expression of target genes. The various NFκB dimers differ in binding affinity and activation. p65 and cRel are the most potent transcriptional activators (13), and p65 together with p50 constitutes the most common NFκB heterodimer (14). While inflammation is critical in development of CRC, the function of NFκB complex in CRC is, however, poorly studied. Only one study describes the genome-wide chromatin binding of p65 in colon cancer, in cell line SW480 in the context of its interaction with p53 (TP53) mutants (15).

The hormone estrogen has been shown to reduce CRC incidence (16–20). Estrogen mainly acts through three receptors, of which estrogen receptor beta (ERβ, *ESR2*) is present in epithelial colon and rectal cells (21, 22). We have recently shown that intestinal epithelial ERβ *in vivo* protects from the epithelial damage caused by TNFα and prevents tumor formation (22). Also, when reintroduced into CRC cell lines, ERβ has antiproliferative and tumor-suppressive activity (22, 23). ERβ is a ligand-activated nuclear receptor which binds to genomic ERE (estrogen response elements). Its homologue, ERα (*ESR1*) is upregulated in breast cancer, where it promotes cell proliferation and interacts with p65 (24, 25). ERβ expression, in contrast, decreases during CRC development, and ERα is not expressed in the colon epithelial cells, nor tumors (26). Our hypothesis is that ERβ in the normal colon opposes NFκB-mediated inflammatory signaling and that this is an essential part of its tumor protective mechanism.

A crosstalk between the related ERα and NFκB has been extensively studied in breast cancer, albeit with some contradicting findings. A few studies report that ERα represses NFκB activity (27, 28), whereas other reports that ERα, in the same cell lines (MCF7, T47D, ZR-75), enhances NFκB activity (24, 25). Specifically, TNFα in ERα-positive MCF-7 breast cancer cells was shown to profoundly modify the ERα enhancer-binding landscape in an NFκB-dependent manner (29). Based on the homology between the two ERs DNA-binding domains, along with previous findings that ERβ regulates NFκB key targets and reduces inflammatory signaling in colon, we speculated that ERβ may impact the colon p65 cistrome. In the present study, we used p65 chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-Seq) to test this hypothesis, and to detail the p65 landscape in CRC cell lines.

MATERIALS AND METHODS

Cell Culture

SW480 (Research Resource Identifier RRID: CVCL_0546) and HT29 (RRID: CVCL_0320), previously generated to express ERβ and corresponding mock control cells (23, 30, 31), authenticated and mycoplasma tested, were cultured in Dulbecco's modified Eagle's medium (D6429, Sigma Aldrich) supplemented with 10% FBS (F9665, Sigma Aldrich), 1% penicillin-streptomycin (P/S) and 1% blasticidin (D429, Sigma Aldrich). A day before ChIP, the media was changed to Dulbecco's Modified Eagle Medium (DMEM)-phenol-red free with 1% charcoal-stripped fetal bovine serum (FBS, 12676011, ThermoFisher). Cell lines were not treated with E2 since previous studies has revealed that transduced ERβ functions ligand independent in CRC cell lines, possibly due to activation through growth factors and phosphorylations (23, 32, 33).

p65 ChIP

For each ChIP experiment, $60x10^6$ cells were used. Cells were treated with TNFα (30 ng/ml, 11088939001, Roche, lot no: 25885600) Sigma-Aldrich) for 30 min and washed with PBS before cross-linking. Cells were first cross-linked with 2 mM disuccinimidyl glutarate (DSG) (20593, lot TF263080, Thermo Scientific) for 45 min during shaking. After washing (three times, with PBS) to remove DSG, they were cross-linked again with formaldehyde (1%) for 10 min during shaking. The double crosslinking was used to capture both short- and long-range p65 chromatin interactions. Glycine (final concentration 0.125M) quenched the cross-linking reaction. After washing (twice with PBS), cells were collected and pelleted by centrifugation. Cell pellets were further processed at 4°C using ice cold reagents. After lysis in LB1 (50Mm HEPES, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5% NP-40 and 0.25% triton-x) for 10 min, and centrifugation (4500 rpm, 5 min), pellets were suspended in LB2 (10 mM Tris-Hcl, 200 mM NaCl, 1 mM EDTA) for 5 min, centrifuged, and dissolved in LB3 buffer (10 mM Tris-Hcl, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate and 0.5% Na-lauroylsarcosine) to separate nuclear chromatin. Sonication generated 200-500 bp long fragments of chromatin. Following centrifugation (13000 rpm, 5 min), supernatants were collected in low-binding DNA tubes and incubated overnight with p65 antibody [Invitrogen, mouse monoclonal, cat no: 33-9900, lot no: QJ216251, RRID : AB_2533153, validated in (34)] or IgG (Santa Cruz, mouse polyclonal, cat no: sc-2025, lot no: J1514, RRID : AB_737182) as control. Next, samples were incubated with 30 µl protein G Dynabeads (cat no: 10004D, Invitrogen) for 3h. Beads were washed in sequential steps using TSE1 (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.1% SDS and 0.1% Triton-X),

TSE2 (20 mM Tris-HCl, 500 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton-X), LiCl buffer (20 mM Tris-HCl, 1 mM EDTA, 250mM LiCl, 1% NP-40 and 1% Na-deoxycholate) and TE buffer (10 mM Tris-HCl and 1 mM EDTA), and eluted (NaHCo3 (0.75%) SDS (1%), proteinase K (200 ng/μl)) overnight at 65°C, and finally treated with RNase A (1h at 37°C). QIAquick PCR purification columns (Qiagen, cat no: 28104) were used to purify DNA.

ChIP-Sequencing

Libraries of the ChIP DNA were prepared and sequenced by the National Genomic Infrastructure (NGI) for Bioinformatics and Expression Analysis (BEA). DNA libraries were prepared using the NEB Next Ultra II DNA Library Prep Kit for Illumina (p/n NEB #E7645) and quality confirmed using TapeStation (DNA D1000 ScreenTape, Agilent). Libraries were loaded (1.8 pM end concentration of 1%) and sequenced (75 cycles, single read) using NextSeq 550 (Illumina).

ChIP-Seq Data Analysis

Spliced Transcripts Alignment to a Reference (STAR) was used to map unique ChIP-Seq reads to the human reference genome assembly hg38 (GRCh38) with the alignIntronMax flag set to 1. Peak calling was performed using Hypergeometric Optimization of Motif Enrichment (HOMER) over input with a four-fold enrichment as cutoff and applying a false discovery rate (FDR) less than 0.001. Peaks which overlapped within 200 bp and were present in at least two out of three biological replicates were used for downstream analysis. Raw tag counts were normalized using R and binding pattern differences were identified with edgeR package. To cluster and visualize the different peaks, Complex heatmap from R was used. Promoter regions were defined as -1kb to +100bp from TSS and genomic distribution of binding sites were identified by HOMER. Gene functional annotation was performed using Database for Annotation Visualization and Integrated Discovery (DAVID), with $P < 0.05$ considered as significant.

Data Availability

The p65 ChIP-Seq data is deposited in the Gene Expression Omnibus (GEO) repository (GSE160856), TNFα bead array gene expression data was published previously (available at GSE65979), and SW480ERβ input and HT29ERβ input controls (GSE149979).

RESULTS

First, to understand the role of p65 transcriptional activity and oncogenic functions in CRC, we aimed to characterize its genome-wide binding in human CRC cells and correlate this to the TNFα-mediated transcriptional impact in the same cells. We used two well-characterized human colorectal adenocarcinoma cell lines: HT29 from a female primary tumor and SW480 from a male Dukes' type B primary tumor.

The p65 Cistrome of Colon Cancer Cells

After optimizing the protocol for antibody specificity and including a double cross-linking (DSG-formaldehyde) procedure to capture long-range interactions of p65, we analyzed the chromatin bound by p65 in triplicate experiments of each cell line, HT29 and SW480. The sequencing produced between 24M and 65M (80%) of high-quality mapped reads per sample (**Table S1**). We identified a total of 12,504 (HT29) and 5004 (SW480) significantly enriched p65 peaks compared to input (**Table S1**). Out of these, 3151 and 1459 binding sites were found in HT29 and SW480 cells, respectively (**Figure 1A**). Whereas more p65 chromatin-binding sites were detected in HT29 cells overall, comparison between the two cell lines revealed that 63% (919) of sites found in SW480 were also found in HT29 (**Figures 1A, B**). We next used HOMER to determine DNA motifs of the identified peaks. As expected, we identified RELA as the top motif, followed by JUN-AP1, in both cell lines (**Figure 1C**). This corroborates the specificity of the antibody and the protocol. Further, the transcription factors HNF4A and NFAT motifs were present in 3-5% of the HT29 binding sites, and FOXA1 and RUNX1 were relatively abundant (12% and 17%) in the SW480 p65 cistrome. Thus, we present the p65 cistrome of two different CRC cell lines and identify a shared common core, as well as cell-line specific differences.

p65-Bound Genes Are Involved in Migration and Circadian Clock

In order to decipher how the above identified p65 binding may impact gene expression, we analyzed where the binding sites were located in relation to known genes. We found a highly similar pattern in both cell lines, with about 38% of sites located within introns, 34% in intergenic chromatin regions, and 22% within the promoter area (-1kb to +100bp from the transcription start sites, TSS) of genes (**Figure 1D**). The top-20 most enriched promoter sites in both cell lines include well-known p65 targets such as NFκB regulators *NFKBIB*, *NFKBIZ*, *TNFAIP3* (35), *BCL3* (36), and *BIRC3* (37, 38), NFκB subunits *NFκB2* (p52) and *RELB*, tumor suppressor p53, *TNIP1*, and *CREB1* (**Table S2**). p65-binding sites unique to either cell line also included well-known p65 target genes (HT29: *BCAR3*, *BIRC7*, *DUSP16*, *PTGS2*, and *TNFAIP8*; SW480: *CDX2*, *CDH10*, *CLRN3*, *ESR1* (ERα), and *KCNH3*, **Figures 2A, B**). Pathway analysis of genes bound by p65 (-50kb to +2kb of TSS) revealed that genes with transcriptional regulatory functions (e.g. *JUND*), cell adhesion and migration (e.g. *WNT5B*, *BCAR1*, *TGFB1*, *CXCL16*), NFκB signaling, TNFα signaling, and apoptosis were enriched. This is in accordance with general NFκB functions (39, 40). We also identified a novel pathway, not previously associated with p65, including circadian rhythm in both cell lines (**Figure 1E**). Circadian rhythm genes bound by p65 included the central circadian regulator *CLOCK*, *BMAL2*, *CREB1*, and *KLF10*. In conclusion, we note highly concordant binding to cis-regulatory chromatin in proximity of genes within expected functions and further identify potential mechanism for p65 regulation of the colon circadian rhythm.

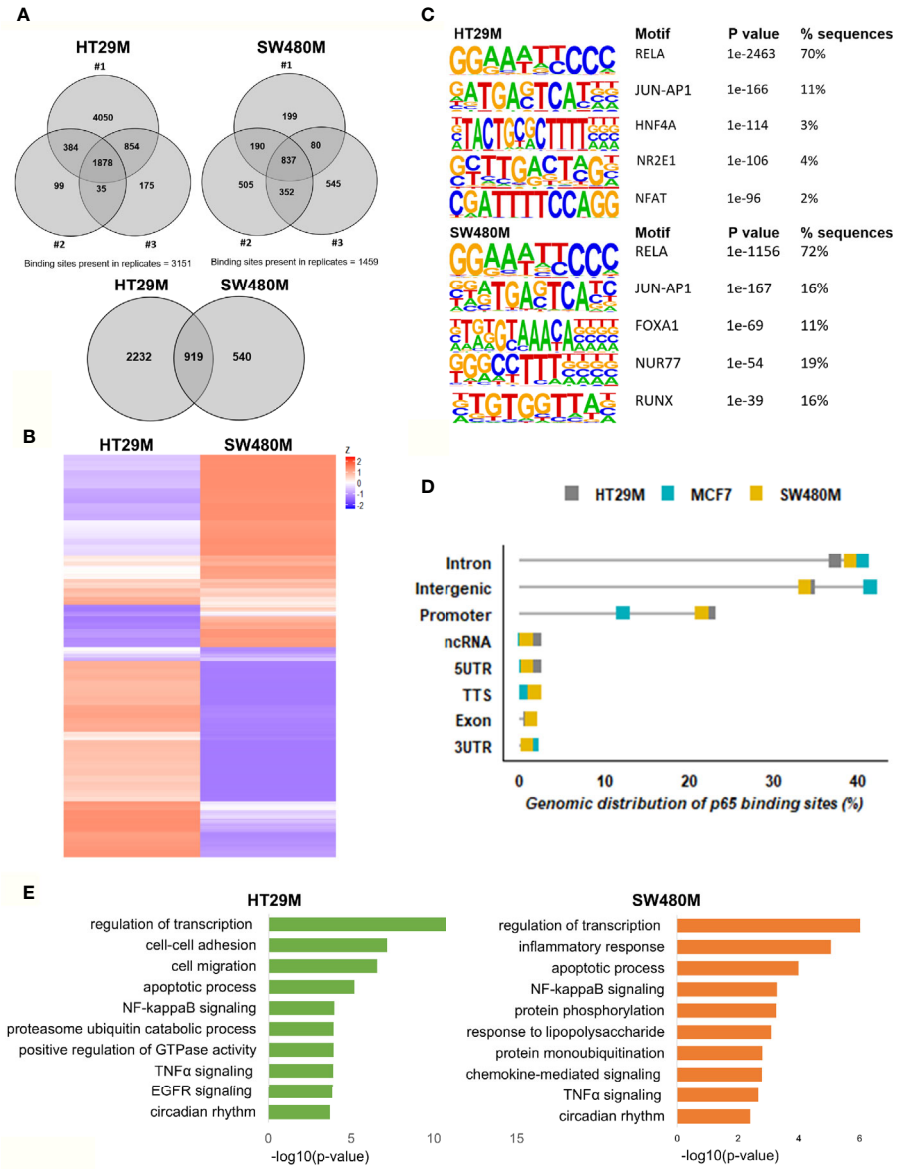

FIGURE 1 | Genomic distribution of p65 binding sites in colon cell lines. **(A)** Identified p65 binding sites in three replicates of colon cancer cell lines HT29 and SW480 with those detected in at least two replicates used for further analysis and highlighted (top), and their overlap between cell lines (bottom), represented using Venn diagram. **(B)** Heatmap representing p65 binding sites in the two cell lines. **(C)** Motifs highly enriched in p65 binding sites identified by HOMER using *de novo* motif analysis and sorted by p-value. **(D)** Genomic distribution of p65 binding sites in relation to gene locations. **(E)** Biological functions enriched in genes nearest p65 binding sites (-50kb +2kb).

p65 Recruitment Correlates With TNFα-Regulated Gene Expression in CRC Cells

While binding of a transcription factor indicates a potential gene regulation of nearby or distant genes, all such bindings do not translate into actual gene regulation. To determine the effect p65 binding has on transcriptional regulation of corresponding genes in CRC cells, we linked the p65 ChIP-Seq data to our previously generated TNFα (2-h treatment) transcriptome data set of the same cells (22). We found coordinated p65 binding and short-term TNFα regulation of 274 genes in HT29 and 82 genes in SW480 cells (**Figure 3A**, **Table S3**). Out of these, 59 genes (72% of those identified in SW480) were bound by p65 and regulated by TNFα in both cell lines (**Table S3**). All 59 core genes were upregulated by TNFα in both cell lines. Motif enrichment analysis of the p65-bound and TNFα-regulated core genes demonstrated significant enrichment for the p65 motif. Overall, the TNFα-regulated genes associated with p65-binding sites were mainly involved in the gene ontology functions of

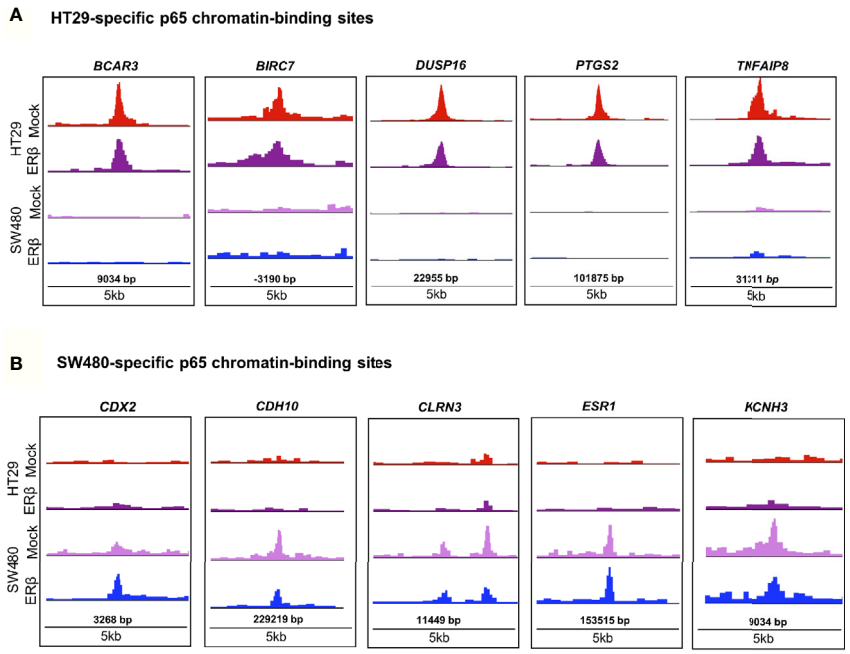

FIGURE 2 | p65 chromatin binding sites. Peaks of p65 chromatin binding mapped to gene sequences in HT29 **(A)** and SW480 **(B)** cells using UCSC genome browser. Bp indicates distance from corresponding gene's TSS.

NFκB signaling, TNFα signaling, and inflammatory pathways. These included regulations and binding of known NFκB target genes (*BCL3*, *CCL20*, *CXCL1*, *CXCL8*, and *NFKBIA*, Fig 3B-C). Other known targets were differently bound and regulated in HT29 (*BCL11B*, *DUSP16*, *KLF6*, *RELA*) and SW480 (*IL23A*, *PRRG1*) cells (**Table S3**). In conclusion, this data clearly shows a strong transcriptional activity by p65 in both CRC cell lines, with p65-bound and regulated genes involved in critical CRC pathways, including apoptosis and cell migration.

p65 Cistrome Differs Between Colon and Breast Cancer Cell Lines

To explore the extent that p65 binding is conserved between colon and breast cancer, we compared our generated p65 cistrome (colon) with previously published p65 ChIP-Seq data of the ER-positive breast cancer cell line MCF7. We selected a data set that also used double crosslinking (41). A heatmap illustrating p65 chromatin-binding sites in MCF7, HT29 and SW480 cells are shown in Fig 4A. Only 22% (230 sites) of MCF7 p65 binding sites were present in either CRC cell line (**Figure 4B**). Also, a markedly lower fraction of p65 sites were located by promoters in MCF7 cells (12% versus 21-22%, **Figure 1D**). The predominant motifs in MCF7 were NFκB (p65) itself, supporting accuracy of this data set, but were otherwise different (FOXA and AP2, **Figure 4C**) from colon (AP1, **Figure 1C**). The enriched biological functions for p65 sites specific for MCF7 also included apoptosis, transcription regulation, cell cycle, and circadian clock (**Figure 4D**). Thus, our study shows a cell specificity of p65 binding, where it binds different motifs and regulates different genes in different tissues or cell lines, but the biological functions of the regulated genes appear to have similar roles in cancer cell lines.

ERβ Diminishes p65 Chromatin Binding in HT29 Cells

Next, we aimed to study whether the mechanism whereby estrogen impacts inflammatory signaling in colon involves p65 chromatin binding. As we have previously found that ERβ can attenuate pro-inflammatory cytokine IL6 signaling in CRC cell lines (23) and regulate several important NFκB target genes and TNFα signaling *in vivo* (22), we explored whether ERβ impacts the p65 cistrome. We performed p65 ChIP-Seq in the same CRC cells, with and without (mock) expression of ERβ. In HT29, we found that whereas 1721 sites remained bound by p65 in both conditions, 1430 p65 binding sites were no longer detected in presence of ERβ. Further, a smaller fraction of 228 new binding sites were identified, only in presence of ERβ (**Figure 5A**). Using density plot, we noted that ERβ reduced the overall p65 binding in all three replicates (**Figure 5B**). We also analyzed this using a sliding window approach, with a window of 200 bp and calling for enriched regions between mock and ERβ, and identified the same trend (**Figure 5C**). Next, to investigate whether specific p65-binding motifs were affected by ERβ, we performed *de novo* motif analysis. The predominant p65 motifs in HT29 remained both in the absence and presence of ERβ (p65, AP-1, HNF4A, **Figure 5D**). Thus, the presence of ERβ reduced p65 chromatin

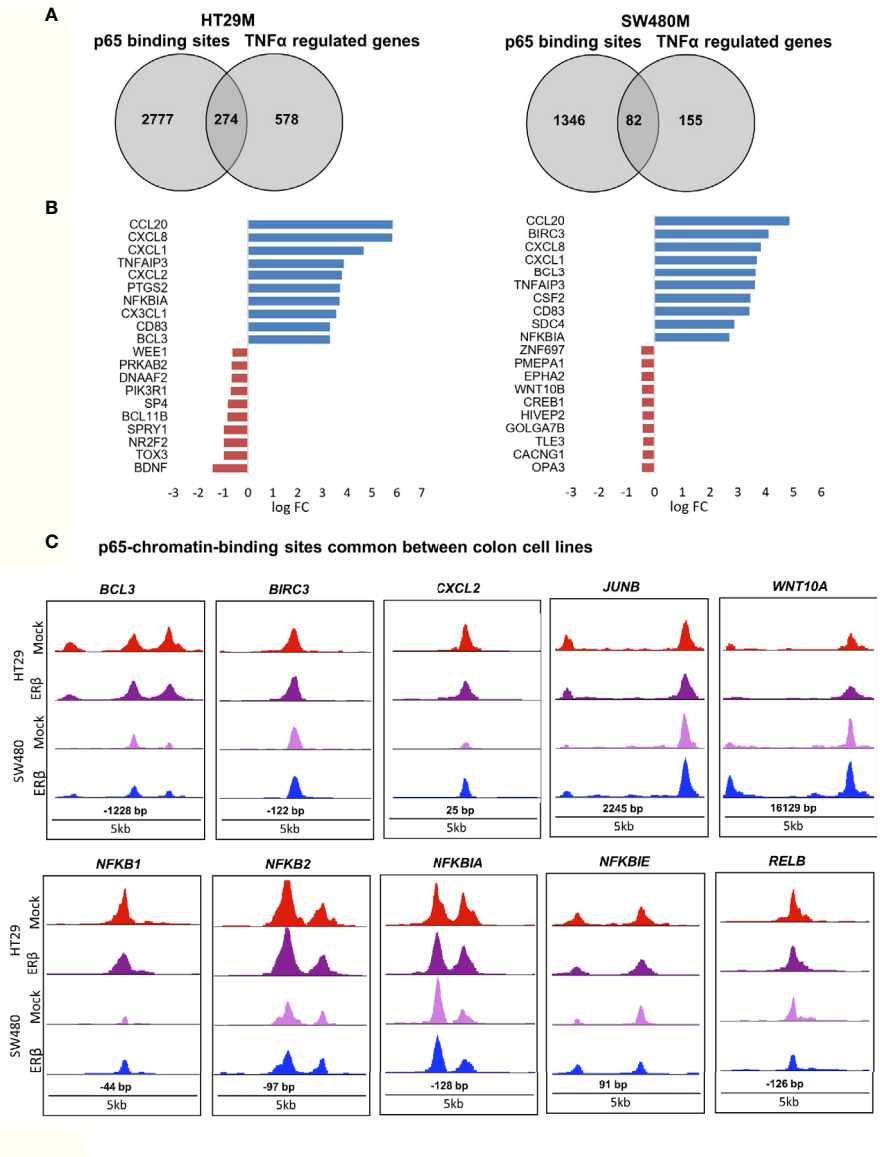

FIGURE 3 | p65 transcriptional regulation in colon cell lines. **(A)** Number of genes with p65 chromatin binding sites and corresponding transcriptional regulation upon TNFα (10ng/ml, 2 h) treatment, per cell line. **(B)** The top-10 TNFα upregulated and downregulated genes with p65 binding sites in both cell lines (HT29, SW480). **(C)** Enrichment signal of p65 binding sites present in both cell lines, illustrated using UCSC genome browser.

binding and affected its distribution (numerous sites disappeared) but did not affect the type of motifs bound. Corresponding pathway analysis indicated that ERβ hindered p65 chromatin binding to genes with activities in cell adhesion, migration and circadian clock, while enabling binding by genes related to cell proliferation and Notch signaling (**Figure 5E**).

We also compared the p65 ChIP-Seq data with corresponding TNFα gene expression data, with and without ERβ (**Figure 5F**). We identified that 162 of p65-bound genes were regulated by TNFα differently in presence of ERβ. Among those, ERβ also inhibited p65 binding by 51 genes and enhanced binding of 7 genes. A heatmap illustrates how ERβ and resulting lack of p65 binding, affects TNFα-mediated regulation of these 51 genes (**Figure 5G**). Notably, presence of ERβ either inhibited TNFα-mediated response (50%), or enabled TNFα induction (31%) for the majority of these genes. These genes were mainly involved in functions such as negative regulation of transcription, negative regulation of cell proliferation, and chromatin remodeling. The seven genes where ERβ enhanced p65 binding were also impacted in terms of TNFα-mediated gene expression (**Figure 5H**). For example, ERβ enabled p65 binding and TNFα upregulation of *TSC22D1* and binding followed by downregulation of *ZNF341*, but blocked (presumably through p65 recruitment) TNFα-mediated upregulation of *VEGFA*,

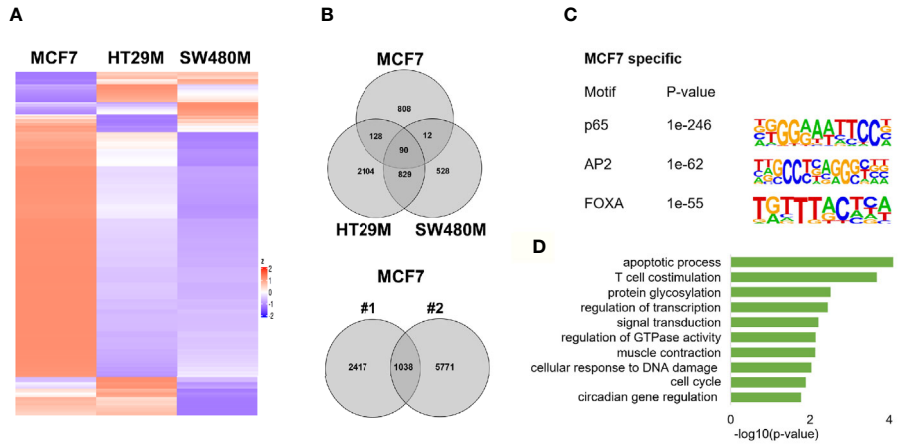

FIGURE 4 | p65 cistrome in colon vs breast. **(A)** Heatmap illustrating p65 binding sites in breast (MCF7) and colon cancer cell lines (HT29, SW480). **(B)** Venn diagram comparing the p65 binding sites in MCF7, HT29, and SW480 cells. **(C)** DNA motifs located in MCF7-specific p65 binding sequences **(D)**. Pathways enriched among the gene ontology functions assigned to genes located nearest to MCF7-specific p65 binding sites.

AGO2 and *IGFL4*. We thus identified that ERβ reduces a sizeable fraction of p65 binding, modifying TNFα regulation in HT29 cells, especially for genes involved in e.g. cell proliferation and cell-cell adhesion.

ERβ Enhances p65 Chromatin Binding in SW480 Cells

Similarly, we explored the impact by ERβ on p65 chromatin binding in SW480 cells. Contrary to HT29, few binding sites were decreased upon introduction of ERβ and nearly all (1433 sites) remained. However, a high number (5796) of new p65 binding sites appeared in presence of ERβ (**Figure 6A**). This enhanced p65 binding was also evident for all replicates in the density plot (**Figure 6B**). Pathway analysis showed that p65 binding sites dependent on ERβ were located by genes involved primarily in transcription regulation, GTPase activity, apoptotic process, protein phosphorylation, cell migration, and MAPK cascade (**Figure 6C**, right panel). The p65 and AP-1 motifs remained highly enriched in SW480 ERβ, but we note that RUNX2 motifs were more common when ERβ was present (**Figure 6D**). RUNX2 is also upregulated by ERβ (23).

In terms of gene expression, ERβ modulated transcription of 110 TNFα-regulated genes that also had p65 binding sites in SW480ERβ cells (**Figure 6E**). About half of these (53 genes) had p65 bound by regulatory chromatin only in presence of ERβ. The corresponding TNFα-mediated gene regulation is illustrated in a heatmap (**Figure 6F**). Interestingly, ERβ (presumably by recruiting p65 to the chromatin) inhibited the TNFα activation of the majority of these genes (56%, or 30 genes). Another 16 TNFα-response genes required ERβ and resulting p65 binding for their induction (**Figure 6F**), including *DUSP5* [which regulates inflammatory gene expression of TNFα (42)], nuclear receptor *NR2F6*, and *KLF9*. Gene ontology enrichment reveals that genes within cell proliferation and cell migration were enriched among the p65 regulations modified by ERβ in SW480 cells. Despite the finding that ERβ enhanced p65 binding in SW480 cells, the resulting transcription of these genes was mostly inhibited. ERβ also modulated expression of p65-bound TNFα-regulated genes, without impacting p65 chromatin binding (57 genes). Among these, expression was attenuated in most (24), and enhanced in some (14). Genes suppressed by ERβ included *BCL3*, *BIRC3*, *CCL20*, *NFκB2* and *RELB* (**Figure 6G**), all of which are associated with poor prognosis in CRC (43–47).

Thus, ERβ clearly modulates p65 binding and TNFα response also in SW480 cells. ERβ appeared to enhance p65 binding, but still repress TNFα transcriptional activity.

ERβ Modulates p65 Signaling in Colon Cells

From the above findings, we conclude that ERβ impacted p65 binding in both CRC cell lines, partly in different ways but with similar outcome in terms of TNFα-mediated gene regulation. Here, we compare p65 chromatin binding (ChIP-Seq data) between the two cell lines, with and without ERβ (**Figure 7**). A heatmap illustrates the reduced p65 binding upon ERβ expression in HT29, and the enhancement noted in SW480 (**Figure 7A**). A Venn diagram comparing p65 binding in the four conditions (HT29 and SW480 with and without ERβ, **Figure 7B**) shows that a large fraction of p65 sites (727 sites) are bound in all conditions, and 77 sites are enhanced by ERβ in both HT29 and SW480. ERβ appears to enable p65 binding 56 kb downstream of TSS of *PROX1* in both cell lines (**Figure 7D**). PROX1 is a transcription factor highly upregulated in colon cancers and previously shown to be regulated by ERβ post-transcriptionally through miR-205 (21). Overall, 15 genes were bound by p65 and regulated by TNFα, both in absence and presence of ERβ, in both cell lines (**Table S4**). Out of these, the TNFα response of 12 genes was modulated in opposite direction by ERβ in HT29 and SW480 cells, which include

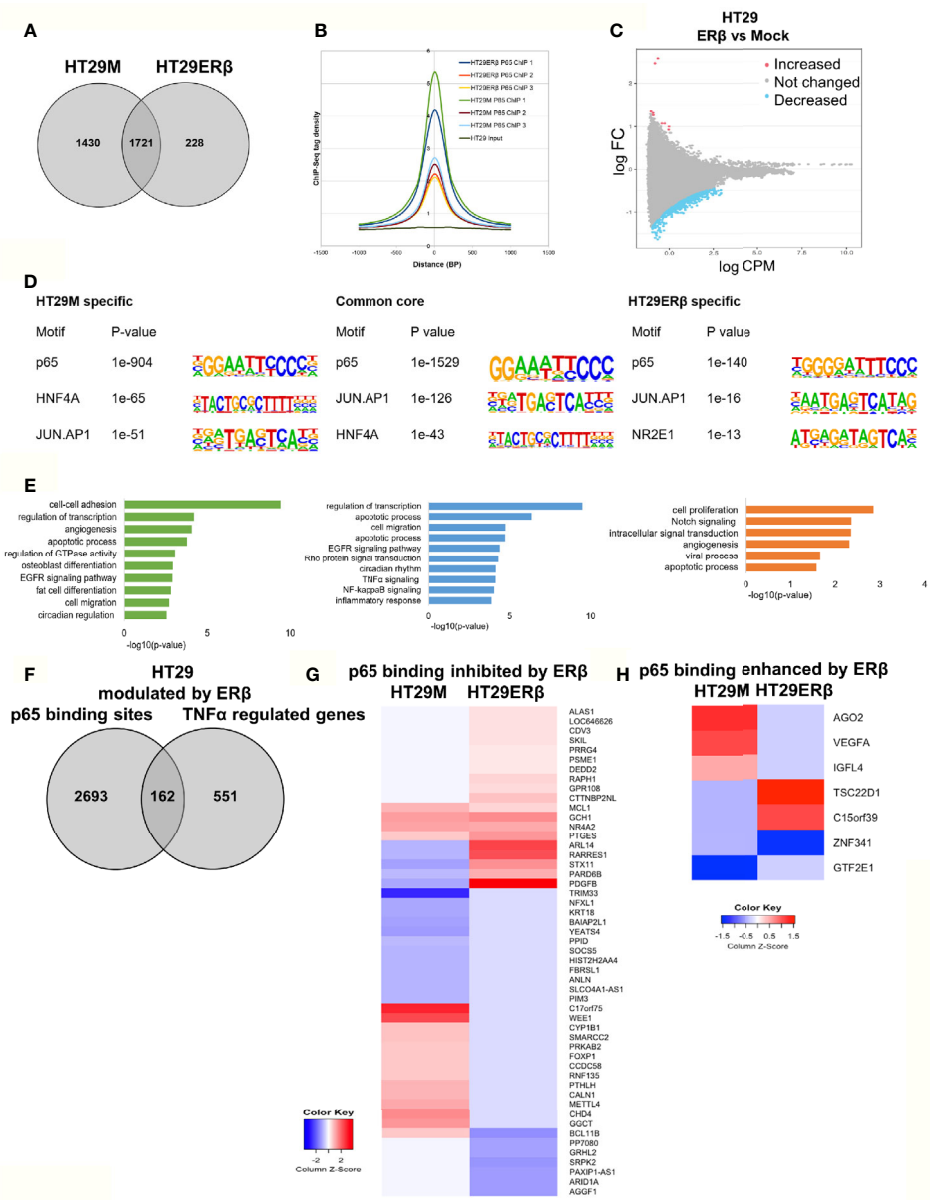

FIGURE 5 | ERβ diminishes p65 chromatin binding in HT29 cells. **(A)** Venn diagram comparing p65 binding sites in HT29 cells with and without expression of ERβ. **(B)** Density plot representing the distribution of p65 tag densities in three replicates each of HT29 cells with and without ERβ. **(C)** Volcano plot highlighting statistically significant differences of p65 binding in HT29 cells in presence and absence of ERβ, using sliding window approach. **(D)** Motifs highly enriched in p65-bound sequencing in HT29 cells only in absence of ERβ, regardless of ERβ expression (core genes), and only in presence of ERβ, respectively. HOMER was used to identify genomic distribution and motifs of p65 binding sites across the genome. **(E)** Biological functions enriched among genes located nearest to p65 binding sites in HT29 cells depending on ERβ expression. **(F)** Overlap of genes located nearest to the p65 binding sites and those genes where ERβ expression impacted TNFα gene response in HT29 cells. **(G, H)** Heatmap representing ERβ modulation of the TNFα-regulated genes, of genes located nearest to p65 binding that was **(G)** inhibited and **(H)** enhanced by ERβ in HT29 cells. Z score values were calculated from the logarithmic fold changes, which represent a value's relationship to the mean of a group of values. A positive Z score indicates the values above the mean and negative if it is below the mean.

important target genes such as *BIRC3, CXCL1, CXCL8* and *PDGFB* (**Figure 7C**). We further identified 596 p65 binding sites opposed by presence of ERβ in HT29 but, in contrast, bound only in presence of ERβ in SW480 cell line (**Figure 7B**). This core set of genes includes the well-known p65 interacting protein AP-1, NFIB, and circadian clock genes (*CLOCK, CXCL10, RUNX1, TP53I11, NFIB, BMAL2/ARNTL2*, **Figure 7D**). Altogether, these results indicate that in addition to conserved patterns there are also considerable cell specific differences in p65 binding between HT29 and SW480 cells, and that ERβ impacts the p65 cistrome and TNFα response in both cell lines.

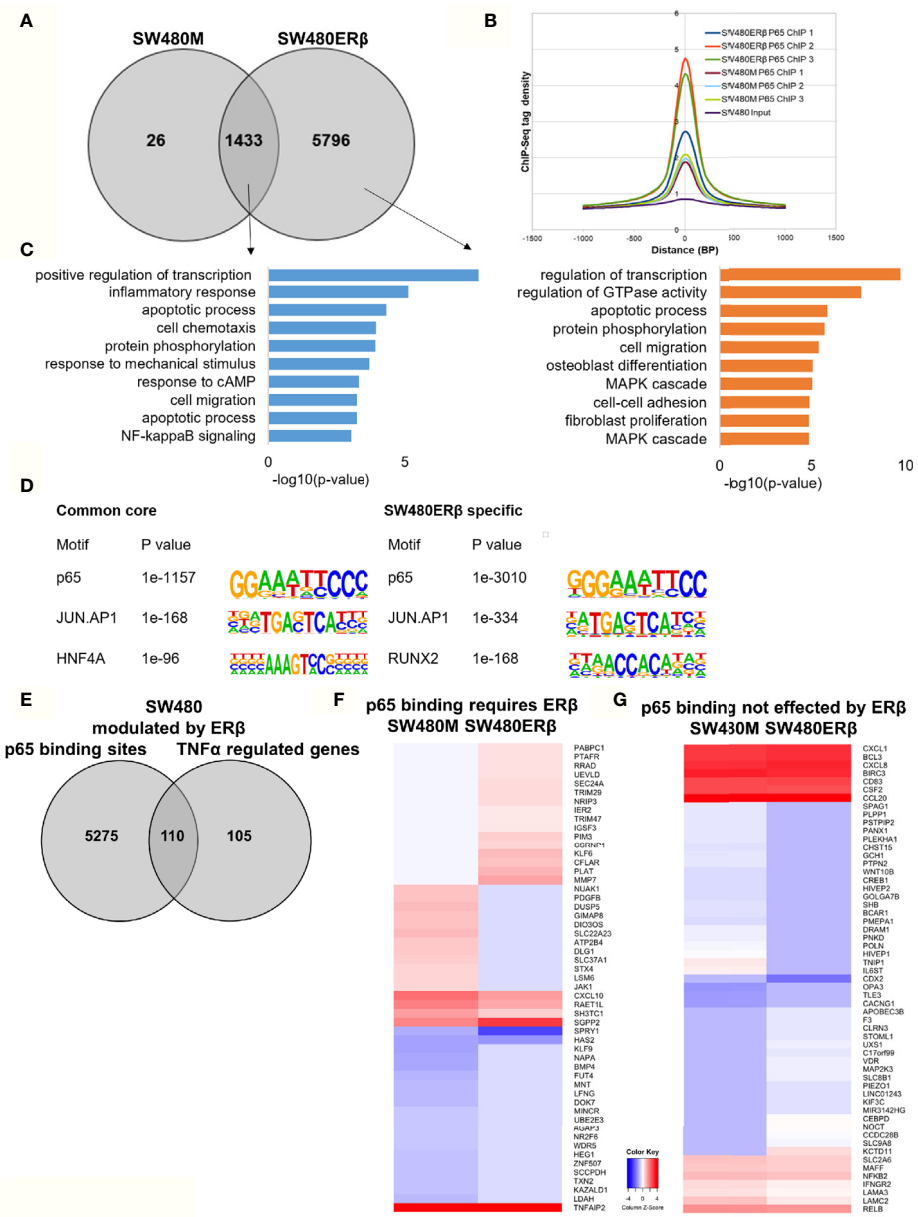

FIGURE 6 | ERβ enhances P65 chromatin binding in SW480 cells. **(A)** Venn diagram of p65 binding sites in SW480 cells with and without expression of ERβ. **(B)** Density plot representing the distribution of p65 tag densities in three replicates each of SW480cells with and without ERβ. **(C)** Biological functions enriched among genes located nearest to p65 binding sites in SW480 cells depending on ERβ expression. **(D)** Motifs highly enriched in p65-bound sequencing in SW480 cells only in absence of ERβ, regardless of ERβ expression (core genes), and only in presence of ERβ, respectively. HOMER was used to identify genomic distribution and motifs of p65 binding sites across the genome. **(E)** Overlap of genes located nearest to the p65 binding sites and those genes where ERβ expression impacted TNFα gene response in HT29 cells. **(F, G)** Heatmap representing ERβ modulation of the TNFα-regulated genes, of genes located nearest to p65 binding that **(F)** required ERβ or **(G)** was not affected by ERβ. Z score values were calculated from the logarithmic fold changes, which represent a value's relationship to the mean of a group of values. A positive Z score indicates the values above the mean and negative if it is below the mean.

DISCUSSION

Activation of NFκB in the intestinal epithelia can lead to colitis-induced CRC (48). Our study attempts to understand the molecular mechanisms behind the CRC promoting role of inflammation, by studying the cistrome of p65 and investigating how this is impacted by ERβ. We characterize the p65 genome-wide chromatin binding in two different CRC cell lines, and specify similarities and differences. We find that presence of ERβ impacts p65 binding and corresponding TNFα-mediated transcription.

Our work emphasizes that NFκB binds primarily through RELA and JUN-AP1 motifs in cells with a colon origin. Genes

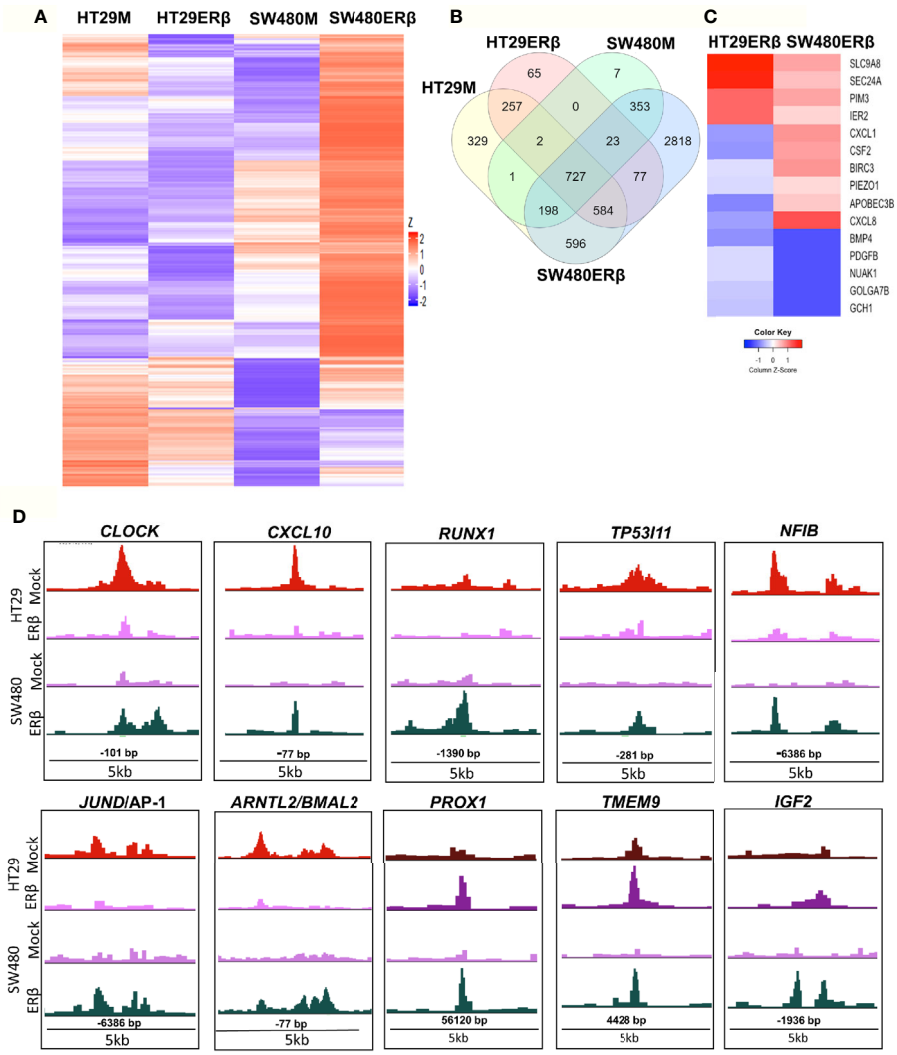

FIGURE 7 | ERβ influences P65 chromatin binding in colon cancer cells. **(A)** Heatmap representing p65 chromatin binding over the genome in absence or presence of ERβ, in HT29 and SW480 cells. **(B)** Venn diagram comparing p65 chromatin binding sites in HT29, SW480 cell lines with and without ERβ. **(C)** Heatmap illustrating the impact of ERβ on TNFα modulated genes with identified p65 chromatin binding sites in HT29 and SW480 cells, respectively. Z score values were calculated from the logarithmic fold changes, which represent a value's relationship to the mean of a group of values. A positive Z score indicates the values above the mean and negative if it is below the mean. **(D)** Examples of p65 chromatin binding sites that was identified only in presence of ERβ in SW480 cell lines, but bound the same sites in HT29 in absence of ERβ and p65 chromatin binding sites enhanced by ERβ in both HT29 and SW480 cells illustrated using the UCSC genome browser.

nearest to p65-bound chromatin were involved in inflammatory response, cell proliferation, cell migration, and, interestingly, circadian clock (*BMAL2*, *CLOCK*). A previous study has shown that dysregulation of circadian rhythm increases the risk for colorectal cancer (49). Indeed, *CLOCK* gene mutations have been identified in 53% of CRC with microsatellite instability (MSI) (50). Moreover, another study identified that mutations in the CLOCK1 gene increased the risk of developing CRC (51). In breast cancer cells, several studies identified a link between circadian genes and NFκB signaling pathway (52, 53). In colon cancer, two studies have shown that REV-ERB-α through NFκB modulates circadian clock and reduced DSS-induced colitis (54, 55). In our recent studies, we have showed that ERβ can modulate the impact of TNFα-NFκB activity in CRC cell lines and *in vivo* using the AOM-DSS mouse model (22). We have also demonstrated that intestinal ERβ regulates the expression of the circadian clock gene *Bmal1* (*Arntl1*) in colon of HFD-fed mice (56). Here, in addition to identifying p65-binding sites, we demonstrate that the activation of the TNFα-NFκB axis impacts the expression of circadian genes. Moreover, we show that ERβ interferes with the general p65 chromatin binding, including the circadian genes *CLOCK* and *BMAL2* (**Figure 7C**). Taken all this into account, our interpretation is that p65 modulates circadian genes in the colon

in the pro-inflammatory pro-tumorigenic condition, and that ERβ can change this and thereby oppose the inflammatory condition that drives development of colon cancer.

Interestingly, p65 chromatin binding appears relatively distinct between the two CRC cell lines, in support with the fine-tuned cell-specific manner whereby NFκB controls transcriptional regulation. The cell lines are indeed different in several respects. While both are derived from primary colon adenocarcinomas, the HT29 cell line is derived from a likely pre-menopausal (44-year-old) woman, whereas SW480 originates from a 50-year-old man (57, 58). We have also reported sex differences in the non-tumor and tumor transcriptome of CRC patients, which impacted biomarker discovery (59). The different female-male origin of the cell lines used here, may indeed impact the different regulation of p65 cistrome or its modulation by ERβ. However, further studies are needed to clarify this. Moreover, HT29 cells are CIMP (CpG island methylator phenotype) positive, and SW480 cells are CIMP negative. Aberrant methylation of the CpG islands has been shown to impact chromatin binding and accessibility to transcription factors (60, 61). Their mutational profile also differs, with HT29 having mutations in BRAF (V600E), PIK3CA (P449T), and p53 (R273H), and SW480 in KRAS (G12V) and p53 (double mutant alleles R273H and P309S, however still retaining functionality of many p53-associated pathways) (62, 63). These proteins are important transcriptional regulators that can also influence the binding of transcription factors (64, 65). In parental CRC cell lines HT29 and SW480, neither ERα nor ERβ is expressed, while MCF7 cell line expresses ERα, which has been shown to interact with p65 (24). These factors may all modulate the p65 cistrome.

While the p65 binding pattern was similar between the two CRC cell lines, the p65 cistrome of breast cancer cell line MCF7 was more distinctly different. One of the well-known interaction-partner of p65 is the p53 protein (65). Recently, it was shown that mutant p53 enhances NFκB activity in mice, leading to chronic inflammation and associated CRC (65). Another study demonstrated that p53 mutants directly interact with NFκB in SW480 cells (15). MCF7 cell line has wild type p53, whereas both HT29 and SW480 cells express the R273H p53 mutant protein, which inhibits DNA binding (66, 67). Hence it is possible that the p53 status impacts p65 cistrome in these cell lines, and further studies are needed to explore this hypothesis.

We have previously shown that TNFα triggers a transcriptional response in both CRC cell lines, and that ERβ modulates this (22). Here, we correlate the transcriptional response with p65 chromatin binding sites, and how ERβ modifies the p65 cistrome. To be noted, in order to optimize experiments, different treatments times and concentrations of TNFα were used in ChIP-Seq (30 ng/ml, 30 min) and for transcriptional analysis (10 ng/ml, 2h). Further, the transduced ERβ is expressed at higher levels in SW480 compared to HT29 (1.8 times more), and previous data suggests that TNFα may increase transactivation of ERβ (22). These factors may all influence the kinetics of the mechanisms described here, but are not expected to have a major influence on the mechanism per se. Our findings offer mechanistic underpinnings of how inflammation modulates specific signaling pathways and how ERβ can attenuate cytokine-induced carcinogenic response in CRC cells.

The strength of this study includes the genome-wide approach to decrypt these interactions, which together with the validated high-quality ChIP-Seq data generates unbiased and reliable data. The significance of these findings is reinforced by the use of two different CRC cell lines and the comparison with transcriptional impact, as well as comparisons between our results and published data generated from cells of other origin. A shortcoming includes the use of exogenous expression of ERβ in the CRC cell lines. However, cell lines mostly lack endogenous expression of ERβ (21, 22). Further, it may be preferable to use non-tumor colonic cell lines, as one aim of the study was to investigate how p53 can prevent CRC through its impact on NFκB signaling. However, a key interest was also to decipher the oncogenic NFκB signaling in CRC, and along with the lack of suitable non-tumor cell lines at hand, and our access to highly characterized cell lines with exogenous expression of ERβ, this is the balance we chose. Further, the difference of antibodies used between our study and the breast cancer tissue study (41), may contribute to the differences found.

In conclusion, we provide a mechanistic foundation for a better understanding of how estrogen influences inflammatory signaling through NFκB in CRC cells.

DATA AVAILABILITY STATEMENT

The p65 ChIP-Seq data is deposited in the Gene Expression Omnibus (GEO) repository (GSE160856), TNFα bead array gene expression data was published previously (available at GSE65979), and SW480ERβ input and HT29ERβ input controls (GSE149979).

AUTHOR CONTRIBUTIONS

CW contributed to conceptualization. Methodology performed by RI, LH, and AA. Validation done by RI and LH. Formal analysis was done by RI and LH. Curation, RI. RI and CW wrote the first original draft and all authors commented or edited. Visualization, RI. Supervision, CW and AA. Project administration, CW. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.650625/full#supplementary-material

Supplementary Table 1 | ChIP-Seq statistics.

Supplementary Table 2 | p65 promoter binding sites in colon cancer cells.

Supplementary Table 3 | p65-bound and TNFα-regulated genes in colon cancer cells.

Supplementary Table 4 | Differently modulated TNFα-regulated genes by ERβ in colon cancer cells.

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Single Hormone Receptor-Positive Metaplastic Breast Cancer: Similar Outcome as Triple-Negative Subtype

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Background: Metaplastic breast cancer (MBC) is a rare and aggressive subtype of the breast. To understand the characteristics and prognosis of single hormone receptor-positive (HR+) MBC (estrogen receptor-positive [ER+]/progesterone receptor-negative [PR-] and ER-/PR+), we compared these tumors to double HR+ tumors as well as HR- tumors.

Patients and Methods: The Surveillance, Epidemiology, and End Results database was used to analyze MBC between 1975 and 2016. The effect of HR status was evaluated using a multivariate Cox regression model.

Results: We included 3369 patients with a median follow-up time of 42 months (range 0-322 months). In this study, 280 (8.3%) cases were double HR+ tumors, 2597 (77.1%) were double HR- tumors, and 492 (14.6%) cases were single HR+ tumors, of which 159 (4.7%) cases were ER-/PR+ tumors and 333 (9.9%) were ER+/PR- tumors. On multivariate Cox analysis, the prognosis was related to age, race/ethnicity, tumor grade, TNM stage, and surgery. HR status remained no impact on breast cancer-specific survival (BCSS). In the Kaplan-Meier curve, HR status was not associated with better BCSS or overall survival (OS). In patients without HER2 overexpression, the BCSS and OS of ER+/PR- and ER-/PR+ tumors were not significantly different from that of ER-/PR- and ER+/PR+ tumors. The difference remains no significant in patients with HER2 overexpression.

Conclusions: In comparison with both ER-/PR- and ER+/PR+ tumors, we have identified clinically and biologically distinct features of single HR+ tumors. In patients with or without HER2 overexpression, the prognosis of single HR+ tumors was similar to ER-/PR- and ER+/PR+ tumors.

Keywords: metaplastic breast cancer, hormonal receptor, HER2, prognosis, SEER database

INTRODUCTION

Metaplastic breast cancer (MBC) is a rare and aggressive subtype accounting for <1% of all breast cancers (1). Previous studies have reported histologic MBC characterized by either homogenous or mixed components (2–6). MBC was not identified as a unique pathological type by the World Health Organization until 2000 (7). Since then, as pathologists' understanding of MBC has considerably improved, the incidence has also increased (8). However, given its rarity, the clinical characteristics and prognosis of single hormonal receptor-positive MBC (single HR+ MBC, ER+/PR-, and ER-/PR+) are unclear.

In the National Comprehensive Cancer Network (NCCN) breast cancer guidelines, the management of MBC is similar to that of invasive ductal carcinoma (IDC) (9). However, MBC is characterized by larger tumor size, lesser regional node involvement, and higher tumor grade than breast cancers with more common histology (10–12). The pathway of metaplastic cancer metastasis was hematogenous but not lymphatic spread (13). A previous study with data from 2001 to 2010 of the Surveillance, Epidemiology, and End Results (SEER) database found that patients with stage I–III MBC had significantly worse 5-year breast cancer-specific survival (BCSS) than those with synchronous IDC (14). Some studies reported that MBC is chemorefractory, regardless of whether the included patients received neoadjuvant or adjuvant settings (8, 15–17). Although the common molecular subtype is the triple-negative (TN) phenotype in MBC, HR+ and human epidermal growth receptor 2 positive (HER2+) tumors do exist (18). A population-based study reported that HR status was not associated with survival of metaplastic carcinoma, which was different from IDC and infiltrating lobular carcinomas (19).

Although the technique of immunohistochemistry has now considerably improved, the incidence of MBC with estrogen receptor-negative (ER-)/progesterone receptor-positive (PR+) phenotype has not decreased (20). Generally, HR+ breast cancers have a favorable prognosis. To understand the characteristics and prognosis of single HR+ MBC, we compared these tumors to double HR+ tumors (ER+/PR+) as well as HR- tumors (ER-/PR-) by using the database of the whole population.

MATERIALS AND METHODS

Patients

Data were retrieved from the SEER database and included all cases of pathologically confirmed MBC diagnosed between 1975 and 2016. This database collects data on cancer incidence, demographics and clinicopathologic data, management, and survival from 18 population-based cancer registries. According to the third edition of the International Classification of Diseases for Oncology (ICD-0-3), carcinoma histology was identified in metaplastic cancers with ICD-0-3 codes: 8560, 8562, 8570–8572, 8575, and 8980–8982 (19). The inclusion criteria were as follows: female sex; age ≥18 years; breast cancer as first and the only cancer diagnosis; unilateral breast cancer; histologically or cytologically confirmed diagnosis (instead of autopsy-confirmed); available information regarding survival time and HR status; and stage exception of T0 and Tis. Accordingly, 3369 patients were finally enrolled.

Demographics and Clinicopathologic Features

The demographic parameters included age at diagnosis; race/ethnicity recorded in the SEER database (White, Black, other); and insurance status. The clinicopathologic parameters included tumor grade; tumor size (T1, T2, T3, T4); regional node status (N0, N1, N2, N3); chemotherapy (CT); radiotherapy (RT); type of surgery (no surgery, lumpectomy, mastectomy); and biomarker profile (ER, PR, HER2). The definition of TNM (T-tumor, N-node, and M-metastasis) stage was according to the sixth/seventh edition of the Union for International Cancer Control/American Joint Committee on Cancer Pathologic Staging System. According to the SEER, HR status was stratified as single HR+, double HR+ tumors, and double HR- tumors.

The primary clinical outcome was BCSS, defined as the date of diagnosis to the date of death from breast cancer. The secondary clinical outcome was overall survival (OS), defined as the date of diagnosis to the date of death from any cause.

Detection of ER, PR, and HER2

In the SEER database, in cases where ER/PR is reported on more than one tumor specimen, the highest value is recorded. If any sample is positive, that record as positive. If neoadjacent therapy was received, the assay was recorded from tumor specimens prior to neoadjuvant therapy. If neoadjuvant therapy was given and there were no ER/PR results from pre-treatment specimens, these findings were reported from post-treatment specimens. If ER/PR was positive on an *in situ* specimen and ER/PR was negative on all tested invasive specimens, code ER/PR was considered negative. If ≥1% cells stained positive, the test results were considered positive. HER2 positivity was defined as an intensity of 3+ by IHC, while a score of 2+ was interpreted as equivocal. A negative test was defined as staining with a score of 0/1+. For equivocal stating, silver *in situ* hybridization (SISH) or fluorescence *in situ* hybridization (FISH) were performed; the results were positive for HER2 amplification when the ratio of HER2 to CEP17 was >2.2. We provided four MBC patients with different ER/PR phenotype (**Figure S1**).

Statistical Analysis

The $\chi 2$ test was carried out to analyze the differences between groups. The Cox proportional hazards model was used to assess the risk factors related to BCSS. Survival curves were constructed using the Kaplan–Meier method. Hazard ratios were presented with 95% confidence intervals (CIs). All statistical analyses were performed using SPSS statistical software (version 24.0; IBM Corporation, Armonk, NY, USA), and $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Patient Characteristics

Of the 4672 MBC patients in the SEER registry, our final sample comprised 3369 patients. In this study, 280 (8.3%) patients had double HR+ tumors, 2597 (77.1%) had double HR- tumors, and 492 (14.6%) had single HR+ tumors, of which 159 (4.7%) cases were ER-/PR+ tumors and 333 (9.9%) were ER+/PR- tumors. The median age of the entire cohort was 61 years (range, 20–89 years). Most patients were white women (n=2565, 76.1%) and had poor differentiation (n=2274, 67.5%). In patients with available tumor size information, 46.0% were stage T2. A total of 3199 (95.0%) and 170 (5.0%) patients had stage I–III and stage IV disease, respectively. In addition, 2450 (72.7%), 576 (17.1%), 131 (3.9%), and 75 (2.2%) patients had N0, N1, N2, and N3 stage disease, respectively. A total of 1194 deaths were recorded, including 791 breast cancer related-deaths.

The clinicopathological characteristics of the four subtypes are summarized in **Table 1**. Compared with ER-/PR- tumors, ER+/PR- tumors were not significantly different with respect to ethnicity, tumor grade, tumor stage, and CT, but ER+/PR- tumors exhibited more regional node involvement (P = 0.004). However, compared with ER+/PR+ tumors, the clinicopathological characteristics of ER+/PR- tumors did not show a significant difference. ER-/PR+ tumors were found more in Black women (ER-/PR+ 25.2% vs. ER-/PR- 17.4%, P = 0.021)

TABLE 1 | Characteristics in MBC patients with ER-/PR-, ER+/PR-, ER-/PR+, and ER+/PR+ tumors.

Variables	ER-/PR-	ER+/PR-	ER-/PR+	ER+/PR+
Age (years)	61.25 ± 13.95	61.63 ± 14.07	60.21 ± 15.71	60.40 ± 15.45
Follow-up time (median, months)	43 (0-322)	34 (0-321)	45 (0-320)	42 (0-273)
Race (n, %)				
Black	421 (16.2)	55 (16.5)	36 (25.2)	34 (12.1)
White	1998 (76.9)	240 (72.1)	107 (74.8)	220 (78.6)
Other	178 (6.9)	38 (11.4)	16	26 (9.3)
Insurance (n, %)				
No	928 (35.7)	55 (16.5)	44 (27.7)	97 (34.6)
Yes	1669 (64.3)	278 (83.5)	115 (72.3)	183 (65.4)
Grade (n, %)				
Undifferentiated	141 (5.4)	13 (3.9)	6 (3.8)	9 (3.2)
Poorly differentiated	1758 (67.7)	228 (68.5)	120 (75.4)	168 (60.0)
Moderately differentiated	261 (10.1)	43 (12.9)	17 (10.7)	50 (17.9)
Well differentiated	91 (3.5)	11 (3.3)	9 (5.7)	14 (5.0)
Unknown	346 (13.3)	38 (11.4)	7 (4.4)	39 (13.9)
Tumor size (n, %)				
T1	642 (24.7)	83 (24.9)	47 (29.6)	82 (29.3)
T2	1205 (46.4)	152 (45.7)	75 (47.2)	118 (42.1)
T3	411 (15.8)	41 (12.3)	21 (13.2)	37 (13.2)
T4	211 (8.2)	41 (12.3)	13 (8.2)	27 (9.6)
Unknown	128 (4.9)	16 (4.8)	3 (1.8)	16 (5.7)
Regional node status (n, %)				
N0	1927 (74.2)	217 (65.2)	120 (75.5)	186 (66.4)
N1	431 (16.6)	71 (21.3)	26 (16.4)	48 (17.2)
N2	89 (3.4)	18 (5.4)	3 (1.9)	21 (7.5)
N3	47 (1.8)	13 (3.9)	4 (2.5)	11 (3.9)
Unknown	103 (4.0)	14 (4.2)	6 (3.7)	14 (5.0)
TNM stage (n, %)				
I-III	2356 (90.7)	294 (88.3)	146 (91.8)	257 (91.8)
IV	129 (5.0)	24 (7.2)	9 (5.7)	8 (2.9)
Unknown	112 (4.3)	15 (4.5)	4 (2.5)	15 (5.3))
HER2				
Positive	1154 (44.4)	211 (63.4)	74 (46.5)	122 (43.6)
Negative	65 (2.5)	13 (3.9)	10 (6.3)	14 (5.0)
Unknown	1378 (53.1)	109 (32.7)	75 (47.2)	144 (51.4)
Chemotherapy (n, %)				
No	937 (36.1)	119 (35.7)	52 (32.7)	121 (43.2)
Yes	1660 (63.9)	214 (64.3)	107 (67.3)	159 (56.8)
Radiotherapy (n, %)				
No	1474 (56.8)	188 (56.5)	79 (49.7)	153 (54.6)
Yes	1123 (43.2)	145 (43.5)	80 (50.3)	127 (45.4)
Type of surgery (n, %)				
No	201 (7.7)	29 (8.7)	9 (5.7)	24 (8.6)
Lumpectomy	1045 (40.2)	133 (39.9)	73 (45.9)	110 (39.3)
Mastectomy	1351 (52.0)	171 (51.4)	77 (48.4)	146 (52.1)

MBC, metaplastic breast cancer; ER, estrogen receptor; PR, progesterone receptor.

and had higher tumor grade (P = 0.010) than ER-/PR- tumors. Further, ER-/PR+ tumors were also found in more black women (ER-/PR+ 25.2% vs. ER-/PR- 13.4%, P = 0.012), had higher tumor grade (P = 0.003), and received more CT treatment (ER-/PR+ 67.3% vs. ER-/PR- 56.8%, P = 0.030) than ER+/PR+ tumors. There was no difference in stage (P = 0.139) or type of surgery (P = 0.288). Furthermore, there was no difference in the expression of HER2 (P = 0.831). Both ER-/PR+ and ER+/PR- tumors had similar HER2 overexpression to ER+/PR+ tumors (P = 0.831). However, ER-/ER+ tumors showed higher HER2 overexpression than ER-/PR- tumors (P = 0.028). The characteristics of single HR+ tumors were more distinct in HER2-negative tumors than in HER2 overexpressing tumors. (**Tables S1** and **S2**)

Prognostic Factors for MBC

We further analyzed the independent prognostic factors associated with BCSS using the multivariate Cox proportional hazards model. HR status was not an independent prognostic factor related to better BCSS (hazard ratio: 0.839; 95%CI: 0.679–1.036; P = 0.102). Patients with stage IV disease had a worse prognosis than patients with stage I–III disease (hazard ratio: 7.594; 95%CI: 6.308–9.289; P < 0.001). In addition, patients could not benefit from CT (hazard ratio: 0.993; 95%CI: 0.839–1.176; P = 0.937) and RT (hazard ratio: 0.895; 95%CI: 0.552–1.535; P = 0.687). Patients who underwent mastectomy had worse prognosis than those who underwent lumpectomy (hazard ratio: 2.131; 95%CI: 1.795–2.530; P < 0.001). Furthermore, age, race/ethnicity, and tumor grade were independent indicators for BCSS (**Table 2**).

Survival Analysis of Single Hormone Receptor-Positive MBC

In multivariate analysis, in patients with or without HER2 overexpression, HR status was not associated with better BCSS or OS (**Tables 3**, **4**). Survival curves were plotted using the Kaplan–Meier curve. HR status was neither associated with BCSS nor OS (**Figures 1A, B**). In patients without HER2 overexpression, the BCSS and OS of ER+/PR- and ER-/PR+ tumors were not significantly different from those of ER-/PR- and ER+/PR+ tumors (**Figures 1C, D**). In patients with HER2 overexpression, the prognosis of ER+/PR- and ER-/PR+ tumors was not significantly different from those of ER-/PR- and ER+/PR+ tumors (**Figures 1E, F**).

TABLE 2 | Prognostic factors for BCSS in our study cohort.

Variables	Univariate analysis		P	Multivariate analysis		P
	HRs	95% CI		HRs	95% CI	
Age	1.010	1.005-1.015	<0.001	1.016	1.010-1.021	< 0.001
Race (n, %)						
Black	1	[Reference]		1	[Reference]	
White	0.754	0.631-0.901	0.002	0.788	0.657-0.944	0.010
Other	0.640	0.465-0.881	0.003	0.725	0.525-1.001	0.051
Insurance (n, %)						
No	1	[Reference]		1	[Reference]	
Yes	1.009	0.873-1.166	0.903	1.051	0.904-1.222	0.515
HR status						
ER-/PR-	1	[Reference]		1	[Reference]	
ER+/PR-	0.966	0.753-1.239	0.788	0.914	0.712-1.172	0.478
ER-/PR+	0.896	0.673-1.259	0.896	0.942	0.670-1.326	0.734
ER+/PR+	0.985	0.766-1.268	0.907	0.934	0.725-1.203	0.597
Grade (n, %)						
Undifferentiated	1	[Reference]		1	[Reference]	
Poorly differentiated	0.683	0.527-0.886	0.004	0.805	0.619-1.047	0.105
Moderately differentiated	0.358	0.247-0.520	<0.001	0.416	0.285-0.607	<0.001
Well differentiated	0.243	0.131-0.451	<0.001	0.333	0.179-0.619	0.001
Unknown	0.803	0.594-1.086	0.154	0.810	0.598-1.099	0.176
TNM stage (n, %)						
I-III	1	[Reference]		1	[Reference]	
IV	10.378	8.554-12.592	<0.001	7.594	6.308-9.289	<0.001
Chemotherapy (n, %)						
No	1	[Reference]		1	[Reference]	
Yes	1.035	0.895-1.196	0.642	0.993	0.839-1.176	0.937
Radiotherapy (n, %)						
No	1	[Reference]		1	[Reference]	
Yes	0.798	0.469-1.357	0.404	0.895	0.552-1.535	0.687
Type of Surgery (n, %)						
Lumpectomy	1	[Reference]		1	[Reference]	
Mastectomy	2.438	2.057-2.889	<0.001	2.131	1.795-2.530	<0.001
No	4.173	3.299-5.278	<0.001	3.092	2.418-3.952	<0.001

BCSS, breast cancer-specific survival; HR, hormonal receptor; HRs, hazard ratios; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.

TABLE 3 | Multivariate analysis of BCSS and OS in 1561 women with HER2-negative MBC.

		B coefficients	Standard error	Wald	*P*	HRs	95% CI
BCSS	ER-/PR- vs. ER+/PR-	-0.131	0.179	0.534	0.465	0.877	0.618-1.246
	ER-/PR- vs. ER-/PR+	0.254	0.271	0.881	0.348	1.289	0.758-2.192
	ER-/PR- vs. ER+/PR+	-0.113	0.238	0.224	0.636	0.893	0.561-1.424
	ER+/PR+ vs. ER+/PR-	-0.018	0.281	0.004	0.949	0.982	0.566-1.705
	ER+/PR+ vs. ER-/PR+	0.367	0.348	1.112	0.292	1.443	0.730-2.852
OS	ER-/PR- vs. ER+/PR-	-0.058	0.153	0.143	0.706	0.944	0.700-1.273
	ER-/PR- vs. ER-/PR+	0.023	0.252	0.008	0.927	1.023	0.624-1.677
	ER-/PR- vs. ER+/PR+	-0.113	0.205	0.305	0.581	0.893	0.598-1.334
	ER+/PR+ vs. ER+/PR-	0.055	0.241	0.053	0.818	1.057	0.659-1.696
	ER+/PR+ vs. ER-/PR+	0.136	0.314	0.188	0.665	1.146	0.619-2.122

BCSS, breast cancer-specific survival; OS, overall survival; MBC, metaplastic breast cancer; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; HRs, hazard ratios; CI, confidence interval. Adjusted for age, race, insurance, T stage, N stage, nuclear grade, and treatment.

TABLE 4 | Multivariate analysis of BCSS and OS in 102 women with HER2-positive MBC.

		B coefficients	Standard error	Wald	*P*	HRs	95% CI
BCSS	ER-/PR- vs. ER+/PR-	-0.093	1.040	0.008	0.929	0.912	0.119-7.001
	ER-/PR- vs. ER-/PR+	-0.710	1.270	0.313	0.576	0.492	0.041-5.927
	ER-/PR- vs. ER+/PR+	1.074	1.087	0.976	0.323	2.927	0.348-24.657
	ER+/PR+ vs. ER+/PR-	-1.167	1.424	0.671	0.413	0.311	0.019-5.072
	ER+/PR+ vs. ER-/PR+	-1.784	1.543	1.337	0.248	0.168	0.008-3.457
OS	ER-/PR- vs. ER+/PR-	-0.093	1.040	0.008	0.929	0.912	0.119-7.001
	ER-/PR- vs. ER-/PR+	-0.710	1.270	0.313	0.576	0.492	0.041-5.927
	ER-/PR- vs. ER+/PR+	1.074	1.087	0.976	0.323	2.927	0.348-24.657
	ER+/PR+ vs. ER+/PR-	-1.167	1.424	0.671	0.413	0.311	0.019-5.072
	ER+/PR+ vs. ER-/PR+	-1.784	1.543	1.337	0.248	0.168	0.008-3.457

BCSS, breast cancer-specific survival; OS, overall survival; MBC, metaplastic breast cancer; HER2, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor; HRs, hazard ratios; CI, confidence interval.
Adjusted for age, race, insurance, T stage, N stage, nuclear grade, and treatment.

DISCUSSION

In the current study, we evaluated tumor response to treatment with CT, RT, and surgery and compared differences in the clinical process, tumor characteristics, and prognosis among the four subtypes, namely ER-/PR-, ER+/PR-, ER-/PR+, and ER+/PR+. We found that CT and RT could not improve the prognosis of MBC. Patients that underwent mastectomy had a worse prognosis than those that underwent lumpectomy. Of concern was the finding that HR status was not associated with a better prognosis in the entire cohort. In patients with or without HER2 overexpression, the prognosis of single HR+ tumors was similar to that of ER-/PR- and ER+/PR+ tumors.

The data presented in this paper represent the largest cohort of patients with MBC, and this is the first descriptive report on the survival prognosis of MBC related to single HR status. For traditional breast cancer, patients with ER+/PR+ tumors had a better prognosis than those with ER+/PR- tumors, who in turn had a better prognosis than patients with ER-/PR- tumors (21). However, to our knowledge, no previous research has investigated the prognosis of single HR+ tumors in case of metaplastic carcinoma.

Ahmed et al. (20) reported that ER-/PR+ breast cancers exist, but are very rare. Itoh et al. (22) reported that among the ER-/PR+ patients, 65% of them were basal-like tumors. Bae et al. (23) pointed out that in single HR+ breast cancers, the ER+/PR- subtype accounts for 10%–15% of all breast cancers, while the ER-/PR+ subtype accounts for 2–4% of all breast cancers. Based on the SEER records, the frequency of the ER-/PR+ phenotype in our series was 4.7%. On the one hand, the results of immunohistochemistry from the SEER database were confirmed by pathologists. On the other hand, MBC tended to have poor differentiation accounting for 67.5% of all cases. In addition, Weigelt et al. (24) showed that MBCs are basal-like breast cancers. These reports propose that ER-/PR+ breast cancers are a biologically and clinically distinct subtype.

Although TN-subtype is the most common in MBC, the HR+ subtype also occurs (18). A study by Wright et al. (19) including 2,338 MBC cases concluded that contrary to traditional breast cancers, HR+ MBC did not have superior clinical outcomes. In our study, 2597 (77.1%), 333 (9.9%), 159 (4.7%), and 280 (8.3%) patients expressed ER-/PR-, ER+/PR-, ER-/PR+, and ER+/PR+, respectively. There was no difference in the prognosis among the four subtypes. In addition, He et al. (25) concluded that patients with TN-subtype had a worse prognosis than those with non-TN MBC. However, our study results showed that regardless of HER2 overexpression, the prognosis of ER+/PR- and ER-/PR+ tumors were not significantly different from those of ER-/PR- and ER+/PR+ tumors. The results using multivariate analysis may be more convincing than those obtained with Kaplan–Meier analysis that they using.

Although the rate of adjuvant CT was quite high (63.9% in ER-/PR-, 64.3% in ER+/PR-, 67.3% in ER-/PR+, and 56.8% in

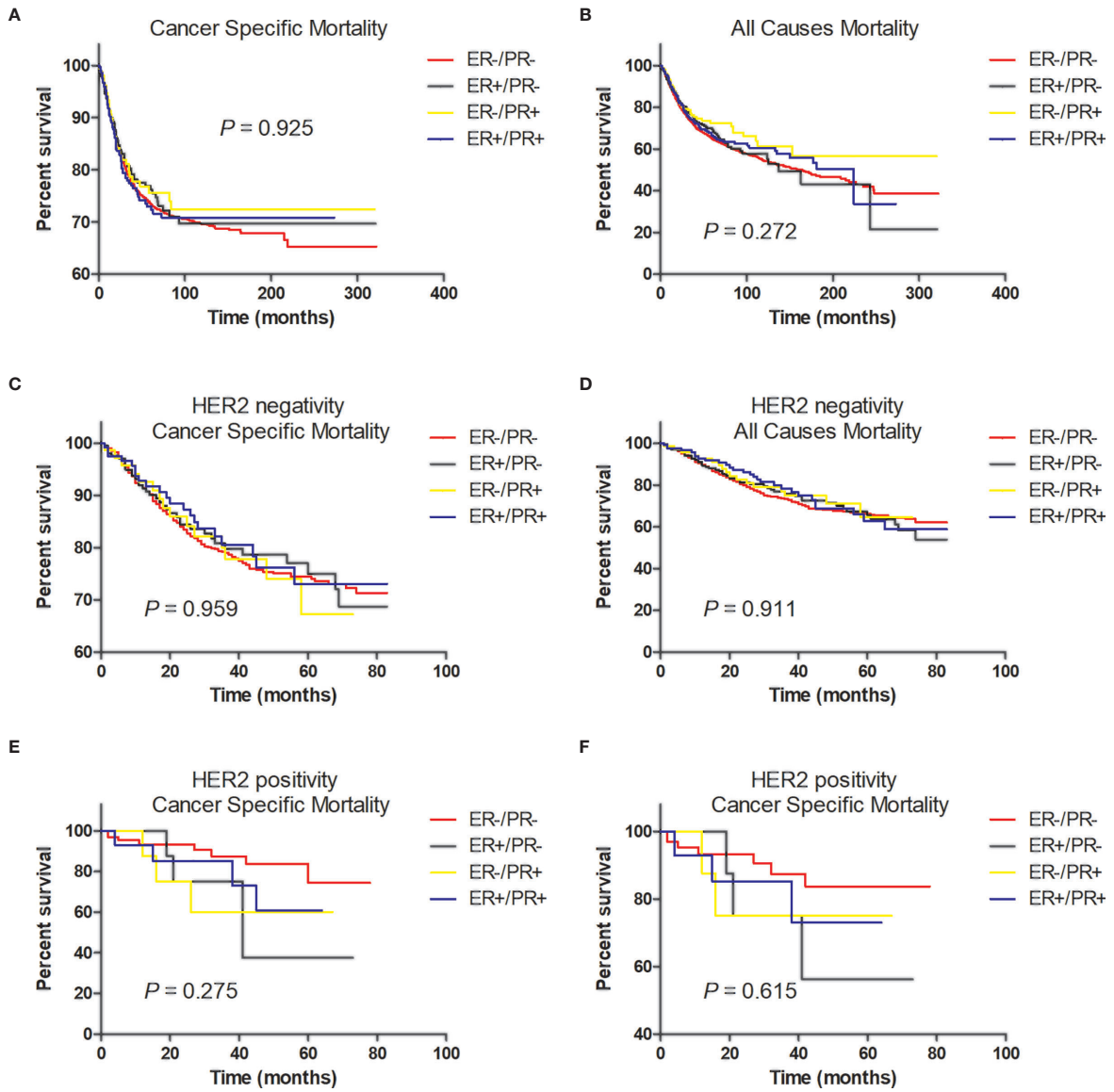

FIGURE 1 | Tumor survival based on hormone receptor status. **(A)** Breast cancer-specific survival (BCSS) and **(B)** overall survival (OS) of all patients; **(C)** BCSS and **(D)** OS of patients with HER2-negative tumors; **(E)** BCSS and **(F)** OS of patients with HER2-positive tumors.

ER+/PR+; P=0.081), there was no significant difference among the four subtypes in the entire cohort or in patients with or without HER2 overexpression. However, previous research has shown that the response rate of MBC to CT regimens was relatively low. MBC might be a type of basal breast cancer, characterized by higher grade and more rapid growth (24, 26–28). The expression levels of ER, PR, and HER-2 receptor in MBC cells were lower than that of IDC, while the expression levels of Ki-67 and p-53 were higher (29, 30). In MBC patients, DNA repair pathways such as TOP2A, PTEN, and BRCA1 showed downregulation upon genomic profiling. These findings might explain the low incidence of lymph node metastasis and resistance to conventional CT regimens. This may be one of the causes of the poor prognosis of patients with MBC.

A recent retrospective analysis showed that RT was related to improvements in OS and BCSS (25). However, some authors pointed out that the role of RT in the prognosis of MBC was related to the types of surgical methods. As we know, post-lumpectomy RT is a standard component of lumpectomy for treating IDC to minimize local recurrence. Dave et al. (31) and Yu et al. (32) found that RT was beneficial for MBC patients undergoing a lumpectomy, but not a total mastectomy. Additionally, a few studies illustrated that the role of RT in prognosis was related to clinical characteristics of MBC besides the types of surgical methods. However, our study

found that receipt of RT was not an independent factor for improved survival.

Notably, mastectomy was performed more often for patients with MBC, likely due to the presentation of larger tumors than those with other types of breast cancer. Tseng and Martinez explained that mastectomy or lumpectomy had no effect on OS or disease-specific survival for patients with MBC (33). In our study, the rate of patients receiving mastectomy was higher than that of patients receiving lumpectomy (51.8% vs. 40.4%), but mastectomy was an independent risk factor for BCSS. This may be another cause for poor prognosis with MBC.

Although detailed endocrine treatment strategies were not available in this analysis, previous studies have reported that the prognosis of HR+ patients receiving antiestrogen therapy showed no difference in outcome as compared to that of patients who did not receive antiestrogen therapy (8, 16, 34). The prognosis of single HR+ MBC is as poor as that of TN-MBC, which may be due to some factors.

Our study has some limitations. First, the retrospective nature of the study may have resulted in some selection bias. Second, detailed chemotherapy regimens, radiotherapy information, and endocrine treatment strategies could not be available from the SEER database; hence, a further case-control analysis could not be performed. However, we believe that our results will help researchers to understand the role of single hormonal receptor status in the prognosis of MBC.

CONCLUSION

We assessed a large cohort of patients with metaplastic breast cancer and found that HR status was not associated with prognosis. Furthermore, regardless of HER2 overexpression, the prognosis of ER+/PR- and ER-/PR+ tumors was not significantly different from those of ER-/PR- and ER+/PR+ tumors. When patients diagnosed with this rare and aggressive tumor were treated with surgery, physicians need to be careful with selecting the type of surgery. Furthermore, the role of anti-hormone therapy in HR+ MBC may need to be further investigated.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://seer.cancer.gov/data

ETHICS STATEMENT

This study was exempt from the approval processes of the Institutional Review Boards because the SEER database patient information is de-identified. Also, a patient consent form was not applicable.

AUTHOR CONTRIBUTIONS

XR, TH, and JMi contributed to the conception and design of the study. JH and JMa organized the database. YZ, JS, FD, and XZ performed the statistical analysis. JH wrote the first draft of the manuscript. JMa, YZ, JS, FD, and XZ wrote sections of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.628939/full#supplementary-material

Supplementary Table 1 | Clinicopathologic characteristics of patients with HER2-negative tumors.

Supplementary Table 2 | Clinicopathologic characteristics of patients with HER2-positive tumors.

Supplementary Figure 1 | Four MBC patients with different estrogen receptor (ER)/progesterone receptor (PR) phenotype. a, H&E, ×200; b, ER (-),×200; c, PR (-),×200; d, H&E, ×200; e, ER (+),×200; f, PR (+),×200; g, H&E, ×200; h, ER (-),×200; i, PR (+),×200; j, H&E, ×200; k, ER (+),×200; m, PR (-),×200.

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Pre-Menopausal Women With Breast Cancers Having High AR/ER Ratios in the Context of Higher Circulating Testosterone Tend to Have Poorer Outcomes

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Purpose: Women with breast tumors with higher expression of AR are in general known to have better survival outcomes while a high AR/ER ratio is associated with poor outcomes in hormone receptor positive breast cancers mostly in post menopausal women. We have evaluated the AR/ER ratio in the context of circulating androgens specifically in patients younger than 50 years most of whom are pre-menopausal and hence have a high estrogenic hormonal milieu.

Methods: Tumor samples from patients 50 years or younger at first diagnosis were chosen from a larger cohort of 270 patients with median follow-up of 72 months. Expression levels of ER and AR proteins were detected by immunohistochemistry (IHC) and the transcript levels by quantitative PCR. Ciculating levels of total testosterone were estimated from serum samples. A ratio of AR/ER was derived using the transcript levels, and tumors were dichotomized into high and low ratio groups based on the third quartile value. Survival and the prognostic significance of the ratio was compared between the low and high ratio groups in all tumors and also within ER positive tumors. Results were further validated in external datasets (TCGA and METABRIC).

Results: Eighty-eight (32%) patients were ≤50 years, with 22 having high AR/ER ratio calculated using the transcript levels. Circulating levels of total testosterone were higher in women whose tumors had a high AR/ER ratio (p = 0.02). Tumors with high AR/ER ratio had significantly poorer disease-free survival than those with low AR/ER ratio [HR-2.6 (95% CI-1.02–6.59) p = 0.04]. Evaluation of tumors with high AR/ER ratio within ER positive tumors alone reconfirmed the prognostic relevance of the high AR/ER ratio with a significant hazard ratio of 4.6 (95% CI-1.35–15.37, p = 0.01). Similar trends were observed in the TCGA and METABRIC dataset.

Conclusion: Our data in pre-menopausal women with breast cancer suggest that it is not merely the presence or absence of AR expression but the relative activity of ER, as well as the hormonal milieu of the patient that determine clinical outcomes, indicating that both context and interactions ultimately influence tumor behavior.

Keywords: breast cancer, androgen receptor, AR/ER ratio, pre-menopausal, testosterone

INTRODUCTION

Breast cancer in the young is more commonly associated with aggressive features and poorer clinical outcomes when compared to that of an older age group (1). Although the incidence of breast cancer in women ≤50 years is limited to less than a third in most clinical series, proportions seem to vary in different ethnic populations (2, 3). Hormonal risk factors are different in this age group, and younger women tend to have more hormone receptor negative breast cancers with adverse prognostic features (4). Recent studies which have characterized genomic and transcriptomic profile of the breast cancers in the young and pre-menopausal women have shown them as a unique etiologic and biological entity (5).

Circulating androgens are detected during all ages in adult women and hence thought to have biological roles (6). Multiple studies both in pre- and post-menopausal women have reported a significant positive association between higher levels of circulating androgens and the risk of developing breast cancer (7–10). Contrary to the action of androgens which mediate their effects through androgen receptor (AR), expression of AR has been shown to be a favorable prognostic indicator in breast cancer. Women with estrogen receptor (ER) positive, AR positive (ER+AR+) tumors are known to have better survival and more favorable clinicopathological features, like negative lymph node metastasis and lower tumor grade than women whose tumors are negative for AR (11).

The clinical and biological significance of AR expression in breast cancer is not straight forward due to variations in both the levels of AR as well as the intrinsic differences among the multiple subtypes of breast cancer. AR is known to control tumor growth in ER positive tumors and stimulate disease progression in the absence of ER (12). *In vitro* studies have demonstrated that AR might decrease ER transcriptional activity probably by competing to the same binding sites as ER in breast tumors (13). However, Cochrane et al. were the first to report high levels of AR could be associated with a worse prognosis with tamoxifen resistance and defined the relationship between AR and ER expression as AR/ER ratio in ER positive tumors to display the dynamic interplay between the two receptors (14). Multiple other studies since then have evaluated the utility of AR/ER ratio and shown higher ratios were associated with unfavorable features and poor prognosis in breast cancer (15–18). Most of them have however, focused on ER positive, HER2 negative subgroup of breast tumors in elderly women (median age >60 years) in predominant Caucasian women. Breast cancer in the south Asian population is seen to arise at least a decade earlier with half of the women less than 50 years of age at first diagnosis (19, 20). In this study, we have investigated the role of AR by evaluating the AR/ER ratio specifically in patients younger than 50 years of age and who are likely to have a dominant estrogenic environment and the role of this particular hormonal environment on tumor progression.

METHODS

Cohort Details

Tumor samples were chosen from a retrospective cohort of 270 women with primary breast cancer including five women with bilateral tumors. These samples were collected as part of an observational longitudinal study from two tertiary cancer care hospitals in Bangalore, India between 2008 and 2013, and these women were followed-up for up to 9 years, with a total loss to follow-up of less than 5% and a median follow-up duration of more than 72 months. The study was approved by the ethical committee of both institutions, and informed consent was obtained from all the patients to use their tissue and blood sample for research. Information on clinical variables like age, grade, tumor size, lymph node status, stage of the disease with ER, progesterone receptor (PgR), and HER2 was obtained from their clinical records. Treatment information was obtained from clinical records of patients during follow-up. Endocrine therapy was recorded as tamoxifen or aromatase inhibitor, and chemotherapy regimens were noted for intake of anthracyclines or taxanes. Information on trastuzumab was recorded in HER2 positive patients whenever received. Formalin fixed paraffin embedded (FFPE) blocks from tumor tissue having more than 50% of the area of representative tumor were selected for the study.

Immunohistochemistry of AR

Immunohistochemistry for AR was done on each of the tumor sections as per standard protocol using the Ventana Benchmark^{XT} staining system (Ventana Medical Systems, Tucson, AZ, USA). Briefly, 5 µm thick sections were fixed in hot air oven at 60°C for 60 min and loaded on to an IHC staining machine. De-paraffinization was performed using EZ Prep solution (Proprietary-Ventana reagent), and antigen retrieval was done using Cell Conditioning solution 1 (CC1) for 60 min. Primary antibody for AR (Clone AR 441, DAKO, dilution at 1:75) was added manually and incubated for 32 min at room temperature. Optiview DAB Detection Kit (Ventana Medical Systems) was used to visualize the signal, using DAB (3–3′diaminobenzidine) as the chromogen. Further, the sections were automatically counterstained with hematoxylin II (Ventana

Medical Systems) for 12 min. The slides were removed from the autostainer, washed in de-ionized water, dehydrated in graded ethanol, cleared in xylene, and examined by microscopy. Appropriate positive and negative controls were run for each batch. Two pathologists scored the staining for AR protein independently and arrived at a final score. Nuclear staining in more >1% of tumor cells was considered as positive.

RNA Extraction, cDNA Conversion, and Real Time PCR

Total RNA was extracted using the Tri Reagent protocol according to manufacturer's instructions (Sigma Aldrich # T9424) from two 20 μm sections from the selected tumor block. Briefly, tumor block was deparaffinized using heat, and then subjected to overnight digestion using proteinase K (Qiagen #19133). Quantitation of the RNA was done using the Qubit RNA BR (Broad-Range) Assay Kit (Invitrogen # Q10210) on a Qubit 2.0 Fluorometer (Invitrogen #Q32866). Then 500 ng of total RNA was reverse transcribed to cDNA using high capacity cDNA conversion kit from Thermofisher scientific (Cat # 4322171) as per manufacturer's instruction.

Primers were designed for *AR* and *ESR1* genes using primer 3 plus software and further validated on ensemble genome browser, NCBI blast and UCSC genome browser. The primers were synthesized by Juniper Life Sciences, Bangalore, India. The details of the primer sequences are given in the **Supplementary Table 1**. For quantitative real time PCR (qPCR), 5 ng of cDNA template was used per reaction and performed in duplicate using SYBR® Green on the LightCycler® 480 II (Roche Diagnostics). Pre-incubation and initial denaturation of the template cDNA were performed at 95°C for 10 min, followed by amplification for 45 cycles at 95°C for 15 s and 60°C for 1 min. Cycles of threshold (Ct) values for the test genes were normalized to the mean Ct values of the three reference genes—*ACTB, RPLP0*, and *PUM1* for each tumor sample which was normalized for varying abundance of transcripts. Relative normalized expression of test genes was calculated by ΔCT method. The methods used for nucleic acid extraction, quantitative PCR (qPCR), and selection of housekeeping genes (HKGs) and the quality control criteria for inclusion of samples in the analysis have been described in detail in our previous publication (21).

Estimation of Total Testosterone

The estimation of total testosterone in serum samples collected prior to surgery or following surgery of 169 breast cancer patients was done by a chemiluminescence based immunoassay method using the Abbott Architect ci8200 (Integrated) & i2000 (Immunoassay) instrument. In brief, the serum sample with a minimum volume of 300 μl was loaded onto the instrument. The sample was then transferred into multiple compartments where it is mixed, incubated, and washed. In the subsequent steps, the conjugate, pre-trigger and trigger solutions were added. The chemiluminescence emission was measured to determine the quantity of total testosterone in the serum sample. The result was calculated using a four parametric logistic curve fit data reduction method to generate a calibration curve.

Statistical Methods

Descriptive analysis was done to evaluate the cohort characteristics and distribution of the high and low AR/ER ratio groups. Difference in the clinical variables between high and low ratio groups was tested by independent Student's t-test or Mann–Whitney U test for continuous variables, and chi-square test was done for categorical variables. Concordance between the AR transcript and protein was estimated by receiver operating characteristic (ROC) curve analysis. Kaplan–Meier survival curves and log rank tests were used to compare the disease-free and breast cancer specific survival between the high and low AR/ER ratio groups. Disease free survival (DFS) and breast cancer specific survival (BCSS) were calculated as the time from the date of first diagnosis to the time when a local or distant recurrence occurred and death due to disease, respectively. Patients with no event or had death due to non-breast cancer related causes were right censored. The prognostic importance of high AR/ER ratio in comparison to other clinicopathological characteristics was validated by both univariate and multivariate cox-proportional hazard analyses. All tests were two tailed, and P-value <0.05 was considered statistically significant. All statistical analyses were done on statistical software XLSTAT version 2019.4.2 and SPSS software version 20 (Chicago, IL).

RESULTS

Patient Characteristics

A total of 270 patients were included in the study with a median age at first diagnosis of 56.2 years. Nearly 60% of the tumors were associated with spread to the regional lymph-nodes and half of women were at clinical stage 2 and a third stage 3. Less than 10% of the tumors were grade 1 with approximately half being grade 2; 68% were estrogen receptor positive, and 19% were HER2 positive. Clinical variables are shown in **Table 1**.

Most of the patients (>95%) were treated with stage appropriate endocrine and chemotherapy as standard of care except those with stage IV disease who died due to disease before completion of therapy. Of ER positive patients 93% (50/54) received endocrine therapy and received stage appropriate chemotherapy as well. Similarly, in ER negative patients, more than 90% of patients received stage appropriate chemotherapy and one had defaulted. Only 15% (3/20) of the HER2 positive patients received trastuzumab while 95% of them received anthracycline and taxane based regimens as intensive chemotherapy.

Among the 270 patients, 88 women were less than or equal to 50 years at first diagnosis, and the median age of this subset was 43.1 years. In this subset, 60% of the tumors were lymph node positive, and nearly half of the tumors belonged to stage II. Ninety-five percent of the tumors were equally distributed between grades II and III. Sixty percent of the tumors were ER positive. Eighty percent of these patients were pre-menopausal (70/88), and the remainder had been diagnosed with breast cancer on average within 3 years of menopause. No significant

TABLE 1 | Clinicopathological features of all patients and patients ≤50 years in our cohort.

Clinicopathological characteristics		All patients (N = 270) N (%)	Patients≤50 years (N = 88) N (%)
Age	Median	56	43
T size	Median	3	3
	T1	72 (27)	22 (25)
	T2	160 (59)	50 (57)
	T3	29 (11)	12 (14)
	Unknown	9 (3)	4 (4)
Lymph Node	Positive	157 (59)	53 (60)
	Negative	104 (38)	34 (39)
	Unknown	9 (3)	1(1)
Stage	I	41 (15)	15 (17)
	II	133 (49)	43 (49)
	III	86 (32)	23 (26)
	IV	10 (4)	7 (8)
Grade	I	19 (7)	5 (5)
	II	131 (48)	42 (48)
	III	117 (43)	40 (46)
	Not available	3 (1)	1 (1)
Menopausal status	Pre	75 (28)	70 (80)
	Post	195 (72)	18 (20)
Estrogen Receptor	Positive	186 (68)	54 (61)
	Negative	89 (32)	34 (39)
Progesterone Receptor	Positive	172 (63)	55 (63)
	Negative	103 (37)	33 (37)
HER2	Positive	53 (19)	20 (23)
	Negative	192 (70)	58 (66)
	Equivocal	30 (11)	10 (11)

difference in any of the clinical characteristics was observed when this subgroup was compared to the entire cohort (**Table 1**).

Expression of AR Protein, Transcript, and Concordance With ER Protein

Immunohistochemistry for AR could be successfully evaluated in 189 of the 275 tumors. Eighty six tumors were not evaluated either due to insufficient tissue or tumor content. An additional 12 tumors were excluded due to poor tissue preservation, and hence the final evaluation included only 177 tumors from 173 women. There were 59/173 women (34%) less than 50 years, and 114/173 women (66%) were >50 years of age.

Overall, 66/177 (37%) tumors had nuclear staining for AR. There was no difference in the distribution of AR protein by age groups (34% (20/59) in ≤50 and 39% (46/118) in >50 years age group). Of the 177 tumors, 120 were ER positive by IHC; 53/120 of these ER positive tumors were AR positive as well, and this proportion did not differ between the age groups [42% (16/38) *vs* 45% (37/82) in ≤50 years *vs* >50 years respectively]. Overall, only 30% (53/177) of the tumors were dual positive for both ER and AR. Of AR positive tumors, 80% were ER positive in all samples, and similar results were seen in both age groups.

Tumors which were positive for AR protein expression had significantly high levels of AR transcripts than AR negative ($p = 0.003$). Tumors in the >50 years group had higher levels of AR transcripts when compared to ≤50 age group ($p = 0.021$). ROC analysis showed only moderate concordance (AUC of 0.63, $p = 0.07$) between the transcript and the protein across all tumors. No difference in this concordance was observed when stratified by age groups.

AR/ER Ratio by Transcript Levels

The *AR* and *ESR1* transcript levels were evaluated by real time PCR on all the 275 breast tumor samples. A significant positive correlation was observed between the *AR* and *ESR1* transcript levels (Pearson's $r = 0.43$, $p < 0.0001$). Relative normalized units of *AR* and *ESR1* transcripts were used to calculate the AR/ER transcript ratio which ranged from 0.65 to 5.53. In the tumors of women ≤50 years of age, the ratio ranged from 0.65 to 3.53 with a median value of 1.46 and third quartile value of 1.75. In tumors from women over 50 years of age, the ratio ranged from 0.74 to 5.53 and had a median of 1.31 and third quartile of 1.57. Though the level of AR transcript was higher in >50 years group, the AR/ER ratio was significantly higher in tumors ≤50 years than in tumors >50 years ($p = 0.005$).

We further divided the tumors from women ≤50 years of age into high and low ratio groups based on the third quartile cut-off of 1.75, and 22/88 had high AR/ER ratio in this subset. Comparison of clinical characters between the high and low ratio groups showed higher preponderance of ER negative tumors in the high ratio group (68%, $p = 0.001$), and no significant differences were observed in other features like stage, grade, lymph node status, and tumor size as shown in **Table 2**.

TABLE 2 | Comparison of clinical variables between high and low AR/ER ratio groups in our cohort in the patients ≤50 years.

Clinicopathological characteristics		High AR/ER ratio (N = 22) N (%)	Low AR/ER ratio (N = 66) N (%)	p-value
Age	Median	43	43	
T size	Median	3.25	3	0.92
	T1	6 (27)	16 (24)	0.57
	T2	10 (46)	40 (61)	
	T3	4 (18)	8 (12)	
	Unknown	2 (9)	2 (3)	
Lymph Node	Positive	14(64)	39 (60)	
	Negative	8 (36)	26 (40)	0.715
Stage	I	5 (22)	10 (15)	0.807
	II	9 (40)	34 (51)	
	III	6 (27)	17 (25)	
	IV	2 (9)	5 (7)	
Grade	I	0	5 (7)	0.18
	II	9 (40)	33 (51)	
	III	13 (60)	27 (41)	
	Not available		1 (1)	
Estrogen Receptor	Positive	7 (32)	47 (71)	0.001*
	Negative	15 (68)	19 (29)	
Progesterone Receptor	Positive	7 (32)	48 (73)	0.001*
	Negative	15 (68)	18 (27)	
HER2	Positive	6 (27)	14 (21)	0.421
	Negative	15 (68)	43 (65)	
	Equivocal	1 (4)	9 (14)	

**p-value <0.05, statistically significant.*

Patients With High AR/ER Ratio Had Poor Survival in ≤50 Years Age Group

We first examined the prognostic ability of ER and AR independently both at protein and transcript levels in women ≤50 years by Kaplan–Meier survival analysis. No significant difference in survival was seen for ER protein (ER positive *vs* negative, mean survival 76.63 *vs* 76.98 months, log rank test p = 0.65) and ER transcript at mean cut-off (high *vs* low, mean survival time 81.76 *vs* 74.68 months, log rank test p = 0.75). Similarly, no difference in survival was seen with AR protein (AR positive *vs* negative, 75 *vs* 81.98 months, log rank test p = 0.18) or its transcript levels at mean cut-off (AR high *vs* low, 78.4 *vs* 76.7 months, log rank test p = 0.55).

Next, we examined the clinical significance of a higher AR/ER ratio in the age group of patients ≤50 years group by Kaplan–Meier survival analysis. As seen in the **Figures 1A, B**, both DFS and BCCS were significantly lesser in the high ratio group in comparison to low ratio group (mean survival time 64.9 *vs* 83.4 months, log rank test p = 0.01 for DFS and 56.99 *vs* 89.65 months, log rank test p = 0.003 for BCSS). We did not observe this difference in the survival in the >50 years of age group, though trends were indicative of better survival for low ratio group (mean survival time 66.9 *vs* 81.13 months, log rank test p = 0.1 for DFS).

Further, based on the ER protein expression by IHC, we divided the tumors into ER positive and negative and evaluated the prognostic significance of the AR/ER ratio independently within each category. Fifty-four (61%) of the 88 tumors were ER positive and women with tumors with higher ratio had significantly poorer survival when compared to the low ratio (mean survival time 41.8 *vs* 82.7 months, log rank test p = 0.007, **Figure 2A**). As observed in all patients ≤50 years age group, no difference in survival was seen with either ER transcript or AR transcript levels alone within the

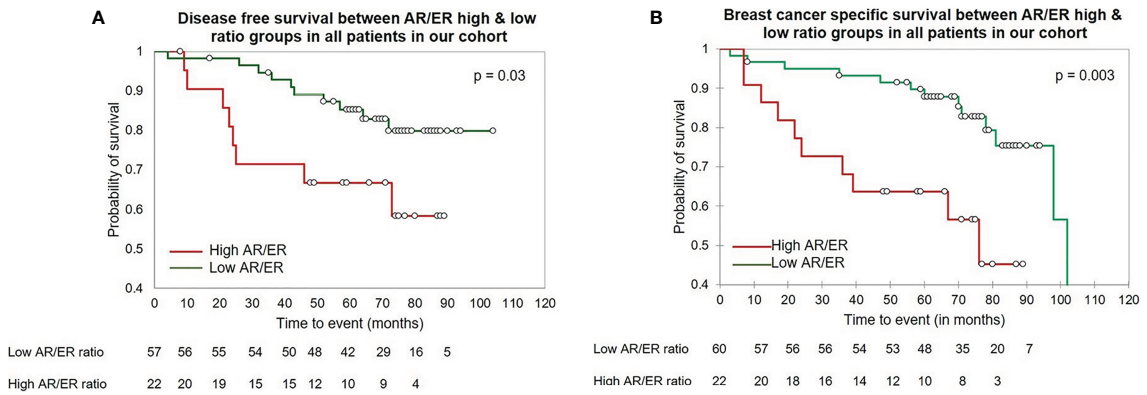

FIGURE 1 | The Kaplan–Meier survival analysis in our cohort in the patients ≤50 years of age **(A)** The disease-free survival between the high *vs* low AR/ER ratio groups. **(B)** The breast cancer specific survival between the high *vs* low AR/ER ratio groups.

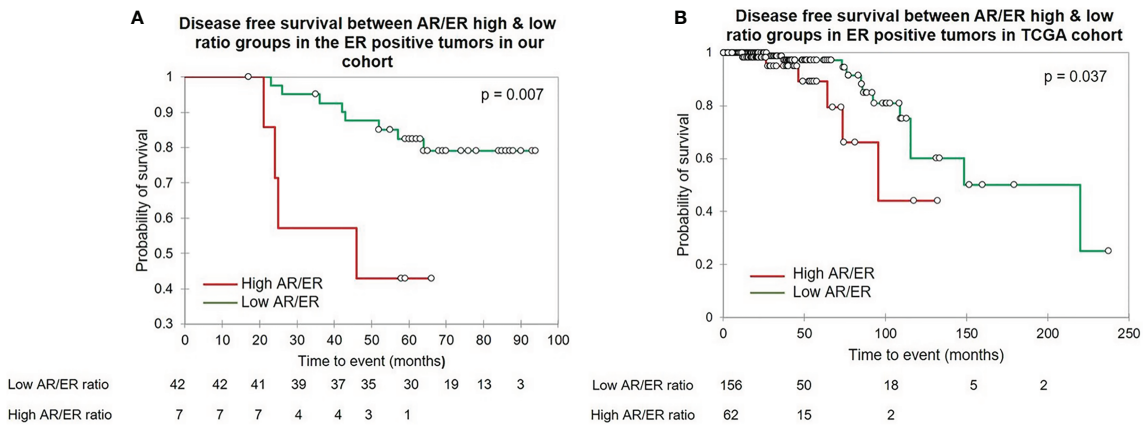

FIGURE 2 | The Kaplan–Meier survival analysis in the ER positive patients ≤50 years of age. **(A)** The disease free survival between the high *vs* low AR/ER ratio groups in our cohort. **(B)** The disease free survival between the high *vs* low AR/ER ratio groups in the TCGA cohort.

ER positive tumors. A similar analysis within the ER negative (by IHC) category did not show any difference in the survival between the AR/ER high and low ratio groups (mean survival time 68 *vs* 72.65 months, log rank test p = 0.68)

To investigate the prognostic significance of the AR/ER ratio in patients ≤50 years of age group, Cox proportional hazard analysis was performed with other known prognostic variables like tumor size, grade, and lymph node status. Univariate analysis showed (**Table 3**) prognostic significance of the high ratio with a hazard ratio of 2.6 (95% CI-1.0–6.5, p = 0.04) and 2.1 in multivariate analysis though not statistically significant (95% CI-0.8–5.8, p = 0.15). Similar analysis within ER positive tumors alone reconfirmed the prognostic relevance of the high AR/ER ratio with a significant hazard ratio of 4.6 (95% CI-1.35–15.37, p = 0.01) in univariate and 3.78 (95% CI-0.87–16.43, p=0.07) in multivariate analyses.

External Validation in TCGA and METABRIC

To check if the results were recapitulated in other cohorts, we accessed the TCGA dataset (https://www.cancer.gov/tcga). This dataset had a total number of 1,082 breast cancer patients, of which 322 patients were ≤50 years. The AR/ER ratio of the transcripts of *AR and ESR1* was calculated and ranged from 0.02 to 4.07. A third quartile cut-off of the ratio at 0.88 was used to divide the tumors into high and low ratio groups. Comparison of clinical characters between the high and low ratio groups showed significantly different distribution of the ER status (p = 0.03) between the two groups, and no significant differences were observed in other features as shown in **Supplementary Table 2**. Kaplan–Meir survival analysis performed in the patients ≤50 years showed that the patients with high AR/ER ratio had a significantly poorer disease free survival than the low ratio tumors (mean survival time 74.8 *vs* 157.3 months, log rank test p = 0.003) similar to what we had seen in our cohort (**Supplementary Figure 1**). The Cox proportional hazard analysis showed prognostic significance of the high AR/ER ratio with a hazard ratio of 2.8 (95% CI-1.4–5.8, p = 0.005) in the univariate analysis and a significant hazard ratio of 3.2 (95% CI-1.4–7.3, p = 0.006) in the multivariate analysis (**Supplementary Table 3**).

Further, we performed similar analysis within ER positive tumors within TCGA; 218/322 were ER positive by IHC. Kaplan–Meir survival analysis in this subgroup showed patients with high AR/ER ratio had a significant poorer disease-free survival than the low ratio tumors (mean survival time 83 *vs* 163 months, log rank test p = 0.037), similar to our results (**Figure 2B**). Cox proportional hazard analysis showed prognostic significance of the high AR/ER ratio with a hazard ratio of 2.9 (95% CI-1.0–8.0, p = 0.046) in the univariate analysis and hazard ratio of 5.96 (95% CI-1.7–20.2, p = 0.004) in the multivariate analysis.

We also attempted to validate our results in the METABRIC dataset (22) (details in the **Supplementary Data**). As seen in our cohort, tumors with high ratio of AR/ER showed poorer survival than the low ratio tumors in both DFS (mean survival time 107.2 *vs* 142 months, log rank test p = 0.001) and BCCS (mean survival time 101.5 *vs* 137.5 months, log rank test p = 0.001) in the ≤50 years age group (**Supplementary Figures 2A, B**). Comparison between clinical variables between high and low ratio groups is shown in **Supplementary Table 4**. Cox proportional hazard analysis showed prognostic significance of the high AR/ER ratio with a hazard ratio of 1.8 (95% CI-1.25–2.52, p = 0.001) in the univariate analysis and hazard ratio of 1.2 (95% CI-0.82–1.78, p=0.33) in the multivariate analysis, though not statistically significant.

Circulating Levels of Total Testosterone Are Higher in Patients ≤50 Years With High AR/ER Ratio

To examine the association between circulating testosterone and AR expression in breast tumors, we estimated the total serum testosterone levels in patients at first diagnosis by chemiluminescence method. Among the total 270 patients, adequate serum was available in 169 patients (63 women were ≤50 years and 106 women were >50 years) for estimation of total testosterone level. The testosterone level ranged from 0.13 to 2.43 ng/ml with the mean value of 0.25 ng/m. No significant difference was observed in the circulating total testosterone levels between women with AR positive *versus* negative tumors (p = 0.847) and between patients ≤50 years and >50 years (p = 0.42). Women ≤50 years, with tumors having high AR/ER ratio tumors had significant high levels of testosterone compared to women with tumors with a low AR/ER ratio (p = 0.02). In contrast, high levels of circulating testosterone were observed in the patients >50 years with tumors having a low AR/ER ratio (p = 0.002) as shown in **Figures 3A, B**.

TABLE 3 | Cox proportional hazard models of AR/ER ratio groups with other clinical variables in the patients ≤50 years in our cohort.

	Reference	Variable	Univariate (95% CI)				Multivariate (95% CI)			
			HR	Low	High	P-value	HR	Low	High	P-value
T-size	≤2 cm	>2 cm	0.7	0.2	1.7	0.38	0.7	0.2	2.1	0.51
LN status	Negative	Positive	1.6	0.6	4.1	0.38	1.6	0.5	4.8	0.43
Grade	Gr I & II	Gr III	1.6	0.58	4.08	0.37	0.6	0.06	5.5	0.65
Ratio groups	Low	High	2.6	1.0	6.5	0.04*	2.1	0.8	5.8	0.15
Treatment	HT	CT	0.61	0.12	3.16	0.56	0.18	0.03	1.06	0.06
		CT+HT	0.79	0.18	3.59	0.76	0.32	0.06	1.68	0.18

LN, lymphnode; HR, hazard ratio; HT, hormonal therapy; CT, chemotherapy.
**p-value <0.05, statistically significant.*

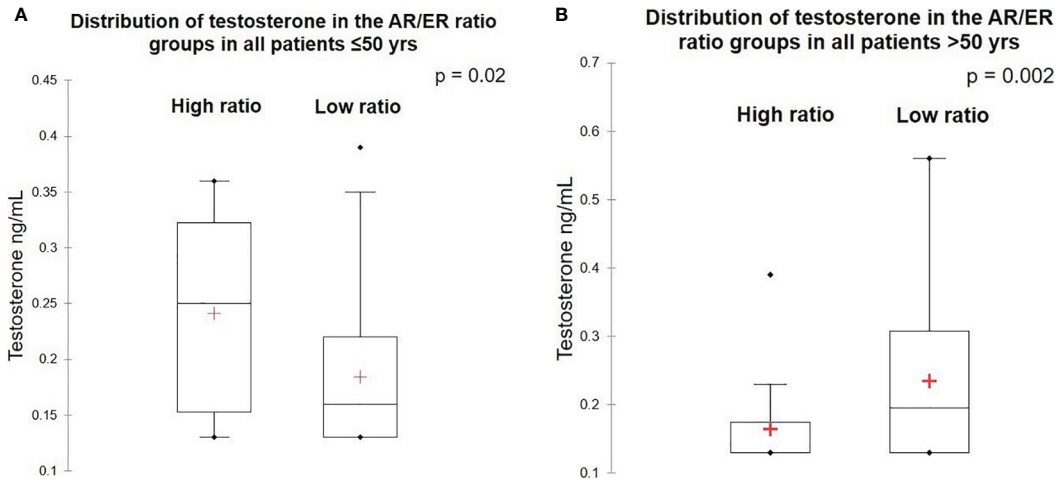

FIGURE 3 | Levels of testosterone in the high and low AR/ER ratio groups. **(A)** Distribution of testosterone in the patients ≤50 years. **(B)** Distribution of testosterone in the patients >50 years.

DISCUSSION

The prognostic value of AR expression in breast cancer has been evaluated in multiple breast cancer cohorts (14, 23–26). Steroid hormone nuclear receptors like estrogen and androgen receptors often crosstalk and influence the action of each other (13, 27). Evidence from *in vitro* studies suggests that AR competes to the same binding sites as ER leading to complex molecular mechanisms of their interaction (12). Despite the favorable prognostic role of AR in ER positive breast cancer, clinical studies have shown a subset of ER+AR+ tumors with a relative higher expression of AR compared to ER, often develops endocrine resistance when treated with tamoxifen (28).

Transcript levels of AR have been earlier used for its relevance as biomarker in clinical trial settings (29, 30). Higher levels of AR expression are seen in breast cancers of older women. Overall, we observed only 37% of all the tumors were positive for AR expression by IHC in the entire cohort. This proportion was not different in ≤50 years (34%). Only a minor proportion of ER positive tumors was AR positive (44%). Other studies from India have also reported similar proportions of AR positive tumors in their cohorts (31, 32). We have used the AR antibody clone AR441 and nuclear staining in >1% of the cells as positive similar to the guidelines for reporting on other nuclear receptors like ER and PR by immunohistochemical assay (33, 34). Higher proportions of AR positivity in other studies might be due to use of different antibody clones. Methodological differences observed in the published reports with use of more sensitive antibodies against AR and subjective interpretation of the AR expression by IHC with varying levels of cut-off, prompted us to use gene expression data for calculation of AR/ER ratio (35). Though moderate concordance was observed between the protein and mRNA of AR, estimation of transcript levels by q-PCR is more quantitative and permitted the estimation of the ratio in tumors which had ER expression below the threshold of detection for protein. In addition, we could also validate our results in a public dataset like TCGA and METABRIC which has limited data on protein expression.

Though ER positive breast cancers with AR expression tend to be well differentiated, Cochrane et al. reported about the prognostic significance of high AR/ER ratio with lower DFS and fourfold higher risk of failure during adjuvant tamoxifen treatment in ER positive breast cancers. Similarly, another study by Rangel et al. has shown that high AR/ER ratio is associated with aggressive features and is an independent indicator of worse prognosis in hormone receptor positive HER2 negative disease (15). Molecular subtyping of the tumors with high ratio in their study showed close to half of the tumors were intrinsically non-luminal though all were ER positive, and more than 60% of these tumors had either intermediate or high risk of recurrence by the PAM50/Prosigna assays. More recently, they further evaluated the gene expression of proliferation genes and showed tumors with high ratio were either luminal B or HER2 enriched with higher rate of proliferation and poor prognosis. Studies in both groups were limited to luminal tumors alone with median age group more than 60 years (18). Another study by Pizon et al. evaluated AR and ER in the circulating epithelial tumor cells (CETCs) in 66 BC tumors and found higher AR/ER ratio in patients with positive lymphnode and tamoxifen resistance (16). Our results are concordant with the findings from these studies in pre-menopausal women as well. Due to uncertainty in establishing menopausal status from medical records, we chose age 50 as a proxy for menopause, and pre-menopausal patients were defined as women younger than 50 years (as per the international average of natural menopause at 50 years, WHO).

Pre-menopausal women who have tumors with high AR/ER ratio had significantly high levels of circulating testosterone.

Testosterone levels are more constant through the menstrual cycle, unlike estrogen and progesterone levels which are cyclical. Though multiple studies have shown the correlation of circulating testosterone with risk of developing breast cancer in post-menopausal women, relatively few studies have established the risk in pre-menopausal women (36). Previous studies in post-menopausal breast cancer women have shown significantly high levels of circulating testosterone than the normal controls (37) and further showed the association of high testosterone levels with worse prognosis in ER positive post-menopausal women (38). Regulation of AR depends on the hormonal milieu, and it is hypothesized that the discordance between AR and ER based signaling may be regulated by relative availability of each receptor (39). Testosterone is a precursor for estrogens and is converted by aromatase to either estradiol or 5α-dihydrotestosterone (DHT) by the enzyme 5α reductase in the tumor microenvironment (40). Our results of higher levels of total testosterone in tumors with high AR/ER ratio in ≤50 years group of tumors indicate these tumors are likely to be driven by the androgens (41). In contrast, higher levels of testosterone associated with lower levels of AR/ER ratio in >50 years tumor group may indicate their preferential conversion to estradiol leading to more ER driven tumors in the post-menopausal age group (42, 43). These results from predominantly pre-menopausal women with breast cancer suggest that it is not merely the presence or absence of AR expression but the relative activity with ER, as well as the hormonal milieu of the patient that determines clinical outcomes, indicating that both context and interactions ultimately influence tumor behavior.

Our study has several limitations. The major limitation is the small number of women under 50. Though our analysis replicated most of the findings in >50 years age group within our cohort, difference in DFS and BCCS between the high and low ratio groups did not reach statistical significance may be due to lack of long term follow-up (extending to median of 120 months or more) for development of endocrine resistance. We have not confined ourselves to ER positive, HER2 negative breast cancer alone as the prognostic utility of high AR/ER ratio is well established within this subtype. Lack of history on menstrual irregularities and information on BMI were other drawbacks due to which significance of higher levels of circulating steroids could not be evaluated further. Though we were able to replicate significance of AR/ER ratio in other external data sets, evaluation of circulating steroids cannot be validated in external data sets due to the absence of information on circulating steroids at the time of diagnosis and paucity of available cohorts with both serum and tissue for analysis associated with data on long term outcomes. These findings obviously need to be validated in larger cohorts along with standardized methods for detection of AR and its signaling.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethical Committee, St John's Medical College and Hospital, Bangalore and Sri Shankara Cancer Hospital and Research Centre, Bangalore. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JSP: Analysis of data, conception and design of the study, performance of histological examination, and drafting the manuscript. SR: performance of experiments, analysis of data, and drafting the manuscript. AK: conception and design of the study. AA: patient consent and follow-up. AE: performance of IHC and sample collection. RR & SBS: surgical oncologist who enabled patient recruitment and tumor collection. TSS: conception and design of the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.679756/full#supplementary-material

Supplementary Figure 1 | The Kaplan–Meier survival analysis in all patients ≤50 years of age for disease free survival (DFS) between the high and low AR/ER ratio groups in the TCGA cohort.

Supplementary Figure 2 | The Kaplan–Meier survival analysis in METABRIC cohort in all the patients ≤50 years of age. **(A)** The disease-free survival between the high and low AR/ER ratio groups. **(B)** The breast cancer specific survival between the high and low AR/ER ratio groups.

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A FACS-Free Purification Method to Study Estrogen Signaling, Organoid Formation, and Metabolic Reprogramming in Mammary Epithelial Cells

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Few *in vitro* models are used to study mammary epithelial cells (MECs), and most of these do not express the estrogen receptor α (ERα). Primary MECs can be used to overcome this issue, but methods to purify these cells generally require flow cytometry and fluorescence-activated cell sorting (FACS), which require specialized instruments and expertise. Herein, we present in detail a FACS-free protocol for purification and primary culture of mouse MECs. These MECs remain differentiated for up to six days with >85% luminal epithelial cells in two-dimensional culture. When seeded in Matrigel, they form organoids that recapitulate the mammary gland's morphology *in vivo* by developing lumens, contractile cells, and lobular structures. MECs express a functional ERα signaling pathway in both two- and three-dimensional cell culture, as shown at the mRNA and protein levels and by the phenotypic characterization. Extracellular metabolic flux analysis showed that estrogens induce a metabolic switch favoring aerobic glycolysis over mitochondrial respiration in MECs grown in two-dimensions, a phenomenon known as the Warburg effect. We also performed mass spectrometry (MS)-based metabolomics in organoids. Estrogens altered the levels of metabolites from various pathways, including aerobic glycolysis, citric acid cycle, urea cycle, and amino acid metabolism, demonstrating that ERα reprograms cell metabolism in mammary organoids. Overall, we have optimized mouse MEC isolation and purification for two- and three-dimensional cultures. This model represents a valuable tool to study how estrogens modulate

mammary gland biology, and particularly how these hormones reprogram metabolism during lactation and breast carcinogenesis.

Keywords: nuclear receptor, estrogen receptor, steroid, breast cancer, organoids, lactation, metabolomics, breast feeding

INTRODUCTION

The mammary gland is known to be highly sensitive to sex-steroid hormones such as estradiol (E_2), the most potent endogenous estrogen. Indeed, E_2 is critical for development of the mammary gland and its evolution through the estrous and reproduction cycles (1, 2). The two estrogen receptors, ERα and ERβ, are transcription factor members of the nuclear receptor family expressed in most tissues in both males and females, including luminal epithelial cells of the mammary gland (3). Interestingly, sex-steroid hormone receptors are increasingly recognized as modulators of cell metabolism (4), even though it is still unknown how ERs reprogram cell metabolism in the mammary gland.

Following pregnancy, lactation in mammals represents a major energy investment. The mammary gland must sustain milk production and is thus a highly active metabolic tissue (1). Mammary luminal epithelial cells produce and secrete milk, which consists of water, proteins, lipids, and carbohydrates (lactose) that are secreted mainly as secretory vesicles. These epithelial cells uptake nutrients from the blood and metabolize them in a specific manner to produce the milk constituents. All the major milk proteins, such as caseins, are synthesized by mammary epithelial cells (MECs) from amino acids, with some exceptions such as serum albumin and immunoglobulins. Consequently, during lactation, the mammary gland exhibits high amino acid uptake and metabolism linked to protein synthesis (1). Branched-chain amino acids are catabolized extensively in this tissue through various cytoplasmic and mitochondrial metabolic pathways (1). Similarly, blood glucose is converted into lactose, and lipids are synthesized in these cells and secreted by secretory vesicles (5). Thus, a specific metabolic program must be regulated in the mammary gland to sustain its specific functions during lactation.

The different regulatory pathways that govern metabolism are mostly unknown at the cellular level. Historically, ERα was mainly associated with the regulation of biological pathways linked to mammary gland development and has not been considered a major metabolic modulator at the cellular level. This contrasts with the well-known relationship between estrogen exposure and whole-body metabolic homeostasis (6, 7). ERs activation has been shown to modulate metabolism in breast cancer cells, notably by differentially altering mitochondrial properties such as respiration, fusion, and fission (8–12). The sparse data available to date mostly come from breast cancer models and not from normal mammary epithelial cells (6, 7). Because metabolic reprogramming is a hallmark of cancer, data from cancer models are most probably not transposable to normal MEC, further highlighting the need to address this question.

Immortalized cell lines from the mammary gland have little or no ERα expression, including the most widely used models, the MCF10A and hTERT cell lines (13, 14). The use of primary mammary epithelial cells is an attractive alternative and is expected to better recapitulate physiological conditions. Still, most published methods to purify these cells often require flow cytometry and fluorescence-activated cell sorting (FACS), which are time-consuming, require specialized instruments, and necessitate specific expertise (15–17). In the current study, we aimed to optimize a FACS-free protocol to purify mammary epithelial cells for two- and three-dimensional culture studies to study ERα metabolic functions. Herein, this protocol for both purification of MECs and their primary culture is described in depth. In addition, we demonstrate that MECs cultured in two- and three-dimensions retain a functional ERα signaling pathway and that they can be used for state-of-the-art metabolomic studies.

MATERIAL AND METHODS

Primary Mammary Epithelial Cell Purification

Mice were bred, housed, and handled at the animal facility of the Centre de recherche du CHU de Québec – Université Laval. C57BL/6 mice were kept in a 12h light:12h dark cycle at 22°C and all protocols were performed according to the Université Laval Research and Ethic Animal Committee's guidelines and regulations.

All the volumes described below are for the purification of mammary epithelial cells from three female mice with an average age of 20 weeks. After sacrifice, the two thoracic and two inguinal mammary glands were collected and conserved on ice in 1X complete HBSS solution (HBSS + 2% FBS + 10 mM HEPES + 100 U/ml penicillin and 100 µg/ml streptomycin (Wisent)) before being cut in small pieces with scissors under a biological hood. The tissues were then transferred to a tube containing a 1X solution of Gentle Collagenase/Hyaluronidase (StemCell) with 1X complete EpiCult-B mouse medium + 5% FBS (EpiCult basal medium (StemCell) + EpiCult proliferation supplement (StemCell) + 10 ng/ml Recombinant human EGF (StemCell) + 10 ng/ml Recombinant human bFGF (StemCell) + 4 µg/ml heparin (Sigma) + 100 U/ml penicillin and 100 µg/ml streptomycin). After overnight incubation at 37°C without shaking, the solution was centrifuged at 350 g for 7 min. The supernatant was discarded by pipetting and the pellet was washed with 1X HBSS complete solution to remove residual fat. The solution was centrifuged at 350 g for 5 min, and the supernatant was discarded by pipetting. The pellet was resuspended with 1 ml of warm 0.25% trypsin-EDTA (Wisent). After 3 min of gentle

pipetting, 4 ml of 0.25% trypsin-EDTA was added to obtain a total of 5 ml and then kept on ice for 1 h. After adding 10 ml of 1X complete HBSS solution, the tube was inverted 2-3 times to gently mix the suspension and then centrifuged at 350 g for 5 min. The supernatant was removed by pipetting, and 2 ml of warm dispase (5U/ml – StemCell) and 0.1-1 mg/ml de DNase I (Roche) were added. Clumps were dissociated by pipetting for 1-3 min, then 10 ml of cold 1X complete HBSS solution was added. The tube was gently inverted 2-3 times, and the suspension was filtered through a 40 µm Cell Strainer (Falcon). The filtered suspension was centrifuged at 350 g for 5 min, and cells were counted. The purification process was performed using the EasySep Mouse Epithelial Cell Enrichment kit II (StemCell) according to the manufacturer's protocol, after which, the cells were counted and plated for 1 h for differential plating at 37°C. After this incubation, the media, which contains epithelial cells that require more time to adhere, was removed and transferred to a new plate. The new plate contained the purified mouse mammary epithelial cells.

Primary Cell Culture Conditions

After purification and differential plating, cells were plated in complete EpiCult-B mouse medium (StemCell) + 5% FBS in a 37°C incubator with 5% CO_2. After 24 h, the medium was replaced by serum-free complete EpiCult-B mouse medium and changed every three days. For three-dimensional culture, the cell suspension obtained after differential plating was centrifuged at 350 g for 5 min. Cells were resuspended in complete EpiCult-B mouse medium with 5% FBS and 75% growth factor reduced Matrigel (Corning) to obtain a droplet. Each droplet contained 30 000 cells in 40 µl of cell suspension + Matrigel and was seeded into a warm 24-well-plate using cold tips. The plate was then turned upside down and incubated for 15 min at 37°C. Finally, the plate was turned upright and 500 µl of warm complete EpiCult-B mouse medium + 5% FBS was added to each well. After 24 h the medium was replaced by a serum-free medium that was changed every three days.

Cell Labeling and Flow Cytometry Analysis

Before and after purification, cells were resuspended in PBS with 5% FBS and stained using CD24-FITC (1:300, M1/69, BioLegend) and CD49f-PE (1:30, GoH3, BD Biosciences). Antibodies were incubated for 30 min at 4°C and then cells were washed twice in PBS + 5% FBS. Data were acquired using a BD FACSCelesta™ Flow Cytometer and BD FACSDiva™ software version 8.0.1.1 (Becton, Dickinson and Company). Data analyses were performed using FlowJo™ version 10.7.1 (Becton, Dickinson and Company, 2019). Ten thousand events were acquired for each sample. Cells were first gated by their forward and side scatter, representing cell size and granularity. Luminal and basal epithelial cells were distinguished respectively using CD24 and CD49f markers.

Immunofluorescence + Microscopy Analysis

The protocol for immunofluorescence was used as previously described (18). Briefly, cells were cultured in Nunc™ Lab-Tek™ II Chamber slide™ system (ThermoFisher Scientific). After three or six days in culture, the cells were fixed with 4% paraformaldehyde for 15 min. Cells were permeabilized for 5 min with 0.5% Triton-PBS. The primary antibodies for CK8/18 (1:1000, MA5-32118, Invitrogen) or vimentin (1:200, 5741T, Cell Signaling) were incubated overnight in 1% FBS-PBS at 4°C. After washing, secondary antibodies (α-rabbit 488 and 555, 1:2000, A-11008 and A-21428, Invitrogen) were incubated for 1 h at room temperature. Finally, Fluoromount + DAPI (ThermoFisher Scientific) were added. For the immunofluorescence and microscopy analyses, images were taken with the EVOS™ M5000 Imaging System (ThermoFisher Scientific) and analyzed using the ImageJ software. Student's T-test was used to evaluate statistical significance.

RNA Extraction and Quantitative Reverse Transcription PCR

For epithelial cells grown in two-dimensions, medium was changed after 2 days in culture and cells were treated with 10 nM E_2 or with 96% EtOH as a control, as previously performed (19). After 24 h of treatment, they were harvested for RNA purification using the RNeasy purification kit (QIAGEN) following the manufacturer's instructions. For organoids, media was changed after 11 days in culture and cells were treated with or without 10 nM E_2. The next day, they were collected in cold PBS and centrifuged at 350 g for 5 min to remove the Matrigel, and RNA was purified as for cells grown in two-dimensions. After purification, RNA was used to synthesize cDNA with the LunaScript RT SuperMix Kit (New England Biolabs). Quantitative RT-PCR (qRT-PCR) was then performed on cDNA samples with the Luna Universal qPCR Master Mix (New England Biolabs) for specific quantification of genes, with duplicate technical replicates performed for every sample. To obtain relative gene expression, normalization was performed using the expression of three housekeeping genes, *Pum1*, *Tbp*, and *Actn*. The primers used can be found in **Supplementary Table S1**. Results are shown as the average of three independent experiments with at least three biological replicates per condition. Student's T-test was used to evaluate statistical significance.

Western Blot

For protein analyses, buffer K was used to obtain whole-cell lysates (WCLs), as described previously (19). WCLs were analyzed by western blots using primary antibodies: CK8/18 (1:1 000, SU0338, Invitrogen), Vimentin (1:1 000, D21H3, Cell Signaling), ERα (1:1 000, E115, Abcam), Tubulin (1:5 000, 11H10, Cell Signaling Technology), and S6 (1:1 000, C-8, Santa Cruz Biotechnology).

Extracellular Flux Analyses

Purified mammary gland epithelial cells (40 000 per well) were plated in a Seahorse XF96e microplate with 200 µl of complete EpiCult-B mouse medium + 5% FBS. After 24 h, the medium was replaced by a complete EpiCult-B mouse medium and cells were treated with vehicle (EtOH 96%) or 10 nM E_2. After 48 h of treatment, cells were rinsed with Seahorse XF assay medium (RPMI with no phenol red) and a final volume of 175 µl of Seahorse XF assay medium was added to each well. After a 1 h

equilibration at 37°C in a CO_2-free incubator, the XF96e microplate was inserted into the XF96e instrument for measurements of oxygen consumption and extracellular acidification rates, as previously described (20–22). Student's T-test was used to evaluate statistical significance, with $p<0.05$ considered as significant.

Gaz Chromatography – Mass Spectrometry

Organoids were washed in ice-cold saline and harvested on dry ice with dry ice-cold 80% MeOH. The cells were then lysed by sonication and centrifuged at 20 000 *g* for 10 min (23). In parallel, cell culture media was also harvested for analysis and 400 µl of dry ice-cold 80% MeOH was mixed with 200 µl of culture media before centrifugation. After centrifugation of both media and organoids, the supernatant was transferred into a clean tube containing the internal standard Myristic acid-d27 (CDN isotopes, Canada), to which 700 µl ACN was added. Samples were then vortexed, centrifuged, and the supernatant was recovered to be dried using nitrogen gas. Subsequently, a two-step derivatization was performed according to the method described by Fiehn et al. (24) for methoxiamination, and the modified method from Patel et al. (25) for silylation with MTBSTFA/TBDMCS (Sigma Aldrich, MO, USA; TCI America, Cambridge, MA, USA). Samples were then used for gas chromatography - mass spectrometry (GC-MS) analysis using an Agilent 8890 GC equipped with DB5-MS+DG capillary column connected to an Agilent 5977B MS operating under electron impact (EI) ionization at 70 eV (Agilent Technologies, Santa Clara, CA, USA). One µl of sample was injected in split mode at 250°C, using helium as the carrier gas at a flow rate of 1 ml/min. The GC oven temperature was held at 50°C for 2 min, then was raised from 50 to 150°C at a rate of 20°C/min for 5 min, and from 150 to 300°C at a rate of 10°C/min; the column temperature was then kept constant at 300°C for another 10 min. The MS source and quadrupole were held at 230°C and 150°C, respectively, and the detector was operated in scanning for mass range 50-600 Da at a signal rate 5,1 scans/sec. Agilent MassHunter Workstation Software was used for analysis (Agilent Technologies, CA, USA). Metabolites were found by deconvolution and identified according to spectral match in the NIST/EPA/NIH Mass Spectral Library (NIST 2017, Gaithersburg, MD, USA). Standards for TCA cycle intermediates were used in parallel to perform absolute quantification; other metabolites are shown in relative quantification normalized with myristic acid-d27. Student's T-test was used to evaluate statistical significance between conditions.

RESULTS

Isolation and Purification of Mouse Mammary Epithelial Cells

In the mammary gland, basal epithelial cells form an outer layer surrounding an inner layer of luminal epithelial cells that face the lumen where milk is secreted. These luminal cells comprise both ERα-positive (ERα+) and ERα-negative (ERα-) cells that will form lobular-alveolar structures and will be responsible for secreting milk following pregnancy. During lactation, the epithelial cell compartment expands and forms alveoli, leading to milk production (26). The protocol described herein was designed to isolate these epithelial cells by combining an isolation protocol based on enzymatic digestion followed by cell-specific purification using magnetic beads coupled with specific antibodies and differential plating (**Figure 1A**).

After mechanical dissociation, a slow, gentle dissociation with a mix of collagenase and hyaluronidase was used to dissociate the fat pad and the epithelial branching structures. Secondly, cell-cell interactions were inactivated with trypsin and dispase. DNase I was used to eliminate DNA released by cell death. The cells were then filtered to obtain a single-cell suspension.

The single-cell suspension was incubated in a mix of non-epithelial-specific antibodies coupled to magnetic beads, leading to the capture of non-epithelial cells on the magnetic beads. The suspension is thus enriched in epithelial cells, with a four-fold decrease in total cell numbers after this purification step (**Figure 1B**). To confirm that the epithelial cell population was enriched, the single-cell suspension was examined by flow cytometry before and after purification. Antibodies targeting CD24 and CD49f, markers of luminal and basal epithelial cells, respectively, were used. As shown in **Figure 1C**, less than 5% of all cells were strongly positive for either marker before purification. After purification, we obtained a >10-fold enrichment of mammary epithelial cells. The two well-known epithelial cell populations were observed: the mammary colony-forming cells (Ma-CFC) or luminal cells, which are $CD24^{High}$; $CD49^{Low}$, and the mammary repopulating units (MRU) or basal cells which are $CD24^{Low}$; $CD49f^{High}$ (27). Overall, these results showed that our isolation protocol significantly enriches mammary epithelial cells.

Primary Culture of Mouse Mammary Epithelial Cells in Two-Dimensions

After purification of primary mouse mammary epithelial cells, we proceeded to differential plating (**Figure 1A**). Stromal cells like fibroblasts need <30 min to attach while epithelial cells need >60 min. Consequently, after purification cells were plated for an hour, and then the supernatant with the non-attached (epithelial) cells was collected. The supernatant was then transferred to a new plate, where epithelial cells were allowed to attach and grow (**Figure 2A**).

Cells attached within a day and had epithelial characteristics. At low densities, they organized as colonies and proliferated in the plate to form a cellular monolayer. Cells were cuboidal and well-organized, as is commonly found in epithelial tissue (**Figure 2A**). In comparison, the cells eliminated during the purification process, *i.e.*, cells attached to the magnetic beads, had stromal characteristics specific to connective tissue. These cells had a spindle-shaped form and proliferated faster than epithelial cells. In two-dimensions, our primary cell culture protocol allows epithelial cells to retain their epithelial phenotype for up to six days (**Figure 2A**). However, with longer culture time, we

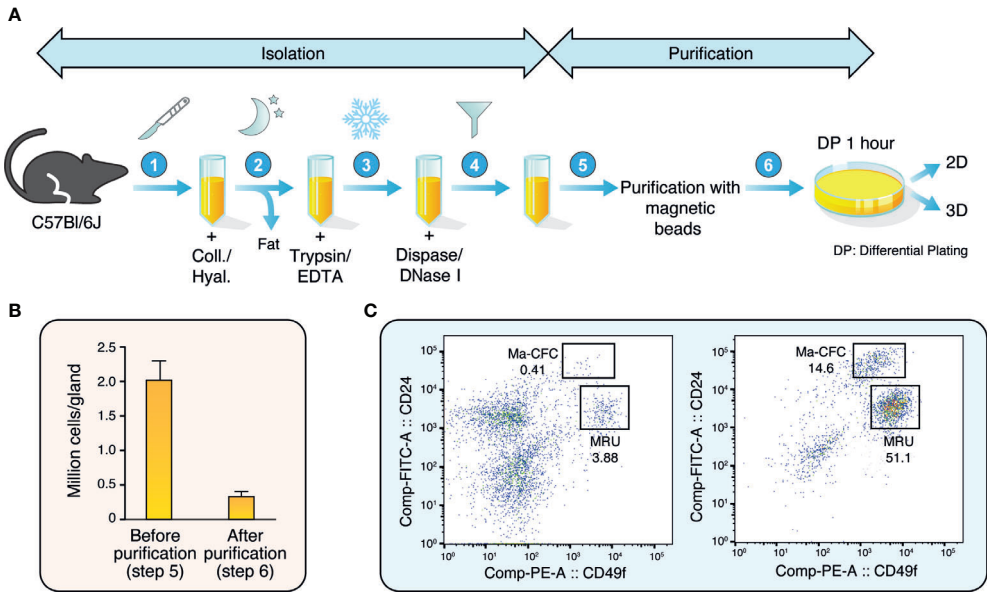

FIGURE 1 | Isolation and purification of mouse mammary epithelial cells. **(A)** Schematic representation of the isolation and purification protocols to obtain mouse mammary epithelial cells. **(B)** Number of mammary epithelial cells obtained before and after isolation and purification (between steps 5 and 6 in A). Results are shown as mean ± SEM of 11 independent experiments. **(C)** Distribution of mammary epithelial cells before (left) and after (right) purification according to their CD24 and CD49f expression. The mammary colony-forming cell (Ma-CFC) fraction was defined as the $CD24^{High}$; $CD49f^{Low}$ and the mammary repopulating units (MRU) fraction as the $CD24^{Low}$; $CD49f^{High}$. Numbers are percentages. One representative experiment out of three independent experiments is shown.

observed a cell transition toward a "fibroblast-like" phenotype, as shown on day 8 in **Figure 2A**.

To confirm that purified cells in culture were epithelial cells, we performed western blots with specific antibodies targeting cytokeratin 8 and 18 (CK8/18), known to be specific to luminal epithelial cells, and vimentin, an intermediate filament found in non-muscle cells including fibroblast and endothelial cells (**Figure 2B**). After three days in culture, purified epithelial cells showed a high signal of CK8/18, indicating a high content of epithelial cells, and barely detectable levels of vimentin. Instead, vimentin was high in the cell fraction eliminated through differential plating (DP), a necessary step to avoid fibroblast contamination. CK8/18 remained high at six days, but vimentin levels increased over time in the purified epithelial cell fraction. This is consistent with results obtained by immunofluorescence analyses. Notably, 80% of the cells were positive for CK8/18 at three days, a ratio maintained up to six days in culture (**Figures 2C** and quantified in **D**). Cells positive for vimentin represented less than 10% of the cells after three days in culture, and slightly increased after six days. Altogether, these results indicate that the mammary epithelial cell purification protocol we performed leads to a significant enrichment of the epithelial cell compartment. However, the epithelial phenotype is lost over time after prolonged two-dimensional cell culture.

Organoid Culture Recapitulates Mammary Gland Organization/Structure

For long-term culture of primary mammary epithelial cells, we then tested our purified cells in three-dimensional cell culture to obtain primary mammary organoids. Briefly, cells purified during step 6 in **Figure 1A** were plated after differential plating in a Matrigel disk to allow cells to grow in three-dimensions. Organoids started to be easily visible after three days in culture and continued growing for several days (**Figures 3A–C**). Once visible, their numbers remained stable over time (**Figure 3D**), and they could be maintained in culture more than one month (data not shown).

These organoids differentiated into two major types. Luminal organoids have bigger lumens and are more spherical and brighter in brightfield visualization, as previously described by others (27). Opaque organoids are considered to have a smaller lumen and thus appear denser and more heterogeneous in shape. Recent studies have shown that organoids with lumen are mainly made of luminal epithelial cells whereas opaque organoids are primarily constituted of basal cells (27). Other structures associated with mammary gland biology started to be visible after a few days in culture. For instance, after six days, contractile cells were found (**Figure 3B**). They could organize themselves in the Matrigel disk (**Figure 3B**, panels 2 and 3) or be attached to an organoid (**Figure 3B**, panel 1). Their contraction started without any stimulation and the movement initiated by one cell seemed to enhance the contractions of nearby cells (see videos in **Supplemental Figure S1**). In the mammary gland, basal epithelial cells could be myoepithelial contractile cells to help with milk expulsion (16, 28). After 10 days of culture, lobular structures appeared (**Figure 3E**). These structures developed from the center of the organoids and expanded over time. Similar structures were found during the branching morphogenesis occurring at puberty and pregnancy (26).

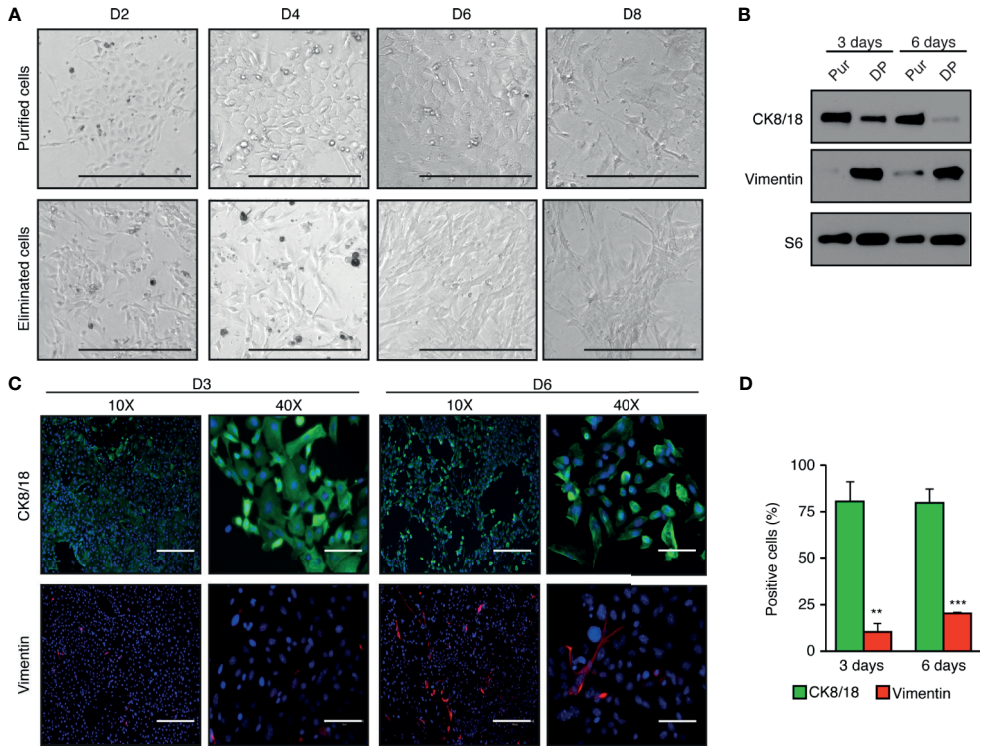

FIGURE 2 | High enrichment of mammary epithelial cell for primary culture in two-dimensions. **(A)** Brightfield images of mammary epithelial cells obtained after purification compared with the cells eliminated during the purification process. Scale bars = 300 µm. **(B)** Western blot analysis of purified cells normally discarded with differential plating (DP) compared to purified epithelial cells (Pur). Protein expression of the cytokeratin 8 and 18 (CK8/18), a marker of epithelial cells, and vimentin, a marker of fibroblasts, after three and six days in two-dimensional culture. S6 was used as the loading control. **(C)** Immunofluorescence showing the expression of CK8/18 (green) and vimentin (red) at three or six days in two-dimensional culture. Nuclei were stained with DAPI (blue). Scale bars = 300 µm and 75 µm. **(D)** Ratios of positive cells for CK8/18 or vimentin per the total number of cells (counts of nuclei) in percentage. Data are shown as mean ± SEM of one representative experiment (n = 6 images per condition). $^{**}p < 0.01$; $^{***}p < 0.001$.

We could also monitor how the different types of organoids changed in culture over time (**Figure 3F**). On day 3, an equivalent number of luminal and opaque organoids could be seen. While luminal organoids tended to be more frequent on day 6. On day 9, when lobular structure appears, the number of luminal organoids decreased, suggesting that luminal organoids are the ones in which lobular structures arise. Opaque organoids were stable between days 9 and 12, showing that they were not affected by the development of lobular structures. These results demonstrate that the current protocol to purify mammary epithelial cells for primary culture recapitulates the structures commonly found in the mammary gland.

Cultured Epithelial Cells in Two- and Three-Dimensions Retain an Active Estrogen Signaling Pathway

Because ERs expression is often lost in immortalized MECs (29), we wanted to validate that the current procedure for primary culture allowed the stable expression of these sex-steroid hormone receptors. We first assessed ERα protein expression levels in primary MECs cultured in two-dimensions (**Figure 4A**). We used MCF7, a human breat cancer cell line that exepresses ERα as positive control; for the negative control, we used MCF10A, a human non-tumorigenic mammary epithelial cell line that has no detectable expression of the receptor. After three and six days in two-dimensional culture, MECs conserved detectable ERα protein expression. Next, we wanted to make sure that the ERα signaling pathway was functional given its established role in the mammary gland (2). In two-dimensional culture, E_2 treatment for 24 h did not significantly modulate *Esr1* or *Esr2* (**Figure 4B**); this is expected, since ERs have not been reported to modulate themselves at the transcriptional level in the mammary gland (30). However, E_2 significantly induced the expression of the progesterone receptor (*Pgr*), a well-known ERα target gene (**Figure 4B**). In parallel, E_2 treatment significantly repressed the expression of *Foxa1*, as ERα does *in vivo* (30). *Krt4*, reported to be induced by E_2 *in vivo* in the mammary gland, was not regulated by this hormone in the current settings. The protein expression of ERα was also investigated in organoids after 12 days in culture. As for MECs, ERα protein was still expressed at significant levels (**Figure 4C**). The estrogenic response of mammary gland organoids was also tested, both with a transient E_2 treatment of 24 h and following chronic activation of ERα (10 days; **Figure 4D**). In both contexts, *Pgr*, *Foxa1*, and *Krt4* were significantly modulated by E_2 treatment as expected, with

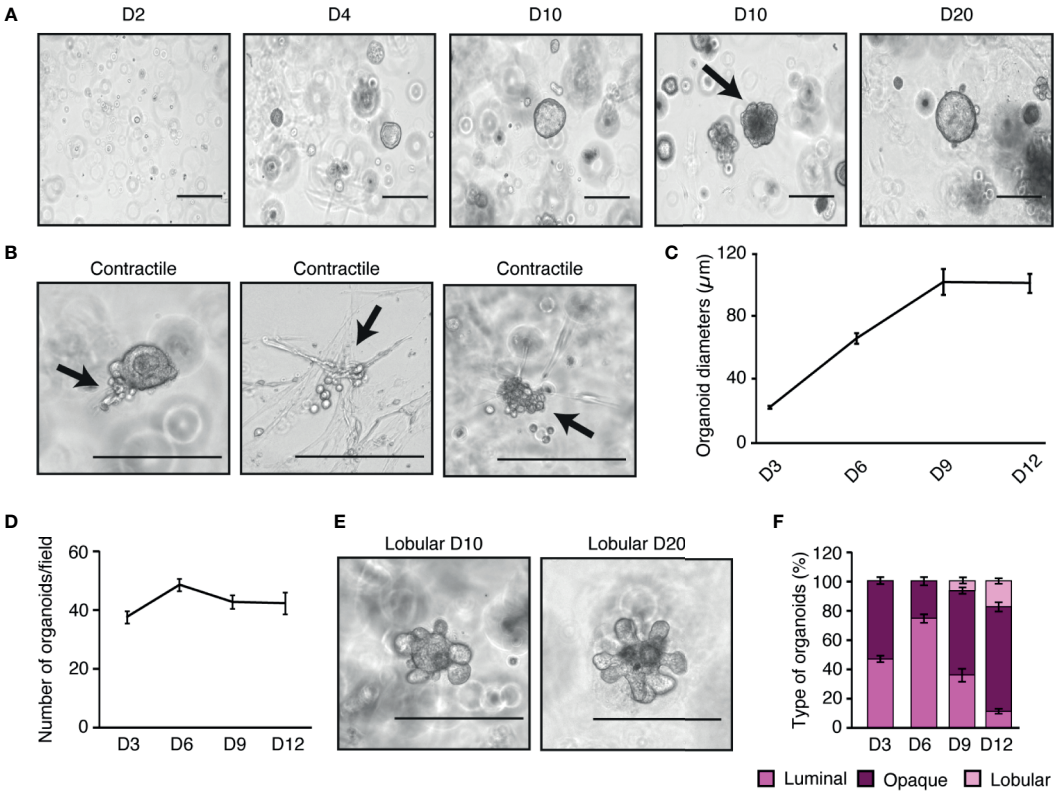

FIGURE 3 | *Ex vivo* mammary epithelial cell organoids recapitulate the *in vivo* architecture of the mammary gland. **(A)** Brightfield visualization of organoids over 20 days in three-dimensional culture. At 10 days, spheroid-like organoids can be observed along with the beginning of lobular structures (black arrow). **(B)** Contractile structures are indicated by black arrows (see videos in **Supplemental Figure S1** to observe contractions). **(C)** Diameter measurements of organoids over time (45 organoids measured/day). **(D)** Number of visible organoids through time in three-dimensional culture. **(E)** Brightfield visualization of lobular organoids after 10 and 20 days in three-dimensional culture. **(F)** Percentage of luminal, opaque, and lobular organoids over time. For **(C, D, F)**, results are shown as mean ± SEM of one out of three independent experiments. Scale bars = 300 μm for **(A, B, E)**.

similar responses between treatments of 24 h or 10 days, indicating that the hormonal response remains functional in primary mammary organoids over time. Finally, we assessed the functional impact of E_2 on the different types of organoids observed in three-dimensional culture over time. As shown in **Figure 3**, we observed similar levels of luminal and opaque organoids after three days in culture, while there was a peak in luminal organoids at day 6 in both the control and E_2 treatment (**Figure 4E**). Interestingly, a shift was observed in the control condition, with a decrease in luminal organoids and the apparition of lobular organoids on days 9 and 12. E_2 significantly impaired this process, favoring the maintenance of luminal organoids as the major type of organoids observed through time and decreasing the formation of lobular organoids. These changes in organoid structures are unlikely related to total organoid number nor diameter alterations, since no significant differences over time was observed with E_2 treatment (**Figure 4F**). These results indicate that primary MECs obtained with our methods express ERα with a functional estrogen signaling pathway, and that E_2 exposure significantly alters organoid formation and structural organization in three-dimensional culture.

Optimization of Culture Conditions for Primary Epithelial Cell Metabolic Analyses

Given that lactation requires high energy levels, we hypothesized that sex-steroid hormones would reprogram mammary gland epithelial cell metabolism to support this process and that MECs in primary culture could be a good model to study this phenomenon. To examine the functional impact of estrogens on cell metabolism, we used an XFe96 extracellular flux analyzer to measure oxygen consumption rates (OCR) and extracellular acidification rates (ECAR). OCR allows the measurement of mitochondrial respiration. ECAR is a readout of lactate production and secretion, which are indicative of aerobic glycolysis, a metabolic pathway often hyperactivated in highly proliferative cells such as cancer cells (also known as the Warburg effect) (4). OCR and ECAR were assessed in mammary epithelial cells purified using the complete protocol we have described (**Figure 1A**). After 24 h in culture, fresh media without serum was added to the cells. After 48 h, to allow steroid deprivation, cells were then treated with vehicle or E_2 for an additional 48 h before metabolic flux analysis. Interestingly, E_2 treatment induces a significant change in the usage of these two

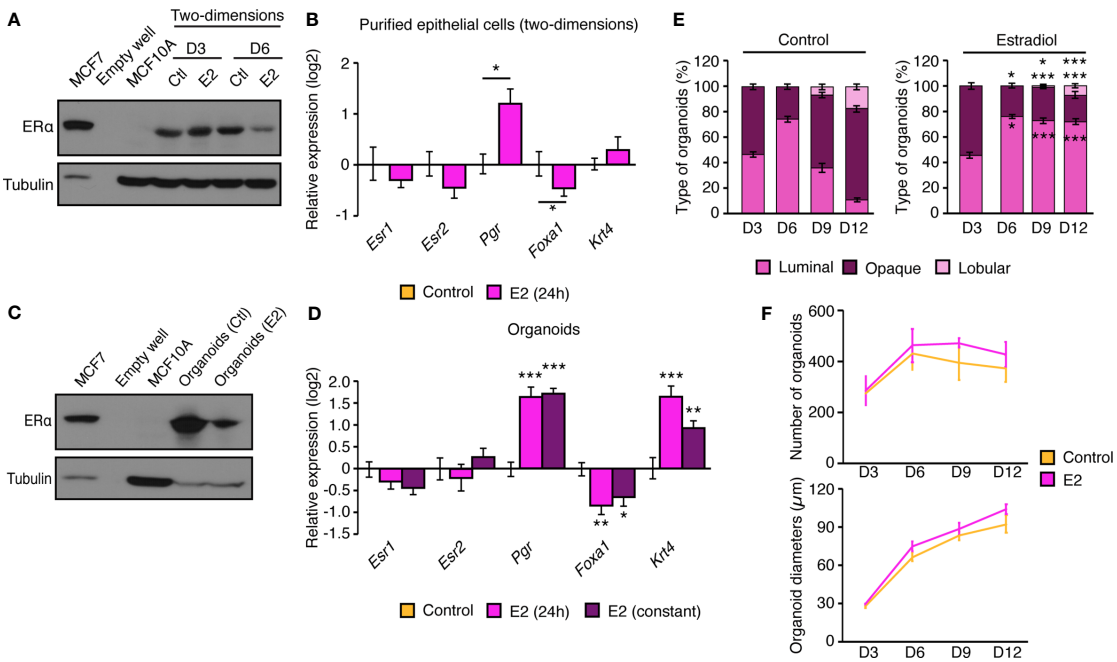

FIGURE 4 | Primary mammary epithelial cells in two- and three-dimensions express ERα. **(A)** Western blot analysis of ERα protein expression in mammary epithelial cells in two-dimensional culture after 3 and 6 days in culture with E_2 or vehicle. MCF7 human breast cancer cells and MCF10A human immortalized mammary epithelial cells were used as positive and negative controls for ERα protein expression, respectively. **(B)** qRT-PCR analysis of genes regulated by estrogens in two-dimensional culture following a 24 h treatment with E_2 or vehicle. **(C)** Western blot analysis of ERα protein expression in mammary organoids after 12 days in culture. MCF7 human breast cancer cells and MCF10A human immortalized mammary epithelial cells were used as positive and negative controls for ERα protein expression, respectively. **(D)** qRT-PCR analysis of genes regulated by estrogens in primary mammary gland organoids following 24 h or 10 d of treatment with E_2 or vehicle. For **(B, D)**, results are shown as mean ± SEM of three independent experiments performed at least in duplicate. **(E)** Percentage of luminal, opaque, and lobular organoids over time, maintained in culture with and without E_2. Results are shown as the mean ± SEM of one out of three independent experiments. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. **(F)** Number of visible organoids treated with E_2 or vehicle. Results are shown as the mean ± SEM of two independent experiments performed in triplicate.

pathways, which represent the two major cellular pathways for ATP synthesis. Indeed, the reliance of mammary epithelial cells on OCR decreased to favor aerobic glycolysis, as monitored by ECAR (**Figure 5A**).

The XFe96 apparatus has four injection ports that allow modulation of the mitochondrial machinery in real-time. In mammary epithelial cells, oligomycin first inhibits ATP synthase and blocks mitochondrial respiration coupled to ATP synthesis (**Figure 5B**). FCCP is then injected to allow maximal respiration of these cells, and finally rotenone and antimycin A allow a complete blockade of mitochondrial respiration, leaving only the non-mitochondrial respiration signal. Interestingly, E_2 treatment decreased basal respiration (before injections), but also decreased the maximal respiration (reserve capacity) of mammary epithelial cells. In parallel, ECAR can be measured to study how cells reprogram their metabolism following inhibition of mitochondrial respiration (**Figure 5C**). Basal ECAR levels (before injections) were higher in E_2-treated cells, and they remained at higher levels even after the different mitochondrial stresses. Overall, our results demonstrate that E_2 reprograms mammary epithelial cell metabolism by promoting the Warburg effect.

MS-Based Metabolomics in Primary Mammary Organoids Reveal a Specific Reprogramming of Metabolism by Estrogens

Organoids have been shown to be more complex structures and to alter cell metabolism compared to traditional monolayer cell culture (31–33). Thus, we hypothesized that our mouse mammary gland organoids could also have a different cell metabolism and a distinct metabolic response to estrogens compared to purified MECs in two-dimensions. To test this hypothesis, organoids were firstly treated with E_2 or vehicle, then both organoids and the extracellular media were harvested for targeted metabolomics using gas chromatography - mass spectrometry (GC-MS). As XFe96 results in two-dimensions showed a Warburg effect with estradiol (**Figure 5**), we expected to observe an increase in lactate levels. Surprisingly, lactate levels in both organoids and media were significantly lower following E_2 treatment (**Figure 6A**). To test the impact of E_2 on the mitochondrial respiration in organoids, several tricarboxylic acid (TCA) cycle intermediates were also measured. We did not detect any changes in citrate levels, the first intermediate of the cycle, but downstream metabolites were all significantly decreased (**Figure 6B**). Intriguingly, one of these metabolites,

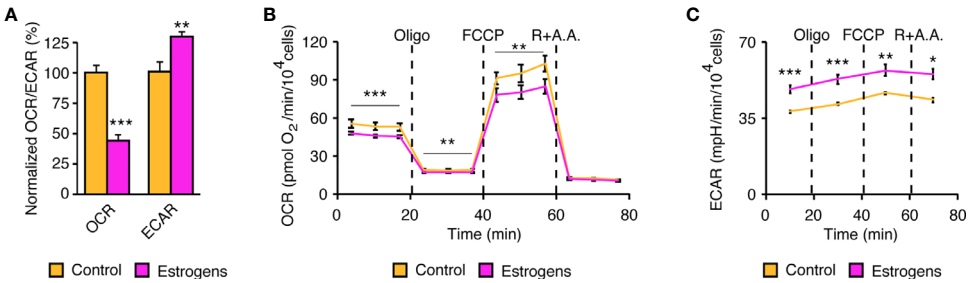

FIGURE 5 | Reprogramming of mammary epithelial cell metabolism in two-dimensions by estrogens. **(A)** Extracellular flux analysis of mammary epithelial cells using an XFe96 Seahorse apparatus. Oxygen consumption rate (OCR), an indicator of mitochondrial respiration, and the extracellular acidification rate (ECAR), an indicator of aerobic glycolysis, are shown normalized to their respective controls. (n= 3 biologically independent samples). Metabolic flexibility of OCR **(B)** and ECAR **(C)** after injections of mitochondrial modulators. Dashed lines indicate when in the assay the different drugs were injected and followed by the metabolic response. Oligo, oligomycin; R, rotenone; AA, antimycin. **(A)** Results are shown as the mean ± SEM of one out of four independent experiments, each with 4-5 biological replicates per group. All experiments were performed in primary mammary epithelial cells in two-dimensions after 3 days in culture and 48 h of treatment with estradiol or vehicle. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

succinate, was detected at high concentrations in the extracellular media (~10 nmol/ml) and was decreased by 3.6-fold following E_2 treatment (**Figure 6C**). Metabolites from other pathways in organoids were also quantified, notably showing a significant decrease in urea and alanine levels following E_2 treatment (**Figure 6D**). This decrease in organoids was paralleled by a significant decrease of urea and alanine levels in the extracellular media by 9- and 2.6-fold, respectively (**Figure 6E**). On the contrary, other metabolite levels in organoids were increased following E_2 treatment, like aspartate and glutamine (**Figure 6D**). Altogether, these results demonstrate that ERα activation reprograms cell metabolism. Important differences were also observed compared to two-dimensional culture, suggesting that how ERα reprograms metabolism is dependent on the three-dimensional cellular organization and cell-cell interactions.

DISCUSSION

In this study, we describe in detail a FACS-free protocol to purify and culture epithelial cells from the mouse mammary gland. This protocol can be used to obtain cells for primary culture in both two-

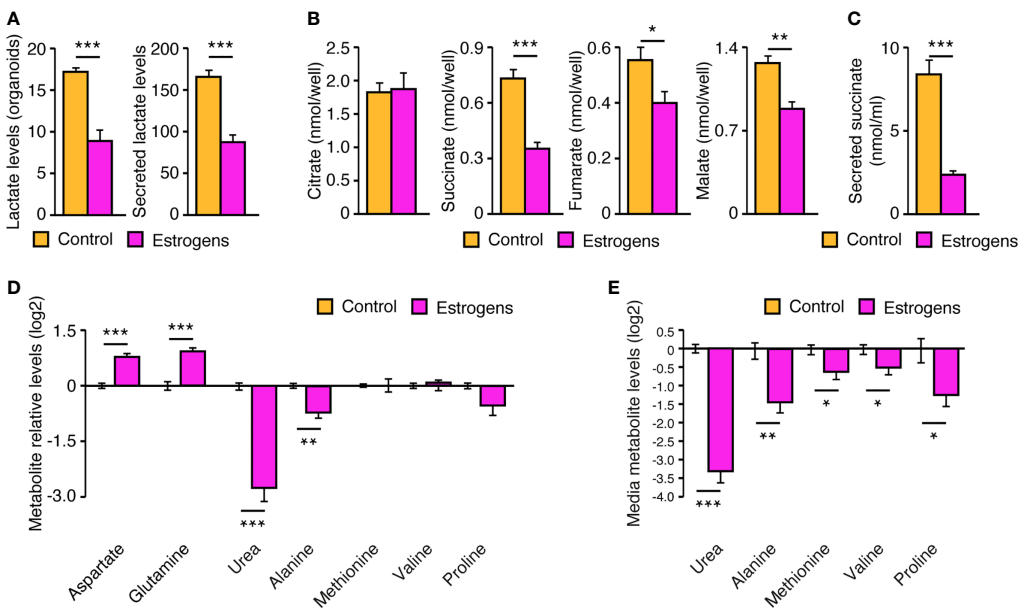

FIGURE 6 | Reprogramming of cell metabolism by estrogens in mouse mammary organoids. After 12 days in culture, with media changed every 72h, organoids and extracellular media were harvested for GC-MS targeted metabolomics to measure lactate **(A)**, TCA cycle intermediates **(B)**, secreted succinate **(C)**, and other metabolite levels in organoids **(D)** or in the extracellular media **(E)**. Organoids were either treated with E_2 or vehicle. All results are shown as the mean ± SEM of two independent experiments performed in triplicate. *$p < 0.1$; **$p < 0.05$; ***$p < 0.001$.

and three-dimensions, in which they can form complex organoid structures that recapitulate the mammary gland structure *in vivo*. Furthermore, we demonstrate that these MECs retain ERα expression, allowing *ex vivo* studies of this critical hormonal signaling pathway in the mammary gland. Finally, as a proof-of-principle, we show that these cells can be used for metabolic studies in primary culture and that ERα activation promotes specific metabolic reprogramming of primary mammary epithelial cells in two- and three-dimensions. To our knowledge, this is the first time ERα is shown to modulate cell metabolism in MECs and mammary organoids.

Mouse MECs are commonly purified using FACS, differential centrifugations, or antibody selection using magnetic beads. For simplicity and maximal purity, the protocol we describe focused on the latter type of protocol. Regarding the cell numbers obtained after purification, our protocol is comparable with FACS methods. We found about half a million MECs per mammary gland, which is similar to what was described in FVB mice by Smalley et al. (34). Our percentage of Ma-CFC and MRU populations is higher than what has been found using other protocols (3.88% MRU in total cells for us compared to an MRU frequency of 1 in 200 sorted cells using a sorting strategy) (34); this difference could be explained by the fact that we only used two markers (CD24 and CD49f). Furthermore, many parameters could influence these numbers, like the age of the mice (around 8 to 10 weeks whereas our average age was 20 weeks) or the strain (34). For example, some stromal cells in C57BL/6 mice, especially in young mice, express CD24, which decreases the ability to resolve the luminal and basal cell populations on a flow dot plot (34). Otherwise, the protocol we describe has several advantages, including no requirement of specialized equipment such as a flow cytometer, the high purity of MECs obtained for primary culture, and the maintained expression of ERα and estrogenic response through time.

ERα is essential for mammary gland development. In fact, its knockout (KO) blocked mammary gland development after puberty, with a lack of epithelial branching and lobuloalveolar development in *Esr1* KO mice (35). Following puberty, sex-steroid hormones promote ductal elongation in mouse mammary gland (36). E_2 is known to stimulate growth and expansion of the ducts into the mammary fat pad as $ER\alpha^+$ cells promote proliferation of surrounding cells by a paracrine mechanism (2). We had assumed that E_2 would increase branching morphogenesis in our organoid system, but we observed the opposite result. In our experiments, other hormones essential for the proper functioning of the mammary gland were missing. For instance, progesterone is known to increase the proliferation of MECs during the diestrus phase of the mouse's cycle whereas our culture condition—with only a peak of estrogen—is more similar to the less-proliferative proestrus phase. Indeed, ovarian hormones in the absence of pituitary hormones have little or no mammogenic activity in rodents (2, 37). Consequently, future studies with more complex hormone combinations are required to fully recapitulate mammary gland organogenesis *ex vivo*.

Despite being a major metabolic investment for females, the metabolic reprogramming of MECs during lactation as well as the different regulatory factors required to sustain cell metabolism for the lactation process remain mostly unknown. Two key signaling pathways, namely the AMP-activated kinase (AMPK) and the mechanistic target of rapamycin (mTOR), have been linked to this metabolic regulation (5, 38). For example, AMPK activation by pharmacological compounds has been shown to decrease lipid synthesis through phosphorylation of acetyl-CoA carboxylase (ACC) and to decrease processing of the SREBP1 lipogenic transcription factor (37, 39). Not much is known about how estrogens and their receptors participate in this reprogramming of cell metabolism in the mammary gland. Recent research by the Maggi group has clearly established that ERα is a key determinant that promotes lipid synthesis using amino acids as a source of fuel in hepatocytes in females, which distinguish liver metabolism between males and females (40). In the liver, this leads to an energy partition strategy that is thought to be the result of selective pressure to tailor reproductive functions to the nutritional status (40). In prostate cancer, androgens—through activation of their receptor AR—have also been shown to be major orchestrators of specific metabolic pathways, such as mitochondrial respiration, lipid synthesis and usage, and glycolysis modulation (4, 20, 21, 41–43). Thus, sex-steroid hormones appear to be key modulators of cell metabolism and to have specific functions in distinct peripheral tissues.

We showed that the estrogen signaling pathway promotes aerobic glycolysis over mitochondrial respiration in primary MECs grown in two-dimensions, a phenomenon known as the Warburg effect. Surprisingly, we observed a different modulation of metabolism when MECs were grown in organoids, with a negative regulation of lactate production and secretion by E_2 in three-dimensional culture. Contrary to MECs grown in two-dimensions, organoids are composed of several cell types, including epithelial luminal and basal cells, and thus represent a more complex environment. E_2 does not alter the number of organoids and their diameters compared to the vehicle (**Figure 4F**), but it does alter the types of organoids (**Figure 4E**). Consequently, it most probably alters the relative fraction of the different cell types composing these organoids, as well as the cell-cell interactions occuring in these organoids. In addition, the three-dimensional structure probably promotes nutrients and oxygen-gradients that will also modulate cell metabolism, as this was shown to be the case in other cellular contexts (31). In organoid culture, E_2 treatment also induced significant alterations of metabolite levels from several metabolic pathways, including the TCA cycle, urea cycle, and amino acid metabolism. Succinate, fumarate, and malate of the TCA were all significantly decreased by E_2 treatment, but not citrate levels, possibly suggesting a global decrease in mitochondrial respiration. The important changes of urea intra-cellular and secreted levels also suggest an important modulation of the urea cycle in MECs. Given that the results shown herein are from a combination of different cell types interacting together, further mechanistic studies are required to fully understand how ERα reprograms cell metabolism, as it is highly probable that it reprograms both luminal and basal cell metabolism in a direct and indirect (paracrine) manner, respectively. In any case, it is

clear that ERα is an important regulator of cellular metabolism in mammary organoids.

In conclusion, purified MECs can be used both for two- and three-dimensional *ex vivo* culture analyses, and they recapitulate different mammary gland structures when cultured to form organoids. These purified MECs are also compatible with sex-steroid hormone signaling studies and their impact on normal mammary epithelial cell metabolism. This study could provide a simple and evolutive tool to better understand the relation between hormones and metabolism in the mammary gland. Notably, our results demonstrate that the estrogen signaling pathway is a powerfull modulator of cell metabolism, but future studies are required to fully decipher the metabolic functions of ERα in mammary glands.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Université Laval Research and Ethic Animal Committee.

AUTHOR CONTRIBUTIONS

Conception and design of the experiments: AL and EA-W. Collection, assembly, analysis and interpretation of data: AL, CJ, CW, LB, DB, IL, MP, and EA-W. Drafting the article: AL and EA-W. Revising the manuscript for critically important intellectual content: AL, CJ, CW, LB, DB, IL, MP, and EA-W. Study supervision: EA-W. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.672466/full#supplementary-material

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Association Between Estrogen Receptors and GATA3 in Bladder Cancer: A Systematic Review and Meta-Analysis of Their Clinicopathological Significance

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Background: Estrogen receptors alpha (ERα) and beta (ERβ) and the cooperating protein GATA-binding factor 3 (GATA3) have been implicated in bladder carcinogenesis and tumour progression. GATA3 and ER have been functionally linked in the establishment of luminal fate in breast tissue, but to date their relationship in bladder cancer has not been established. This information will be useful to advance diagnostic and prognostic markers.

Aim: To determine the relationship between the expression of ERα, ERβ and GATA3 in bladder cancer, disclose their prognostic and diagnostic value and their association with clinicopathological characteristics.

Methods: A comprehensive literature search in PubMed database was performed for all immunohistochemical studies of ERα, ERβ and/or GATA3 in bladder cancer patients. We selected eligible studies in accordance with the PRISMA guidelines and evaluated methodological quality and risk of bias based on quality criteria from the reporting recommendations for tumour MARKer (REMARK) prognostic studies. Risk of bias assessment was performed using Review Manager 5. R software was used for all statistical analysis, the packages used were meta and dmetar for the standard meta-analysis, and netmeta for the network meta-analysis.

Results: Thirteen studies were eligible for ERα, 5 for ERβ and 58 for GATA3 meta-analysis. Low grade tumours showed significantly lower ERα expression. GATA3 was widely expressed in bladder tumours, especially urothelial carcinomas, with higher expression of GATA3 in low grade and low stage tumours. Data was insufficient to determine the prognostic value of either ERα or ERβ, but GATA3-positivity was associated with higher recurrence free survival. A negative correlation between ERα or

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ERβ positivity and GATA3 expression was disclosed. Additionally, several sources of heterogeneity were identified, which can be used to improve future studies.

Conclusion: The clinicopathological value of ERα and ERβ was inconclusive due to low availability of studies using validated antibodies. Still, this meta-analysis supports GATA3 as good prognostic marker. On the contrary, ERα-positivity was associated to higher grade tumours; while ERα and ERβ were inversely correlated with GATA3 expression. Considering that it has previously been shown that bladder cancer cell lines have functional ERs, this suggests that ERα could be activated in less differentiated cells and independently of GATA3. Therefore, a comprehensive analysis of ERα and ERβ expression in BlaCa supported by complete patient clinical history is required for the identification of BlaCa subtypes and subgroups of patients expressing ERα, to investigate if they could benefit from treatment with hormonal therapy.

Systematic Review Registration: Prospero, CRD42021226836.

Keywords: bladder cancer, estrogen receptors, GATA3, tumour markers, immunohistochemistry

INTRODUCTION

Bladder cancer (BlaCa) arises and progresses along two distinct pathways with distinct behaviour and molecular profile (1–3). Low grade, non-muscle invasive cancers (NMIBC) account for 75% of the cases at diagnosis and are characterized by good prognosis. However, patients frequently develop local recurrences requiring lifelong cystoscopy surveillance, and around 25% of the cases will ultimately progress to invasive disease (4). In contrast, muscle invasive tumour (MIBC) progress rapidly and have a high propensity for metastasis with 5-year survival rate less than 15%, even after radical cystectomy and systemic treatment (5–7). Cisplatin based chemotherapy has been the standard of care for MIBC for the past three decades. Recently, immune check point inhibitors and erdafitinib, an FGFR antagonist, have been approved and show therapeutic benefit for a small group of patients (8, 9). Still, the relative lack of molecular biomarkers and targeted therapies for BlaCa diagnosis and treatment (10, 11), renders the pathological assessment currently used insufficient to predict disease progression and response to therapy (12).

BlaCa risk is mainly associated with cigarette smoking and gender (13). It is 3 to 4 times more frequent in men than in women, with the excess risk in males remaining even after adjustment for known risk factors (14). Gene expression studies identified intrinsic basal and luminal subtypes of BlaCa that closely resemble corresponding subtypes of breast cancer (BC) (15–17). Luminal BlaCa is characterized by high expression of PPARγ and active estrogen receptor (ER) signalling pathway including expression of FOXA1, GATA3 and TRIM-24 (17). GATA3 is a marker of luminal cell differentiation in the breast and bladder (18) and together with FOXA1 are important mediators of PPARγ signalling to drive luminal fate in BlaCa (19). GATA3 loss is associated with an invasive less differentiated phenotype (20) and is mutated in ~5% of sporadic and ~13% of familial BC (21–23). It is unclear if estrogens have any protective effect because women are more likely to be diagnosed with invasive disease and have less favourable outcomes after treatments (24). However, ER activation requires both GATA3 and FOXA1 (25). Disclosing the functional connection between GATA3 and ER expression in BlaCa may improve the current tools for patient management, namely their eligibility for endocrine therapy used to inhibit ER-mediated proliferation.

The two ERs (ERα and ERβ) are expressed in the normal urothelium of both sexes (26). Analysis of the TCGA urothelial cancer data set (n=406) showed that ERα and ERβ mRNA expression is low (median FPKM 0.2 and FPKM 0.1, respectively) but detected in about 80% of the samples. Moreover, several independent studies showed that, BlaCa-derived cells lines are responsive to anti-estrogenic therapy (27, 28). To date, few studies have assessed the association between ERα and ERβ protein with the clinicopathological features of BlaCa. The reports are inconsistent, and the role of ERs in BlaCa development and progression remains controversial, partly because many of the studies dealt with small and heterogeneous patient cohorts and used antibodies that were not validated for clinical diagnosis of ERα, or anti-ERβ antibodies that were proved to be unspecific at a later stage (29, 30).

A previous meta-analysis of immunohistochemical studies correlated ERβ expression with high grade (OR=2,169; p<0,001) and muscle-invasive (OR=3,104, p<0,001) tumours (31) and revealed associations between ERβ expression and worse recurrence-free (HR=1,573; p=0,013) and progression-free (HR=4,148; p=0,089) survivals in patients with NMIBC. However, these results are compromised due to inclusion of studies that used anti-ERβ antibodies that are unspecific (29). In the same study, incomplete information hampered conclusive evaluation of associations between ERα expression and patient's clinicopathological features. Regarding GATA3, much effort has

Abbreviations: BlaCa, bladder cancer; ER, estrogen receptor; SCC, squamous cell carcinoma; UC, urothelial carcinoma; UCDD, urothelial carcinoma with divergent differentiation; VH, variant histologies; MIBC, muscle invasive bladder cancer; NMIBC, non-muscle invasive bladder cancer; FPKM, fragments per kilobase million; TUR, Transurethral resection; TMA, tissue microarray; CYS, cystectomy.

been devoted into understanding its prognostic value as immunohistochemical marker, but to date there is no systematic evaluation and meta-analysis of such findings. Additionally, there is no study assessing the relationship between these functionally related proteins. In this work, we present a systematic review of the literature and meta-analysis to investigate the associations between immunohistochemical detection of ERα, ERβ and GATA3 with clinicopathological features such as patient's gender, age, tumour stage, grade and survival and explore the relationship between the expression of these three makers.

METHODS

This study was submitted to PROSPERO on January 7, 2021 and registered on February 7, 2021 (CRD42021226836).

Search Strategy

The aim was to identify all primary literature that reported immunohistochemical detection of ERα, ERβ and GATA3 in BlaCa. All potentially relevant articles were identified by a search in PubMed/Medline database using both Medical Subject Headings (MeSH) terms and free text words in the search queries. Singular and plural forms of the key terms, searched in Title and Abstract, were combined with MeSH terms. For GATA3 the queries were (transitional cell carcinoma OR urothelial tumor OR urothelial cancer OR urothelial carcinoma OR bladder tumor OR bladder cancer OR bladder carcinoma OR urinary bladder neoplasms [MeSH Terms]) AND (GATA OR GATA3 OR GATA transcription factors [MeSH Terms]). For ERs, the queries combined all the MeSH Terms listed above for bladder cancer AND (receptors, estrogen OR estrogen OR estradiol OR oestrogen OR estrogen receptor ESR1 OR estrogen receptor beta ESR2 [MeSH Terms]). The search was unlimited for articles published up to December 2020. Existing reviews and reference lists were hand searched for studies missed by the initial query.

Eligibility and Data Collection

All retrieved references were screened for eligibility based on the title and abstract analysis by two of the authors. Potentially eligible full-text articles were retrieved for full-text assessment. The articles were reviewed against the following inclusion criteria: (1) expression level of ERα, ERβ or GATA3 analysed in human BlaCa samples by immunohistochemistry (IHC); (2) reports with sufficient data to evaluate the methodological quality of the trial and to carry out a meta-analysis, including a clear description of the study population and IHC methods (i.e. tissue handling, antibodies used, positive controls), and description of the methodology and cut-off used to assign expression status; (3) Correlation between ERα, ERβ and/or GATA3 expression and clinicopathological data discussed; (4) when different papers reported ERα, ERβ and GATA3 expression from the same patient cohort, the most recent or the most complete study was included. Only original reports were considered. Letters, reviews, case reports, editorials and comments were excluded. Selected references for which a full-text report was not available after contact with dedicated libraries and with corresponding authors were also excluded. For ERs, only published studies using validated antibodies were included. A flowchart depicting the literature search and selection process is represented in **Figure 1**.

For ERs, a total of 331 articles were identified, 298 were excluded after title and abstract screening for relevance. Of the 33 studies included in the qualitative analysis, 17 were excluded after full text analysis due to insufficient data, duplicated report of the same cohort, or use of non-validated or non-specific antibodies (**Table S1**). This resulted in 16 studies included of which 2 included information on ERα and ERβ (27, 32), 11 on ERα (33–41) and 3 on ERβ (42–44). For GATA3, 211 articles were retrieved, of which 129 were excluded after title and abstract screening. Of the 83 studies included in the qualitative analysis, 24 were excluded after full-text analysis due to insufficient data, duplicated report of same sample cohort, contradictory data between text and tables, and lack of information about antibody used (**Table S1**), resulting in 58 studies included. Three studies

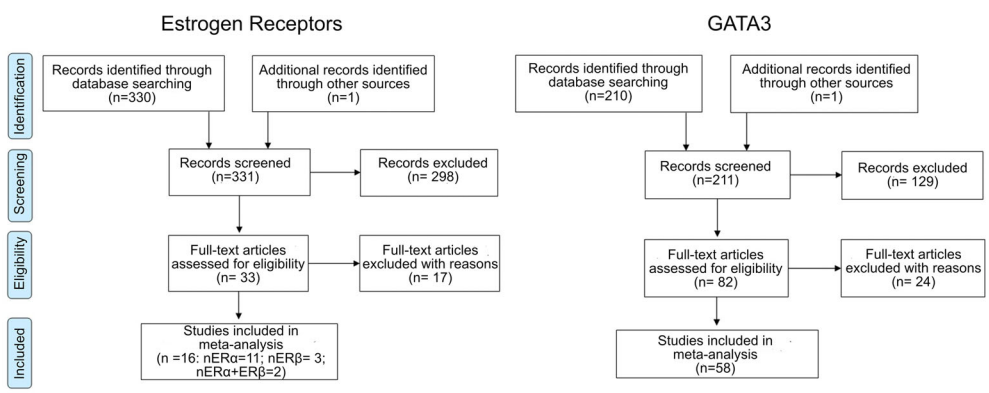

FIGURE 1 | Flowchart summarizing the literature selection process for Estrogen Receptor (ERα and ERβ) and GATA-binding factor 3 (GATA3).

reported ERα and GATA3 in the same tumour sample cohort (35, 45, 46).

Data was extracted from all relevant articles independently by two authors using a predefined data collection template which included identification details (surname of first author, year of publication), number of cases (total number and number of positive cases), primary antibody and dilution used, cut-off for positivity, subcellular localization of the staining (cytoplasmic or nuclear), tissue used for analysis [whole section or tissue microarray (TMA)], tissue collection method [transurethral resection (TUR) and/or cystectomy (CYS)], expression levels according to clinicopathological features such as age, gender, tumour grade, stage, lymph node metastasis and histology. Tumour histology was grouped as pure urothelial carcinomas (UC), UC with divergent differentiation (UCDD) and variant histologies (VH) such as adenocarcinomas and pure squamous cell carcinomas. Prognostic data (duration of follow-up after surgery or treatment, endpoint, overall survival (OS), recurrence and progression-free survival) and the statistical analysis used in each study (type of statistical test, P-value, hazard or risk ratio, 95% confidence interval (CI), univariate or multivariate analysis) were also collected.

The methodological quality and the risk of bias of each study were assessed independently by two of the authors using a list of quality criteria derived from the reporting recommendations for tumour MARKer (REMARK) prognostic studies and any disagreement was resolved by consensus. Four areas of potential bias were assessed: study design, assay methodology, results reporting and methods for statistical analysis. Risk of bias assessment was performed using Review Manager 5 (RevMan 5.3, Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). The overall risk of bias for an individual study was categorized as low (green: risk of bias low in all domains), unclear (yellow: risk of bias is unclear in at least one domain, but no domains with high risk) or high (red: high risk of bias in at least one domain) as shown in **Figure S1**. The weight of all studies on the overall risk of bias for each specific domain is shown in **Figure 2**.

Data Analysis

All analysis were performed using R software (version 3.6.2) and the packages meta, dmetar (47) for statistics and netmeta for network meta-analysis (48). The prevalence, odds ratio (OR), Cohen's d and relative risk (RR) were calculated as point estimates of the association between expression of ERα, ERβ or GATA3 and the patients' clinicopathological characteristics. Pooled prevalence indicates the proportion of positive staining for each marker. Pooled OR was used to evaluate differences in the proportion of positive cases between pre-defined groups. Cohen's d effect size was calculated relative to differences between the average age of the patients reported to be positive or negative where d = 0 means that distribution of ages in one group overlaps the distribution of ages in the other group. The effect size can further be interpreted as small (0.1), medium (0.5) and large (0.8), with higher values indicating less overlap between the groups (49). Pooled RR was calculated for differences in GATA3 positivity regarding Relapse-Free Survival (RFS). Between-studies heterogeneity was estimated using heterogeneity index (I^2) statistics (50). In case of substantial heterogeneity between studies (I^2>50%), only the results from random effects model were considered for further analysis; otherwise, a fixed effect model was used for the pooled statistical analysis and a meta-regression analysis (mixed-effects model) was performed using an 'adjusted effect' to potential moderators. All results were considered statistically significant at the level of 5% (p <0.05). Sensitivity analysis was carried out to assess the robustness of the results by removing individual studies from the meta-analysis and assessing the effect on the pooled results. The publication bias was evaluated using funnel plots and two-sided Egger's tests (**Figure S2**).

Subgroup meta-analysis and/or meta-regression were performed to explore sources of heterogeneity using five factors: 1) antibody used, 2) cut-off for positivity, 3) tumour histology, 4) sample type and 5) sample collection. Meta-regression was also used to assess the influence of the following seven factors in the ERs and GATA3 proportion of positive cases: 1) gender, 2) tumour stage, 3) lymph node metastases, 4) tumour grade, 5) tumour histology, 6) therapy pre-collection, and 7) deaths by cancer.

To assess a possible relationship between the expression of ERα, ERβ and GATA3, we estimated OR using pairwise and network meta-analysis with random effects using frequentist methods. Moreover, we evaluated the inconsistencies between direct and indirect comparison using the z-value of test for disagreement (direct versus indirect) in network meta-analysis.

RESULTS

Characteristics of the Eligible Studies for the Systematic Review

All studies were retrospective, 13 were eligible for ERα comprising a total of 1616 tumour samples (1998-2020; 20 -

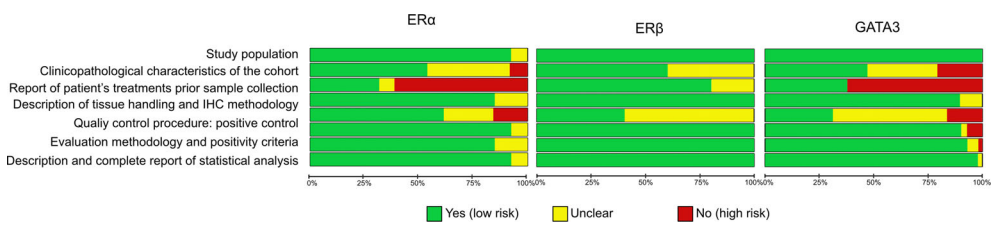

FIGURE 2 | Risk bias assessment per item presented as percentages across all included studies.

317 patients per cohort), 5 for ERβ consisting of 675 samples (2006-2020; 80-224 patients) and 58 for GATA3 covering a total of 4254 samples (2011-2021; 4-303 patients), as shown in **Tables 1**–**3**, respectively.

Methodological Quality and Risk of Bias

REMARK (106) based risk of bias assessment is shown **Figure 2** and **Figure S1**. The most common factor in the bias analysis was lack of information on pre-operative treatment status (high risk for ERα and GATA3 in over 50% of studies). Followed by positive controls (unclear in 25% of ERα studies and in over 50% of ERβ or GATA3 studies), incomplete description of clinicopathological characteristics of the cohort (specially for GATA3, with nearly 50% studies unclear or not reporting) and no information about the quality controls including positivity criteria (above 50% of ERβ or GATA3). The main differences in the methodology between studies included: use of different antibodies and antibody dilutions or different scoring systems.

Meta-Analysis of ERα Expression in BlaCa

The pooled proportion of ERα-positive cases was 7%, (0-38%; **Figure S3**). Despite the high level of variation between the 13 studies (I2 = 93%), the sensitivity analysis did not identify any study as having a significant influence in the overall heterogeneity (**Figure S4A**). However, it is worth mentioning that data from Imai (2019) stood out and influenced pooled results, most likely due to the use of a lower cut-off (1%) and inclusion of UCDD tumours. Subgroup analysis could not explain the heterogeneity between studies (**Table S2**); Meta-regression disclosed lymph node metastases as a significant source of variation associated with ERα expression (p-value = 0.0275; mixed-effects model; **Table S3**).

Correlation of ERα Immunostaining With Clinicopathological Parameters

We conducted a binary meta-analysis to establish the correlation of ERα-positive cases with clinicopathological parameters: gender, age, tumour grade, tumour stage and histology (**Table 4**).

TABLE 1 | Characteristics of the studies included for meta-analysis of ERα expression in BlaCa.

Study	N	Positive Cases	Antibody	Collection	Sample	Cutoff criteria	Age (range)	Gender M/F	<T2/ ≥T2	LG/ HG	Mets/ no Mets	Histology UC/ UCCD/VH	Treated no/yes	Country
Basakci (2002) (33)	121	15	K1900	TUR	tissue	10%	Med 62 (19-87)	99/22	121/0	112/9	NA	121/0/0	NA	NA
Bernardo (2020) (27)	80	14	6F11	CYS+TUR	tissue	1%	Mean 69.2 (38-86)	71/9	40/40	12/68	NA	80/0/0	67/13	Portugal
Bolenz (2009) (34)	198	9	1D5	CYS	tissue	NA		156/42	NA	14/184	63/135	198/0/0	138/60	NA
Borhan (2017) (35)	45	0	SP1	CYS+TUR	tissue	score	Mean 69.6 (51-83)	37/8	NA	NA	NA	0/0/45	NA	USA
Croft (2005) (36)	92	10	6F11	NA	tissue	10%	Mean 65 (30-93)	60/32	43/49	50/42	NA	92/0/0	92/0	USA
Imai (2019) (37)	125[A]	48	6F11	CYS+TUR	tissue	1%	(37-93)	89/26	81/44	63/62	NA	100/20/5	NA	Japan
Kaufmann (1998) (38)	185	34	6F11	NA	tissue	10%	Mean 68.3 (29-94)	84/101	138/47	140/45	NA	185/0/0	NA	Germany
Mashhadi (2014) (39)	120	3	1D5	CYS+TUR	tissue	10%	Mean 66.2 +- 12.1	105/15	61/59	20/100	14/106	120/0/0	120/0	Iran
Pena (2019) (46)	58[B]	14	SP1	TUR	TMA	1%	Mean 68 (47-89)	41/19	57/3	26/34	NA	60/0/0	NA	USA
Shen (2006) (32)	224	2	6F11	CYS+TUR	TMA	10%	NA	NA	145/79	114/96[C]	20/204	224/0/0	NA	NA
Tan (2015) (40)	317[B]	12	1D5	CYS	TMA	10%	Med 69 (37-90)	259/59	98/218[C]	28/262[C]	59/215[C]	314/0/4	242/76	USA
Wang Y (2020) (45)	31	3	1D5	NA	tissue	10%	NA	NA	NA	NA	NA	31/0/0	NA	USA
Wei (2009) (41)	20	0	6F11	NA	TMA	10%	NA	NA	NA	NA	NA	20/0/0	NA	TMA purchased from US Biomax (Rockville, MD)

M, male; F, female; <T2, non-muscle invasive tumours; ≥T2, muscle invasive tumours; LG, Low Grade; HG, High Grade; Mets, metastasis; UC, urothelial carcinoma; UCDD, urothelial carcinoma with divergent differentiation; VH, variant histology; NA, not available; TUR, transurethral resection of the bladder; CYS, cystectomy; TMA, tissue microarray; Med, median.
[A]Number of samples doesn't correspond to number of patients; [B]Not all samples were analysed for ERα; [C]data not available for all samples, missing information for some samples.

TABLE 2 | Characteristics of the studies included for meta-analysis of ERβ expression in BlaCa.

Study	N	Positive Cases	Antibody	Collection	Sample	Cutoff criteria	Age (range)	Gender M/F	<T2/ ≥T2	LG/ HG	Mets/no Mets	Histology UC/ UCCD/ VH	Treated no/yes	Country
Bernardo (2020) (27)	80	73	14C8	CYS+TUR	tissue	1%	Mean 69.2 (38-86)	71/9	40/ 40	12/ 68	NA	80/0/0	67/13	Portugal
Izumi (2016) (42)	72	39	14C8	TUR	tissue	10%	Med 73 (63-80)	NA	72/0	50/ 18[A]	NA	72/0/0	36/36	Japan
Kontos (2011) (43)	111	84	14C8	CYS+TUR	tissue	10%	Mean 70 (23-90)	74/37	70/ 41	57/ 54	NA	111/0/0	111/0	NA
Miyamoto (2012) (44)	188	93	14C8	CYS+TUR	TMA	1%	Mean 65.9 (30-89)	148/40	97/ 91	56/ 132	32/53[A]	178/10/0	160/28	USA
Shen (2006) (32)	224	141	MYEB	CYS+TUR	TMA	10%	NA	NA	145/ 79	114/ 96[B]	20/204	224/0/0	NA	NA

M: male; F: female; <T2: non-muscle invasive tumours; ≥T2: muscle invasive tumours; LG: Low Grade; HG: High Grade; Mets: metastasis; UC: urothelial carcinoma; UCDD: urothelial carcinoma with divergent differentiation; VH: variant histology; NA: not available; TUR: transurethral resection of the bladder; CYS: cystectomy; TMA: tissue microarray; Med: median. [A]*data not available for all samples, missing information for some samples.* [B]*data not available for all samples, missing information for some samples.*

TABLE 3 | Characteristics of the studies included for meta-analysis of GATA3 expression in BlaCa.

Study	N	Positive Cases	Antibody	Collection	Sample	Cutoff criteria	Age (range)	Gender M/F	<T2/ ≥T2	LG/ HG	Mets/ no Mets	HistologyUC/ UCCD/ VH	Treated no/yes	Country
Agarwal H. (2019) (51)	74	57	EPR16651	TUR	tissue	1%	Mean 55.9 (21-83)	65/9	NA	24/ 47 [C]	NA	74/0/0	NA	India
Aphivatanasiri (2020) (52)	137	109	L50-823	NA	TMA	1%	Mean 70.5 (34-92)	101/36	NA	NA	NA	137/0/0	NA	Thailand, China and Indonesia*
Barth (2018) (53)	156[A]	151	CM405A	NA	TMA	10%	Med 70 (42–93)	104/28	156/ 0	NA	0/156	156/0/0	96/51[C]	Germany
Beltran (2014) (54)	20	20	L50-823	CYS+TUR	tissue	1%	Mean 63 (45-75)	14/6	0/20	NA	6/8[C]	0/20/0	NA	Spain, Portugal, Italy and USA*
Beltran (2014) (55)	28[B]	28	L50-823	CYS+TUR	tissue	1%	Mean 66 (45-83)	45/11	NA	NA	14/19	0/0/28	NA	Portugal, USA, Italy, Spain and France*
Bernardo (2019) (56)	205	191	D13C9	NA	TMA	10%	NA	156/49	163/ 40[C]	119/ 86	NA	194/10/1	NA	Portugal
Bertz (2020) (57)	33[B]	10	L50-823	CYS+TUR +Biopsy	tissue	NA	Mean 66.6 (24-88)	27/7	NA	NA	NA	0/16/18	NA	Germany
Bezerra (2014) (58)	22	7	L50-823	NA	tissue+ TMA	1%	Med 69.5 (34-88)	16/6	7/15	NA	4/18	0/22/0	NA	USA
Bontoux (2020) (59)	184[A]	94	L50-823	CYS	TMA	10%	Med 68 (40-86)	141/46	2/ 185	0/ 184 [C]	87/100	101/38/34 [C]	187/0	France
Borhan (2017) (35)	45	37	L50-823	CYS+TUR	tissue	Score (>1)	Mean 69.6 (51-83)	37/8	NA	NA	NA	0/45/0	NA	USA
Broede (2016) (60)	25	21	L50-823	NA	TMA	Score (>2)	NA	NA	NA	NA	NA	16/0/9	NA	NA
Chang (2012) (61)	35	28	L50-823	NA	TMA	score	NA	NA	NA	0/35	NA	35/0/0	NA	NA
Clark (2014) (62)	27	23	L50-823	NA	TMA	score	NA	NA	NA	NA	NA	22/0/5	NA	TMA purchased from US Biomax (Rockville, MD)

(Continued)

TABLE 3 | Continued

Study	N	Positive Cases	Antibody	Collection	Sample	Cutoff criteria	Age (range)	Gender M/F	<T2/ ≥T2	LG/ HG	Mets/ no Mets	HistologyUC/ UCCD/ VH	Treated no/yes	Country
Comperat (2017) (63)	32[B]	29	L50-823	CYS+TUR	tissue	10%	Mean 66.7 (38-84)	32/4	3/33	NA	7/17 [C]	0/32/0	NA	France, Germany Czechia, USA and Canada
Davis (2016) (64)	79	56	L50-823	NA	TMA	1%	NA	NA	NA	NA	NA	79/0/0	NA	USA
Ellis (2013) (65)	49	12	L50-823	CYS	TMA	score	Mean 54 (30-79)	39/10	NA	NA	NA	0/0/49	NA	USA
Eckstein (2018) (66)	89[B]	46	L50-823	NA	TMA	score	Mean 69.7 (41-88)	69/26	0/95	0/95	58/29 [C]	41/52/2	68/27	Germany
Fatima (2014) (67)	22	16	L50-823	CYS	tissue	10%	NA	NA	NA	NA	NA	0/22/0	NA	USA
Guo (2020) (68)	74	52	HG3–31	NA	tissue	NA	NA	NA	NA	NA	NA	74/0/0	NA	USA
Gruver (2012) (69)	37	29	HG3-35	TUR	TMA	5%	NA	NA	NA	NA	NA	37/0/0	NA	USA
Gulmann (2013) (70)	50	22	HG3-31	TUR	tissue	5%	(34-96)	31/19	31/ 19	11/ 39	NA	15/23/12	NA	USA and Spain
Gürbüz (2020) (71)	300	297	L50-823	TUR	tissue	20%	Mean 69 (28-100)	265/35	150/ 150	75/ 225	NA	300/0/0	300/0	Turkey
Hoang (2015) (72)	103	86	L50-823	NA	TMA	5%	NA	78/25	NA	26/ 77	NA	103/0/0	NA	USA
Jangir (2019) (73)	40	18	L50-823	CYS	tissue	20%	Mean 56.6	37/3	NA	0/40	17/23	22/18/0	40/0	NA
Johnson (2020) (74)	28	28	L50-823	CYS+TUR	tissue	1%	Med 66	24/3	1/16 [C]	NA	NA	0/0/28	4/23	USA
Kandalaft (2016) (75)	21	21/20	L50-823/ HG3-31	NA	tissue	1%	NA	NA	NA	NA	NA	21/0/0	NA	USA
Kim (2020) (76)	166	92	L50-823	CYS+TUR	TMA	20%	Mean 76 (37-87)	139/27	0/ 166	7/ 159	NA	166/0/0	166/0	South Korea
Kim (2013) (77)	43	29	L50-823	TUR	TMA	5%	Mean 64.2 (52-79)	NA	NA	NA	NA	22/10/11	5/5[C]	South Korea
Leivo (2016) (78)	89	88	L50-823	CYS	TMA	5%	Mean 64 (43–85)	71/18	2/87	NA	43/46	89/0/0	56/33	USA
Liang (2014) (79)	244	114	HG3-31	CYS	TMA	10%	(32-90)	187/57	11/ 225[C]	NA	NA	103/141/0	NA	USA
Liu (2012) (80)	72	62	HG3-31	NA	TMA	5%	NA	NA	NA	NA	NA	72/0/0	NA	USA
Lobo (2020) (81)	70	62	HPA029731	CYS+TUR	tissue	10%	Mean 69.5 (45-91)	58/12	47/ 23	28/ 42	9/61	70/0/0	NA	Portugal
Lu (2020) (82)	176	176	UMAB218	CYS+TUR	tissue	score	Mean 62.1 (28-90)	153/23	176/ 0	40/ 136	7/169	100/76/0	33/143	China
Manach (2018) (83)	60	31	CM405B	CYS+TUR	TMA	10%	Mean 64.6 (41-91)	46/14	NA	NA	NA	32/28/0	54/6	France
Miettinen (2014) (84)	54	49	L50-823	NA	TMA	NA	NA	NA	NA	22/ 32	NA	49/5/0	NA	NA
Mitra (2018) (85)	5	5	390M-15	CYS+TUR	tissue	10%	Mean 66.8 (52-75)	5/0	NA	NA	NA	5/0/0	NA	NA
Miyamoto (2012) (86)	145	125	L50-823	CYS+TUR	TMA	1%	Mean 66 (30-89)	110/35	80/ 65	51/ 94	21/47[C]	145/0/0	128/17	USA
Mohammed (2016) (87)	79	56	L50-823	NA	TMA	20%	NA	NA	0/79	0/79	NA	79/0/0	NA	USA
Mohanty (2014) (88)	16	16	HG3-31	TUR	tissue	score	Mean 74.5 (45-79)	NA	0/16	0/16	NA	16/0/0	16/0	USA

(Continued)

TABLE 3 | Continued

Study	N	Positive Cases	Antibody	Collection	Sample	Cutoff criteria	Age (range)	Gender M/F	<T2/ ≥T2	LG/ HG	Mets/ no Mets	HistologyUC/ UCCD/ VH	Treated no/yes	Country
Paner (2014) (89)	7	6	HG3-31	CYS	tissue	1%	Mean 67 (47-87)	6/1	0/7	NA	3/4	0/7/0	5/2	USA and Spain
Paner (2014) (90)	111	67	HG3-31	NA	TMA	5%	NA	NA	NA	NA	NA	10/20/81	NA	USA, Spain and South Korea
Patriarca (2014) (91)	11	11	L50-823	TUR	tissue	10%	Mean 74 (61-86)	7/4	11/0	10/1	NA	11/0/0	7/4	Italy and France
Rodriguez Pena (2019) (46)	58[B]	58	CM405B	TUR	TMA	1%	Mean 68 (47-89)	41/19	57/3	26/ 34	NA	60/0/0	NA	USA
Perrino (2019) (92)	26[B]	25	L50-823	CYS+TUR	tissue	1%	Med 68 (36-91)	56/13	1/68	NA	14/36 [C]	0/69/0	44/25	USA
Priore (2018) (93)	15	14	L50-823	NA	tissue	5%	Mean 72 (55-84)	15/1	10/5	9/6	NA	0/0/15	NA	USA
Rao (2013) (94)	36	3	L50-823	NA	tissue	1%	NA	NA	NA	NA	NA	0/0/36	NA	NA
Raspollini (2011) (95)	4	4	HG3-31	CYS+TUR	tissue	score	Mean 68.5 (53-78)	3/1	0/4	NA	2/2	0/0/4	2/2	NA
Samaratunga (2015) (96)	10	9	L50-823	TUR	tissue	score	NA	6/4	5/5	NA	NA	0/0/10	NA	Australia
Sanfrancesco (2016) (97)	26	16	L50-823	CYS+TUR	TMA	score	NA	NA	NA	NA	NA	0/0/26	NA	USA
Sjodahl (2017) (98)	303	194	D13C9	TUR	TMA	10%	NA	236/28 [C]	56/ 241 [C]	41/ 262	NA	257/5/41	NA	Sweden
So (2013) (99)	12	10	L50-823	NA	tissue	score	Med 60.5 (26-85)	NA	NA	NA	NA	0/0/12	NA	USA
Verduin (2016) (100)	86[A]	43	L50-823	NA	TMA	1%	Mean 66.7 (39-91)	53/25	NA	NA	NA	0/17/69	NA	NA
Wang (2019) (101)	91	80	L50-823	CYS+TUR	tissue	10%	Mean 66 (39–89)	64/27	0/91	NA	31/60	91/0/0	91/0	Taiwan
Wang (2018) (102)	30[B]	1	HG3-31	CYS+TUR	TMA	NA	Mean 68 (34-90)	69/12	2/ 46[C]	42/ 39	27/54	0/0/30	NA	USA
Wang (2020) (45)	31	31	L50-823	NA	tissue	10%	NA	NA	NA	NA	NA	31/0/0	NA	USA
Yuk (2019) (103)	100	92	156-3C11	CYS+TUR	TMA	1%	Mean 65.1	83/17	0/ 100	NA	20/80	100/0/0	90/10	South Korea
Zhao (2013) (104)	69	62	HG3-31	NA	TMA	5%	Mean 68.7 (25-89)	45/24	NA	NA	69/0	48/18/3	NA	USA
Zinnall (2018) (105)	94	79	L50-823	NA	TMA	1%	Med 68 (41-99)	61/14[C]	7/ 74[C]	0/94	NA	0/0/94	NA	Germany

M: male; F: female; <T2: non-muscle invasive tumours; ≥T2: muscle invasive tumours; LG: Low Grade; HG: High Grade; Mets: metastasis; UC: urothelial carcinoma; UCDD: urothelial carcinoma with divergent differentiation; VH: variant histology; TUR: transurethral resection of the bladder; CYS: cystectomy; TMA: tissue microarray; Med: median. NA: not available. [A]*Number of samples doesn't correspond to number of patients;* [B]*Not all samples were analysed for GATA3;* [C]*data not available for all samples, missing information for some samples. *patients are from participating institutions but is doesn't specify if all or just few and which ones.*

Gender analysis (n=849 pooled cases from 7 studies; I2 = 0%) disclosed no significant differences between males and females CI= [0.43; 1.02], however, there was a tendency for a lower ERα expression in males (p=0.06). There was no difference in the age at diagnosis (n=230 from 3 studies; I2 = 0%) between ERα-positive and negative cases. ERα expression was significantly higher in high grade tumours (n=661 from 6 studies; I^2 = 41%, CI= [0.21-0.78], p-value< 0.01; **Figure 3**). For stage analysis, data from 4 studies (I^2 = 5%) was divided as Ta+T1 (218 cases) and >=T2 (136 cases) and no significant association was found (CI= [0.31-1.04], although there was tendency for higher ERα-positivity in late-stage tumours. The association with histology could only be inferred from a single study (n=125) with low number of VH cases and showed that the proportion of ERα-positive cases was lower in UC tumours when compared with either VH or UCDD (**Table 4**).

Meta-Analysis of ERβ Expression in BlaCa

Four hundred and thirty samples pooled from 5 studies were ERβ-positive (**Figure S5**), corresponding to 69% of the cases (range: 49–91%; I2 = 94%). Neither subgroup analysis nor meta-

TABLE 4 | Meta-analysis summary table.

Stratification	Protein	No. Of Studies	Patients (n)	Pooled OR (95% CI)		Heterogeneity	
				Random	p value	I2 (%)	p value
Gender	ERα	7	849	0.66 [0.43; 1.02]	0.06	0	0.92
	ERβ	2	268	1.80 [0.91; 3.57]	0.09	0	0.79
	GATA3	10	961	1.53 [1.02; 2.29]*	0.04	0	0.73
Tumour Stage	ERα	4	354	0.57 [0.31; 1.04]	0.07	5	0.37
	ERβ	4	583	0.77 [0.18; 3.33]	0.72	91	<0.01
	GATA3	7	1040	4.73 [2.18; 10.28]*	< 0.01	38	0.14
Lymph node metastases	ERα						
	ERβ	2	309	2.62 [1.25; 5.48]*	0.01	0	0.40
	GATA3	5	453	0.88 [0.37; 2.10]	0.78	54	0.07
Tumour Grade	ERα	6	661	0.41 [0.21; 0.78]*	< 0.01	41	0.13
	ERβ	5	657	1.08 [0.34; 3.49]	0.89	86	<0.01
	GATA3	9	1253	4.14 [1.79; 9.54]*	< 0.01	38	0.11
Histology UC *vs* VH	ERα	1	105	2.55 [0.41; 16]	0.32	NA	NA
	ERβ						
	GATA3	9	991	0.08 [0.03; 0.18]*	<0.01	52	0.03
Histology UC *vs* UCDD	ERα	1	120	1.14 [0.43; 3.03]	0.80	NA	NA
	ERβ						
	GATA3	10	758	0.21 [0.08; 0.53]*	<0.01	50	0.03
Histology UCDD *vs* VH	ERα	1	25	0.44 [0.06; 3.29]	0.43	NA	NA
	ERβ						
	GATA3	8	354	2.55 [0.45; 14.66]	0.29	82	<0.01
Therapy pre-collection	ERα						
	ERβ	1	72	1.12 [0.44; 2.83]	0.81	NA	NA
	GATA3						
				Pooled MD (95% CI)			
				Random	**p value**		
Age	ERα	3	230	0.77 [-3.08; 4.62]	0.69	0	0.97
	ERβ	2	268	-2.22 [-5.64; 1.20]	0.20	0	0.43
	GATA3	5	283	7.41 [1.90; 12.92]*	<0.01	66	0.02

*UC, urothelial carcinoma; UCDD, urothelial carcinoma with divergent differentiation; VH, variant histology; NA, not applicable. *significant association.*

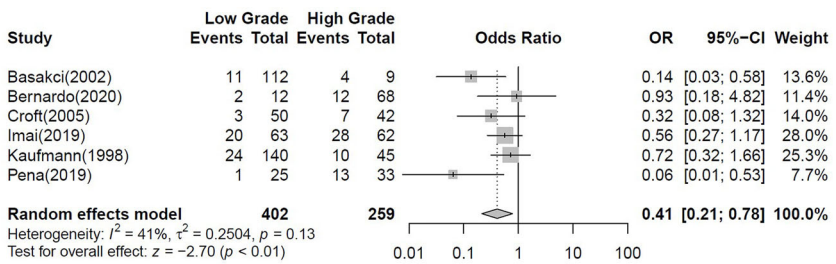

FIGURE 3 | Forest plot for the binary meta-analysis stablishing the association between ERα positivity and tumour grade. Individual study estimates of crude odds ratios (OR) and 95% confidence intervals (CI). The diamond at the bottom of the plot denotes the random effects estimate. Error bars indicate confidence intervals. Heterogeneity was assessed using I2.

regression could explain the source of heterogeneity (**Tables S2** and **S3**) and a sensitivity analysis showed that selective omission of each study did not influence the overall heterogeneity (**Figure S4B**).

Correlation of ERβ Immunostaining With Clinicopathological Parameters

A binary meta-analysis was conducted to evaluate the association of ERβ positivity with patients' gender, tumour stage, grade, presence of lymph node metastasis and patients' pre-operative treatment (**Table 4**). Variation between studies was high and no significant association was found between ERβ expression and the clinicopathological parameters evaluated except for lymph node metastases. ERβ-positive cases were significantly correlated with the presence of lymph node metastasis [n=309 from 2 studies (32, 44)] (**Figure 4**).

Meta-Analysis of GATA3 Expression in BlaCa

GATA3 was expressed in 85% of the 4275 pooled cases from 58 studies (range: 3-100%; **Figure S6**). Despite the high level of

heterogeneity (I2 = 97%), the sensitivity analysis did not identify any study as having a large influence in the overall results (**Figure S4C**). However, data from Liang (2014) stands out and influences pooled results possibly due to the higher number of UCDD cases analysed (79). However, this trend did not reach significance in the subgroup meta-analysis (**Tables S2**, **S3**) indicating that technical variations or cohort composition were not the main drivers of heterogeneity. Meta regression was used to estimate whether the heterogeneity between studies was explained by clinicopathological covariates (**Table S3**). Interestingly, tumour stage, grade and pre-operative therapy significantly affected GATA3 positivity (p-value for mixed-effects model, p=0.0409, p=0.0056, p=0.0006, respectively).

Correlation of GATA3 Immunostaining With Clinicopathological Parameters

The association of GATA3 positivity with patients' gender, tumour stage, grade, histology and the presence of lymph node metastasis was evaluated in a binary meta-analysis (**Table 4**). Gender analysis (n=961, from 10 studies; I2 = 0%) disclosed a significantly higher proportion of GATA3-positive cases in males (CI= [1.02; 2.29]; **Figure 5A**). There was significantly higher expression in tumours from older patients (n=282, from 5 studies; I2 = 66%, CI= [1.90; 12.92]; **Figure 5B**). Two studies were included in the meta-analysis of GATA3 expression and recurrence free survival (RFS), analysing a total of 172 positive samples in a cohort of 192 patients. GATA3 expression was significantly associated with lower risk of recurrence (I2 = 0%; RR= 0.33; CI = [0.19; 0.58], p-value< 0.01; **Figure 5C**). Although this conclusion deserves to be followed up with a higher number of studies, the effect was strong and reflected the results of the individual studies included in this analysis (101, 103).

GATA3 expression was found significantly higher in low stage (Ta+T1) compared with invasive tumours (>=T2) (CI= [2.18; 10.28], p-value< 0.01; **Figure 6A**) in the stage analysis (n=1040, from 7 studies; I2 = 38%). However, no significant correlation was found between GATA3 expression and lymph node metastasis. Similarly, GATA3 expression was significantly higher in low grade tumours as shown in the tumour grade analysis (n=1253, from 9 studies; I2 = 38%, CI= [1.79; 9.54], p-value< 0.01; **Figure 6B**). Tumour histology analysis revealed significantly higher GATA3 positivity in UC when compared to UCDD (n=880, from 10 studies; I2 = 50%, CI = [0.08; 0.53], p-value< 0.01; **Figure 6C**) or VH tumours (n=991, from 9 studies; I2 = 52%, CI = [0.03; 0.18], p-value< 0.01) (**Figure 6D**). No difference was found between UCDD and VH tumours.

Association Between ERα, ERβ and GATA3

Network meta-analysis was performed to assess a possible relationship between the expression of ERα, ERβ and GATA3 (**Table 5**). The model was based on direct evidence pooled from studies evaluating at least two of the proteins in the same study: 3 studies for ERα and GATA3 (35, 45, 46), 2 studies for ERα and ERβ (27, 32) and 1 study for ERβ and GATA3 (86). The model showed that both ERβ (0.014; 95%; CI: 0.007-0.030) and GATA3 (0.002; 95%CI: 0.001- 0.005) positive cases negatively correlate with ERα-positivity. GATA3 positivity was also negatively associated with ERβ positive cases (0.168; 95%; CI: 0.098 - 0.290), even though the association wasn't as strong as for ERα. Still, this associations should be interpreted with extreme caution as even though the studies evaluating ERβ used antibodies that to date were not found to be unspecific, there is still great controversy as to how to best detect ERβ by IHC and the number of studies is low. No disagreement/inconsistency between direct and indirect comparison were detected as significant (p = 0.936).

DISCUSSION

BlaCa is a heterogeneous disease for which to date, limited histopathological markers and therapeutic options exist. Gene expression signatures with GATA3 and active ER signalling characterize luminal BlaCa (15, 17) and disclose some similarities between luminal BlaCa and BC (107). In the breast, GATA3 is a necessary transcriptional coactivator of ERα-mediated proliferation (25, 108), both proteins cooperate to maintain the epithelial lineage and are diagnostic tools for luminal BC (109). However, it is unclear whether these proteins collaborate or have a role in luminal BlaCa pathophysiology. Since ERα is the gold standard for indication of hormonal therapy and both ERs can be targeted with hormonal therapy (110), disclosing the relationship between ERs and GATA3 is a necessary step to advance BlaCa diagnostics and therapeutics. To date, this is the first systematic review and meta-analysis addressing a potential relationship between ERs and GATA3 in BlaCa. Moreover,

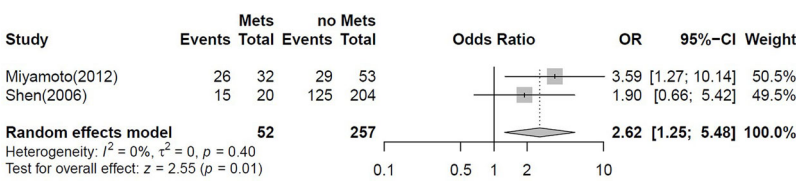

FIGURE 4 | Forest plot for the binary meta-analysis stablishing the association between ERβ positivity and lymph node metastasis. Individual study estimates of crude odds ratios (OR) and 95% confidence intervals (CI). The diamond at the bottom of the plot denotes the random effects estimate. Error bars indicate confidence intervals. Heterogeneity (I2).

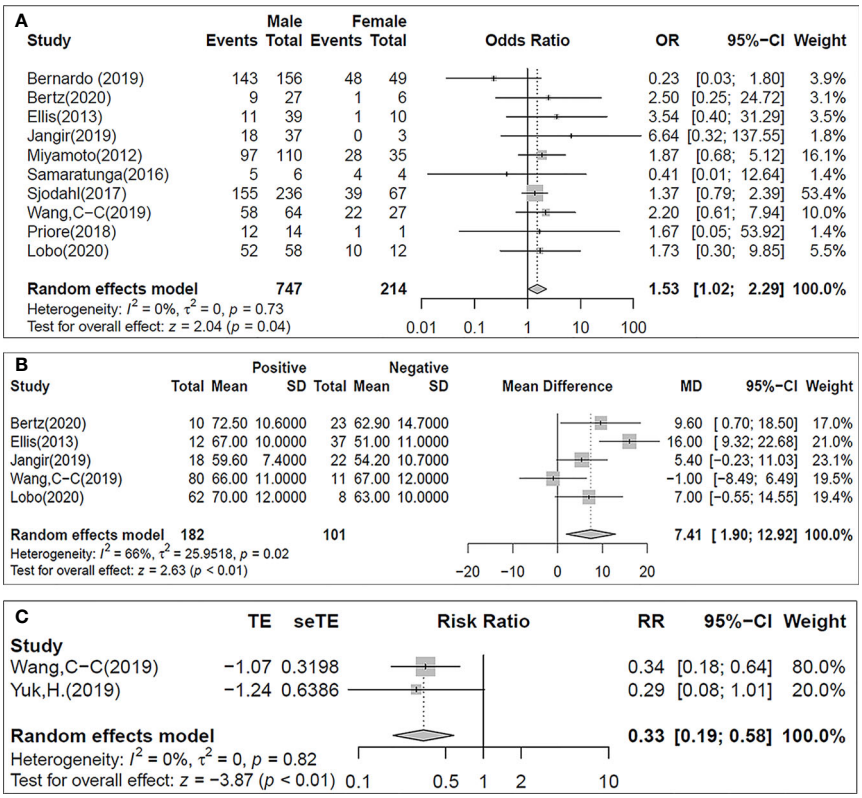

FIGURE 5 | Forest plots for the binary meta-analysis stablishing the association between GATA3 positivity and the patients' sex **(A)**, age at the time of surgery **(B)** and the recurrence free survival [RFS; **(C)**]. Individual study estimates of crude odds ratios (OR) and 95% confidence intervals (CI). The diamond at the bottom of the plot denotes the random effects estimate. Error bars indicate confidence intervals. Heterogeneity (I2).

based on recent findings that disclosed a vast amount of anti-ERβ antibodies as unspecific (29, 30), we restricted the inclusion criteria to only include validated anti-ERβ antibodies.

To improve our understanding ERα, ERβ and GATA3 roles in BlaCa pathophysiology we defined the following questions *a priori*: 1) What is their prognostic value? 2) What is their diagnostic value? 3) How do clinicopathological parameters impact their expression? 4) What are the sources of heterogeneity and which can be controlled in future studies? and 5) Is expression of these three markers associated in any way?

Prognostic Value

Six studies analysed the association between ERα and patients' prognosis, with no significant association between ERα expression and tumour recurrence/progression or survival observed in each individual study (27, 33, 34, 39, 40). An exception was Pena et al. that showed less likelihood for tumour recurrence in ERα -positive cases using unadjusted logistic regression (46). Due to differences in the methodology and information reported, it was not possible to carry out a meta-analysis. Therefore, current data is still insufficient to determine the prognostic value of ERα. However, higher ERα-positivity was observed in late-stage and high-grade tumours not only in the present meta-analysis but also in individual studies (33, 36–38), which support the hypothesis that ERα positivity may be a marker of poor prognosis. Our analysis disclosed an association between ERα expression and higher-grade tumours. Moreover, cell line studies showed that blocking ERα signalling with antiestrogens reduces cancer cell viability (27, 28), and a case study reported regression of metastatic transitional cell carcinoma in response to tamoxifen (111). Aromatase expression in the tumour parenchyma and stroma has been found significantly associated with more than a 2-fold risk of bladder cancer recurrence and may be associated with advanced tumour stage and poorer survival outcomes (112), while aromatase in the tumour stroma was significantly associated to adverse pathologic variables and poorer overall survival (113). On the other hand, the predictive value of ERβ is debatable, one study found ERβ-positivity to be associated with worse prognosis for low-grade tumours and lower CSS in high-stage tumours (114), while another study didn't find any correlation between ERβ positivity and tumour recurrence (42). Additionally, Kauffman et al. found that higher ERβ levels were predictive of worse RF and OS following cystectomy (115). In the current meta-analysis data was insufficient to determine the prognostic value of ERβ due to differences in the methodology and data reported among individual studies. Regarding GATA3, pooled

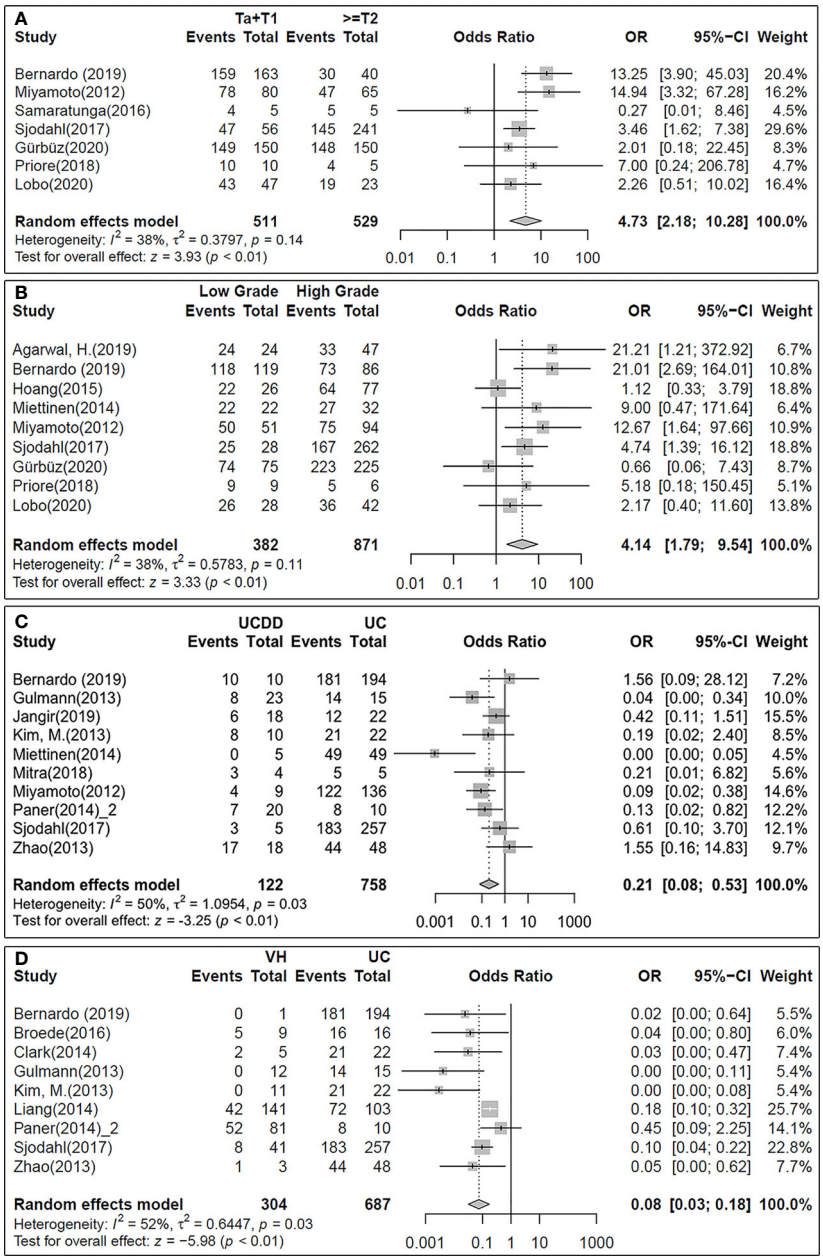

FIGURE 6 | Forest plots for the binary meta-analysis showing the association between GATA3 positivity and the clinicopathological parameters tumour stage **(A)**, tumour grade **(B)** and histological differentiation of the tumours **(C, D)**.

TABLE 5 | Network meta-analysis summary table.

OR (95%CI)	ERα	ERβ	GATA3
ERα		0.014 (0.007;0.028)*	0.002 (0.001; 0.005)*
ERβ	Consistency (0.936)		0.168 (0.098; 0.291)*
GATA3	Consistency (0.936)	Consistency (0.936)	

**Significant association.*

analysis indicated that positive expression was significantly associated with lower risk of recurrence, which is in agreement with the results of the individual studies included in this analysis (112, 113) and others that didn't meet including criteria (73). This result independently confirms the prognostic value of GATA3 immunohistochemical determination in BlaCa.

Diagnostic Value

Out of the 13 eligible studies for ERα, 8 analysed the association between ERα and tumour stage, grade, histological type and/or presence of lymph node metastasis. Two studies evaluated ERα expression in cohorts with multiple histologies and none found significant differences among the different histological types (37, 40). In the current meta-analysis, the association between ERα and tumour histology type was inferred from a single study (37) due to mathematical limitations. However, ERα-positivity was found to be higher in VH and UCDD histological types and less frequent in UC. From the 6 studies analysing ERα among different grade and stage, four found ERα expression to be significantly associated with high grade and muscle invasive tumours (33, 36–38) as in this meta-analysis. These results suggest that ERα-positivity is associated with more advanced tumours. No significant associations were found between ERα positivity and lymph node metastasis in individual studies (34, 40), and the available data was not suitable for meta-analysis. In the case of ERβ, previous studies based on evaluation of mRNA showed that ERβ expression was associated with tumours of the luminal subtype (107). None of the eligible studies provided data to allow the investigation ERβ expression among different histological types. The correlation between ERβ expression and tumour grade was inconsistent among the 5 eligible studies. Two studies found significant association between positive expression and tumour grade but in opposing directions. One noted higher expression in low grade tumours (44) an another in high grade tumours (43). A trend association between positive expression and high grade (32) and no relationship found in the remaining 2 (27, 42). Out of the 5 studies, 4 investigated the relationship between ERβ expression and tumour stage. ERβ expression was found significantly associated with high stage tumours in 2 of them (32, 44). In the same cohorts, ERβ-positivity was also associated with lymph node metastasis as also observed in this meta-analysis. The association between ERβ expression and other clinicopathological variables remains to be investigated.

GATA3 is an established marker of luminal papillary bladder tumours which are the least aggressive tumours and still retain some of the features of the urothelial differentiation (98). Similar to the results of this meta-analysis, four studies reported significant association between GATA3 expression and low grade (51, 59, 71, 86) and low stage (51, 59, 71, 86) tumours. GATA3 expression showed a significant association with tumour histology, with higher expression in UC as opposed to VH and UCDD, both in individual studies (57, 60, 70, 77, 79, 84, 86, 90) and in our meta-analysis. This is not surprising given its role in urothelial differentiation. Contradictorily, individual studies found significant correlation between increased GATA3-positivity and cases with lymph node metastasis (86) while in another data set it was associated with lymph node negative cases (104). Only one of these studies had data suitable for our pooled analysis (86) which found no significant correlation between GATA3-positive cases and lymph node metastases.

Impact of Age and Sex

The relationship between age and sex and ERα (27, 33, 36–39,) and ERβ-positivity was investigated in seven and two independent studies, respectively, revealing no significant associations. The meta-analysis of the pooled data didn't reach statistical significance but, suggest that both ERα and ERβ are more frequent in tumours from female patients as compared to males, and no differences were observed regarding age. Considering the higher estrogen levels in females, even after menopause, this observation is aligned with epidemiological data showing less frequent but more aggressive BlaCa in females (24). In the case of GATA3, 10 studies reported expression levels by age and sex (57, 65, 73, 81, 86, 93, 96, 98, 101), but no significant associations were reported. In the current meta-analysis, GATA3 expression was more frequent in tumours from older patients and, although not significant, there was a trend for higher GATA3 expression in males. These results are in line with a recent study that identified differences in BlaCa molecular subtypes based on sex, with tumours from females expressing higher levels of basal genes and more frequently from the basal/squamous subtype, while tumours from male patients expressed higher levels of luminal markers (116). A reduction of estrogen levels, as observed in menopause, causes urogenital side-effects (117, 118) and may participate in the carcinogenic process by promoting an inflammatory environment (119, 120). Therefore, it can be argued that antiestrogen therapy as used in BC treatment, which report similar urinary side-effects as menopausal and post-menopausal women would result in higher risk of developing BlaCa. We found a case study reporting a 65-year-old woman who developed non-muscle invasive low-grade papillary urothelial carcinoma grade 1, one and a half year after starting on endocrine therapy with aromatase inhibitors (121). However, this would not be related to ERα signalling directly initiating urothelial cell transformation, but to the inflammatory environment resulting from lower estrogen levels or higher androgen/estrogen ratio. Moreover, a large cohort prospective study found no overall associations of HRT use and oral contraceptive use with reduced risk of BlaCa (122).

Limitations of the Meta-Analysis

We found several sources of heterogeneity common to evaluation of ERα, ERβ or GATA3, which may limit this meta-analysis but will certainly elucidate variables to consider in future research studies. These involve the inclusion of tumour samples from patients previously submitted to local or systemic therapy, which varied across different studies and most of the times it was not possible to stratify results by therapy. This might contribute to protein expression fluctuations in response to treatment. Another source of heterogeneity might be the publication bias related to lower number of non-statistically significant results, which can be explained by lack of reporting or less detailed description of results (123). Heterogeneity in stage

and grade may be explained by inclusion of recurrent tumours which may have the same stage and grade of a firstly diagnosed tumour but distinct expression profile (124). Differences between primary and recurrences were not taken into consideration for analysis as this information is not available. The exclusion of cases with preoperative treatment and *carcinoma in situ* was also observed in some studies, which affect the stage and grade of the tumours under analysis. Similarly, the source of tissue contributes to variations as observed by a lower proportion of positive cases in TMA cohorts than in studies using whole-tissue sections. Regarding GATA3 and ERβ, differences in tumour histology explained part of the heterogeneity, with more GATA3 positive cases among UC and UCDD than in VH tumours. Furthermore, for ER detection, the antibodies are also an important source of variability as, even though they are validated for IHC and clinical use, come may detect more than one ER isoform. For ERα, the use of the clones 6F11 and SP1 provided higher dispersion in ERα positive cases, while the 1D5 gave more consistent results. These monoclonal antibodies recognize different epitopes, 6F11 was raised against the full length ERα, 1D5 recognizes the N-terminus, while SP1 antibody recognizes the C-terminus of human ERα. Others have shown that 6F11 and 1D5 antibodies only bind the full-length protein (66kDa) and SP1 could in principle also detect splice variants of smaller size (36 kDa, 46 kDa) (116). In the case of ERβ, all studies using non-specific antibodies were excluded, limiting the meta-analysis to the clone 14C8, which has been independently validated by different groups (29, 117), and the polyclonal MYEB which, to date, has not been probed unspecific. However, clone 14C8 detects ERβ isoforms 1 and 2 which, at least *in vitro*, have different biological effects (118) and may be differentially expressed. For GATA3, antibody usage does not seem to have much influence in the results obtained as evidenced by *Kandalaft et al* that used two different antibodies (119). Additional sources of heterogeneity that weren't explored in this meta-analysis might also be at play such as different technologies to perform IHC, sensitivity to recognize positivity by different pathologists, among others. Finally, we were not able to include absolute positive/negative proportions, leaving some studies out of the pooled analysis for individual clinicopathological parameters.

Correlation Between ER and GATA3 Positivity

The network meta-analysis model showed that there is a negative correlation between ERα or ERβ positivity with GATA3 expression, being the effect stronger for ERα. This agrees with *Miyamoto et al* (60) that showed a negative correlation between GATA3 and ERβ expression. Furthermore, both individual studies and our meta-analysis, propose that ERα and ERβ are markers of bad prognosis (33, 36–38, 60, 120), while GATA3 is associated to lower risk of recurrence and more differentiated tumours (60, 73, 98, 115, 121). Moreover, while GATA3 is higher in males, ERα appears to be higher in females. Therefore, in BlaCa, ERs and GATA3 do not appear to cooperate as observed in BC. Interestingly, ERα and ERβ expression were also negatively correlated. ERβ has been shown to counteract ERα activation, at least in some contexts (118, 122), so it is possible that lower ERβ contributes to an even more aggressive phenotype in ERα-positive BlaCa. Notably, this small subset of tumours may be eligible for hormonal treatment.

CONCLUDING REMARKS

This systematic review confirmed that ERα is expressed in a small proportion of bladder tumours (3 – 13%) and is associated with higher tumour grade and stage independently of tumour histological type. Even if the % of positive cases is low, the possibility of benefiting these subgroup of worse prognosis patients with endocrine therapy should be further explored.

Our analysis and evidence from cell lines and aromatase expression points to a role of ERα in the progression of the disease. Functional studies are needed to identify if ERα is in fact a driver of proliferation in this subgroup of high-grade tumours and the relationship with aromatase expression in order to understand if these patients can benefit from antiestrogen therapy. No conclusion could be reached regarding ERβ even though it is a signature marker for luminal BlaCa and detected by IHC in 69% cases. On the other hand, GATA3 is expressed in about 80% cases and associated with low grade and low risk of recurrence. Therefore, while we were able to confirm the prognostic value of GATA3 using data from two studies, more studies correlating these biomarkers with time to event endpoints are needed to establish their prognostic value. Interestingly, this meta-analysis highlighted that ERα expression is dissociated from GATA3. In fact, higher positivity for each protein was identified in different groups of tumours with GATA3 positive expression associated with well differentiated tumours and ERα with loss of urothelial differentiation. Therefore, these two proteins do not collaborate to maintain epithelial luminal differentiation as observed in BC (109) and instead, they either participate in different stages of tumour progression or may be required for growth of different cancer cell types. This should be further confirmed in prospective studies considering both markers in advanced tumours and pre-resection treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CB: Conceptualized the work, carried out literature and statistical analysis for ERs and drafted the manuscript. ID: updated the literature search for ERs carried out the search for GATA3 and statistical analysis of all data and helped draft the manuscript. FLM: updated the literature search for ERs carried out the search for GATA3 and statistical analysis of all data and helped draft the manuscript. FA: discussed results and critically read the manuscript. VA: coordinated the meta-analysis and conceptualized the work. LS: discussed results and critically read

the manuscript. LH: conceptualized the work, analysed the results and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.684140/full#supplementary-material

Supplementary Figure 1 | Risk of bias in individual studies. +, low risk;?, unclear risk; -, high risk.

Supplementary Figure 2 | Funnel plots showing the asymmetry and publication bias for ERα, ERβ and GATA3.

Supplementary Figure 3 | Forest plot showing pooled results for ERα positive expression in bladder cancer samples.

Supplementary Figure 4 | Baujat plots comparing the weight of each study to the overall heterogeneity for ERα **(A)**, ERβ **(B)** and GATA3 **(C)**.

Supplementary Figure 5 | Forest plot showing the pooled results for ERβ positive expression in bladder cancer samples.

Supplementary Figure 6 | Forest plot showing the pooled results GATA3 positive expression in bladder cancer samples.

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MINI REVIEW
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The Role of Ki67 in Evaluating Neoadjuvant Endocrine Therapy of Hormone Receptor-Positive Breast Cancer

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Ki67 is a proliferation marker. It has been proposed as a useful clinical marker for breast cancer subtype classification, prognosis, and prediction of therapeutic response. But the questionable analytical validity of Ki67 prevents its widespread adoption of these measures for treatment decisions in breast cancer. Currently, Ki67 has been tested as a predictive marker for chemotherapy using clinical and pathological response as endpoints in neoadjuvant endocrine therapy. Ki67 can be used as a predictor to evaluate the recurrence-free survival rate of patients, or its change can be used to predict the preoperative "window of opportunity" in neoadjuvant endocrine therapy. In this review, we will elaborate on the role of Ki67 in neoadjuvant endocrine therapy in breast cancer.

Keywords: neoadjuvant endocrine therapy, breast cancer, Ki67, hormone-positive breast cancer, clinical marker

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INTRODUCTION

Ki67 is a nuclear antigen that is an excellent marker of active cell proliferation in the normal and tumor cell populations (1). It has been proposed as a useful clinical marker for breast cancer subtype classification, prognosis, and prediction of therapeutic response (2–4). But the questionable analytical validity of Ki67 prevents its widespread adoption of these measures for treatment decisions in breast cancer (5). Previous study suggested that baseline Ki67 and its change after short-term endocrine treatment (e.g., 2 weeks) have predictive value of recurrence-free survival (6). Currently, several studies have investigated the possible use of Ki67 assessment in neoadjuvant endocrine therapy (NET). This review assessed the role of Ki67 in NET of breast cancer.

KI67 STRUCTURE AND BIOLOGICAL FUNCTION

Ki67 is expressed in all active phases of the cell cycle (late G1 phase and subsequent S, G2, and M phases), peaks in M phase, dissipates rapidly after mitosis, and is not expressed in stationary G0 phase (7). It is encoded by MKI67 and maps to human 10q26.2. It has a potential phosphorylation site for a range of essential kinases, PEST[1] sequences, and a forkhead-associated domain (8) (**Figure 1**). It acts as an early protein to bind the perichromosomal layer in mitosis at the transition

[1] PEST: The amino acid sequences of ten proteins with intracellular halflives less than 2 hours contain one or more regions rich in proline (P), glutamicacid (E), serine (S), and threonine (T).

from prophase to prometaphase (9). During mitosis, Ki67 stabilizes and maintains the mitotic spindle and prevents chromosomes from collapsing into a single chromatin mass after nuclear envelope disassembly, thus enabling independent chromosome motility and efficient interactions with the mitotic spindle (10, 11). The tandem repeat group of Ki67 contains residues of Cyclin dependent kinase 1 (CDK1) phosphorylation during mitosis (12, 13), and many biological functions of Ki67 have subsequently been shown to be related to phosphorylation (8).

KI67 IN BREAST CANCER

Ki67 is a marker of cell proliferation. In normal healthy breast tissue, very low levels of Ki67 (<3%) have been reported (14, 15). Previous research indicated that estrogen receptor (ER)-positive cells did not proliferate in rodent mammary gland; ERα receives the proliferation signal from E2, initiates DNA synthesis, and is then lost from cells (16). The subsequent steps in proliferation can proceed in the absence of either ERα or ERβ (16). Ki67 is expressed exclusively in ER-negative cells in normal breast tissue (15, 17, 18). Ki67 expression is significantly higher in hyperplastic enlarged lobular units than in adjacent normal terminal duct lobular units (average 6.3% vs. 2.0%; $P < 0.0001$) (19) and is related to the subsequent risk of breast cancer (14, 20, 21). The exclusive Ki67 expression pattern with ER is disrupted during breast carcinogenesis (22, 23). Numerous studies have indicated that early-stage breast cancer patients with high Ki67 expression have a higher risk of recurrence and poorer survival rate (3, 24–27). The International Ki67 in Breast Cancer Working Group (IKWG) accepted that Ki67 immunohistochemistry (IHC) as a prognostic marker in breast cancer has limited clinical validity at present. Ki67 IHC is used as a prognostic marker in early breast cancer regarding whether further adjuvant chemotherapy is warranted to predict or monitor chemotherapy response (28). Ki67 IHC is a useful tool in assessing the risk of recurrence for ER-positive human epidermal growth factor receptor 2 (HER2)-negative breast cancers, where it may be considered a surrogate of molecular assays for distinguishing luminal A from luminal B breast cancer subtypes. High Ki67 has been reported to be associated with a good clinical response to chemotherapy (3), especially in triple-negative breast cancer (15, 29). But it had limited independent significance and does not merit measurements in most routine clinical scenarios. A clinical trial from the European Institute of Oncology indicated that high Ki67 (≥32%) can benefit from adjuvant chemotherapy in luminal B breast cancer with positive lymph node metastasis (30). Penault-Llorca et al. (31) reported that a high Ki67 index (≥20%) in the PACS01 trial was linked with a higher efficacy of docetaxel in adjuvant therapy for ER-positive breast cancer. The BCIRG001 clinical trial found that docetaxel, doxorubicin, and cyclophosphamide (TAC) chemotherapy regimen had a significant complementary effect on endocrine therapy for patients with a high Ki67 index (≥13%), ER positivity, and lymph node positivity (32). In IBCSG trials VIII and IX, high Ki67 index (≥19%) correlated with poor disease-free survival among 1,521 patients with endocrine-reactive breast cancer (33). Ki67 index is a valuable prognostic indicator in endocrine-responsive breast cancer without lymph node metastasis, but it is not a predictive factor of better response to adjuvant chemotherapy in these studies (30, 34).

KI67 IN NEOADJUVANT ENDOCRINE THERAPY

The efficacy evaluation of neoadjuvant chemotherapy (NAC) is mainly based on the clinical response and pathologic response tumor and lymph nodes after treatment (35). In NET, Ki67 has been tested as a predictive marker for chemotherapy using clinical and pathological responses as endpoints (36). Several large clinical trials of NET have assessed the change of Ki67 as an endpoint (37–39) (**Table 1**). Two important clinical trials of NETs, the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) trial and P024, established Ki67 as the evaluation index of NETs. IMPACT compared the efficacy of NET with anastrozole, tamoxifen, and a combination of anastrozole and tamoxifen in postmenopausal women with ER-positive invasive primary breast cancer (45). P024 compared letrozole with tamoxifen in NET (40, 46). IMPACT is a clinical trial similar to the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial, which compared 5 years of the aromatase inhibitor anastrozole alone, tamoxifen

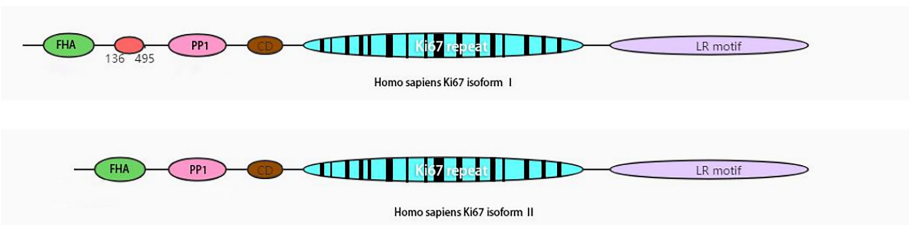

FIGURE 1 | Schematic view of human Ki67 protein structure. The isoform II lacks amino acid 136-495. FHA, forkhead-associated domain; PP1, PP1-binding domain; CD, conserved domain; LR, leucine-arginine-rich domain.

TABLE 1 | Main neoadjuvant endocrine trials.

Clinical trials	Clinical response	Ki67 outcome
P024 (40–42)	ORR letrozole 55% vs. tamoxifen 36% (P < 0.001); ultrasound response letrozole 35% vs. tamoxifen 25% (P < 0.05); mammographic response letrozole 34% vs. tamoxifen 16% (P < 0.001); breast-conserving surgery letrozole 45% vs. tamoxifen 35% (P = 0.022).	No interaction with treatment-induced changes in Ki67 or absolute posttreatment Ki67 levels in either tamoxifen- or letrozole-treated tumor samples. Letrozole inhibited Ki67 to a greater extent than tamoxifen did (Ki67 geometric mean reduction 87% vs. 75%, respectively; P = 0.0009).
IMPACT (39)	There were no significant differences in OR in anastrozole, tamoxifen, or combination.	Greater Ki67 reduction in anastrozole arm. Ki67 geometric mean reduction: anastrozole 76% at 2 weeks/82% at 12 weeks; tamoxifen 59% at 2 weeks/ 62% at 12 weeks; combination 64% at 2 weeks/61% at 12 weeks.
ACOSOG Z1031 (43)	CRR letrozole 75% vs. exemestane 63% vs. anastrozole 69%.	No significance difference in Ki67 geometric mean reduction. Anastrozole 79% vs. exemestane 79% vs. letrozole 82%. Ki67-based data are closely equivalent with the data in adjuvant endocrine trials, therefore predicting similar activity as adjuvant therapies.
PROACT (44)	In hormonal therapy-only patients, ORR favored anastrozole arm (anastrozole 33% vs. tamoxifen 27%, P = 0.04), feasible surgery at baseline improved after 3 months in 43% of patients receiving anastrozole and 31% receiving tamoxifen (P = 0.04).	No data about Ki67

ORR, overall response rate; CRR, complete response rate.

alone, and their combination as adjuvant therapy in postmenopausal women with localized breast cancer. IMPACT was designed to test the hypothesis that the clinical response or the change in Ki67 predicts the outcome of ATAC (39). The ATAC trial is the largest adjuvant trial with the longest follow-up data to date, with 24,522 woman-years of follow-up in the anastrozole group and 23,950 woman-years in the tamoxifen group (47, 48). The results of this study are valuable, and its data continue to demonstrate improved efficacy for 5 years of anastrozole over tamoxifen alone. IMPACT has a similar design to ATAC in NET, avoiding a large number of patients and long follow-up time required for the efficacy evaluation of adjuvant trials, and aims to compare the recurrence and death risk of hormone receptor-positive patients in three NET regimens. The IMPACT trial required only 330 patients and a follow-up of just 3 months to provide its primary endpoint (40). In IMPACT, the change of Ki67 was greater in the anastrozole group than in the other groups at 2 weeks and 12 weeks, which closely parallels the results of the relative recurrence-free survival with adjuvant endocrine therapy after long follow-up in the ATAC trial in 9,366 patients. The short-term changes in Ki67, not the clinical evaluation (tumor size) in NET, might predict the long-term outcome during adjuvant use of the same treatments.

P024 was a randomized, multinational, double-blind study comparing 4 months of letrozole vs. tamoxifen in postmenopausal women with hormone-responsive primary untreated breast cancer (41). P024 indicated that the percentage of Ki67-positive cells, pathological tumor size, lymph node status, and ER status were independently associated with breast cancer-specific survival and relapse-free survival. Based on these factors, Ellis et al. (46) obtained a clinically valuable prognostic model of preoperative endocrine prognostic index (PEPI) score for the outcome prediction of hormone-positive breast cancer with NET. The Ki67 and PEPI triage approaches can predict the risk of relapse. NET was initially an option for breast cancer patients who were too frail to have surgery or cytotoxic chemotherapy. It is very difficult to evaluate the efficacy of adjuvant endocrine therapy because of its long-term follow-up, and NET offers useful clues. The initial endocrine neoadjuvant therapy clinical trial collected data to evaluate the long-term outcome of adjuvant endocrine therapy indirectly rather than as a neoadjuvant treatment (49–51). Future adjuvant endocrine therapy clinical research designs should be based on a biological superiority hypothesis generated by a neoadjuvant endocrine study (52).

After almost 20 years of clinical studies on NETs, with considerable response rates in HR-positive breast cancer, NETs could be a significantly less toxic alternative to NAC for a subgroup of endocrine therapy-responsive breast cancer. The Z1031A trial enrolled postmenopausal women with large (stage II/III) ER-positive breast cancer with random anastrozole, exemestane, or letrozole NET. Subsequently, in Z1031B, the trial protocol was amended to include Ki67 determination after 2–4 weeks of neoadjuvant aromatase inhibitor therapy (53). If Ki67 was >10%, patients were switched to neoadjuvant chemotherapy on the basis of a presumptive lack of hormonal therapy benefit. A pathologic complete response (pCR) rate of >20% was the predefined efficacy threshold. With >5 years of median follow-up, only 3.7% (4/109) with a PEPI score of 0 relapsed vs. 14.4% (49/341) with a PEPI score >0. The Ki67 and PEPI algorithms can be used to evaluate relapse risk after NET. Miller et al. (54) collected 63 postmenopausal breast cancer patients with neoadjuvant letrozole for 3 months. Reduction in Ki67 >40% between pretreatment and 10–14 days was related to pathological responses. A pooled analysis of two multicenter, randomized, noncomparative, phase 2 clinical trials (HORGEN and CARMINA02) evaluating neoadjuvant anastrozole and fulvestrant efficacy for postmenopausal HR+/HER2- breast cancer indicated that PEPI can identify a subset of patients with poorer prognosis who should be offered all appropriate adjuvant therapy (55). Ki67 in neoadjuvant trials predicted the long-term outcomes of large adjuvant trials; Ki67 and PEPI can be predictors for evaluating the recurrence-free survival of breast cancer patients with NET (50). Early breast cancer patients with a PEPI = 0 have little to gain from adding additional adjuvant systemic therapy to their endocrine therapy (46).

The postmenopausal women with hormone-sensitive early breast cancer (POETIC) study was a phase 3 trial in which postmenopausal hormone receptor-positive early breast cancer patients were randomly assigned to POAI (letrozole or anastrozole) for 14 days before and following surgery or no POAI (control) (38). The data from POETIC showed that the patients with a low baseline Ki67 (<10%) had a low risk of recurrence (4.3% in HER-2-negative breast cancer, 10.1% in HER-2-positive breast cancer), and those with a high baseline Ki67 (≥10%) with conversion to low Ki67 after 2 weeks of NET had a high recurrence (21.5% in HER-2-negative breast cancer, 15.7% in HER-2-positive breast cancer). In patients with low baseline Ki67 or POAI-induced low Ki67 associated with good prognosis, adjuvant standard endocrine therapy and high POAI-induced Ki67 might benefit from further adjuvant treatment or trials of new therapies. The Ki67 change after 2 weeks of NET provided substantially more prognostic information for those who had high baseline Ki67.

Clinical practice is unequal to clinical trials, and every patient is unique. In our clinical practice, some patients need time to accept their disease and the subsequent treatment. Perhaps it is just a temporary choice for some ER-positive HER-2-negative breast cancer patients who refuse chemotherapy because of its side effects. The NET, Ki67, and PEPI systems are useful tools that provide useful information about screening for de-escalation treatment in low-risk patients. Especially in times of crisis, such as during the coronavirus disease 2019 (COVID-19) pandemic, it is of paramount importance for most patients to reduce or postpone visits to the hospital (56, 57). The NET, Ki67, and PEPI systems are alternative choices for ER-positive HER-2-negative breast cancer. However, 5%–20% of ER-positive HER-2-negative breast cancers have clinical progression (58). As we know, the data about axillary lymph nodes after NET remain limited; no research majored on the relationship between the Ki67 index and axillary lymph node response to NET. A previous study indicated that NETs can have equivalent clinical benefit to neoadjuvant chemotherapy in appropriately selected patients (59). According to the subtype of breast cancer, the attitudes of the patients and family members, and the information provided by clinical trials, the determination of NET should be cautious and followed up closely. For patients who demonstrate early endocrine resistance to NETs, additional adjuvant systemic therapy should consider alternative treatment approaches to reduce recurrence risk and aggression.

KI67 MEASUREMENT IN NEOADJUVANT ENDOCRINE THERAPY

Ki67 measures the proportion of proliferating cells in breast cancer. Ki67 IHC has been used for many years and is reported by pathologists as a Ki67 index in the clinic. However, Ki67 is not completely integrated in clinical decision-making because of a lack of a standardized procedure for Ki67 assessment as well as persistence of several issues of debate with regard to the Ki67 assay interpretation and the marker's clinical utility. With the goal of establishing a uniform Ki67 evaluation system, the International Ki67 in Breast Cancer Working Group of the Breast International Group and North American Breast Cancer Group conducted a Ki67 reproducibility study. They found that tumor region selection, hot spot analysis, counting method, and subjective assessment of staining positivity resulted in interlaboratory discordance (60–62). A set of guidelines for staining, analysis, and reporting of Ki67 is recommended by the IKWG (5, 28).

The cutoff for Ki67 is still under debate. Published Ki-67 data from the IMPACT and P024 were used for the development of cutoff points for prospective validation. In the IMPACT trial, the geometric mean percentage change of Ki67 after 2 and 12 weeks of NTS was greater in the anastrozole group (76.0% and 81.6%) than in the tamoxifen group (59.5.0% and 61.9%) or the combination group (63.9% and 61.1%) (47). In P024, letrozole inhibited Ki67 to a greater extent than tamoxifen did (reduction in geometric mean Ki67 level 87% vs. 75%, respectively; P = 0.0009) (42, 46). The PEPI score was established in the P024 trial and validated in IMPACT trial (46, 63). It combines the residual Ki67 score, which was analyzed as the natural log interval, or per 2.7-fold increase according to the original scale of percentage values (53, 63). The Z1031 study established a Ki67 cutoff point for triage to chemotherapy after 2 weeks of AI therapy (56). If Ki67 ≤10%, the patient continued AI therapy for another 12–14 weeks and then proceeded to surgery. If the Ki67 level was >10%, the patients were offered either neoadjuvant chemotherapy or surgery. In HORGEN and CARMINA02, the cutoff of Ki67 expression is ≤10% vs. >10% (55). In the POETIC clinical trial, the cutoff was <10% vs. ≥10% (28). The change in Ki67 is of predictive value in NET (28, 38). Currently, the evaluation of Ki67 is considered important in clinical practice, especially in neoadjuvant endocrine clinical trials, and standardized and accurate evaluation under strict quality control is needed. Unless the assessment is carried out in an experienced laboratory with its own reference data and strict quality control, it is not reliable to directly apply a specific cutoff value to make decisions.

CONCLUSION

Ki67 is a useful proliferation marker; its potential usefulness in predicting response and long-term outcome is explored in NET. It cannot represent or predict the regression of the primary tumor or lymph node after NET. It can be used as a predictor to evaluate the recurrence-free survival rate of patients, or its change can be used as the preoperative "window of opportunity" in NET. At present, a set of guidelines for staining, analysis, and reporting of Ki67 is recommended in breast cancer, but the uniformity among different centers needs to be improved. Standardized NET, Ki67, and PEPI systems require further standardization and subsequent clinical validation.

In clinical practice, the aim of neoadjuvant therapy is to shrink or downstage breast cancer, increase the breast conservation rate, and help to screen appropriate patients for de-escalation or escalation therapy, regardless of neoadjuvant chemotherapy or NET. For triple-negative and HER-2-positive breast cancer, neoadjuvant chemotherapy is the first choice.

For ER-positive and HER-2-negative breast cancer, is NAC or NET the best choice or first choice? With large tumor burden, should NAC or NET be selected? With lymph node metastasis, should NAC or NET be selected? Ki67 may offer clues. Previous reports indicated that a higher pretreatment Ki67 was more likely to attain pCR after NAC and can be used as a predictor of NAC in luminal subtypes only (3, 4, 64). This suggests that higher pretreatment Ki67 may improve the prognostic significance of clinical response in NAC. Due to the uniformly low pCR and slow response (65, 66), NETs are not the first choice for the quick downstaging of large tumor burden. Due to the limited data on axillary management or outcomes in NET clinical trials, most patients selected for NETs have limited nodal burden. More research is needed.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to articles reviewed in this manuscript, were involved in the drafting and revision, and approved the final version of this manuscript.

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