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Women in molecular and cellular oncology 2021

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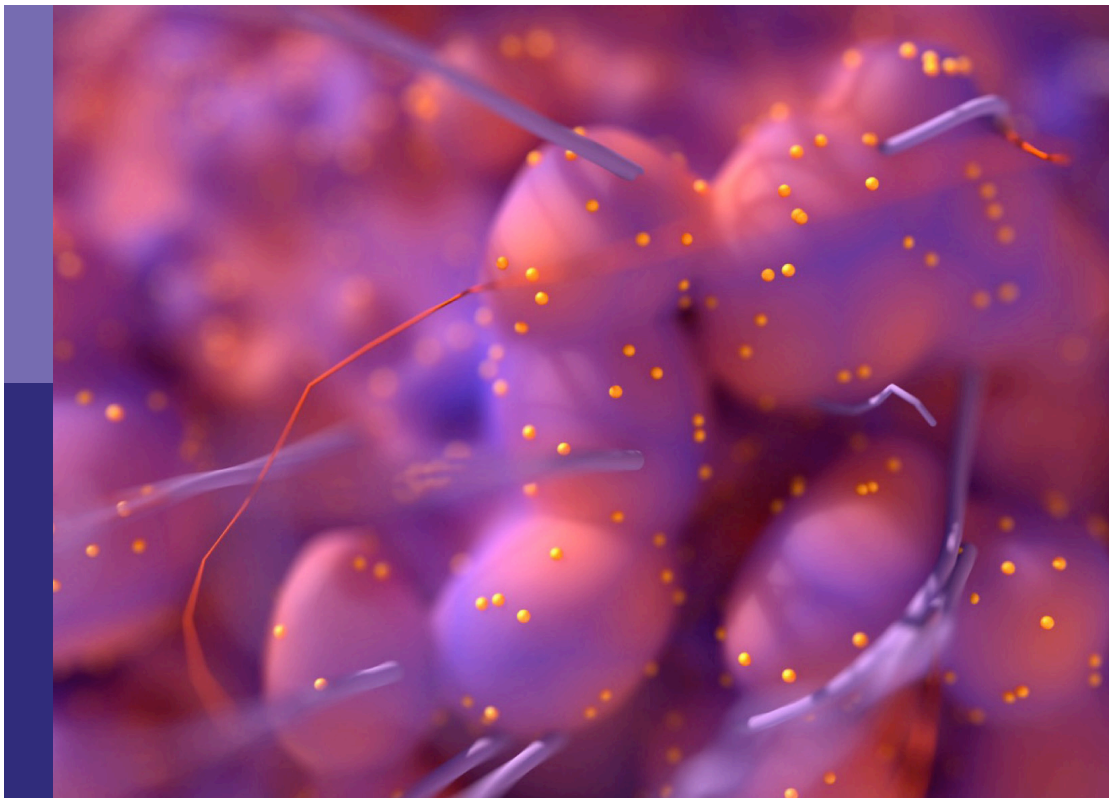

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Women in molecular and cellular oncology: 2021

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Table of contents

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Editorial: Women in molecular and cellular oncology

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Editorial on the Research Topic
Women in molecular and cellular oncology

Despite clear evidence that diversity increases the quality and impact of science (1–3), there remains more to be done to achieve gender equality. While the number of female students and post-doctoral fellows can equal or even exceed that of males in most organizations, the ratios are reversed at more senior levels with a much greater representation of scientists identifying as male (4). Male scientists are also more likely to be cited in papers and to successfully secure research grants (5, 6). In relation to awards and recognition, less than 4% of Nobel Prizes for science have been awarded to women (7).

Various organizations and associations are endeavoring to bridge this gap by implementing new approaches that support women, such as achieving equitable conditions of work, recruitment and promotion, appraisal, training, and pay without discrimination. This inaugural Research Topic "*Women in molecular and cellular oncology*" is one of such initiatives providing a platform that promotes STEMM research by women, by inviting first or senior author contributions from scientists identifying as female.

In this Research Topic, there are 14 articles led by women on various aspects of several cancer types. Waldhorn et al. have compiled data from clinical trials from the last two decades, highlighting women underrepresentation in leading positions in oncology clinical trials. Although the percentage of female principal investigators in oncology clinical trials has been slowly increasing, mainly with respect to cancers more common in females, such as breast cancer, the increase is slow worldwide and more so in Asia.

Breast cancer (BC)

Cholesterol metabolism is gaining increasing attention in BC, although its role remains controversial. Centonze et al. discussed new understanding of cholesterol homeostasis and

summarized key findings of recent preclinical and clinical studies investigating cholesterol metabolism and its derivatives. They provided discussion on the therapeutic effects of natural compounds and cholesterol-lowering drugs in BC treatment, opening a window for new innovative combinatorial therapies, although future work will be needed to evaluate their effective therapeutic potential.

Wang et al. examined the predictive power of breast cancer staging based on positive lymph node ratio (LNR), demonstrating that patients with apical or infraclavicular/ipsilateral supraclavicular lymph node metastasis (APN(+)) had a significantly worse prognosis than APN(−) patients in the same LNR staging group. Accordingly, exclusion of APN(+) patients from the LNR classification significantly improved its predictive power. This study contributes to improving the precision of LNR classification for APN (–) patients.

Cervical and endometrial cancer

Wen et al. reported that the prevalent genomic mutations in Chinese cervical cancer patients were not significantly different when compared to TCGA data of patients from western countries. In both groups, DNA damage repair (DDR) gene alterations were significantly correlated with hypoxia features and increased Tumor Mutational Burden, but not with immunosuppression as previously proposed. The authors therefore suggest that DDR alterations may not be robust predictors of Immune Checkpoint Inhibitor responsiveness in cervical cancer.

Mahajan et al. explored the changes in the expression of TET enzymes and steroid hormone receptors in response to hormones in endometrial cancer cells. Their results suggest that TET gene expression and protein levels are cell-specific and imply possible co-regulation of the expression of steroids and steroid receptors, prompting future studies on how these expression patterns could regulate endometrial biology and interrelate in endometrial cancers.

Perivascular epithelioid cell tumors (PEComas)

PEComas are rare and mostly benign soft tissue neoplasms, only rarely presenting as malignant with poor prognosis, in part due to resistance to conventional chemotherapy. Sui et al. described a patient with chemotherapy resistant metastatic uterine PEComa displaying a partial response to combined treatment with the mTOR and VEGR inhibitors Everolimus and Apatininb. Treatment was chosen after targeted next-generation sequencing, corroborating work by others supporting target-specific therapy for malignant PEComas.

Butz et al. reported a novel TP53 germline splice mutation in a metastatic PEComa and a sinonasal carcinoma. This discovery contributes to the growing number of newly identified germline TP53 variants identified through Next Generation Sequencing, which expands the understanding of Li-Fraumeni syndrome and its association with a wider range of cancer predispositions. The study demonstrated locus-specific loss of heterozygosity in the PEComa, suggesting that the splicing mutation plays a causal role in its development. This study represents the first evidence linking an abnormal TP53 mutation to PEComa.

Lung cancers

Mixed small cell lung cancer (SCLC) and large cell neuroendocrine lung carcinomas (LCNEC) are rare and poorly characterized tumors. Zhu et al. described a tumor containing 35% LCNEC and 65% SCLC, suggesting a common clonal origin with dual mutations in TP53 and RB1. This is an important contribution towards the understanding of this type of cancer, characterized by high genomic stability and with few therapeutic options.

ROS1 rearrangements occur in 1-2% of non-small cell lung cancer (NSCLC) cases, with about 10 fusion partners identified so far. Wei et al. reported a case where a stage IV NSCLC patient harboring a novel TPR-ROS1 fusion showed a rapid but transient response to Crizotinib but resistance to Ceritinib, with a pulmonary nodule negative for PD-L1 staining but displaying the TPR-ROS1 fusion. After the transient Crizotinib response, the patient responded well to chemotherapy. This case highlights TPR-ROS1 as an oncogenic driver, encouraging further research to understand resistance mechanisms and develop effective treatments.

Retinoblastoma

Ke et al., by combining simplified RNAseq data with functional studies in a human retinoblastoma cell line, hypothesize that the downregulation of miR-211-5p is associated with the upregulation of GDNF and of a metabolic pathway leading to carboplatin excretion and drug resistance.

Hematological malignancies

Scripicca et al. described the impact of cyclin-dependent kinases (CDK) inhibitors (CKI) on cancer progression, providing a systematic overview of the key alterations of INK4 or CIP/KIP family members and their function in hematological malignancies. They noted the need for development of novel CDK inhibitors with reduced side effects for cancer treatment.

Resistance to BCR-ABL Tyrosine Kinase Inhibitors, a game changer treatment in Chronic Myeloid Leukemia (CML), is mainly, but not always, due to mutations in BCR-ABL. Elias et al. systematically reviewed the literature on CML focusing on differential expression of miRNAs, bioinformatically identifying their main target genes and associated pathways linked to resistance, which included genomic instability, proliferation, apoptosis, differentiation, and migration.

Chronic Lymphocytic Leukemia (CLL) is a common lymphoid malignancy linked to dysregulated expression of anti-apoptotic and

pro-apoptotic members of the Bcl-2 family. Boncompagni et al. demonstrated that glycerophosphoinositol (GroPIns) can induce expression and activity of the pro-apoptotic family member Bax *via* both binding and modulating SHP-1 and directly interacting with Bax to promote its activation and recruitment to the mitochondria. These data suggest that GroPIns treatment may help overcoming the apoptosis defect of CLL cells, enhancing the effects of other drugs including the Bcl-2 inhibitors.

An emerging common issue across multiple cancers

Treatment resistance is a common problem in cancer therapy as mentioned in some of the articles outlined above. With the recent approval of many new therapeutics, it is common for patients to receive a variety of different treatments throughout their cancer journey and acquire cross-resistance. Discussing the current literature on drug resistance and focusing on cross-resistance to sequential therapeutics and the underlying molecular mechanisms in diverse tumor types, Loria et al. suggest that real-world patient data is often more complex than predicted from clinical trials and offer perspectives for the development of more effective personalized treatment strategies.

There is still much more to be done in the field of molecular and cellular oncology to improve our understanding of the underlying biological characteristics of cancers, particularly in relation to treatments. As we face some major challenges, revealed by 'real world' observations of cross-resistance to sequential treatments, there is an on-going need to drive meaningful progress.

Since diversity in scientific teams enhances creativity and innovation (8), and increases the quality and impact of science, having input and contribution from a broad spectrum of researchers, irrespective of gender or other differences, to develop, produce and present quality research is an advantage. Accordingly, it is essential to implement strategies that actively support underrepresented groups, particularly women that would like to pursue roles in the STEMM field now and in the future.

Waldhorn et al. discussed the necessity of affirmative action to increase female leadership representation in medicine and science in general. As a group of women who have co-edited this inaugural 1st edition of *Women in molecular and cancer oncology 2021*, we feel strongly about supporting the need for more action to gain gender equality at all levels and across all sectors that contribute to the advancement of the oncology field. As we strive to build more opportunities, such as this topic that highlights and showcases the research advances being led and driven by women in the field, we believe that by supporting the path to gender equality, the quality and impact of research will also improve, providing benefits for all cancer patients.

Author contributions

PF: Conceptualization, Project Administration, Writing – original draft, Writing – review & editing. LR: Writing – original draft, Writing – review & editing. VP: Writing – original draft, Writing – review & editing. SD: Writing – original draft, Writing – review & editing. AL: Writing – original draft, Writing – review & editing.

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Conflict of interest

PF is the director of Corpallium Pty Ltd. and Plena Vitae Therapies Pty Ltd.

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

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Genomic Profiling of Chinese Cervical Cancer Patients Reveals Prevalence of DNA Damage Repair Gene Alterations and Related Hypoxia Feature

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Background: Cervical cancer is responsible for 10–15% of cancer-related deaths in women worldwide. In China, it is the most common cancer in the female genital tract. However, the genomic profiles of Chinese cervical cancer patients remain unclear.

Materials and Methods: A total of 129 cervical cancer patients were enrolled in this study (113 squamous, 12 adenocarcinoma, 2 adenosquamous, and 2 neuroendocrine carcinoma). To classify the clinical features and molecular characteristics of cervical cancer, the genomic alterations of 618 selected genes were analyzed in the samples of these patients, utilizing target next-generation sequencing (NGS) technology. Furthermore, the findings from the Chinese cohort were then compared with the data of Western patients downloaded from The Cancer Genome Atlas (TCGA) database, in terms of gene expression files, mutation data, and clinical information.

Results: All studied patients had valid somatic gene alterations, and the most frequently altered genes were *PIK3C*, *TP53*, *FBXW7*, *ARID1A*, *ERBB2*, and *PTEN*. Comparison of genomic profiling showed significantly different prevalence of genes, including *TP53, KMT2C*, and *RET*, between the Chinese and the TCGA cohorts. Moreover, 57 patients (44.19%) with 83 actionable alterations were identified in our cohort, especially in PI3K and DNA damage repair (DDR) pathways. After an in-depth analysis of cervical cancer data from the TCGA cohort, DDR alteration was found to be associated with extremely higher tumor mutation burden (TMB) (median mutation count: 149.5 vs 66, $p < 0.0001$), and advanced stages ($p < 0.05$). Additionally, DDR alteration, regardless of its function, was positively correlated with hypoxia feature and score. Moreover, patients with a high hypoxia score were positively correlated with a high abundance of mast cell resting, but lower abundance of CD8+ T cells and activated mast cell. Finally, *CDHR5* was identified as the hub gene to be involved in the DDR–hypoxia network, which was negatively correlated with both the DDR alteration and hypoxia score.

Conclusions: Overall, a unique genomic profiling of Chinese patients with cervical cancer was uncovered. Besides, the prevalent actionable variants, especially in PI3K and DDR pathways, would help promote the clinical management. Moreover, DDR alteration exerted the significant influence on the tumor microenvironment in cervical cancer, which could guide the clinical decisions for the treatment. *CDHR5* was the first identified hub gene to be negatively correlated with DDR or hypoxia in cervical cancer, which had potential effects on the treatment of immune checkpoint inhibitors (ICIs).

Keywords: cervical cancer, Chinese cohort, Western cohort, genomic alterations, actionable alterations, DDR, hypoxia, tumor microenvironment

INTRODUCTION

Cervical cancer is the fourth most common cancer among women worldwide, affecting nearly 600,000 women annually (1). The application of the human papillomavirus (HPV) vaccine and screening programs have significantly reduced the incidence of cervical cancer; however, it is still highly prevalent in developing countries as the second most common cause of cancer-related deaths in women (2). Even though the disease at its early stages can be amenable to surgery or radiotherapy, recurrent or metastatic cervical cancer is still incurable and calls for novel therapeutic approaches (3). In the past decade, the ICI, pembrolizumab, was the only novel treatment approved by the FDA for treating PD-L1-positive, recurrent or metastatic cervical cancer patients with disease progressing on or after chemotherapy. Though it offers new hope for advanced disease, it is notable that its efficacy was still poorly limited, with an objective response rate of 14.6% in patients with PD-L1–positive tumors (4). Thus, a better understanding of the genomic feature of cervical cancer is a fundamental part for the identification of biomarkers for the development of novel therapeutic approaches and improvement of the efficacy of ICIs.

With the great advances of next-generation sequencing, it enables the researchers to find a comprehensive genomic feature and identify the treatment-related biomarkers in cervical cancer patients. The genomic profiles of Western patients with cervical cancer have been revealed by the TCGA project in 2017 (5). A high prevalence of genes, namely, *PIK3CA*, *EP300*, *FBXW7*, and *PTEN* was identified as the genomic feature of Western cervical cancer patients, and inferred as novel potential therapeutic targets for drug development in future. Meanwhile, in a pan-cancer study, researchers from the Memorial Sloan-Kettering Cancer Center (MSKCC) uncovered that over one third of metastatic cervical cancer patients harbored at least one actionable alteration (6). However, comparing with other tumor types, the number of studies in genetic profiling on cervical cancer is relatively limited. Furthermore, previous studies were predominantly on the Caucasian patients, leaving an unsolved question on whether there were genetic differences between Chinese and Western cervical patients.

Recently, it has been found that DDR alteration(s) could influence the inflammatory signaling pathways which have the ability of reshaping tumor microenvironment (7), and are emerging as an effective biomarker for predicting the response of ICI, for example, (1) DDR alterations were significantly correlated with clinical benefit in urothelial carcinoma patients who received the therapeutic treatment of anti-PD1/PDL1 (8), (2) ICIs therapy could improve the survival of non-small cell lung cancer patients having co-mutations of DNA damage response and repair pathways (9), and (3) DDR mutations were correlated with improved overall survival of patients with colorectal cancer (10). Meanwhile, it has been comprehensively studied in other gynecological tumors, especially in ovarian and endometrial carcinoma as the hallmark event for precision medicine or prognosis classification. However, the role of DDR in cervical cancer has not been specifically clarified yet. In cervical cancer, HPV could manipulate DDR genes to improve its viral life and prevent the viral apoptosis (11). In addition, the progression of cervical cancer is significantly associated with the increased genetic instability, which is primarily caused by the abnormal regulation of DDR genes (12). Thus, the latest Clinical Trials Planning Meeting from the National Cancer Institute (NCI) in 2020 have stressed the development of clinical trials to explore the potential role of DDR in the treatment of cervical cancer (13).

To our knowledge, there existed several studies describing genomic features of Chinese cervical cancer patients, namely, 13 cervical cancer cases of Chinese Hong Kong women (14), 20 endocervical adenocarcinoma cases (15), 32 cervical cancer cases (16), 32 advanced cervical cancer (17), and 74 cervical cancer cases (18) of Chinese mainland women. However, the latter two studies focused on the molecular profiles of integrated gynecologic cancers containing ovarian cancer, endometrial cancer, and cervical cancer. In the present study, we performed the NGS to determine the genomic profiling of 129 Chinese cervical cancer patients, especially the actionable alterations to explore some potential therapeutic strategies. Furthermore, by comparison with the data from Western cohort, it was the first time to figure out the genetic difference(s) between Chinese and Western patients with cervical cancer. Subsequently, we further explored the DDR alteration and tumor microenvironment based on the public dataset.

MATERIALS AND METHODS

Sample Source and Ethic Data

A total of 129 cervical cancer patients were enrolled in the Fudan University Shanghai Cancer Center, from 2018 to 2020. A total

of 72 of enrolled patients (55.81%) had sufficient achieved tumor tissues, while the rest provided blood samples instead for genetic testing, mostly for the following reasons: (i) tumor samples were pathologically reviewed and having tumor cells less than 20%; (ii) no valid or sufficient archived tumor tissue samples; and (iii) diagnosed as metastatic or recurrent disease and more willing to have liquid biopsy testing to exclude potential heterogeneity. Blood samples were drawn into Streck Cell-Free DNA collection tubes and stored at 4°C. Demographics and clinical data were collected for analysis. All patients had provided with signed informed consent and agreed to publish related genomic data without revealing personal identity.

DNA Isolation and Targeted Next-Generation Sequencing

Genomic DNA (gDNA) of tumor samples and germline DNA (from white blood cells) were isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, CA, USA) according to the manufacturer's instruction. Circulating cell-free DNA (cfDNA) was extracted using a QIAamp Circulating Nucleic Acid Kit (Qiagen, CA, USA). Quantity and quality of the purified DNA were checked using Qubit 2.0 Fluorometer (Thermo Fisher Scientific, MA, USA) and Bioanalyzer 2100 (Agilent Technologies, CA, USA). A total of 100 ng of gDNA was sheared with a Covaris E210 system (Covaris, MA, USA) to target fragment sizes of 200 bp. We performed library preparation for tumor gDNA (>30 ng), cfDNA (>20 ng) and matched germline gDNA (>100 ng) using Accel-NGS 2S DNA Library Kit (Swift Biosciences, MI, USA) and target enrichment using xGen Lockdown Probes kit (Integrated Device Technology, Inc., CA, USA). The custom xGen Lockdown probe was synthesized by IDT, Inc. for the exons and parts of introns of 618 genes of interest. Samples underwent paired-end sequencing on an Illumina Novaseq 6000 platform (Illumina, CA, USA) with a 150-bp read length. The minimum coverage of 1,000×, 3,000×, and 500× were achieved for tumor gDNA, plasma cfDNA, and germline DNA, respectively.

Database and Genomic Analysis

Raw sequencing data were aligned to the reference human genome (UCSC hg19) through Burrows–Wheeler Aligner and producing a binary alignment/map (BAM) file. After the duplicate removal and local realignment by using Picard (http://broadinstitute.github.io/picard/), the Genome Analysis Toolkit (GATK) was used for single nucleotide variation (SNV), short insertions/deletions (indels) calling. Variants were annotated using the ANNOVAR software tool. Variants identified in gDNA from white blood cell (WBC) with allele fraction (AF) beyond 25% were determined as germline variants. Germline variants were filtered with following rules: (i) allele frequency (AF) below 25%; (2) variants were synonymous or not in the coding region (not including the splice-site variants); (3) occurred in over 1% population in the ExAC database (http://exac.broadinstitute.org/); and (4) known benign or likely benign variants (Clinvar). Interpretation of germline variants followed the standards and guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP).

After filtering out the germline variants identified in the matched WBC samples, variants with allele frequency (AF) beyond 1% were generated from each tumor gDNA and AF beyond 0.5% for plasma cfDNA, and further annotated according to the Catalog of Somatic Mutations in Cancer (COSMIC) database. The functional classification of each somatic alteration followed the interpretation and reporting standards and guidelines recommended by the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists (ASCO/CAP) and the Oncokb database (through cBioPortal for Cancer Genomics at http://www.cbioportal.org/) (19). Somatic mutation data, gene expression profiles, and clinical information of cervical cancer patients from the TCGA cohort were downloaded from the cBioPortal.

Analysis of the Functional Enrichment, Hypoxia Feature, and Tumor Environment

The "limma" package was used to screen the differentially expressed genes (DEGs) in the two groups using False Discovery Rate (FDR) <0.05 and Fold Change (FC) >1.5. Heatmaps were visualized using the "pheatmap" package. The Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were conducted by using the "ClusterProfiler" package (20) in the R studio (v. 3.4.3, https://rstudio.com/). The tumor mutation burden of each sample was calculated according to a published and widely applied method (21). The hypoxia feature was quantified by the previously described buffa hypoxia score (22) and ragnum hypoxia score (23). The CIBERSORT algorithm was used to calculate the proportion of infiltrating immune cells in cervical cancer samples (24).

Statistical Analysis

Differential mutations analysis was performed using the Chi-Square test or Fisher exact test under a dominant model. Two-sided P values less than 0.05 were considered to be statistically significant. All analyses were performed using SPSS 25.0 software.

RESULTS

The Characteristics of Cervical Cancer Patients in the Chinese Cohort

One hundred and twenty-nine Chinese patients diagnosed with cervical cancer were enrolled in this study with a median age of 48 (n = 97, range: 21 to 78 years). The subtypes included squamous cell carcinoma (SCC, n = 113, 87.60%), adenocarcinoma (AC, n = 12, 9.30%), adenosquamous carcinomas (ASC, n = 2, 1.55%), and neuroendocrine carcinoma (NEC, n = 2, 1.55%). In addition, 75 of the patients (58.14%) have diseases of FIGO (International Federation of Gynecology and Obstetrics [FIGO] staging system) stages III–IV (**Table 1**).

Somatic and Germline Alterations in Chinese Cervical Cancer Patients, and Correlation Between Genomic Alterations and Histologic Types

All surveyed samples had been identified with valid somatic alterations, and the mean and median counts of somatic

alterations per sample were 8.35 and 7, respectively. The most frequently altered genes in the patients were *PIK3CA* (27.13%), *TP53* (15.50%), *FBXW7* (11.63%), *ARID1A* (10.85%), and *PTEN* (10.08%), respectively (**Figure 1A**). We noticed that 9.30% of patients had *ERBB2* alterations and 6.97% of those had oncogenic alterations which were only identified in the tissue samples. Additionally, the most recurrent altered signaling pathways included RAS/RAF/MAPK (70.54%), DDR (60.47%), PI3K/ATK/MTOR (59.69%), cell cycle (36.43%), and epigenetic modifiers/chromatin remodelers (34.11%) (**Figure 1B**). Of note, PI3K/ATK/MTOR pathway had the most oncogenic alterations (47.15%). Moreover, three patients (2.33%) harbored pathogenic or likely pathogenic germline variants, including one *ATR*-K704*, one *BRCA1*-S1841fs, and one *POLE*- S2173fs, respectively.

In addition, the correlation analysis was further conducted to investigate whether one specific histologic subtype of cervical cancer was associated with the most frequently altered genes. It was found that there was a statistically significant difference in the alteration frequency of *TP53* among these four histologic types, showing that *TP53* alteration happened more frequently in ACs and ASCs ($p = 0.003$, **Table 2**). Moreover, it could be obviously observed that all *ARID1A* alterations happened in SCCs, but with no statistically significant difference ($p = 0.214$, **Table 2**).

The Comparison of Genomic and Actionable Alterations of Cervical Cancer Patients Between Chinese Cohort and Western Cohort

To determine the potential differences of genomic feature between Chinese and Western cervical cancer patients, we conducted a comparison of the genomic alterations data of the selected 618 genes between the Chinese and the Western cohort (published by the TCGA project) to identify the genetic differences. The genomic feature between the Chinese and the Western cohorts was similar, except the significant different prevalence of alterations in *KMT2C* (Chinese cohort vs Western cohort: 3.88% vs 18.56%), *RET* (Chinese cohort vs Western cohort: 6.20% vs 0.69%), and *TP53* (Chinese cohort vs Western cohort: 15.50% vs 7.90%) ($p < 0.05$, **Figure 2A**).

Next, we compared the frequency of actionable alterations between the Chinese and Western cohorts. Based on the OncoKB Levels of Evidence V2 (12/20/2019), 57 patients (44.19%) with 73 actionable alterations were identified in the Chinese cohort (**Figure 2B**), of which the ratio was approximately similar to the prevalence of actionable alterations in the Western cohort (47.42%). Besides, more patients had actionable variants of level 3 than level 4 (39.53% vs 4.65%), as nearly a quarter of the cervical cancer patients had actionable of alterations in *PIK3CA*, which may confer sensitivity to the PI3K or mTOR inhibitors. The rest actionable alterations were mainly enriched in the DDR and RAS/RAF/MAPK pathways, associated with increasing sensitivity to the poly (ADP-ribose) polymerase (PARP) Inhibitors and receptor tyrosine kinases (RTKs) inhibitors.

TABLE 1 | Characteristics of 129 patients with cervical cancer.

Variables		n (%)
Total		129
Age	Mean (range)	48 (21–78)
Histologic type		
	Squamous cell	113 (87.60%)
	Adenocarcinoma	12 (9.30%)
	Adenosquamous	2 (1.55%)
	Neuroendocrine	2 (1.55%)
FIGO Stage		
	I	11 (8.53%)
	II	43 (33.33%)
	III	38 (29.46%)
	IV	37 (28.68%)
Sample Type	Blood	57 (44.19%)
	Tumor	72 (55.81%)

FIGO stage, International Federation of Gynecology and Obstetrics [FIGO] staging system.

Alterations in DNA Damage Repair Pathway

A total of 61 patients (47.29%) harbored at least one alteration in 34 DNA repair genes defined by MSKCC (25), and the prevalence of specific genes in DDR was exhibited in **Figure 3A**. In addition, the frequently altered DDR signaling pathways were Homologous recombination (32.71%), Damage sensor (17.76%), Fanconi anemia (15.89%), Base excision repair (14.95%), Mismatch repair (13.08%), and Nucleotide excision repair (5.61%) (**Figure 3B**). Genes with known or likely deleterious variants among cervical cancer patients with DDR gene alterations were *ATM* (n = 3, 2.33%), *BRCA2* (n = 3, 2.33%), *ATR* (n = 2, 1.55%), *CHEK2* (n = 2, 1.55%), followed by *BRCA1* (n = 1, 0.78%), *FANCA* (n = 1, 0.78%), *MSH6* (n = 1, 0.78%), and *RAD51D* (n = 1, 0.78%) (**Figure 3C**).

The Comparison of Clinical Features of Cervical Cancer Patients With or Without DDR Alteration(s)

We identified a total of 92 cervical cancer patients (31.62%) from the TCGA cohort harboring DDR alterations, including 47 (16.15%) and 45 (15.46%) patients having deleterious DDR alteration and non-deleterious DDR alteration, respectively. The prevalence of total DDR alterations in the Western cohort was significantly lower than the Chinese cohort ($p < 0.05$). Next, we investigated the clinical features of cervical cancer patients with any DDR alteration (DDRmt group, N = 92) and without DDR alteration (DDRwt group, N = 199). Interestingly, a significantly older age at diagnosis was observed in the DDRmt group (average age at diagnosis: 51.18 vs 46.64 years old, $p = 0.01$, **Figure 4A**), and also more genetic mutations (median mutation count: 149.5 vs 66, $p < 0.0001$, **Figure 4B**). However, according to the histological grading for cervical cancer, there was no statistically significant difference between the two groups (DDRmt vs DDRwt group, G1–G2: 58.22% vs 53.88%, G3–G4: 41.78% vs 46.12%, $p = 0.39$, **Figure 4C**). Besides, we found a significantly decreased number of patients with T1 stage disease but a significantly increased patient number at T2 or T4 stage in the DDRmt group (stage T1: 44.29% vs 62.11%; stage T2: 40.00% vs 26.71%, $p < 0.05$; stage T3: 8.57% vs 9.31% at T3; stage

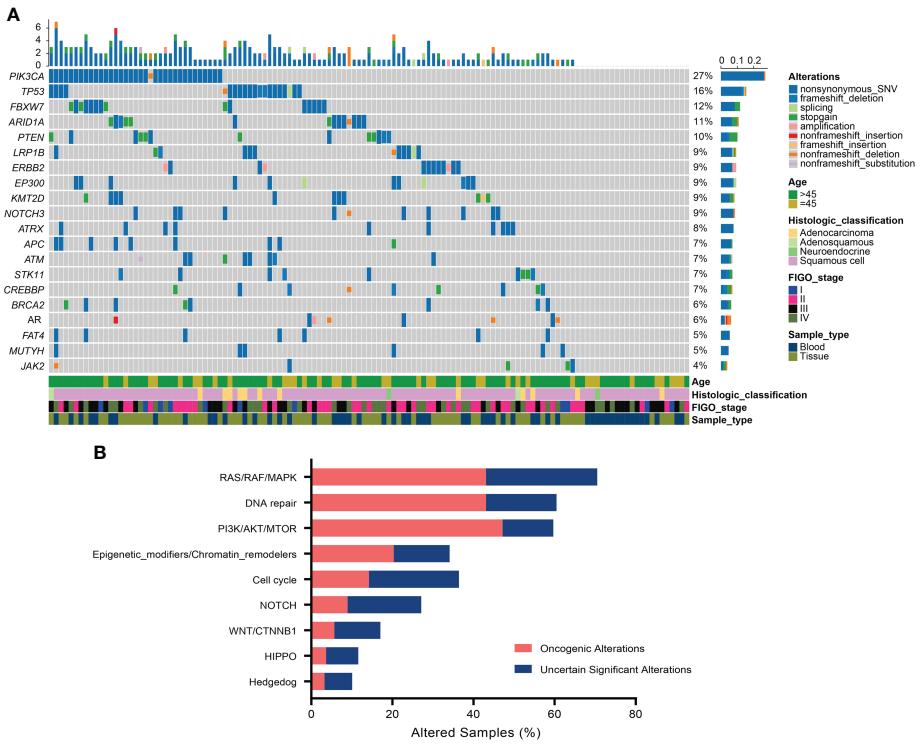

FIGURE 1 | Somatic alterations in Chinese cervical cancer patients. **(A)** Oncoprint of the top 20 frequently altered genes in 129 cervical cancer patients. **(B)** The distribution of most recurrent altered signaling pathways in the Chinese cervical cancer.

TABLE 2 | Genomic alterations in four histologic types among 129 cervical patients.

Altered gene (Patient number)	Histologic type				p-value
	SCC[1]	AC[2]	ASC[3]	NEC[4]	
	n = 113	n = 12	n = 2	n = 2	
PIK3CA (n = 35)	33 (29.20%)	1 (8.33%)	1 (50.00%)	0 (0.00%)	0.232
TP53 (n = 20)	13 (11.50%)	6 (50.00%)	1 (50.00%)	0 (0.00%)	0.003
FBXW7 (n = 15)	13 (11.50%)	2 (16.67%)	0 (0.00%)	0 (0.00%)	1.000
ARID1A (n = 14)	14 (12.39%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0.214
PTEN (n = 13)	10 (8.85%)	1 (8.33%)	1 (50.00%)	1 (50.00%)	0.205

[1]*Squamous cell carcinoma.*
[2]*Adenocarcinoma.*
[3]*Adenosquamous carcinoma.*
[4]*Neuroendocrine carcinoma.*

T4:7.14% vs 1.86%, p <0.05, **Figure 4D**). Also, no significant correlation between DDR mutation and the lymph node metastasis or long-distance metastasis status was found (p >0.05, **Figures 4E, F**).

In addition, we further explored the clinical features of the groups with deleterious DDR alteration (N = 47) or without (N = 244) this genomic feature. Similar to patients with any DDR alteration, we identified a significantly higher age at diagnosis in the patients with deleterious DDR alteration (average age at diagnosis: 52.66 vs 47.16 years old, p = 0.034, **Figure 4A**), and also a higher mutation count (median mutation count: 149 vs 78, p <0.0001, **Figure 4B**). By the statistical analysis of the patient number in high or low histological grading, we found no significant difference between two groups (p = 0.10, **Figure 4C**). Furthermore, we surveyed the specific associations between TNM stages and cervical cancer patients with deleterious DDR alteration but found neither tumor, lymph node nor long distant metastasis stage was significantly associated with deleterious DDR alteration (p >0.05, **Figures 4E, F**).

DDR Alteration, Hypoxia Feature, and Tumor Microenvironment

Signaling pathway analysis found that DDR alteration, regardless of its function, was significantly associated with hypoxia feature

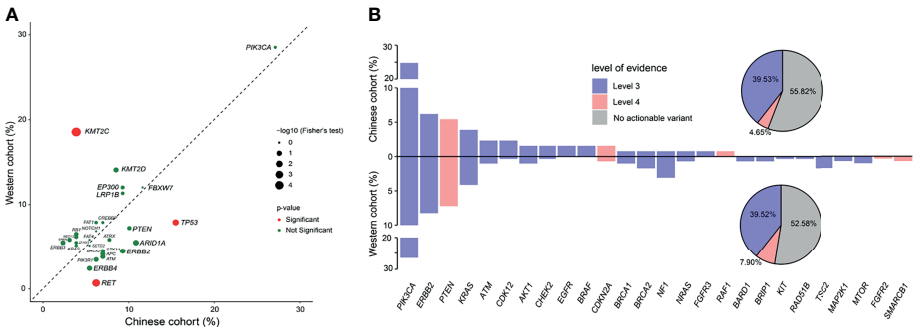

FIGURE 2 | Comparison of genomic and actionable alterations of cervical cancer patients between the Chinese cohort and the TCGA cohort. **(A)** Comparison of the prevalence of gene alterations identified between Chinese and Western cervical cancer patients. **(B)** Comparison of the actionable alterations identified between Chinese and Western cervical cancer patients.

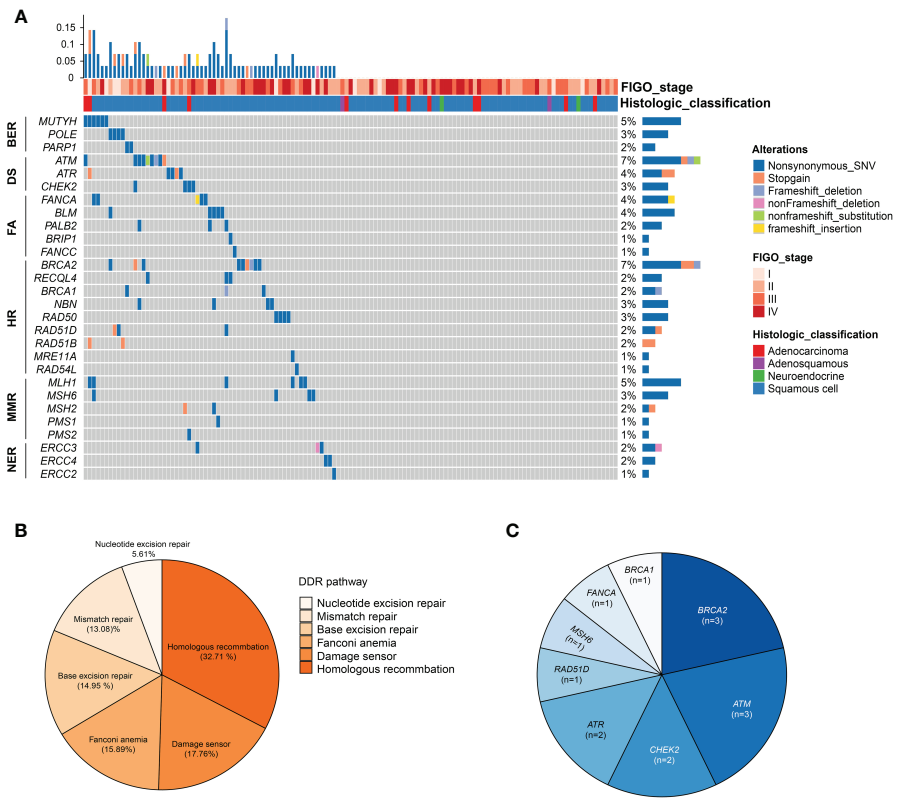

FIGURE 3 | Alterations in DNA damage repair (DDR) pathway. **(A)** Oncoprint of the DDR alterations in 129 cervical cancer patients. **(B)** Frequency of altered pathway of DDR. **(C)** The distribution of known or likely deleterious DDR alterations. HR, homologous recombination; FA, fanconi anemia; MMR, mismatch repair; NER, nucleotide excision repair; BER, base excision repair; DS, DNA sensor.

(**Figures 5A, B**). Subsequently, we found a significant difference in the hypoxia score between patients with or without DDR alteration(s). Remarkably, there was a significantly higher buffa hypoxia score in the DDRmt group (buffa hypoxia score: 26.32 vs 21.70, p = 0.024; ragnum hypoxia score: 16.61 vs 15.34, p = 0.026, **Figures 5C, D**). The findings were concordant when we compared this feature between cervical cancer patients with or without deleterious DDR alteration (buffa hypoxia score: 27.04 vs 22.36; ragnum hypoxia score 17.00 vs 15.49, p = 0.025, **Figures 5E, F**).

Though DDR alteration was not associated with cervical cancer patients' outcomes, the high hypoxia score or feature

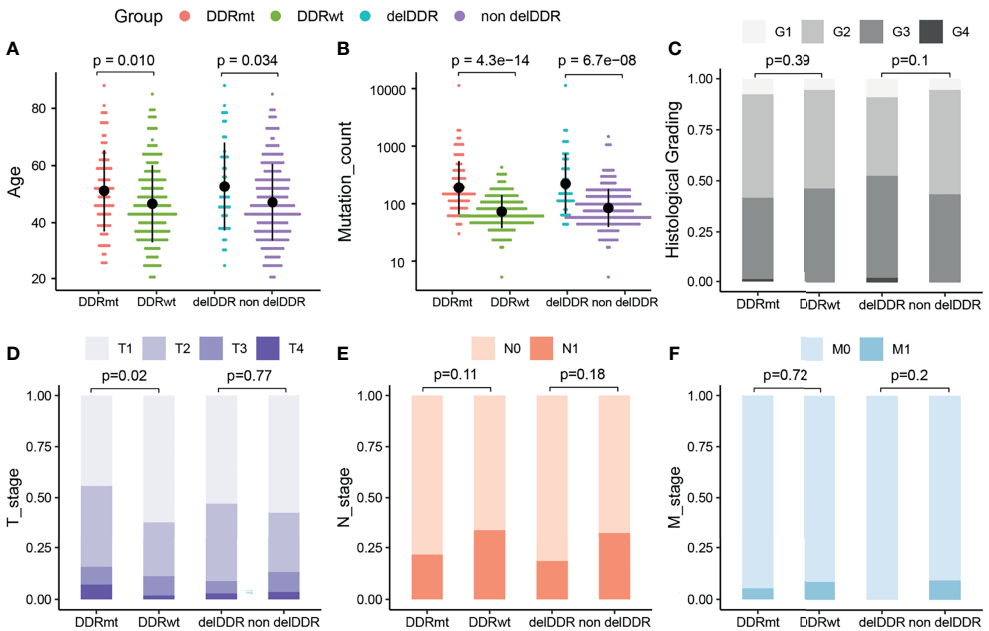

FIGURE 4 | The analyses of clinical features of cervical cancer patients with DDR and DDR alteration from the TCGA cohort. **(A)** Age at diagnosis of cervical cancer patients with and without any DDR alteration, and patients with deleterious DDR alteration or not. **(B)** Mutation count of cervical cancer patients with and without any DDR alteration, and patients with deleterious DDR alteration or not. Histological grading **(C)**, tumor stage **(D)**, lymph node stage **(E)**, and metastasis stage **(F)** of cervical cancer patients with and without any DDR alteration, and patients with deleterious DDR alteration or not. DDRmt, patients with any DDR alteration; DDRwt, patients without any DDR alteration; delDDRmt, patients with deleterious DDR alteration; nondelDDRmt, patients without any deleterious DDR alteration.

was associated with a worse outcome in the cervical patients from TCGA database (**Supplemental Figure 1**). Moreover, as hypoxia condition is usually connected with the tumor microenvironment, we evaluated the infiltrated immune cells level in cervical cancer patients with high or low hypoxia feature. Both the buffa and ragnum hypoxia scores were significantly associated with a decreasing level of CD8 positive T cells, activated mast cells but a higher level of resting mast cells and M0 macrophages (**Figures 6A–C**). Moreover, there is a significant abundance of NK cell resting, mast cell resting, and M0 macrophage in patients with high ragnum hypoxia score, while a significant abundance of CD8+ T cells, NK cell activated, mast cell activated, and M2 macrophage in patients with low ragnum hypoxia score ($p < 0.05$, **Figure 6C**).

Hub Gene(s) Identification

We conducted DEGs analysis between samples with and without DDR alteration in the TCGA cohort (**Figure 7A**), and samples with high and low hypoxia scores (**Figure 7B**), respectively. Notably, there were only three genes were identified in both the DDRmt and high hypoxia groups, namely, *CDHR5*, *MYO7B*, and *ANKS4B* (adjust $p < 0.01$, **Supplemental Table 1**), which were all downregulated. The protein–protein interactions (PPI) network of DDR and hypoxia score was constructed by the STRING database, and hub genes were selected from the PPI network by using Maximal Clique Centrality algorithm of CytoHubba plugin, respectively (**Figures 7C, D**). The top 10 high-scored hub genes were selected, but only one gene (*CDHR5*) was shared by the two PPI network. The expression of *CDHR5* was not associated with cervical cancer patients' survival (**Figure 7E**). However, a significant higher count of B cell, CD8 positive T cells, resting CD4 positive T memory cells, regulatory T cells, gamma delta T cells, and resting NK cells were presented in cervical cancer samples with high *CDHR5* expression. On the contrary, more M1 and M2 macrophage and myeloid dendritic cells were in the samples with low *CDHR5* expression (**Figure 7F**).

DISCUSSION

Over the past decades, the overall survival of advanced cervical carcinoma has not been strikingly improved, mainly attributing to slow drug development. Unlike ovarian carcinoma, the most prevalent genes in cervical carcinoma patients have poor relationship with any target therapy with high efficacy (5). Furthermore, the genetic feature of Chinese cervical carcinoma patients has not been clarified yet.

Initially, we found significant differences in the genetic features between our cohort and the TCGA database, namely, a different prevalence of *KMT2C*, *RET*, and *TP53*. The frequency of *TP53* in our cohort was nearly equivalent with previous result in a 32-patient cohort (15.50% vs 15.60%, $p > 0.05$) (17), both of which were more prevalent among Chinese cervical patients in comparison with those (7.90%) in the Western cohort ($p < 0.05$). Meanwhile, in a 32-sample cohort the frequency of

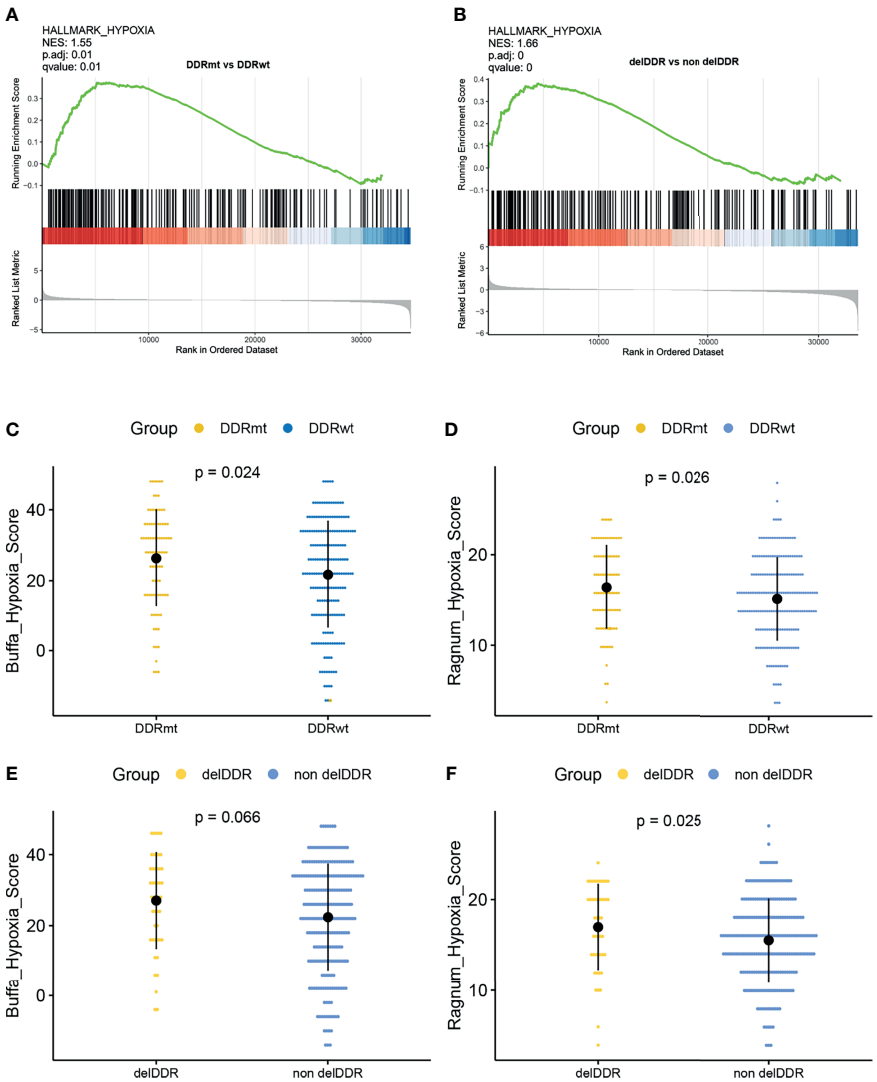

FIGURE 5 | The correlation analyses between the DDR alterations and hypoxia features. **(A)** Gene set enrichment analysis identified hallmark_hypoxia in patients with DDR alteration. **(B)** Gene set enrichment analysis identified hallmark_hypoxia in patients with deleterious DDR alterations. Comparison of the buffa_hypoxia score **(C)** and ragnum_hypoxia score **(D)** between patients with and without any DDR alteration. Comparison of the buffa_hypoxia score **(E)** and ragnum_hypoxia score **(F)** between patients with or without deleterious DDR alterations.

KMT2C was also significantly lower than the result in the TCGA cohort (<9.00% vs 18.56%, p <0.05), but the Western groups had similar frequency of *KMT2C* (TCGA cohort vs 182-patient cohort: 18.56 vs 16.00%, p >0.05) (26). Of note, the prevalence of *RET* in Chinses cohort was first found in the present study. Our study revealed the different genetic profiles of cervical cancer patients with different genetic backgrounds. However, the prevalence of the most recurrent genes and actionable genes were similar, and notably, over 40% of investigated Chinese and Western cervical cancers patients harbored at least one actionable genomic alteration, which was also close to the previous findings of Zehir et al. (6). The most prevalent actionable alterations were in PI3K signaling pathway, especially for *PIK3CA* and *PTEN*. A recent study also demonstrated the high prevalence of *PIK3CA* alterations in cervical carcinoma patients with 31.30% altered samples, suggesting the promising targeted therapy with related PI3K or mTOR inhibitor (27). However, only limited evidences supported the correlation of *PIK3CA* alterations with the response to the mTOR inhibitors in cervical carcinoma (28). Besides, *ERBB2* is widely altered in solid tumors, especially breast and gastric cancers. Previous studies found that nearly 5.5 and 3.15% of Western and Chinese invasive cervical carcinoma patients had *ERBB2* alterations, which were associated with a worse prognosis (29, 30). In our study, we found 9.30% of the patients had *ERBB2* alterations, but 6.97% had oncogenic alterations, including 6 gains of function missense variants and 3 of amplification. Interestingly, all the oncogenic *ERBB2* alterations were only identified in the tissue samples. Early

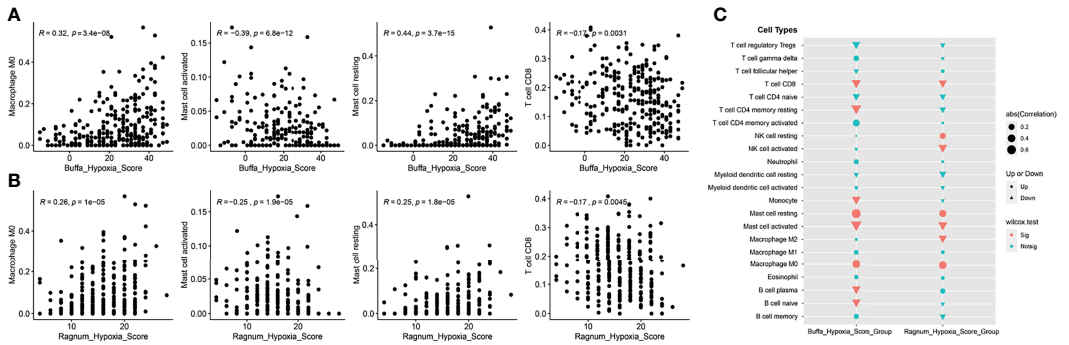

FIGURE 6 | The analysis of tumor microenvironment (TME). The correlation analyses between the buffa **(A)** or ragnum **(B)** hypoxia score and the infiltrated immune cells level. **(C)** Comparison of infiltrating immune cells between high- and low- hypoxia score groups. Up represents "positive correlation", Down represents "negative correlation". Sig represents "significant", Notsig represents "not significant".

research on patient-derived xenograft derived from the cervical carcinoma patients found anti-HER2 therapy, the combination of trastuzumab and lapatinib inhibited tumor growth. Neratinib, an ERBB2 inhibitor, showed a confirmed objective response rate of 25% and progression-free survival of 7.0 months in 10 cervical carcinoma patients from the phase 2 SUMMIT basket trial (31). In addition, 10.88% of Chinese cervical carcinoma patients in our cohort were identified to harbor functional DNA damage repair alterations, similar to the prevalence in Western patients (16.15% in the TCGA cohort and 13.2% in another cohort with 824 Western cervical patients) (32). In the past decades, PARP inhibitors have been the promising targeted therapies for pan-cancers, especially for those with homology recombination deficiency. Though they have made remarkable progress in multiple solid tumors, namely, ovarian, breast, pancreatic, and prostate carcinoma, results of the efficacy of PARP inhibitors in cervical cancer are still quite poor. One study analyzed the combination of chemotherapy (paclitaxel and cisplatin) with PARP inhibitor (Veliparib) in 34 biomarker-unselected persistent or recurrent cervical carcinoma patients, showing a promising ORR of 34%, and the median PFS and OS were 6.2 and 14.5 months, respectively (33). Enlightened by the results of trails on biomarker-guided match-therapy (34), it would be recommended that these cervical cancer patients with actionable alterations in our cohort could try the matched therapy when they progressed following prior treatment or without satisfactory alternative standard treatment options.

In the present study, it was found that DDR alteration was positively correlated with the hypoxia score, especially for the deleterious DDR alteration indicating the higher hypoxia score. In addition, both the buffa and ragnum hypoxia scores, described in previous studies (22, 23), were negatively implicated with the level of CD8+ T cells which play a pivotal role in cancer immunity and are associated with a better response in patients receiving ICIs (35). The immune checkpoint inhibitors are promising treatments for various advanced cancers. FDA had approved pembrolizumab for treating patients with recurrent or metastatic cervical cancer based on the phase II KEYNOTE-158 study, though its objective response rate (ORR) was only 12.2% (4). Given the limited response rate of anti-PD-1 therapy, it is vital to identify robust biomarkers for distinguishing cervical patients who may benefit from ICIs treatment. DDR alteration was widely suggested as an effective biomarker for predicting the potential responder in multiple types of cancer, including lung, bladder, and renal cell carcinoma (36–38). Furthermore, DDR alteration may lead to genomic instability, namely, mismatch instability and chromosomal rearrangements, and further affects the tumor immune microenvironment by activating of T cells and adaptative immune system (39). However, there was no study revealing the relationship among DDR alteration, tumor microenvironment, and ICIs efficacy in cervical cancer. Our study is the first one suggesting that although DDR alteration was associated with a higher TMB value, and it was also positively related to increasing hypoxia feature, which may reshape the immune suppressive tumor microenvironment. DDR alterations, regardless of their specific function, were positively associated with both the higher hypoxia score and hypoxia feature in cervical cancer patients. Previous studies have suggested a complex relationship between hypoxia and DDR function, revealing a multifaceted regulatory role of hypoxia for DDR (40). For chronic tumor hypoxia, it downregulated most DDR pathways to silence their function in maintain genomic stability. Tumor hypoxia is not only associated with the development of malignancy and therapeutic resistance as an indicator for poor outcomes but also serves a vital determinant of tumor microenvironment (41). Previous studies also demonstrated that hypoxia could suppress the NK cell function, affect the contents of effective and regulatory T cells, and promote the polarization of macrophages to M2, a immunosuppressive phenotype (42). Thus, it could be suggested that DDR alteration could not function as a robust determinant for predicting the efficacy of ICIs in cervical cancer patients as other types of cancers, which need to be further verified.

Furthermore, we identified *CDHR5* as the significant hub gene solely related to both DDR alteration and hypoxia score. This gene belongs to the superfamily of cadherin, and participates in multiple physical processes including cell

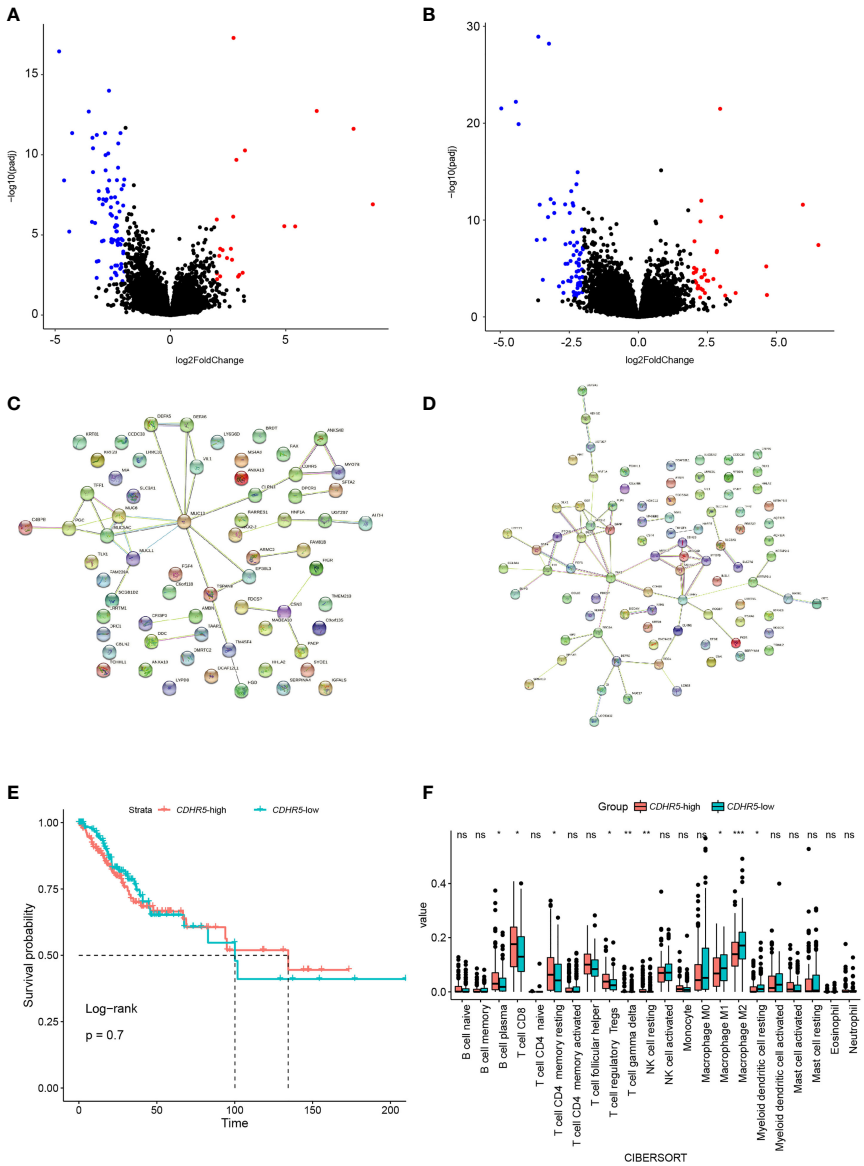

FIGURE 7 | DEGs analysis between samples with and without DDR alteration **(A)**, and samples with high and low hypoxia scores **(B)**, respectively. The protein–protein interactions (PPI) network of DDR **(C)** and hypoxia score **(D)**, respectively. **(E)** The overall survival analysis between the groups with high or low expression of hub gene *CDHR5*. **(F)** Analysis of tumor infiltrated lymphocytes in cervical cancer samples with high and low *CDHR5* expression. *p < 0.05, **p < 0.01, ***p < 0.001, ns, not significant.

adhesion and branching morphogenesis of organs (43). Previous studies have suggested controversial roles of *CDHR5* in the cancer progression in different cancer types, but according to the decreased expression level in tumor tissues than the adjacent non-tumor tissues, it's more likely to function as tumor suppressor (43–46). Its decreased expression in the tumor was associated with hypermethylation and transcriptional regulation. Though Beck and his colleagues found the negative correlation between *CDHR5* and DNA replication and repair (44), the explicit relationship between *CDHR5* and DDR or hypoxia has not been established yet. This is the first study that suggested the negative correlation between *CDHR5* and DDR or hypoxia in the cervical cancer, which merited further study.

The work presented here has several limitations. Firstly, it is limited by the sample size to comprehensively understand the genetic profiling of Chinese cervical cancer patients, and further study with a larger sample size is required to fully evaluate the findings. Secondly, we just investigated the potential correlation between DDR alteration, hypoxia feature, and tumor microenvironment, but whether the efficacy of ICIs in cervical cancer patients with or without DDR alteration is different merits further study.

CONCLUSIONS

In summary, this study provided a comprehensive analysis of genomic alterations in Chinese patients with cervical cancer. Genomic profiling of Chinese patients uncovered a unique genomic feature and widely prevalent actionable variants, especially in PI3K and DDR pathways, which could guide clinical management in future. Moreover, we found the association between DDR alteration, hypoxia feature, and tumor microenvironment in cervical cancer, namely, the negatively regulated hub gene *CDHR5*, suggesting that DDR alteration(s) could not function as a robust predictor of ICIs in cervical cancer patients.

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.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Fudan University Cancer Center Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JL, HW and X-LZ proposed the design of this study. HW and Q-HG collected samples and conducted data analysis. JL, HW, Q-HG and X-LZ wrote the manuscript draft. X-HW and JL revised 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/fonc.2021.792003/full#supplementary-material

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CASE REPORT
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Genomic Feature of a Rare Case of Mix Small-Cell and Large-Cell Neuroendocrine Lung Carcinoma: A Case Report

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Background: Cases of both of small- (SCLC) and large-cell neuroendocrine lung carcinoma (LCNEC) were rarely reported. Although typical cases are morphologically distinct, the distinction between LCNEC and SCLC is still controversial, with some LCNECs showing close morphologies with SCLC. Here, we reported on a patient who had tumor with a mix of SCLC and LCNEC and uncovered these components' histological and genomic features.

Case Presentation: A 59-year-old man was diagnosed with lung cancer and had resection surgery in our hospital. The H&E and immunohistochemistry staining revealed that the tumor had 30%–35% LCNEC and 65%–70% SCLC cells. The whole-exome sequencing (WES) identified no potentially actionable alteration in the tumor sample but found five alterations all with allele frequency over 90%, including *TP53* p.R273H, *MYH8* p.Q1814K, *SLC17A6* p.W505L, *PTPN5* p.M40I, and *RB1* p.L267X. The genomic results supported that these two different components shared a similar dominant clonal origin. Furthermore, fluorescence *in situ* hybridization analysis revealed that the LCNECs have a higher copy number of MET than the SCLC component while without notable difference in the copy number of HER2 and TP53. Chemotherapy with pemetrexed and carboplatin was administrated for two cycles after the surgery. Although the chest CT showed remission in the lung, he was diagnosed with bone metastasis in 1 year later. Then, he received chemotherapy with etoposide and carboplatin but had severe side effect, leading to the discontinuation of the regime. Unfortunately, he returned to the local hospital with supportive care and died shortly after.

Conclusion: Based on these observations, we proposed that LCNEC and SCLC components in this patient may have a common clonal origin with dual mutations in *TP53* and *RB1*, while the chromosome instability may cause multiple independent conversion that leads to LCNEC or SCLC morphologies.

Keywords: small cell lung cancer (SCLC), large-cell neuroendocrine lung carcinoma (LCNEC), genomic feature, FISH, case report, MET

INTRODUCTION

Although only approximately 13% of all lung cancer cases are small-cell lung cancer (SCLC), it remains the sixth most common cause of cancer-related death worldwide due to early metastasis and rapid progression (1). Meanwhile, large-cell neuroendocrine lung carcinoma (LCNEC) represents roughly 3% of all lung cancer cases. According to the fourth edition of the World Health Organization classification of lung tumors, it is categorized as a neuroendocrine tumor with SCLC (2). SCLC and LCNEC are mainly distinguished by morphological features; however, the definitive distinction is still controversial (3, 4). Although typical cases are morphologically distinct, some LCNECs showed close morphologies with SCLC (3, 4). Recent molecular characterization shed new light on the classification of SCLC and LCNEC tumors. Here, we reported on a 59-year-old male patient who had tumor with a mix of SCLC and LCNEC and analyzed their histological and genomic features.

CASE PRESENTATION

A 59-year-old man was transferred to our hospital in May 2015, with a 4.8 × 3.5 cm nodule with clear boundaries in the right lower field revealed by the chest computed tomography (**Figure 1**). Then, surgery was performed with video-assisted thoracoscopic resection of the right lower lobe and lymph nodes. The pathological evaluation showed a 6.0 × 4.0 × 3.3 cm tumor mass, and by hematoxylin–eosin (H&E) staining and immunohistochemistry (IHC) staining, it was demonstrated that 30%–35% of the tumor cells were LCNEC, and the rest 65%–70% were SCLC (**Figure 2A**). Both the small- and large-cell components were positive for NCAM (CD56), synaptophysin (Syn), and thyroid transcription factor-1 (TFF1) but negative for cytokeratin 19 (CK19), which were indicative of neuroendocrine tumor. The Ki67 staining was positive for both the small- and the large-cell components, with the small cells having a high percentage of positive cells (67.5% versus 47.5%, **Figure 2B**).

In order to identify actionable genomic alterations to guide patient's treatment, genetic testing of the whole tumor sample was performed. However, the whole-exome sequencing (WES) identified no actionable alteration in the tumor samples. WES data showed that, in addition to the high allele frequency (AF) of *TP53* R273H (AF, 98.9%), which is a well-studied pathogenic mutation, alterations with high allele frequency were found in *MYH8* (95.3%), *SCL17A6* (93.1%), *PTPN5* (92.1%), and *RB1* (90.0%) (**Table 1**), indicating that both the SCLC and the LCNEC components were of the same mutant genotype. The *RB1* c.799delC mutation was not reported in the ClinVar or COSMIC database, and as it resulted in a premature stop codon (p.L267X) that led to a non-functional protein, so it was classified as a novel pathogenic mutation. The *SCL17A6* p.W505L was also not presented in ClinVar database but had been identified previously in lung cancer as documented in the COSMIC database with a highly pathogenic FATHMM score of 0.99. The *MYH8* p.Q1814K and *PTPN5* p.M40I had not been reported in the COSMIC database, indicating that they are likely to be novel mutations. Furthermore, fluorescence *in situ* hybridization test (**Figure 3**) showed that *MET* was amplified in the large-cell components with an average copy number of 5.51, whereas for the small-cell component, the *MET*

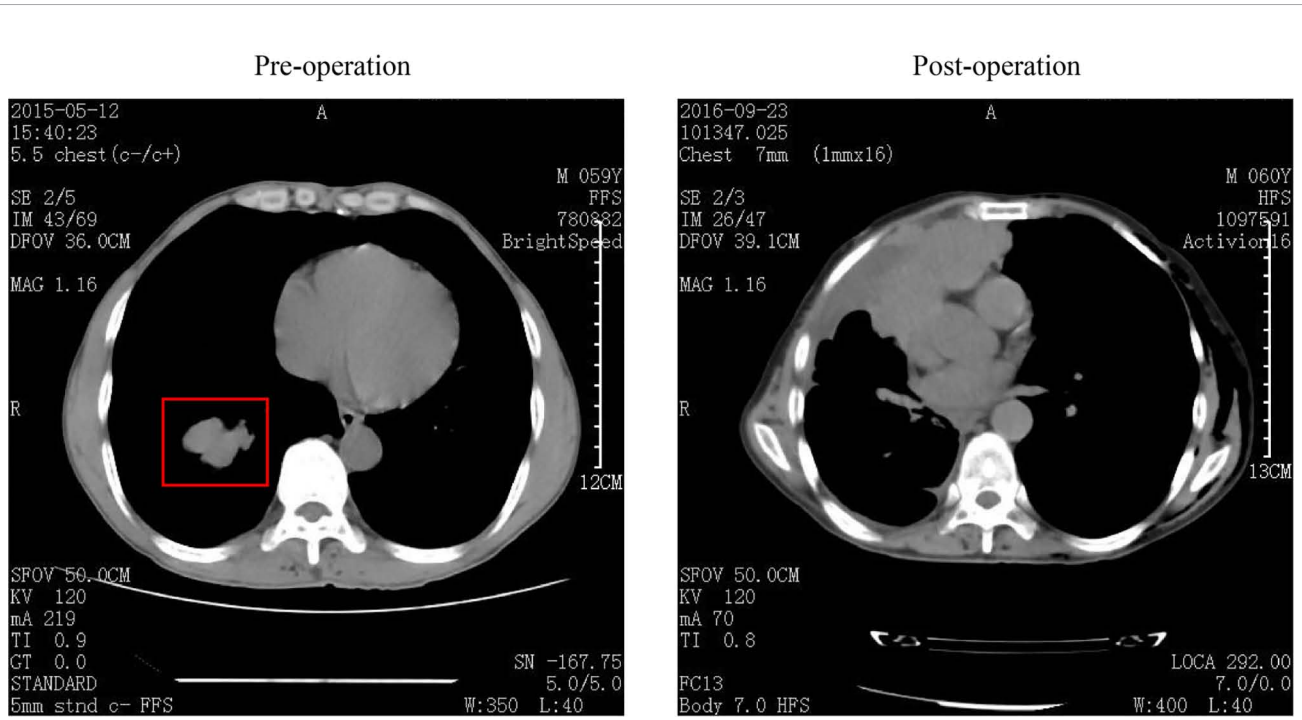


FIGURE 1 | Computed tomography (CT) images of this patient. CT image was collected before (left) and after (right) surgery, and the tumor mass was labeled within the red box.

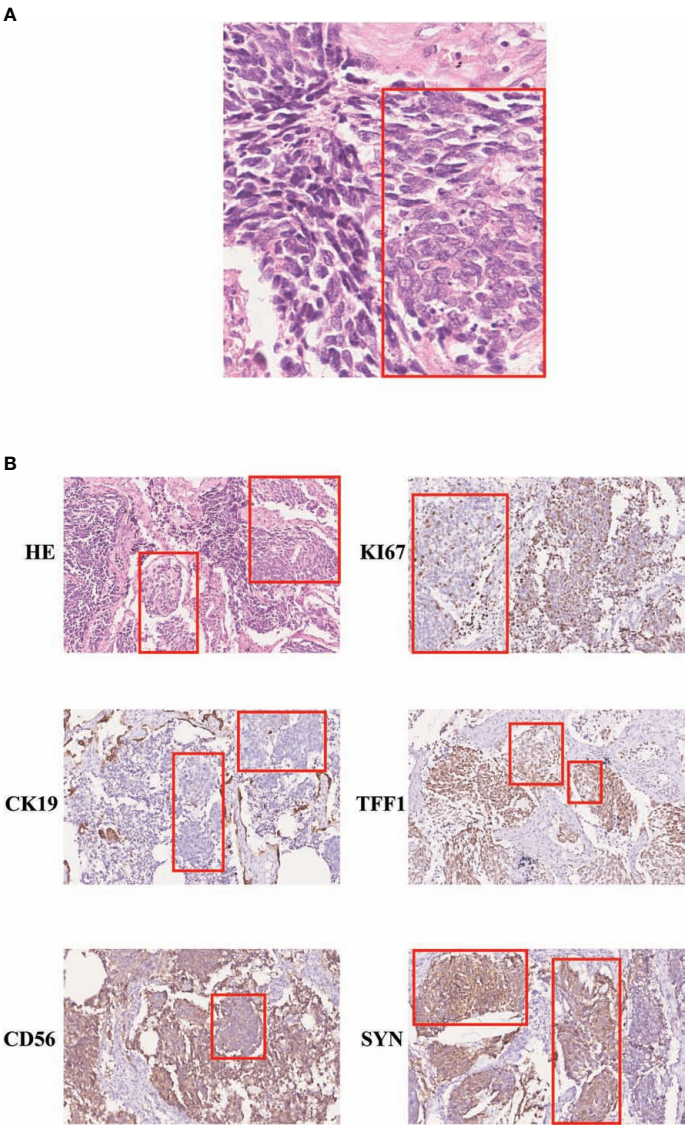


FIGURE 2 | Immunohistochemistry staining of the SCLC and LCNEC regions. **(A)** H&E staining (400×); **(B)** immunohistochemistry staining of KI67, CK19, TFF1, CD56, and SYN (200×). The large-cell components were labeled within the red box.

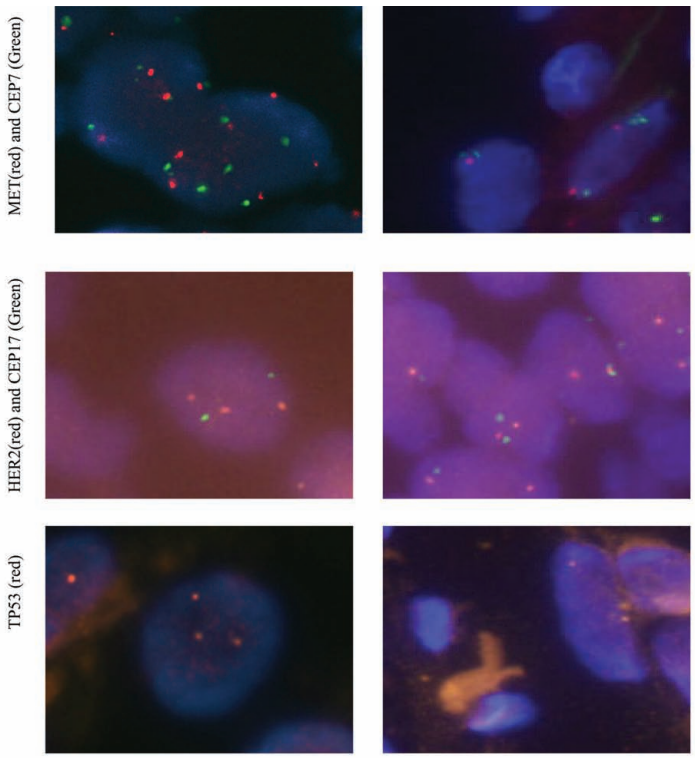
TABLE 1 | High allele frequency mutations identified by WES in the tumor sample.

Chr	Gene	Freq	Mut/Wt	Transcript	cDNA	Protein	COSMIC	FATHMM	χ^2 test
17	TP53	98.9%	117/1	NM_000546	c.G818A	p.R273H	10660	Pathogenic	4.8E–12
17	MYH8	95.3%	212/13	NM_002472	c.C5440A	p.Q1814K	None	Unknown	2.2E–15
11	SLC17A6	93.1%	58/2	NM_020346	c.G1514T	p.W505L	6132215	Pathogenic	6.6E–06
11	PTPN5	92.1%	76/3	NM_006906	c.G120T	p.M40I	None	Unknown	3.7E–07
13	RB1	90.0%	30/3	NM_000321	c.799delC	p.L267X	None	Pathogenic	8.8E–03

The χ^2 test was done against an expected frequency of 70%.

copy number was gained to 4.22 but did not reach the threshold of five copies per cell. To find out whether the cells were polyploid, HER2, CEP17, and TP53 were also tested, and three copies of HER2, CEP17, and TP53 (**Figure 3**) were detected in the large-cell component, but less than three copies of HER2, CEP17, and TP53 were detected in the small-cell component (**Supplemental Figure S1**). These results indicated that the large and small components of the tumor had different ploidy, which were also

A



B

	LCNEC	SCLC		LCNEC	SCLC		LCNEC	SCLC
Evaluated cells	100	100	Evaluated cells	40	40	Evaluated cells	100	100
MET signal	551	422	HER2 signal	128	100	TP53 signal	295	266
CEP7 signal	466	359	CEP17 signal	122	98	TP53 copy number	2.95	2.66
MET/CEP7 ratio	1.18	1.17	HER2/CEP17 ratio	1.04	1.02			
MET copy number	5.51	4.22	HER2 copy number	3.19	2.5			
CEP7 copy number	4.66	3.59	CEP17 copy number	3.06	2.45			
Result	**MET amplification**	**Negative**	**Result**	**Negative**	**Negative**			

FIGURE 3 | Results of MET, HER2, and TP53 FISH in large- and small-cell components. **(A)** FISH images of MET, HER2, and TP53 FISH in LCNEC (left) and SCLC components (right). The magnification was 1,000×. **(B)** Quantification of MET, HER2, and TP53 copy number in FISH. LCNEC, large-cell neuroendocrine lung carcinoma; SCLC, small-cell lung cancer.

validated by the evaluation of the single nucleotide polymorphism (SNP) frequency generated by WES of the tumor and non-tumoral lymph node samples. Additional whole chromosome trisomy was found on Chr3, 21, and 22; regional trisomy was found on Chr5, 9, and 11, and loss of heterozygosity (LOH) was found on Chr11 and 13 (**Supplemental Figure S2**).

Chemotherapy of 800 mg pemetrexed and 400 mg carboplatin was administrated for two cycles after the surgery. A year later, chest CT showed remission in the lung, but he was diagnosed with bone metastasis. Then, chemotherapy with 100 mg × 3 etoposide and 200 mg carboplatin was administrated. Unfortunately, the patient had severe side effect and did not continue with the regime; then, he returned to the local hospital with supportive care but died shortly after.

MATERIAL AND METHODS

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks were cut into 4-μm sections, deparaffinized in xylene, and rehydrated in a graded series of ethanol. Antigen retrieval was performed using citric (CK19, CD56, synaptophysin) or Tris–EDTA buffer (TTF-1 and Ki67). Immunohistochemistry was performed using primary antibodies and ultrasensitive second antibody kit (PV-9000, Zsbio Inc., Beijing). The following primary antibody working solutions were used: CK19(ZM-0074), CD56 (ZM-0057), synaptophysin (Syn) (ZM-0246), TTF-1 (ZM-0250), and Ki67 (ZM-166).

DNA Extraction From FFPE Tissue

The FFPE sections were deparaffinized in dewaxing agent (Wuxi Jiangyuan Industrial and Trade Co., Jiangsu, China) at 60°C for 1 min, washed with 100% ethanol at room temperature, and air dried for 10 min. Genomic DNA was isolated from the tumor and lymph node FFPE samples by using the Biomark FFPE Genomic DNA Kit (Beijing ACCB Biotech, Beijing, China) in accordance with the manufacturer's instructions.

Whole-Exome Sequencing and Data Analysis

DNA from FFPE sections of the tumor or lymph node were sequenced by Bionova (Beijing, China). Briefly, the DNA samples were fragmented and captured by IDT's xGenExome Research Panel (Integrated DNA Technologies, San Diego, USA) and sequenced by using the Illumina HiSeq^{TM}4000 platform with 150 bp pair-end reads with a total coverage of 200×. The sequencing reads were aligned to the human reference genome hg19/GRCh37 using the Burrows–Wheeler Aligner tool, and the PCR duplicates were removed by using Picard v1.57 (http://picard.sourceforge.net/). GATK (https://software.broadinstitute.org/gatk/) were employed for variant calling. Variant annotation and interpretation were conducted through the use of ANNOVAR. Somatic mutations were defined as mutations found in the tumor tissue of the patient but not in the cancer-free lymph node.

FISH

FFPE sections of the tumor and lymph node were pretreated with Vysis Paraffin Pretreatment IV (Abbott Molecular, IL) according to the manufacture's instruction. Probe mixture for HER2, MET, and TP53/CEP17 (Abbott Molecular, IL) was added onto the hybridization area, then coverslipped and sealed with rubber cement. Slides were incubated in Termobrite (Abbott) at 73°C for 5 min (HER2, TP53/CEP17) or 73°C for 3 min (MET) for denaturation, and hybridized at 37°C overnight. The sections were washed by using Post-Hybridization Wash Buffer Kit (Abbott). After gently removing the rubber cement and coverslip, the slides were washed in Washing Buffer II (HER2, TP53/CEP17) at 72°C for 2 min or Washing Buffer II (MET) at 74°C for 2 min. Then, the slides were washed briefly in 70% EtOH, air-dried in darkness, and stained with 4′,6-diamidino-2-phenylindole (DAPI) counterstain and coverslipped. FISH results were examined with a BX43 fluorescence microscope (Olympus), and photographs were taken with a digital camera (CellSens) by using appropriate filters.

DISCUSSION

Although SCLC and LCNEC are distinguished by morphological features, the expression of the neuroendocrine markers such as CD56 and synaptophysin is indicative of a similar origin (3, 4). Recent molecular characterization showed that SCLC and LCNEC tumors had overlapping mutation profiles, which complicated their classification. In this study, the histological and genomic feature of a rare case of mix SCLC and LCNEC was analyzed. Although the tumor sample contained about a third of LCNEC cells, pathogenic alterations *TP53* p.R273H and *RB1* p.L267X were found at an AF of 98.9% and 90%, respectively, indicating that both the SCLC and LCNEC components harbored the pathogenic *TP53* and *RB1* alterations. The dual inactivation of *TP53* and *RB1* is a prominent feature for SCLC as reported by multiple independent studies (5–8). For LCNEC, genetic and gene expression analysis of 45 morphologically identified cases showed that 40% cases were SCLC-like as characterized by *TP53* and *RB1* co-mutation and gene expression profiles, and the rest 56% had the NSCLC-like profiles instead, lacking dual mutation in *TP53* and *RB1* (9). Since both LCNEC and SCLC are neuroendocrine tumors, in a study of 148 lung neuroendocrine tumors that included LCNEC, SCLC, and carcinoids, distinct mutational landscape was noticed for carcinoids and carcinomas, but LCNEC and SCLC showed similar mutational profiles except for the high prevalence of *RB1* mutation in SCLC, and *SMARCA2* mutation is found exclusively in LCNEC (10). A recent study on LCNEC, SCLC, and LC showed that *RUNX1*, *ERBB4*, *BRCA1*, and *EPHA3* distinctively mutated in LCNEC, although the mutation frequency was moderate, and consistent with a previous study, 4/14 of LCNEC cases showed dual inactivation mutation in *TP53* and *RB1* (11). The result of the current study is in line with these reports, which highlighted the similarity of a subset of LCNEC to SCLC. Yet, due to that the SCLC and LCNEC cases were of independent patients, it is hard to conclude whether the SCLC and LCNEC subset had the same oncogenesis path. The current case study offered a unique opportunity to study the origin of SCLC and LCNEC. First, the SCLC and LCNEC components were derived from the same patient, rendering them identical in genetic background and environmental influences. Second, the SCLC and LCNEC components did not originate from separate locations but were present as multiple intermingled nests. Third, in addition to *TP53* and *RB1*, high-frequency mutations in genes such as *MYH8* (95.3%), *SCL17A6* (93.1%), and *PTPN5* (92.1%), which located on different chromosomes, were also identified. This indicated that the similarity of genetic mutation in SCLC and LCNEC components are unlikely to be originated independently; a more likely scenario is that the SCLC and LCNEC components had the same origin of early oncogenesis, and they were derived from the same mutant clone that harbors these mutations.

If the SCLC and the LCNEC components originated from the same clone, why were they of different morphologies? To answer this question, the best study would be to isolate the SCLC and LCNEC components and perform mutation and gene expression analysis on them. The intermingled growth of the SCLC and LCNEC components, however, made the dissection technically difficult. FISH study at the single cell level allowed a preliminary evaluation of the genetic differences of the two components. We found that the LCNEC portion had slightly higher copy numbers in *MET*, which indicated that after the initial clonal growth, subsets of cells diverged. Although *MET* copy number was above the threshold as a biomarker for TKI treatment, the SCLC had a higher Ki67 levels than the LCNEC component.

For patients with mix pathological tumor components, the prognosis was usually poor. The heterogeneity *per se* may indicate a high level of genomic instability, which renders the

tumor a higher chance to mutate and gain drug-resistant features. In addition, the subclones or the heterogeneous components may contain different signal transduction pathways, and the inhibition of one pathway may hinder the growth of a portion of cells but not the rest. The development of drugs that targets different subclones/components may be necessary for the effective control of tumor growth.

In summary, this study reports a rare case of mix SCLC and LCNEC. The molecular analysis indicated that the SCLC and LCNEC were derived from the same early clone that harbors *TP53* and *RB1* null mutations, and mutations in *MYH8*, *SCL17A6*, and *PTPN5*. We propose that LCNEC containing dual mutations in *TP53* and *RB1* can have a common clonal origin with SCLC, with the genomic instability that causes additional mutations for the diversion to LCNEC or SCLC.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Jiaxing University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YZ: manuscript writing. FZ: data collection and analysis. DY: data collection. FW: data collection. MY: data collection. LC: data collection. CX: data collection. YH: project development and data collection. FD: project development and manuscript writing. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.794744/full#supplementary-material

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Downregulation of miR-211-5p Promotes Carboplatin Resistance in Human Retinoblastoma Y79 Cells by Affecting the GDNF–LIF Interaction

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Purpose: To investigate the role of the miR-211-5p-GDNF signaling pathway in carboplatin resistance of retinoblastoma Y79 cells and what factors it may be affected by.

Methods: A carboplatin-resistant retinoblastoma cell line (Y79R) was established *in vitro*. RNA-seq and microRNA-seq were constructed between Y79 and Y79R cells. RNA interference, RT-PCR, Western blot (WB), and flow cytometry were used to verify the expression of genes and proteins between the two cell lines. The TargetScan database was used to predict the microRNAs that regulate the target genes. STING sites and Co-Immunoprecipitation (COIP) were used to study protein–protein interactions.

Results: GDNF was speculated to be the top changed gene in the drug resistance in Y79R cell lines. Moreover, the speculation was verified by subsequent RT-PCR and WB results. When the expression of GDNF was knocked down, the IC50 of the Y79R cell line significantly reduced. GDNF was found to be the target gene of miR-211-5p. Downregulation of miR-211-5p promotes carboplatin resistance in human retinoblastoma Y79 cells. MiR-211-5p can regulate the expression of GDNF. Our further research also found that GDNF can bind to LIF which is also a secreted protein.

Conclusion: Our results suggest that downregulation of miR-211-5p promotes carboplatin resistance in human retinoblastoma Y79 cells, and this process can be affected by GDNF–LIF interaction. These results can provide evidence for the reversal of drug resistance of RB.

Keywords: retinoblastoma, carboplatin resistance, miR-211-5p, glial cell-derived neurotrophic factor (GDNF), leukemia inhibitory factor (ILF)

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INTRODUCTION

Retinoblastoma (RB) is the most common intraocular malignancy in children under 5 years of age (1). In addition, the incidence of RB is one in 15,000–20,000 (2). Although the treatment of RB has been improved obviously, the survival rate of patients is still poor. Chemotherapy is currently recognized as the first-line treatment for RB in children. At present, carboplatin constitutes one of the standard chemotherapeutic agents applied for RB (3), but its clinical application is greatly limited due to acquired

drug resistance upon the long-term treatment. Although many studies have clarified the molecular mechanisms and signal pathways closely related to the carboplatin resistance of retinoblastoma (4), the mechanisms remain incompletely elucidated and require further investigation.

Now more and more evidence shows that microRNA is not only widely involved in the occurrence, development, recurrence, and metastasis of various tumors but also related to the generation of tumor drug resistance. These studies have pointed out that microRNA is closely related to the invasiveness and drug resistance of tumor cells, and regulation of microRNA can inhibit the drug resistance of tumor stem cells and improve their sensitivity to chemotherapy (5). Although some articles have studied the relationship between microRNA and RB resistance mechanism (6–9), the studies are scattered, and it is not clear whether there are other signaling pathways involved in RB resistance mechanism. MiR-211-5p has been demonstrated to play an important role in several cancer types, including colorectal cancer (10), non-small cell lung cancer (11), hepatocellular carcinoma (12), and renal cell carcinoma (13). However, the biological role of miR-211-5p in retinoblastoma Y79R cells is still unclear.

The glial cell-derived neurotrophic factor (GDNF) is a small protein that potently promotes the survival of many types of neurons. GDNF is overexpressed in glioma cancer (14), lung cancer (15), and pancreatic cancer (16). However, the GDNF expression in RB has not been reported. Pretreatment of glioblastoma cell lines with GDNF conferred chemoresistance to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (17). In prostate cancer, exposure to GDNF also induced tumor cell resistance to mitoxantrone and docetaxel chemotherapy (18). GDNF stimulates downstream signal transduction pathways, such as AKT and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways. These two pathways are important for cell invasion, survival, proliferation, and differentiation (19, 20). However, to the best of our knowledge, the relationship between GDNF and RB resistance mechanism has not been reported. In the present study, to elucidate the chemoresistance mechanism, we constructed a retinoblastoma cell line Y79 which is a drug-resistant cell line, and then this cell line was used for RNA-seq and microRNA-seq. We distinguished any candidate differentially expressed genes (DEGs) between the two lines. Then, RNA sequencing revealed that GDNF was a gene enriched in drug transport with obvious differences. Then, it was found that GDNF was the target gene of miR-211-5p. Currently, the research on the signaling pathway of miR-211-5p-GDNF has only been reported in the congenital gastrointestinal atresia (21). We further explore the role of this signaling pathway in the mechanism of RB resistance and what factors it may be affected by.

MATERIALS AND METHODS

Cell Cultures and Treatments

The human retinoblastoma cell line Y79 was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (ZQXZ Biotech, Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Grand Island, USA) , 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, Grand Island, USA) in a humidified atmosphere (95% air, 5% CO_2) at 37°C. The carboplatin-resistant RB Y79 (Y79R) cells were established by intermittently exposing the RB cells to a high concentration of carboplatin (10 μg/ml) (APExBIO, Houston, USA) for 24 h and then with a normal medium. After the surviving cells return to normal growth, the next dosing treatment is performed, and this process is repeated for about 8 months to obtain drug-resistant cell lines.

Detection of Drug Resistance

Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan) was used to detect drug resistance. Y79R cells were seeded at a density of 3.0×10^4 cells/well with 100 μl of medium in 96-well plates and treated with different concentrations of carboplatin for 72 h. Cells without drug and medium without cells were served as the controls. Then, 10 μl of CCK-8 solution was added to each well and incubated for 4 h at 37°C. GraphPad Prism 7.0 software was used to calculate the half-maximal inhibitory concentrations (IC50).

RNA-Seq and MicroRNA-Seq Data Analysis and Pathway Enrichment Analysis

Normal cell lines (C1, C2, C3) and drug-resistant cell lines (D1, D2, D3) were chosen for RNA-seq and microRNA-seq. The differential expression analysis was performed using the DESeq2 (v1.4.5) (22) with Q value ≤ 0.05. The Gene Ontology (GO) (http://www.geneontology.org/) (23) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/) (24) enrichment analyses of annotated differently expressed genes were performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on the hypergeometric test. The significant levels of terms and pathways were corrected by Q value with a rigorous threshold (Q value ≤ 0.05) by Bonferroni (25).

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (Real-Time QRT-PCR) Validation

Total RNA was isolated from cells using RNAiso Plus (TaKaRa, Tokyo, Japan) and was then converted to cDNA using a gDNA Eraser kit (TaKaRa, Tokyo, Japan). RT-qPCR analysis was carried out in triplicate for each sample using SYBR Green Master Mix (TaKaRa, Tokyo, Japan). Reverse transcription and detection primers were purchased from RiboBio (RiboBio, Guangzhou, China). All procedures were performed according to the manufacturer's instructions.

Western Blotting

The total protein was extracted by RIPA (Beyotime, Jiangsu, China), isolated on 4%–20% ExpressPlus™ PAGE Gel (GenScript, Shanghai, China). Then, the protein was blotted onto the PVDF membrane (Millipore, Bedford, MA, USA). The primary antibody blocking solution (Beyotime, Jiangsu, China) was used to block for

1 h, then incubated with mouse monoclonal anti-ACTIN (M02014-5, Boster, China), rabbit anti-GDNF (ab176564, Abcam, Cambridge, MA, USA), and Monoclonal Mouse anti-LIF (MAB250-100, R&D Systems, Abingdon, UK) overnight, and the corresponding secondary antibody was incubated for 1 h. Densitometry of the resulting bands was performed using ImageJ 1.8.0 software.

Transfection and Small RNA Interference of Selected Genes

In order to verify whether the expression of GDNF affects the drug resistance of Y79 cell lines, siRNA was used to interfere with the expression of GDNF in Y79R cell lines. Both small interfering RNA of GDNF and miR-211-5p mimics were purchased from RIBOBIO (Ribobio, Guangzhou, China). Cells (1×10^5) were seeded in a 12-well plate at 37°C, 5% CO_2, and cultured for 12 h. The riboFECTTM CP kit (Ribobio, Guangzhou, China) was used for transfection.

Apoptosis Detection

Cells were inoculated with 3×10^5 cells/well into 6-well plates, cultured overnight, and treated with carboplatin for 48 h. In accordance with Annexin V-APC/PI Apoptosis Detection Kit (BioLegend, San Diego, CA, USA) instructions, the cells were collected, successively Annexin V-APC was added, and the PI was incubated for 10 min in the dark, at room temperature. Flow cytometry was used to detect cell apoptosis.

Analysis of GDNF Binding Target MicroRNA

The TargetScanHuman 7.1 (26) website (http://www.targetscan.org/vert_71/) was used to analyze the GDNF binding target microRNA.

Protein Interaction Analysis

The STING website (https://string-db.org/cgi/input.pl) was used to predict the protein which interacts with GDNF in the transport pathway. Then, Co-Immunoprecipitation (COIP) was used to verify the proteins.

Molecular Docking

AutoDock Vina software is used for this molecular docking work. GDNF (PDB ID: 3FUB) and LIF (PDB ID: 1pvH) were downloaded from the PDB database (https://www.rcsb.org/), respectively. The 3D structure of carboplatin (SDF format files) was downloaded from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/). In addition, the protein was treated with PyMOL 2.4, including the removal of ligand molecules, water molecules, and hydrogen atoms. After the protein and small molecule are ready, the protein core is further defined as the center of the docking pocket, and a cube box that can wrap the protein is set up for the docking conformation search of carboplatin. Finally, the prepared files were used for molecular docking by Vina software.

Statistical Analysis

All experiments were repeated 3 times, and data were expressed as mean ± standard deviation (mean± SD). GraphPad Prism 7.0 software was used for analysis, and the t-test was used for comparison of differences between groups. If the p value is less than 0.05, the difference is statistically significant.

RESULTS

Cytotoxicity Test of Drugs

After nearly 8 months of inducing resistant cell lines by the high-dose shock method, Y79R cells showed significant resistance to carboplatin, as compared to Y79 cells. The IC50 of carboplatin on the Y79R cell line (16.295 μg/ml) increased 6.4 times compared to the normal culture of Y79 cells (2.547 μg/ml), as shown in **Figure 1**. This finding shows that DEGs needed to be identified to elucidate the intrinsic mechanism of chemoresistance in Y79R cell lines.

DEGs and Enrichment Analysis of RNA-Seq Data Between Parental Y79 and Y79R Cells

RNA-seq results show that 1,330 differential expression genes are identified; among these genes, 857 genes are upregulated (**Supplementary Table 1**) and 473 genes are downregulated (**Supplementary Table 2**) in Y79R cells compared to normal Y79 cells. The top 20 genes with the largest fold change in the upregulated group and downregulated group are shown in **Figure 2A**. In KEGG pathway analysis, the upregulated genes are enriched in the cAMP signaling pathway, oxytocin signaling pathway, proteoglycans in cancer, and p53 signaling pathway (**Figure 2B**). The downregulated genes have no obvious enrichment pathways, and the total differential genes are enriched in axon guidance, p53 signaling pathway, arrhythmogenic right ventricular cardiomyopathy, and phototransduction (**Figure 2C**). The GO enrichment results

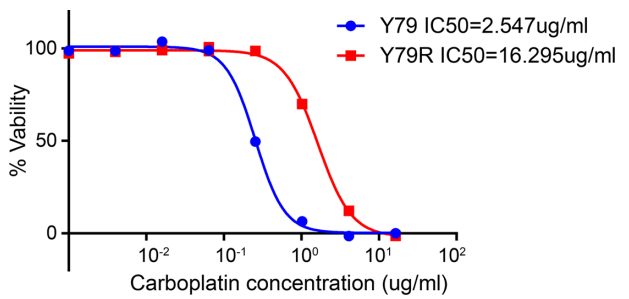

FIGURE 1 | Cytotoxicity of carboplatin in Y79 and Y79R cells. Cell viability was measured by CCK-8 assay. Experiments were done twice in triplicate. Values represent mean ± SD cell viability as percentage of untreated control samples. The half-maximal inhibitory concentrations (IC50) indicate the concentration of a drug required to inhibit 50% cell growth *in vitro*.

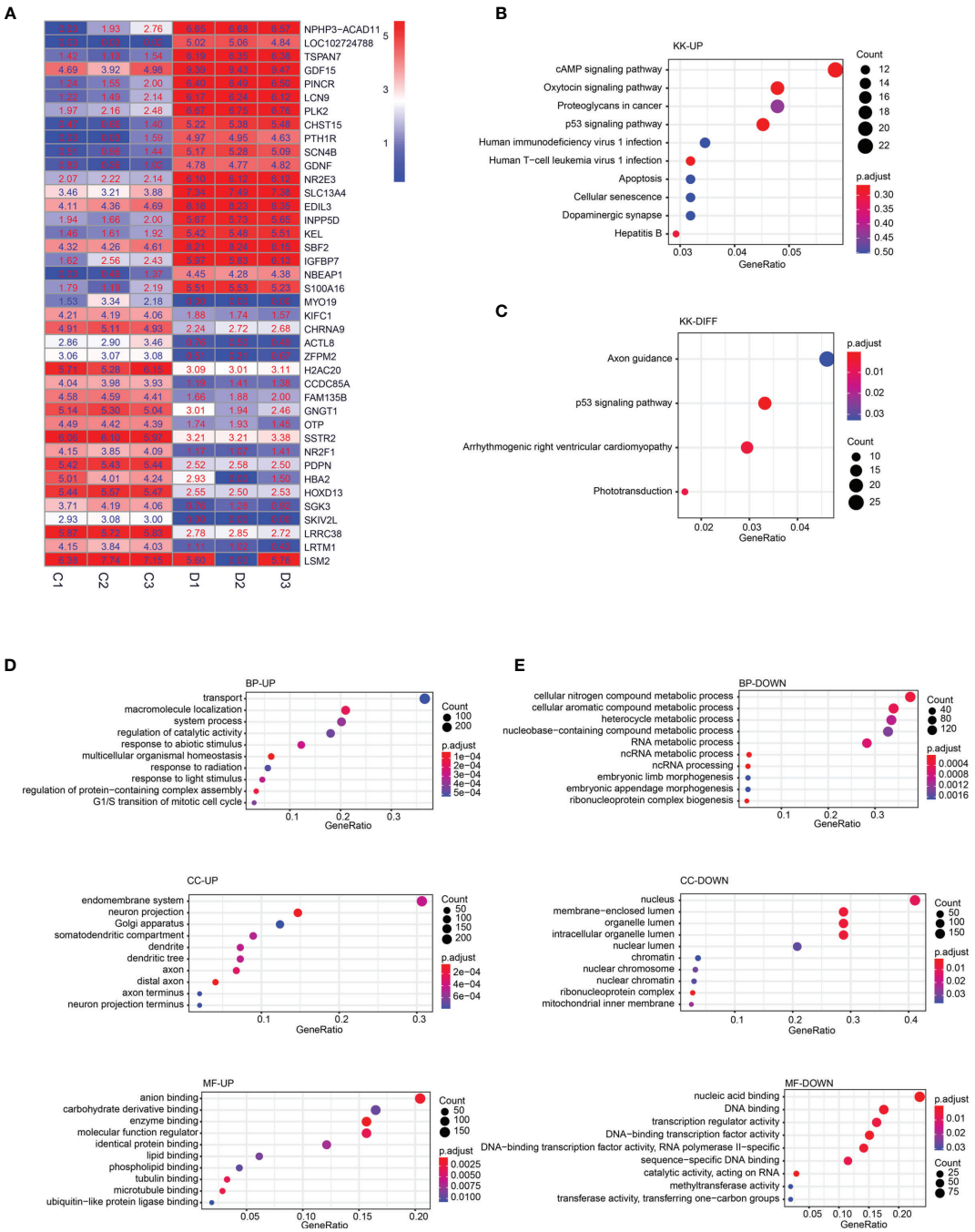

FIGURE 2 | DEGs and enrichment analysis of RNA-Seq data between parental Y79 and Y79R cells. **(A)** Heatmap of 20 upregulated and 20 downregulated genes with top log2FC; FC: fold change. Color indicates the expression level of DEGs with log2(FPKM+1). C:Y79 group; D: Y79R group. **(B)** KEGG pathway enrichment analysis of upregulated DEGs. **(C)** KEGG pathway enrichment analysis of downregulated DEGs. **(D)** GO enrichment analysis of upregulated DEGs. **(E)** GO enrichment analysis of downregulated DEGs (the X-axis is the gene ratio, corresponding to the % column in DAVID's results table. The Y-axis is the enrichment pathway or GO term. The size of the dot is the number of genes; the color of the dot is the p value. BP, biological process; CC, cellular component; MF, molecular function.

shows that in the upregulated group, the biological process (BP) is significantly enriched in transport, the cellular component (CC) is significantly enriched in the endomembrane system, and the molecular function (MF) is significantly enriched in anion binding. Our study suggests that the upregulated genes are significantly enriched in the transport group in BP analysis (**Figure 2D**). In the downregulated group, BP is significantly rich in the cellular nitrogen compound metabolic process, CC is

significantly rich in the nucleus, and MF was significantly rich in nuclear acid binding (**Figure 2E**).

Further Enrichment Analysis of RNA-Seq Data in the Drug Transport Signaling Pathway

We further conducted GSEA analysis and found that drug transport function showed a positive correlation with Y79 drug resistance (**Figure 3A**, **Supplementary Table 3**). Therefore, we decided to further analyze drug transport. We performed a heatmap analysis (**Figure 3B**) and a volcano map analysis (**Figure 3C**) for all genes in drug transport and then selected the gene GDNF, which is the most differentially expressed gene for the next step of verification. RT-PCR results show that GDNF gene expression is significantly increased in drug-resistant cell lines (**Figure 3D**) ($p < 0.001$), and Western results also showed

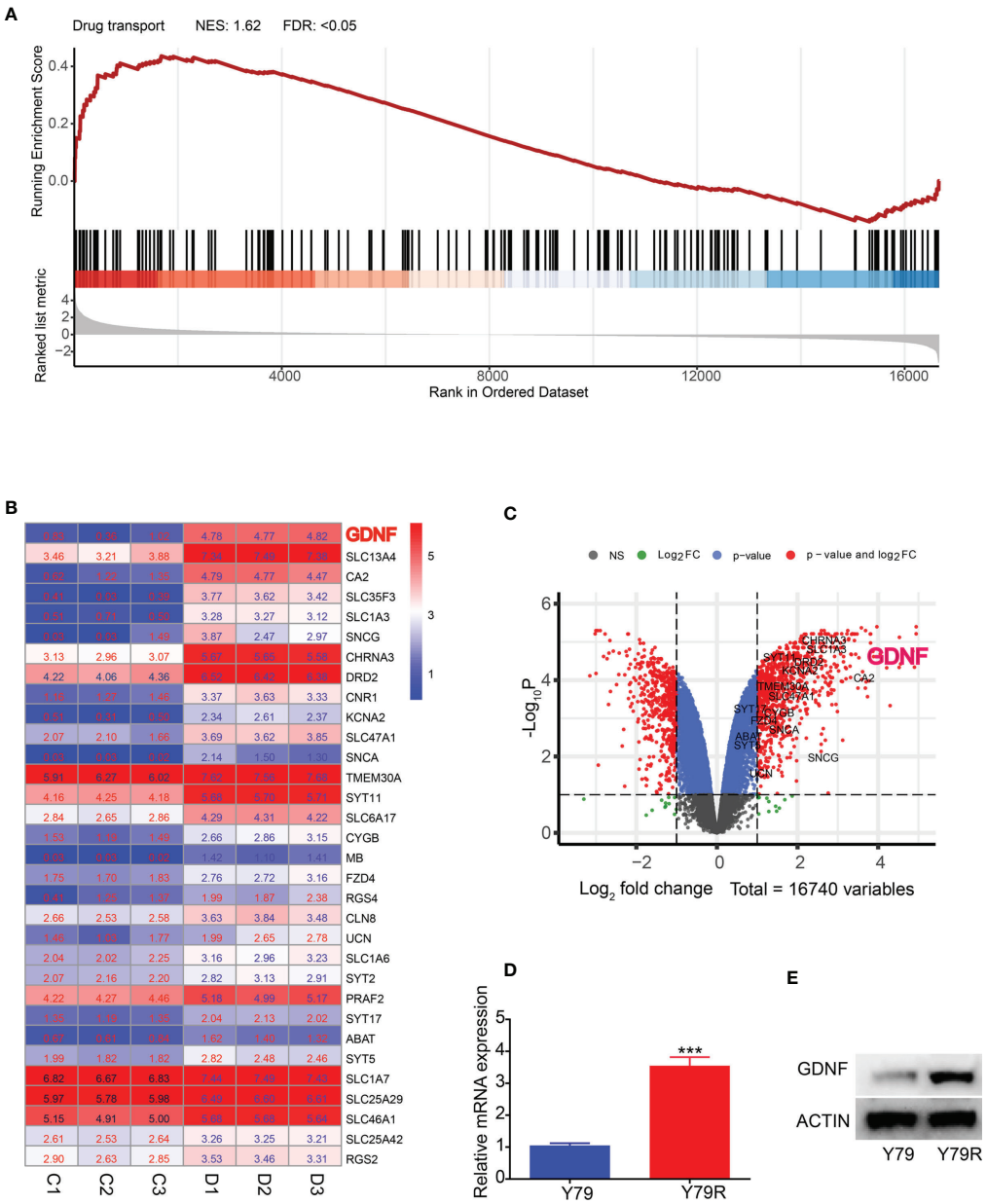

FIGURE 3 | Further enrichment analysis of RNA-Seq data in the drug transport signaling pathway. **(A)** GSEA analysis of drug transport signaling pathway. NES-normalized enrichment score; FDR-false discovery rate. **(B)** Heatmap of DEGs in the drug transport signaling pathway with log2FC. FC, fold change. Color indicated expression level of DEGs with log2(FPKM+1). **(C)** Volcano plot of the distribution of DEGs in the drug transport signaling pathway. NS, No Significant. p: p-value. The red dots indicated DEGs. The other color dots indicated no significantly differential expression. **(D)** QRT-PCR results of GDNF in Y79 and Y79R. **(E)** Western blot results of GDNF in Y79 and Y79R. ***Significance at $p < 0.001$, by t-test between two groups.

that GDNF protein was significantly increased in drug-resistant cell lines (**Figure 3E**).

Effect of GDNF Knocking Down on Drug Resistance of Y79R Cells

In order to verify whether the expression of GDNF affects the drug resistance of Y79 cell lines, siRNA was used to interfere with the expression of GDNF in Y79R cell lines. RT-PCR (**Figure 4A**) and WB results (**Figure 4B**) both show that the expression of GDNF in Y79R cell lines is knocked down. The IC50 of Y79R cell lines that interfered with GDNF was significantly lower compared to the control group (**Figure 4C**). Flow cytometric analysis showed that the proportion of apoptotic cells in the interference group is significantly higher than that in the control group (**Figure 4D**). The above results indicated that GDNF knockdown can weaken the drug resistance of Y79R cell lines.

MiR-211-5p Is Highly Expressed in Drug-Resistant Cell Lines

MicroRNA-seq analysis found that a total of 353 differentially expressed microRNAs were identified in drug-resistant cell lines, of which 55 microRNAs were upregulated (**Supplementary Table 4**) and 298 microRNAs were downregulated (**Supplementary Table 5**). The top 20 differentially expressed microRNAs are displayed by the heatmap (**Figure 5A**) and the volcano map (**Figure 5B**). We analyzed the microRNAs regulating GDNF on the TargetScanHuman 7.1 website, and then we analyzed the intersection between the microRNAs regulating GDNF and differentially expressed microRNAs in the drug-resistant cell line. It is found that there are 34 differentially expressed microRNAs that may regulate GDNF (**Figure 5C**). Among them, miR-211-5p is the most obvious change. Next, qRT-PCR was used to verify the expression of miR-211-5p in drug-resistant cell lines. The results show that miR-211-5p is highly expressed in the Y79R cell line (**Figure 5D**) ($p < 0.001$).

Effect of Overexpression of miR-211-5p on Drug Resistance of Y79R Cell Lines

In order to further verify the results of the information analysis, miR-211-5p mimics was used to transfect drug-resistant cell line Y79R. RT-PCR detection found that the RNA expression of miR-211-5p increased 100 times than that in the normal group (**Figure 6A**). The RNA and protein expression of GDNF was significantly reduced in the miR-211-5p overexpression group (**Figures 6B, C**). There is a binding site of miR-211-5p at the 3′UTR end of GDNF by TargetScanHuman 7.1 analysis (**Figure 6D**). Then, we constructed a dual luciferase vector based on the binding site, where WT is a wild-type sequence vector and MUT is a vector that lacks the sequence of the binding site. Then, a dual luciferase experiment was performed. The luciferase activity was significantly lower in the WT group than that in the mutant group (**Figure 6E**) ($p < 0.001$). Then, the flow cytometric analysis also showed that the proportion of apoptotic cells in the overexpression group was significantly higher than that of the control group (**Figure 6F**). The above results indicate that overexpression of miR-211-5p can reduce the drug resistance of Y79R cell lines.

Interaction of GDNF With LIF

In order to further study the function of GDNF, we analyzed the interaction of all the proteins in the transport by the String

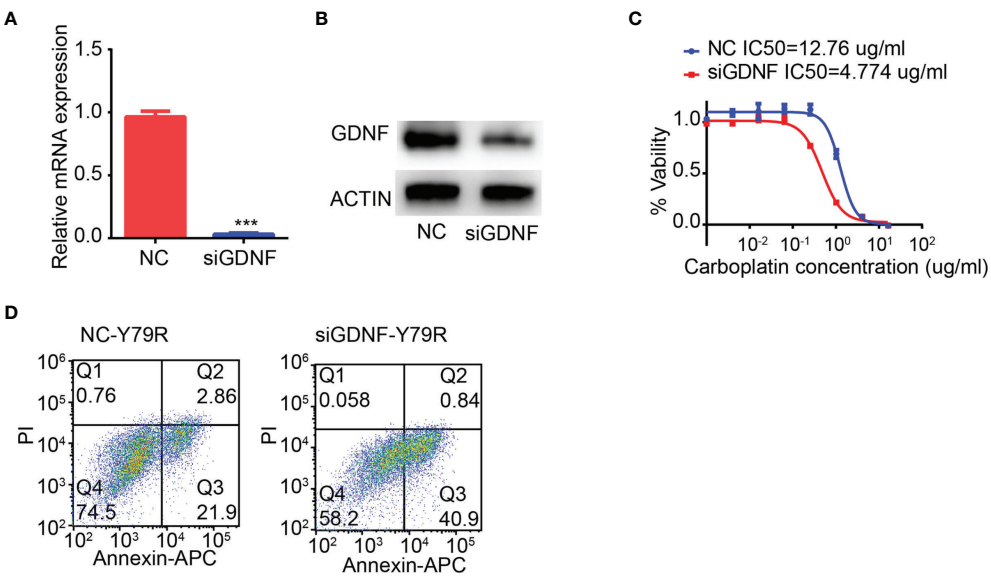

FIGURE 4 | Knocking down GDNF weakens drug resistance of Y79R cells. **(A)** QRT-PCR results of GDNF expression in the Y79R cell line was knocked down by siRNA. **(B)** Western blot results of GDNF in the Y79R cell line was knocked down by siRNA. **(C)** GDNF silencing restored carboplatin sensitivity in Y79R cells. **(D)** GDNF silencing restored carboplatin-induced apoptotic cell death in Y79R cells. ***Significance at $p < 0.001$, by t-test between two groups.

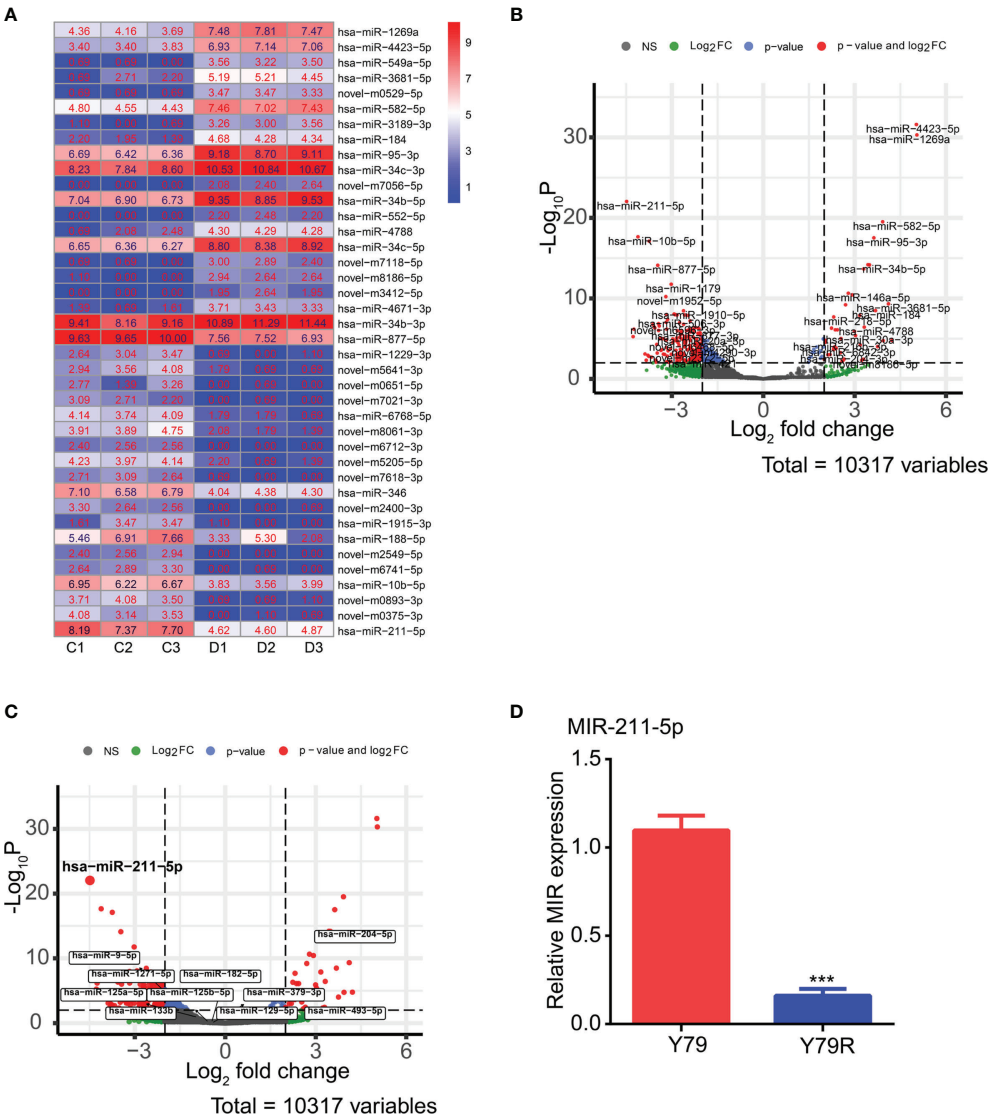

FIGURE 5 | DEG analysis of microRNA-seq data between parental Y79 and Y79R cells and qRT-PCR verification test. **(A)** Heatmap of 20 upregulated and 20 downregulated microRNAs with top log2FC. FC: fold change. Color indicates the expression level of different microRNA expressions with log2(FPKM+1). C:Y79 group, D: Y79R group. **(B)** Volcano plot of differentially expressed microRNAs. NS, No Significant. The red dots indicated different microRNA expressions. The other color dots indicated no significantly differential expression. **(C)** Volcanic map of 34 differentially expressed microRNAs that regulate GDNF. **(D)** QRT-PCR results of MIR-211-5p in Y79 and Y79R. ***Significance at $p < 0.001$, by t-test between two groups.

website (http://string-db.org/) and found that GDNF could interact with PTK3R1, NEFH, MAP2, SLC1A3, LIF, and SNCA (**Figure 7A**). In these proteins, only LIF is a secreted protein which can be secreted out of the cell. Therefore, we selected LIF for COIP analysis and found that it interacts with GDNF (**Figures 7B, C**). Then we predicted that GDNF and LIF proteins could interact with carboplatin. The molecular docking results found that both GDNF and LIF could interact with carboplatin (**Figures 7D, E**). Therefore, we speculated that the reason for GDNF promoting carboplatin resistance in Y79R cell lines may be the following: firstly, GDNF interacted with carboplatin. Then, GDNF and LIF are secreted out of the cell, and a large amount of carboplatin is also taken out of the cell, thereby reducing the concentration of carboplatin in the cell to promote drug resistance.

DISCUSSION

Chemoresistance, either inherent or acquired, is a major constraint of RB treatment. Exploring the mechanisms underlying drug resistance and developing novel therapeutic strategies to overcome such problem are important for RB treatment. Carboplatin is a conventional chemotherapeutic

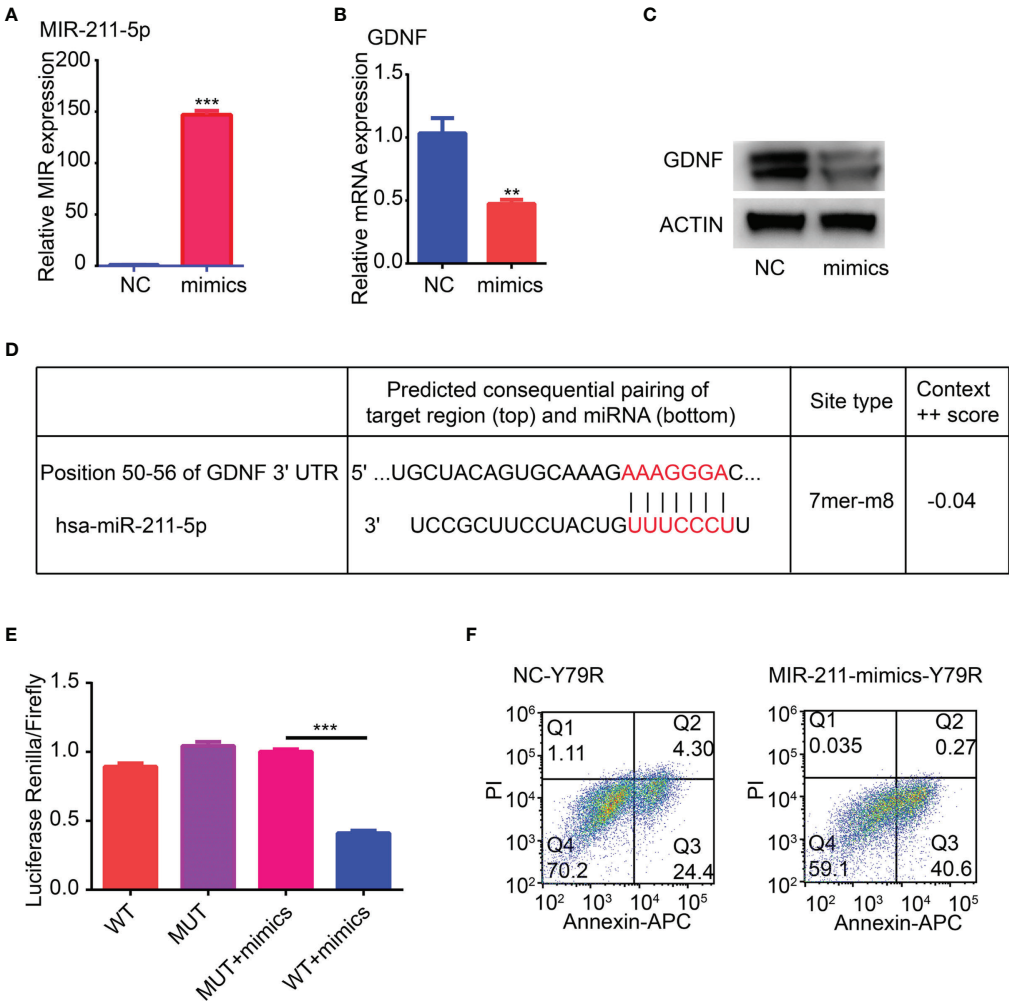

FIGURE 6 | Overexpression of MIR-211-5p weakens drug resistance of Y79R cell lines. **(A)** Overexpression of MIR-211-5p in Y79R. **(B)** QRT-PCR results of GDNF expression in the Y79R cell line overexpressing MIR-211-5p. **(C)** Western blot results of GDNF in the Y79R cell line overexpressing MIR-211-5p. **(D)** Binding target of GDNF and MIR-211-5p was predicted by the TargetScanHuman 7.1 web. **(E)** Luciferase activity was measured using a dual-luciferase reporter gene assay. **(F)** MIR-211-5p overexpression restored carboplatin-induced apoptotic cell death in Y79R cells. **Significance at $p < 0.01$, ***Significance at $p < 0.001$, by t-test between two groups.

drug that has been used in the past few years for the treatment of RB. Carboplatin is a second-generation platinum compound that can directly inhibit DNA repair to attenuate tumor growth (27); it inhibits tumor growth by binding with DNA and affecting DNA replication. As for the chemoresistance mechanism in RB, proteins such as multidrug resistance-associated proteins (MRP) (28), P-gp (29), and glutathione transferase (30) have been demonstrated to be involved. In the present study, to elucidate the chemoresistance mechanism of carboplatin in RB, we generated transcriptome profiles of Y79R and parental Y79 cells and distinguished any candidate differentially expressed genes (DEGs) between the two lines before performing functional and technical validation studies.

To detect the different expression levels of the gene and protein in the relevant signal pathway between Y79 and Y79R cell lines, the DEGs and enrichment analysis of RNA-Seq data suggested that the upregulated genes were significantly enriched in the transport group in BP analysis in the Y79R cells. The GO enrichment showed that the upregulated genes were significantly enriched in the transport group in BP analysis. In further enrichment analysis of RNA-Seq data in the drug transport signaling pathway, GDNF was a gene enriched in drug transport with obvious differences in Y79R cell lines. As drug transport function showed a positive correlation with drug resistance, we speculated that GDNF is the top changed gene in the drug resistance in Y79R cell lines. The above results indicated that GDNF has a great influence on drug resistance; when GDNF was knocked down, drug resistance decreased in drug-resistant cell lines. In Morandi's study, GDNF-RET signaling was established as a rational therapeutic target to combat or delay the onset of aromatase inhibitor resistance in breast cancer (31). GDNF confers chemoresistance in a ligand-

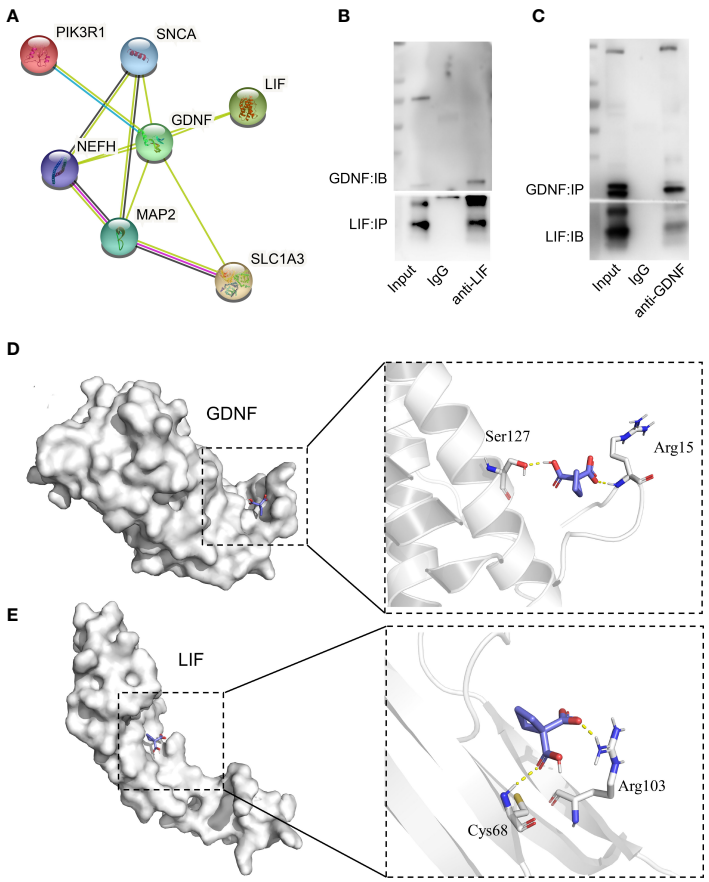

FIGURE 7 | GDNF and LIF interaction. **(A)** The protein interaction with GDNF in DEGs of transport analysis in the sting website. **(B)** Co-Immunoprecipitation analysis between GDNF and LIF (IP : LIF;IB : GDNF). **(C)** Co-immunoprecipitation analysis between GDNF and LIF (IP : GDNF;IB : LIF), **(D)** Vina software predicted the interaction between GDNF and carboplatin. **(E)** Vina software predicted the interaction between LIF and carboplatin.

specific fashion in malignant gliomas (32). Our results are similar to those of the above studies, but the difference is that the sites of influence on the drug resistance mechanism are different, and GDNF acts in cells, while other studies take the next step by acting on receptor RET on the cell membrane (33).

MiR-211-5p functions as a tumor suppressor in hepatocellular carcinoma (34), breast cancer (35), and renal cell carcinoma (36). There are some studies of miR-211-5p on the regulation of tumor drug resistance; miR-211-5p can enable resistance to BRAF inhibitors in melanoma (37). LncRNA KCNQ1OT1 regulates cisplatin resistance in tongue cancer *via* miR-211-5p-mediated Ezrin/Fak/Src signaling (38). Downregulation of circNRIP I suppresses the paclitaxel resistance of ovarian cancer *via* regulating the miR-2 I I-5p/HOXC8 axis (39). However, the biological role of miR-211-5p in retinoblastoma is still unclear. Our results indicate that overexpression of miR-211-5p can weaken the drug resistance of Y79R cell lines. About the signaling pathway involved in miR-211-5p, the miR-211-5p/CENPK axis in tongue squamous cell carcinoma (40) and the miR-211-5pp/BRD4 axis in non-small cell lung cancer (11) have been reported, but not about resistance mechanisms. In our microRNA-seq analysis, miR-211-5p was downregulated in drug-resistant cell lines and directly bound to the 3′ terminal region of GDNF to regulate GDNF degradation. It has been reported that lncrNA-MEG3 has a protective effect on congenital intestinal atretic ganglion cell dysplasia through direct regulation of the Mir-211-5p/GDNF axis, but the role of miR-211-5p/GDNF in carboplatin resistance is still unclear. We further investigated how miR-211-5p regulates GDNF expression upstream. When miR-211-5p was overexpressed, the expression of GDNF decreased significantly. These results can provide evidence for the reversal of drug resistance of RB.

The leukemia inhibitory factor (LIF) is a secreted protein which belongs to the interleukin-6 family of cytokines. LIF has been implicated in many physiological processes including development, hematopoiesis, bone metabolism, and inflammation. Regarding the interaction between GDNF and LIF, the combination of GDNF and LIF could significantly

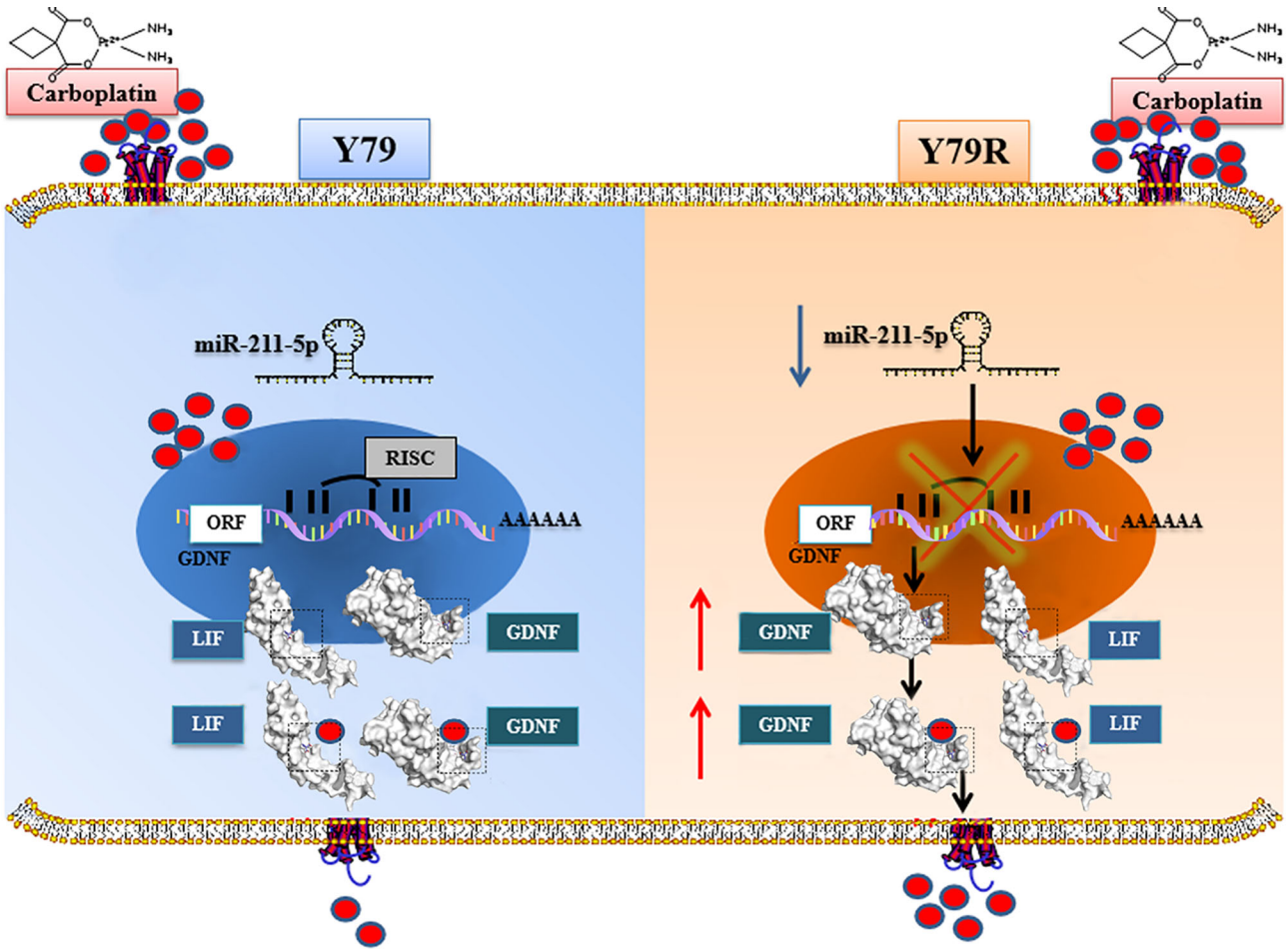

FIGURE 8 | Mechanisms involved in carboplatin resistance in human retinoblastoma Y79R cells. In Y79 cells, downregulation of Mir-211-5p promoted the intracellular expression of GDNF. Highly expressed GDNF binds to more carboplatin and secretes it out of the cell. In addition, GDNF was found to bind to another secreted protein, LIF. It is speculated that LIF can bind carboplatin and excrete carboplatin from cells by secretion. We hypothesize that these processes are involved in cellular drug resistance. ORF, Open Reading Frame; RISC, RNA-induced silencing complex; AAAAAA, 3′polyA tail on behalf of the end of mRNA.

enhance the *in vitro* proliferation of mouse SSCs (41). Upregulation of the receptor components for LIF and GDNF in motoneurons is important for the regeneration of intramuscular motor nerves damaged by muscle contusion (42). LIF may be utilized for signaling mediated by GDNF and may be important in the pathobiology of neuroendocrine tumors (43). We conducted protein interaction analysis and found that there was an interaction between LIF and GDNF, which was consistent with the above research results. The molecular docking results showed that both GDNF and LIF interacted with carboplatin. The cell membrane, cytoplasm, and nuclear protein participate in these resistance mechanisms. Drug resistance at the level of cell membrane reduces drug uptake and increases efflux, leading to a decrease in the absolute concentration of intracellular drugs. For example, P-GP is the earliest ABC transporter discovered, and the high expression of P-GP is also the most classical mechanism of drug resistance (44). Drug resistance at the level of intracellular metabolic processes of drugs strengthens the cell detoxification function, rapidly inactivates the drug, and repairs the DNA damage caused by the drug in tumor cells in time, such as glutathione transferase (GST)-related drug resistance (45). Resistance occurs at the nuclear level such as topoisomerase ii (45). The previous results also showed that the expression of GDNF and LIF significantly increased in the drug-resistant cells (TBALE 1). This supports the hypothesis that the mechanism of GDNF promoting carboplatin resistance might be related to the combination of GDNF and intracellular carboplatin. As GDNF and LIF are secreted into the extracellular environment, a large amount of carboplatin is also taken out of the cell, thus reducing the intracellular concentration of carboplatin and promoting its drug resistance. Nasma D. Eljack's study supports a major role of passive membrane diffusion in the uptake of cisplatin and suggests that reduced cell uptake is unlikely to be a significant mechanism leading to the development of drug resistance (46). Our results suggested that the resistance of carboplatin was about

intracellular metabolic processes of drugs, not at the cell membrane in RB Y79 cells.

In conclusion, our results suggest that downregulation of miR-211-5p can promote carboplatin resistance in human retinoblastoma Y79 cells, and this process can promote GDNF expression. High expression of GDNF will bind to more carboplatin and secrete it out of the cell. In addition, GDNF was found to bind to another secreted protein LIF. It is also predicted that LIF can combine with carboplatin and take carboplatin out of the cell by secretion. Thus, these events lead to drug resistance of Y79 cells (**Figure 8**). However, whether the actual principle is that this needs further study.

However, some limitations must be addressed. Firstly, we only studied the carboplatin resistance mechanism of Y79, the most common cell line of RB. Whether there are other mechanisms in other cell lines of RB needs to be further studied. Secondly, RNA-seq and microRNA-seq analyses showed that there were many different genes between drug-resistant cells and normal cells, indicating that there were many genes involved in the drug resistance process, and the drug resistance process was a network regulation process in the whole process. In this paper, only drug transport pathways were selected for analysis, and finally, only GDNF with the greatest change in the group was selected for analysis. Few molecules were selected in the experiment, which could not fully reflect the principle of drug resistance. Even for the regulation of GDNF expression, there may be other regulation methods besides microRNAs, and the regulation network of GDNF needs to be further studied and expanded. Finally, this paper only predicted the interaction between GDNF and LIF and carboplatin in the aspect of bioinformatics, which requires further experimental verification.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The raw rna-seq data generated in this study have been stored in the NCBI Sequence Read Archive (SRA) with Bioproject No.PRJNA796367 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA796367) and SRA accession number: SRR17567975, SRR17567974, SRR17567971, SRR17567970, SRR17567969, SRR17567968, SRR17567967, SRR17567966, SRR17567965, and SRR17567964 SRR17567973 and SRR17567972.

AUTHOR CONTRIBUTIONS

NK and XZ designed the study. NK did the experiments or collected the data for the study. NK, LC, and QL analyzed the data. NK, LC, QL, XC, and HX contributed to writing the paper. 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/fonc.2022.848733/full#supplementary-material

Supplementary Table 1 | 857 genes were upregulated.

Supplementary Table 2 | 473 genes were downregulated.

Supplementary Table 3 | All results of GSEA analysis.

Supplementary Table 4 | 55 microRNAs were up-regulated.

Supplementary Table 5 | 298 microRNAs were down-regulated.

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SYSTEMATIC REVIEW
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A Systematic Review of Candidate miRNAs, Its Targeted Genes and Pathways in Chronic Myeloid Leukemia–An Integrated Bioinformatical Analysis

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Chronic myeloid leukaemia is blood cancer due to a reciprocal translocation, resulting in a BCR-ABL1 oncogene. Although tyrosine kinase inhibitors have been successfully used to treat CML, there are still cases of resistance. The resistance occurred mainly due to the mutation in the tyrosine kinase domain of the BCR-ABL1 gene. However, there are still many cases with unknown causes of resistance as the etiopathology of CML are not fully understood. Thus, it is crucial to figure out the complete pathogenesis of CML, and miRNA can be one of the essential pathogeneses. The objective of this study was to systematically review the literature on miRNAs that were differentially expressed in CML cases. Their target genes and downstream genes were also explored. An electronic search was performed *via* PubMed, Scopus, EBSCOhost MEDLINE, and Science Direct. The following MeSH (Medical Subject Heading) terms were used: chronic myeloid leukaemia, genes and microRNAs in the title or abstract. From 806 studies retrieved from the search, only clinical studies with *in-vitro* experimental evidence on the target genes of the studied miRNAs in CML cells were included. Two independent reviewers independently scrutinised the titles and abstracts before examining the eligibility of studies that met the inclusion criteria. Study design, sample size, sampling type, and the molecular method used were identified for each study. The pooled miRNAs were analysed using DIANA tools, and target genes were analysed with DAVID, STRING and Cytoscape MCODE. Fourteen original research articles on miRNAs in CML were included, 26 validated downstream genes and 187 predicted target genes were analysed and clustered into 7 clusters. Through GO analysis, miRNAs' target genes were localised throughout the cells, including the extracellular region, cytosol, and nucleus. Those genes are involved in various pathways that regulate genomic instability, proliferation, apoptosis, cell cycle, differentiation, and migration of CML cells.

Keywords: chronic myeloid leukemia, genes, pathways, microRNA, BCR-ABL1

INTRODUCTION

Chronic myeloid leukaemia (CML) is a proliferative disorder of pluripotent stem cells. CML is linked to a specific genetic disorder involving *BCR* and *ABL1* gene translocation, resulting in the Philadelphia chromosome. *BCR-ABL1* fusion gene encodes an active tyrosine kinase BCR-ABL which activates several molecular pathways that cause abnormal cell adhesion, increase cell proliferation, and inhibit apoptosis. Nevertheless, tyrosine kinase plays an essential role in many signalling cascades, including biological processes such as cell growth, differentiation, metabolism, and apoptosis (1). Numbers of studies have revealed the mechanisms of CML pathogenesis which involved several key signalling pathways, including the MAPK, JAK-STAT, PI3K/AKT, EGFR, ERBB, TGF-β and tumour protein p53 pathways (2–4).

Thus, specific treatment for CML has been developed by targeting the *BCR-ABL1* gene. The first molecularly targeted therapy, Imatinib, is a small molecule known as tyrosine kinase inhibitor (TKI) that directly targets *BCR-ABL1* tyrosine kinase activity. Despite the success of Imatinib as the front-line therapy for CML, there were reports on drug resistance that is primarily due to the presence of mutations in the *BCR-ABL* tyrosine kinase domain (TKD) (5–7). Tyrosine kinase inhibitors are not able to fully prevent the progression of CML cells with BCR-ABL TKD mutated CML cells. Therefore, research into alternative treatments for CML remains clinically essential. In recent years, microRNA (miRNA) has been widely studied in human malignancies and chemical compounds. They have received widespread attention as essential regulators of gene expression in leukemogenesis and are linked to resistance to *BCR-ABL1* TKIs (3, 8).

MiRNA is a short, non-coding RNA that regulates gene expression at the post-transcriptional level. It inhibits translation by binding to the 3'untranslated (3'UTR) region of specific target mRNA. MiRNA has been linked to disease pathogenesis in CML and is known to play an essential role in tumorigenesis (9). MiRNA's roles and functions have been highlighted in several studies in a variety of circumstances and scenarios. For instance, miRNAs expression profiles were used as biomarkers and therapeutic tools (10–12). Their expression differences were used in several studies to improve response prediction in diseases, particularly CML. Aberrant miRNA expression is linked to stem cell survival, cell renewal and sensitivity or resistance to TKI therapy, all of which contribute to disease progression (1, 13, 14). Additionally, miRNAs have been discovered to affect genes in signalling pathways involved in cell proliferation, apoptosis, leukemogenesis, and tumour suppression (15–17). Hence, the current study aims to identify and screen differentially expressed miRNAs in CML patients from previous literatures. The application of integrated bioinformatics analysis is essential in predicting miRNA target genes, gene ontology and pathways, and protein-protein interaction networks. Findings from this study will help researchers to better understand the role of miRNA in CML pathogenesis and treatment resistance.

METHODS

Search Strategy

A comprehensive search of information was done using PubMed, Scopus, EBSCOhost MEDLINE, and Science Direct to identify relevant research publications with an unlimited starting publication date until 1st April 2021. The Medical Subject Heading (MeSH) terms like chronic myeloid leukemia, genes and microRNAs were used as the keywords in the title or abstract. The search strategy involved a combination ("AND") of the following two sets of keywords (1): "chronic myelo* leukemia" OR CML OR "BCR*ABL*positive" and (2) mi*RNA. Synonyms for keywords were generated through MeSH terms from the Cochrane Library. Additional text terms were discovered by reviewing collected review articles. Additional references were discovered from the bibliographies of the retrieved studies.

Inclusion Criteria

Case-control and prospective observational studies with abstracts investigating the differentially expressed miRNAs on Philadelphia chromosome-positive chronic myeloid leukemia patients in chronic, accelerated, or blast phases were included. In addition, only clinical studies that have further *in vitro* experimental evidence on the target genes of the studied miRNAs in CML cells were included in this review. Due to limited resources, only manuscripts written in English were included.

Exclusion Criteria

Publications that did not have primary data, such as editorials, case reports, conference proceedings, and narrative review articles, were excluded. In silico, *in vitro*, and *in vivo* studies were excluded. The review focus on the outcome of the differentially expressed miRNAs in CML patients. Therefore, studies that involved responses toward tyrosine kinase inhibitors treatment or any other intervention studies on a new treatment for CML patients were excluded from consideration. These selection criteria were used to achieve the objective of this systematic review in determining the typical miRNA expression signature in CML patients, the miRNAs target genes, and related pathways that could potentially be involved in the pathogenesis of CML.

Screening of Articles for Eligibility

Articles retrieved from all resources were screened in three phases. All articles with titles that did not match the inclusion criteria were excluded, and duplicates were removed in the first phase. The abstracts of the remaining articles were screened, and any articles that did not meet the inclusion criteria were excluded in the second phase. Finally, the full texts of the remaining articles were read and assessed thoroughly. Systematic reviews, meta-analyses, and articles that did not meet the inclusion criteria were excluded in this third phase. All the authors were involved in the screening, selection, and data extraction phase. Any differences in opinions were resolved by discussion between

the authors. All data extraction was performed independently using a data collection form to standardize the data collection, and records on reasons for rejection were kept. **Figure 1** shows the flow chart that summarizes the article selection process and the reasons for article exclusion.

Data Extraction and Study Quality

Data were extracted from the studies that fulfilled inclusion criteria. Data collected from these studies include (1) author name (2), study design (3), study objective (4), study population (5), type of sample used (6), method used in gene expression analysis and experiments performed to validate their targeted genes (7), results (upregulated and downregulated miRNAs, their validated targeted genes and downstream effected genes), and (8) conclusion. The extracted details are listed in **Table 1**.

The analysis details of each report were discussed thoroughly among the reviewers to assess the quality of each study. The authors focused on the reported list of miRNAs as well as their targeted genes. Bias was excluded by adhering to the inclusion criteria. Pairs of reviewers with adequate reliability worked independently to determine the validity of each study.

Prediction of miRNA Target Genes

The miRNAs listed were further analysed using four different bioinformatics tools that include 1) DIANA-microT web server v5.0 with MiTG scores being set at more than 0.95, 2) TargetScan release 7.2 with Cumulative weighted context++ score of more than -0.5, 3) miRDB with a target score more than 90, and 4) mirDIP v5.0.2.3 with score class set at "very high". Genes that are predicted by more than two bioinformatics tools were selected for further analysis.

Gene Ontology and Pathway Enrichment Analysis

Two groups of analysis that include 1) a group of validated target genes and downstream genes of the miRNAs extracted from the studies and 2) a group of predicted target genes of the reported miRNAs was done. These two groups of genes were analyzed using Database for Annotation, Visualization, and Integrated Discovery (DAVID). DAVID was performed to determine the cluster of genes that displayed significant functional annotation enrichment related to CML's pathogenesis. The contribution of genes in the pathway related to CML was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, the Biological Biochemical Image database (BBID), BIOCARTA pathway database, and Reactome.

Protein–Protein Interaction (PPI) Network

The targeted genes were further analyzed at the protein level using protein-protein interaction network functional enrichment analysis through STRING (Protein-Protein Interaction Network Functional Enrichment Analysis) (https://string-db.org/). Results from STRING were further analyzed using Cytoscape to visualize molecular interaction networks and integrating gene

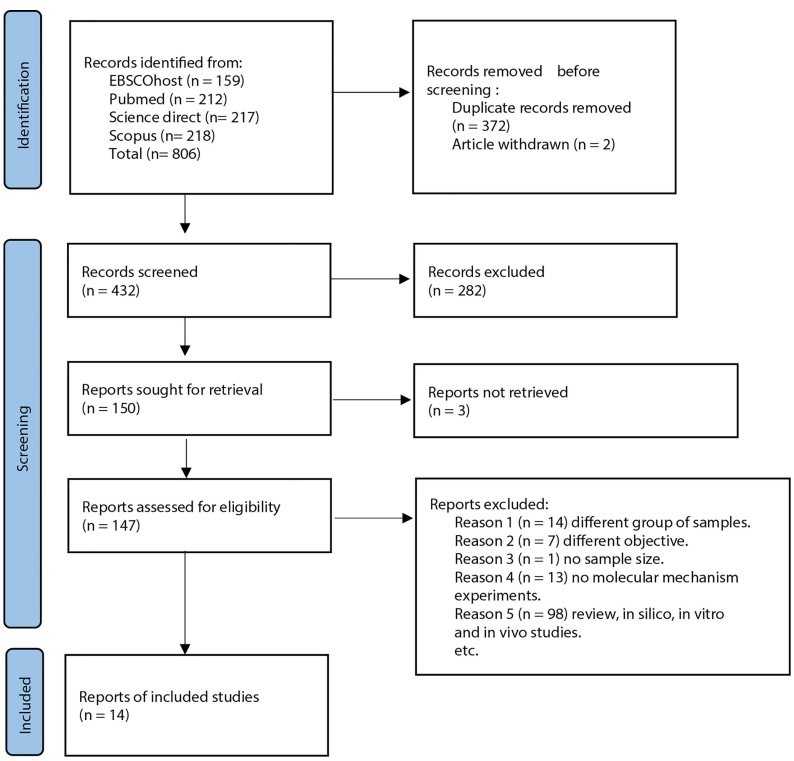

FIGURE 1 | PRISMA flow diagram for studies selection in this systematic review.

TABLE 1 | Summary of selected studies.

Title (References)	Study design	Samples(Type)	Methodology		Findings	
			Gene expression analysis	Target gene analysis	MiRNA (regulation)	Targeted gene
MiR-152-3p promotes the development of chronic myeloid leukemia by inhibiting p27 (18).	Case-control study	CML (n=40) Controls (n=40) (BM)	qPCR	Luciferase assay	miR-152-3p (upregulated)	*CDKN1B*
MiR-362-5p promotes the malignancy of chronic myelocytic leukaemia *via* downregulation of GADD45α (19)	Case-control study	CML (n=40) Controls (n=26) (PB)	qPCR	Luciferase assay	miR-362-5p (upregulated)	*GADD45A*
miR-140-5p induces cell apoptosis and decreases Warburg effect in chronic myeloid leukemia by targeting SIX1 (20)	Case-control study	CML (n=30) Controls (n=30) (PB)	qPCR	Luciferase assay	miR-140-5p (downregulated)	*SIX1*
MiRNA-409-5p dysregulation promotes imatinib resistance and disease progression in children with chronic myeloid leukemia (21)	Case-control study	CML (n=42) Controls (n=40) (PB)	qPCR	Luciferase assay	miR-409-5p (downregulated)	*NUP43*
MiR-570 inhibits cell proliferation and glucose metabolism by targeting IRS1 and IRS2 in human chronic myelogenous leukemia (22)	Cross sectional study	CML (n=15) (PB)	qPCR	Luciferase assay	miR-570-3p (downregulated)	*IRS1* *IRS2*
MicroRNA-320a acts as a tumor suppressor by targeting BCR/ABL oncogene in chronic myeloid Leukemia (23)	Case-control study	CML (n=90) Controls (n=90) (BM)	qPCR	Luciferase assay	miR-320a (downregulated)	*BCR-ABL1*
Restoration of miR-424 suppresses BCR–ABL activity and sensitizes CML cells to imatinib treatment (24)	Case-control study	CML (n=18) Controls (n=10) (PB)	qPCR	Luciferase assay	miR-424-5p (downregulated)	*BCR-ABL1*
The malignancy suppression role of miR-23a by targeting the BCR/ABL oncogene in chromic myeloid leukemia (25)	Case-control study	CML (n=79) Controls (n=25) (BM)	qPCR	Luciferase assay	miR-23a-3p (downregulated)	*BCR-ABL1*
Low Expression of miR-196b Enhances the Expression of *BCR-ABL1* and HOXA9 Oncogenes in Chronic Myeloid Leukemogenesis (26)	Case-control study	CML (n=16) Controls (n=10) (BM)	qPCR	Luciferase assay	miR-196b (downregulated)	*BCR-ABL1* *HOXA9*
Decreased microRNA-30a levels are associated with enhanced ABL1 and *BCR-ABL1* expression in chronic myeloid leukemia (27)	Case-control study	CML (n=16) Controls (n=10) (BM)	qPCR	Luciferase assay	miR-30a (downregulated)	*BCR-ABL1*
miR-29b suppresses CML cell proliferation and induces apoptosis *via* regulation of BCR/ABL1 protein (28)	Case-control study	CML (n=5) Controls (n=3) (BM)	qPCR	Luciferase assay	miR-29b (downregulated)	*BCR-ABL1*
Targeting of the signal transducer Smo links microRNA-326 to the oncogenic Hedgehog pathway in CD341 CML stem/progenitor cells (29)	Case-control study	CML (n=6) Controls (n=4) (BM)	qPCR	Luciferase assay	miR-326 (downregulated)	*SMO*
BCR-ABL mediated repression of miR-223 results in the activation of MEF2C and PTBP2 in chronic myeloid leukemia (30)	Cross sectional study	CML (n=35) (PB)	qPCR	Luciferase assay	miR-223 (downregulated)	*MEF2C* *PTBP2*
Down-Regulation of hsa-miR-10a in Chronic Myeloid Leukemia CD34+ Cells Increases USF2-Mediated Cell Growth (31)	Case-control study	CML (n=6) Controls (n=6) (BM)	qPCR	Luciferase assay	miR-10a (downregulated)	*USF*

expression profiles to identify clusters of protein interaction that are highly related to the pathogenesis of CML. The gene interaction relationship was downloaded in the "TSV" format file and was imported into the Cytoscape software (http://www.cytoscape.org/) for further analysis and clustering. The Cytoscape MCODE plug-in was employed to perform module analysis of the target network and protein clustering. The module selection criteria were as follows: degree cutoff = 2, node score cutoff = 0.2, node density cutoff = 0.1, K-score = 2, and max depth = 100. The list of genes in the cluster was then analyzed again using DAVID for significantly enriched ontology terms.

RESULTS

A total of 806 potentially relevant titles were identified from the database search. EndNote X9 software by Clarivate Analytics (Philadelphia, USA) was used as the reference manager. Upon filtering the titles, 372 articles were identified as duplicates, and two other articles were withdrawn from publication. A total of 432 articles were retrieved for abstract reviewing. Upon screening titles and abstracts, 263 articles were removed, resulting in the selection of 159 potentially relevant articles for full-text review. However, three articles could not be retrieved as

the full text are not in English. Then, 147 potentially relevant articles' full text was thoroughly reviewed, and 130 articles were eliminated based on our inclusion and exclusion criteria. Finally, 17 articles were selected to be included in the present systematic review. All studies were original research articles published between the year 2008 to 2019. Homogeneity of the selected studies was ensured by adhering to the defined inclusion and exclusion criteria to prevent sampling bias. Notably, all the studies performed real-time polymerase chain reaction (qPCR) for miRNA expression analysis. A confirmatory method was done to validate the miRNA binding site on their selected genes. Sample sizes for each study varied from 8 to 180 samples for miRNA expression analysis. The characteristics of these studies are highlighted in **Table 1**.

Patient Recruitment and Sampling

Samples collections were described briefly in most of the study. Six studies collected peripheral blood samples, while eight studies collected bone marrow tissues (**Table 1**). The inclusion criteria listed in the study by Nie et al. (20) include diagnosis *via* bone marrow morphology, immunology, molecular biology, and cytogenetic result, with no chemotherapy treatment before the specimen collection (20). In most studies, samples were collected at diagnosis; thus, most patients were in the chronic phase during sample collection. However, the study by Babashah et al. (29) collected samples from CML patients in the blast crisis phase at diagnosis (29).

Effect of miRNA on Cells

The effect of miRNA on cells, including proliferation, apoptosis, cell cycle, migration, and invasion, were adequately reported in the studies. Among miR-409-5p, miR-424, miR-29b, miR-570, miR-320a, miR-23a, miR-196b, miR-30a, miR-326, miR-223, miR-10a that were downregulated in CML clinical samples, the low expression of miR-409-5p, miR-424, miR-29b miRNAs in CML cells were reported to be the cause of the increase in CML cell viability. The low expression of miR-570, miR-320a, miR-23a, miR-196b, miR-30a, miR-326, miR-223, miR-10a in CML cells contribute to high proliferation rate. On the other hand, overexpression of miR-152-3p, miR-362-5p were reported in CML samples and from the functional analysis done, these miRNAs contributed to the increase in proliferation rate. In the cell cycle analysis, the overexpression of miR-152-3p was reported to reduce the percentage of cells in G0/G1 phase when compared with G2SM phase. However, overexpression of miRNA-409-5p, miR-362-5p, miRNA-196b, miR-30a, miR-29b arrested cell cycle in G0/G1 phase and S phase (21). Thus, downregulation of miRNA-409-5p, miR-362-5p, miRNA-196b, miR-30a, miR-29b increase the cell cycle activities in CML cells.

From the selected studies, overexpression of miRNAs like miR-140-5p, miR-320a, miR-570 induced apoptosis, but overexpression of miRNAs like miR-362-5p reduced apoptosis. Thus, in CML cells, a high level of miR-362-5p and a low level of miR-140-5p, miR-320a, miR-570 contribute to low apoptosis activities. Overexpression of miR-320a was proved in the *in vitro* studies to inhibit CML cell migration and invasion, but overexpression of miR-362-5p increased CML cell migration and invasion. Therefore, in CML cells, upregulation of miR-362-5p and downregulation of miR-320a were proposed in cell migration and invasion pathways. Furthermore, synthetic overexpression of miR-570 suppresses glucose metabolism and reduced ATP generation in CML cells. Thus, in clinical samples, downregulation of miR-570 increases glucose metabolism and ATP generation, producing high available energy for cell growth (22).

Targeted Gene Validation

The targeted gene validation of all miRNAs was adequately reported in all studies by co-transfecting cell lines with the targeted gene 3'-UTR reporter vector and miRNA mimic. In all studies, miRNA mimics used in the luciferase assay reveal decreased luciferase activity in wild-type targeted gene 3'UTR, suggesting that each of the miRNAs could bind to their respected genes. Mutant-type targeted gene 3'UTR did not show significant changes in luciferase activities after miRNA mimic transfection in all studies, suggesting a specific target of the miRNAs.

Effect of miRNAs in the Expression of Downstream Genes

Some studies also reported on the downstream genes that are differently expressed related to the expression changes of their studied miRNA. Overexpression of miR-140-5p was reported to increase BAX protein expression indirectly but decreased the BCL2 protein expression *via* SIX1 in CML cells (20). Overexpression of miRNA-409-5p in CML cells indirectly leads to downregulation of NUP43, leading to downstream downregulation of PCNA, c-Myc and cyclin D1 protein (21). The expression of genes associated with glucose metabolism, namely PGC1α, PCK1 and ABCA1 proteins, were indirectly suppressed by miR-570 overexpression *via* IRS1 and IRS2 (22). Inhibition of miR-362-5p indirectly increased P38 and JNK activity in CML cells *via* GADD45A (19). MiR-320a was reported to regulate the phosphorylation of PI3K, AKT and NF-κ B *via* BCR-ABL (23). Expression of p-Crkl and p-STAT5 was reduced in the presence of miR-424 through BCR-ABL (1).

Overexpression of miR-23a resulted in lower expression of PI3K, Akt and MMP-9, which are the downstream target of BCR-ABL (25). Overexpression miR-326 indirectly downregulates SMO expression, leading to downregulation of Bcl2 expression in CML cells (29). Significant downregulation of the survival gene Bcl-xL was reported to be associated with down-regulation of MEF2C and PTBP2 due to overexpression of miR-223-3p (30). Overexpression of miR-29b led to a significant increase in BCR-ABL expression that upregulates p21 and p27 expression in CML cells (28).

Gene Ontology Analysis of the Downstream Genes

A total of 26 downstream genes that were affected by the miRNAs was extracted from all the studies. The functions and pathway enrichment of these genes were analyzed using DAVID (https://david.ncifcrf.gov/home.jsp). A p-value of <0.05 was used as a cut-off standard. The gene listed were categorized into three functional

categories of gene ontology that include biological process (BP), cellular component (CC) and molecular function (MF), as shown in **Table 2**. In the CC group, the downstream genes are enriched in the intracellular component of cells, including nucleus, cytosol, cytoplasm, nucleoplasm, and mitochondria. In the BP group, the downstream genes are enriched in the regulation of transcription, cell proliferation, apoptosis, and drug response. The downstream genes are enriched in the DNA binding, protein binding, and protein heterodimerization activities in the MF group. The complete list for gene ontology cluster is included in '**Data S1**'.

Signaling Pathway Enrichment Analysis of the Downstream Genes

The miRNA targeted genes and downstream genes signalling pathway enrichment analysis were conducted using DAVID with integrated KEGG PATHWAY, BBID, BIOCARTA, and Reactome. Concerning CML pathogenesis, the genes are mainly enriched in pathways related to cancer (hsa05200), microRNAs in cancer pathway (hsa05206), Hepatitis B (hsa05161), PI3K-Akt signalling pathway (hsa04151) and many other pathways with some directly associated with apoptosis, proliferation, and cell cycle pathways as reported in **Table 3**. The complete list of pathways is included in '**Data S2**'.

Identification of Key Candidate Genes and Pathways in the Protein–Protein Interaction Network (PPI) and Modular Analysis of the Downstream Genes

Using STRING online database (http://string-db.org), a total of 26 proteins from miRNA targets and their downstream genes were

TABLE 2 | The significantly enriched analysis of downstream genes in CML.

Term	Description	Count	p-value
MF_GO:0005515	Protein binding	20	2.18E-04
CC_GO:0005634	Nucleus	14	0.002201
CC_GO:0005829	Cytosol	12	4.13E-04
CC_GO:0005737	Cytoplasm	12	0.01868
CC_GO:0005654	Nucleoplasm	8	0.031381
BP_GO:0043524	Negative regulation of neuron apoptotic process	6	5.12E-07
BP_GO:0042493	Response to drug	6	3.02E-05
BP_GO:0008283	Cell proliferation	6	7.30E-05
MF_GO:0043565	Sequence-specific DNA binding	6	3.61E-04
BP_ GO:0045944	positive regulation of transcription from RNA polymerase II promoter	6	0.006252
BP_ GO:0006355	regulation of transcription, DNA-templated	6	0.034631
BP_ GO:0043066	negative regulation of apoptotic process	5	0.002207
MF_GO:0046982	protein heterodimerization activity	5	0.002344
BP_GO:0008284	positive regulation of cell proliferation	5	0.002408
BP_GO:0006915	apoptotic process	5	0.0048708
BP_GO:0000122	negative regulation of transcription from RNA polymerase II promoter	5	0.0112021
MF_GO:0042802	identical protein binding	5	0.012588

TABLE 3 | Signaling pathway enrichment analysis of downstream genes' function in CML patients.

Pathway	Name	Count	Genes	p-value
hsa05200	Pathways in cancer	9	BCR, SMO, CCND1, MYC, BCL2, ABL1, BAX, MMP9, BCL2L1	2.79E-06
hsa05206	MicroRNAs in cancer	7	CCND1, IRS1, MYC, BCL2, ABL1, IRS2, MMP9	5.96E-05
hsa05161	Hepatitis B	6	PCNA, CCND1, MYC, BCL2, BAX, MMP9	2.67E-05
hsa04151	PI3K-Akt signaling pathway	6	CCND1, IRS1, MYC, BCL2, PCK1, BCL2L1	0.001536
h_P53Pathway	P53 Signaling Pathway	5	PCNA, CCND1, GADD45A, BCL2, BAX	7.72E-06
hsa05220	Chronic myeloid leukemia	5	BCR, CCND1, MYC, ABL1, BCL2L1	3.02E-05
hsa04152	AMPK signaling pathway	5	CCND1, IRS1, IRS2, PCK1, PPARGC1A	2.45E-04
hsa04110	Cell cycle	5	PCNA, CCND1, GADD45A, MYC, ABL1	2.53E-04
hsa04068	FoxO signaling pathway	5	CCND1, IRS1, GADD45A, IRS2, PCK1	3.41E-04
hsa05202	Transcriptional misregulation in cancer	5	MEF2C, MYC, SIX1, MMP9, BCL2L1	7.86E-04
hsa05166	HTLV-I infection	5	PCNA, CCND1, MYC, BAX, BCL2L1	0.003689
hsa05210	Colorectal cancer	4	CCND1, MYC, BCL2, BAX	5.17E-04
hsa04920	Adipocytokine signaling pathway	4	IRS1, IRS2, PCK1, PPARGC1A	7.38E-04
hsa05222	Small cell lung cancer	4	CCND1, MYC, BCL2, BCL2L1	0.001299
hsa04931	Insulin resistance	4	IRS1, IRS2, PCK1, PPARGC1A	0.002587
hsa04722	Neurotrophin signaling pathway	4	IRS1, BCL2, ABL1, BAX	0.003489
h_il2rbPathway	IL-2 Receptor Beta Chain in T cell Activation	4	IRS1, MYC, BCL2, BCL2L1	0.003877
127	Mito-stress	3	BCL2, BAX, BCL2L1	0.002915
152	Altered synaptic signalling-neurodegenerative disorders	3	BCL2, BAX, BCL2L1	0.002915
hsa05219	Bladder cancer	3	CCND1, MYC, MMP9	0.004992

filtered into a PPI network complex, containing 22 nodes and 75 edges (**Figure 2**) with a PPI enrichment p-value is 2.22e-16. At the same time, two other proteins did not fall into the PPI network. The results were transferred from STRING to Cytoscape for further analysis. Through Cytoscape MCODE, a significant module from the PPI network complex were found. Functional annotation clustering showed that this cluster (score = 8.909) consisted of 12 nodes and 49 edges (**Figure 2**). The cluster is mainly associated with protein binding (GO:0005515) as all the 12 proteins are involved in this molecular function. Ten of the proteins can be found in the cytosol (GO:0005829), and nine are involved in cancer pathways (hsa05200). **Table 4** includes a functional annotation cluster with more than six proteins involved. The complete list for functional annotation cluster is included in '**Data S3**'.

Prediction of miRNA's Targeted Genes

Apart from the downstream genes reported from the articles, target genes of the miRNAs were also identified

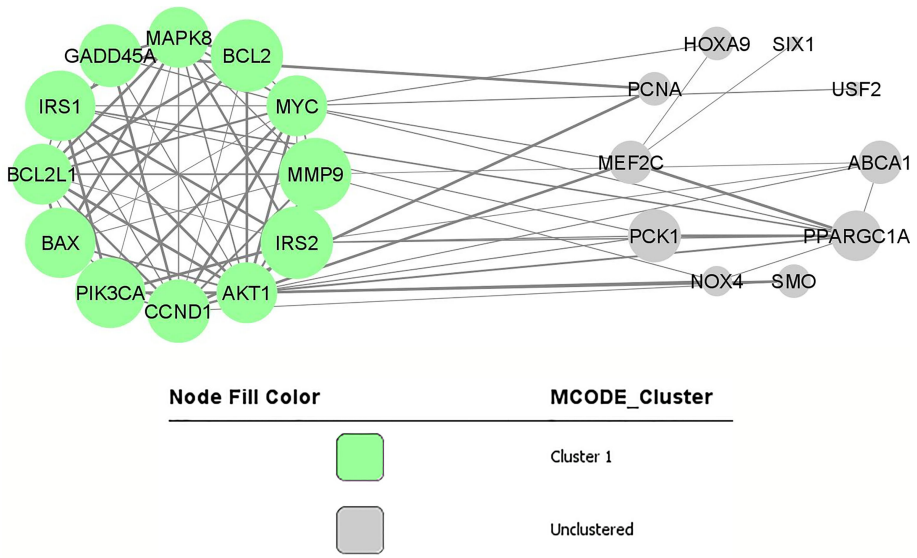

FIGURE 2 | PPI network and modular analysis of downstream genes. From STRING online database analysis, a total of 26 proteins were filtered into a PPI network complex. The green nodes represent a functional annotation cluster that was identified from Cytoscape MCODE. This functional annotation clustering showed a cluster consisted of 12 proteins.

TABLE 4 | Functional annotation clustering on the cluster identified from downstream genes.

Term	Description	Count	p-value
GO:0005515	protein binding	12	7.56E-04
GO:0005829	cytosol	10	8.32E-06
hsa05200	Pathways in cancer	9	1.50E-08
Up_Keywords: Phosphoprotein	Phosphoprotein	9	0.029
hsa05161	Hepatitis B	8	4.92E-10
Up_Keywords: mutagenesis site	mutagenesis site	8	4.06E-05
GO:0005634	nucleus	8	0.020
hsa05210	Colorectal cancer	7	1.87E-10
hsa04068	FoxO signaling pathway	7	2.08E-08
hsa04151	PI3K-Akt signaling pathway	7	5.68E-06
Up_Keywords: Nucleus	Nucleus	7	0.037
hsa05222	Small cell lung cancer	6	1.12E-07
hsa04722	Neurotrophin signaling pathway	6	6.31E-07
h_il2rbPathway	IL-2 Receptor Beta Chain in T cell Activation	6	1.42E-06
hsa04932	Non-alcoholic fatty liver disease (NAFLD)	6	1.98E-06
GO:0043066	negative regulation of apoptotic process	6	5.77E-06
hsa05166	HTLV-I infection	6	2.54E-05
hsa05206	MicroRNAs in cancer	6	4.51E-05
GO:0005739	mitochondrion	6	6.56E-04

from in silico analysis to ensure extensive coverage of miRNAs' targets. One hundred eighty-seven target genes predicted concordantly by four different bioinformatics tools were selected for further analysis. The top fives genes are the DCP2, QKI, S1PR1, NPTN and B4GALT5. The list of genes is included in '**Data S4**'.

Gene Ontology Analysis of the Predicted Target Genes

The functions and pathway enrichment of predicted target genes were analysed using DAVID (https://david.ncifcrf.gov/home.jsp). A p-value of <0.05 was used as a cut-off standard. The genes listed were categorised into three functional categories of gene ontology that include biological process (BP), cellular component (CC) and molecular function (MF), as shown in **Table 5**. In the CC group, the predicted target genes are enriched in the nucleus, proteinaceous extracellular matrix and perinuclear region of the cytoplasm. In the BP group, the genes are enriched in the regulation of transcription, extracellular matrix organisation and angiogenesis. In the MF group, the predicted target genes are enriched in the DNA binding. The list of predicted genes' GO is included in '**Data S5**'.

Signaling Pathway Enrichment Analysis of the Predicted Target Genes

The predicted target genes signalling pathway enrichment analysis were conducted using DAVID with integrated KEGG PATHWAY, BBID, BIOCARTA, and Reactome. The genes were found to be mainly enriched in the PI3K-Akt signalling pathway (hsa04151), focal adhesion (hsa04510), pathways in cancer (hsa05200), and many other pathways, with each pathway, involve from 13 to six predicted target genes (**Table 6**). The complete list for predicted pathways is included in '**Data S6**'.

TABLE 6 | Signaling pathway enrichment analysis of predicted targeted genes' function in CML patients.

Pathway	Name	Count	p-value
hsa04151	PI3K-Akt signaling pathway	13	1.30E-05
hsa04510	Focal adhesion	10	3.13E-05
hsa05200	Pathways in cancer	10	0.003611
hsa04974	Protein digestion and absorption	8	5.43E-06
hsa04512	ECM-receptor interaction	6	6.10E-04
hsa05215	Prostate cancer	6	6.43E-04
hsa05146	Amoebiasis	6	0.001491
hsa04611	Platelet activation	6	0.003644
hsa04068	FoxO signaling pathway	6	0.004149
hsa04910	Insulin signaling pathway	5	0.024022
hsa05214	Glioma	4	0.014896
hsa05211	Renal cell carcinoma	4	0.015518
hsa05222	Small cell lung cancer	4	0.030132
hsa00512	Mucin type O-Glycan biosynthesis	3	0.025168

Identification of Key Candidate Genes and Pathways in the Protein–Protein Interaction Network (PPI) and Modular Analysis of the Predicted Target Genes

Using STRING online database (http://string-db.org), a total of 187 proteins from predicted target genes were filtered into a PPI network complex, containing 136 nodes and 211 edges (Figure 3) with PPI enrichment p-value is less than 1.0E-16. At the same time, 51 other proteins did not fall into the PPI network.

The results were transferred from STRING to Cytoscape for further analysis. Through Cytoscape MCODE, six significant modules from the PPI network complex were found. Functional annotation clustering showed that cluster 1 (score = 9) consisted of 11 nodes and 45 edges (**Figure 3**). Cluster1 is mainly located in the extracellular region and associated with extracellular matrix organisation and the collagen catabolic process. Cluster 2 (score= 5) consisted of five nodes and ten edges (**Figure 3**) associated with homeobox, sequence-specific DNA binding and

TABLE 5 | The significantly enriched analysis of predicted target genes.

Term	Description	Count	p-value
MF_GO:0005515	protein binding	106	0.010477
CC_GO:0005634	nucleus	73	0.001961
BP_GO:0006351	transcription, DNA-templated	30	0.019201
MF_GO:0003677	DNA binding	26	0.033161
BP_ GO:0045944	positive regulation of transcription from RNA polymerase II promoter	24	1.21E-04
BP_GO:0000122	negative regulation of transcription from RNA polymerase II promoter	18	9.06E-04
MF_GO:0003700	transcription factor activity, sequence-specific DNA binding	17	0.036439
MF_GO:0043565	sequence-specific DNA binding	13	0.006885
BP_GO:0030198	extracellular matrix organization	12	4.54E-06
BP_GO:0001525	angiogenesis	12	1.54E-05
CC_GO:0005578	proteinaceous extracellular matrix	12	7.45E-05
BP_GO:0006366	transcription from RNA polymerase II promoter	12	0.014271
CC_GO:0048471	perinuclear region of cytoplasm	12	0.04456
BP_GO:0030574	collagen catabolic process	10	1.39E-08
CC_GO:0005788	endoplasmic reticulum lumen	10	1.26E-04
MF_GO:0000978	RNA polymerase II core promoter proximal region sequence-specific DNA binding	10	0.010699
BP_GO:0008283	cell proliferation	10	0.01151
BP_GO:0043066	negative regulation of apoptotic process	10	0.039644

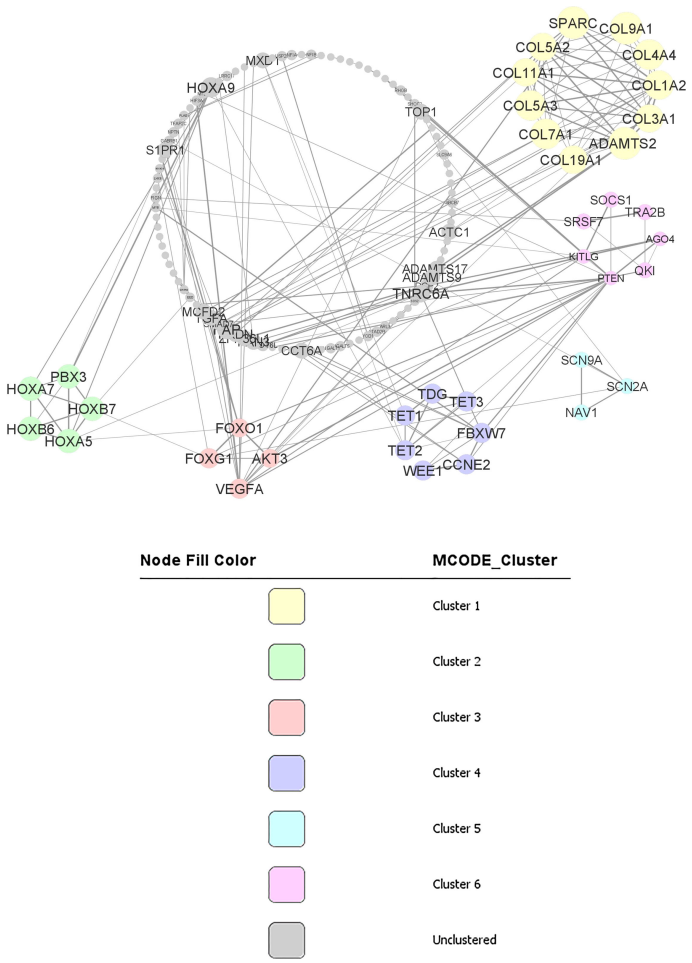

FIGURE 3 | PPI network and modular analysis of downstream genes. From STRING online database analysis, a total of 187 proteins were filtered into a PPI network complex with 136 nodes and 211 edges. Six clusters were identified from Cytoscape MCODE.

transcription regulation. Cluster 3 (score= 3.333) consisted of four nodes and five edges (**Figure 3**) associated with the FoxO signalling pathway, pathways in cancer and mutagenesis site. Cluster 4 (score= 3.33) consisted of seven nodes and ten edges (**Figure 3**) associated with polymorphism, nucleus and DNA methylation. Cluster 5 (score= 3) consisted of three nodes and five edges (**Figure 3**) associated with coiled-coil structure and sodium ion transport channel. Cluster 6 (2.667) consisted of seven nodes and eight edges (**Figure 3**) located in the cytoplasm and nucleus and associated with protein binding, RNA binding and phosphatidylinositol-mediated signalling. **Table 7** includes functional annotation clustering for all six clusters. The list of genes ontology of cluster 1 until 6 are included in '**Data S7**'.

DISCUSSION

Tyrosine kinase inhibitors that target BCR-ABL protein have been successfully used to treat CML. However, there are still many cases that does not response well to the treatment. In those cases, it is postulated that their CML pathogenesis does not only involves BCR-ABL oncogene, it involves other mechanisms. For many decades, numerous molecular and clinical studies involving chromosomal changes, DNA mutation, DNA methylation and mRNA expression have been done to understand the underlying mechanism of CML development and progression. Nevertheless, the complete mechanisms of CML remain unclear. Thus, since many years ago, miRNA has come into the picture and has been studied extensively since then. In this systematic review, we attempted to improve our understanding of the involvement of miRNAs in CML development from fourteen different reports. The studies' dataset was divided into two datasets to make sure high coverage of target genes. **Figure 4** shows the summary of the bioinformatical analysis findings. The analysis of the first dataset started with pooling of the miRNA and their validated downstream genes, followed by gene's ontology analysis, signalling pathway enrichment analysis and finally, the

TABLE 7 | Functional annotation clustering of cluster 1 to 6.

Cluster	Term	Description	Count	p-value
1	Up_Keywords: Extracellular matrix	Extracellular matrix	11	8.05E-20
	CC_ GO:0005576	Extracellular region	11	2.82E-11
	Up_Keywords: Secreted	Secreted	11	6.17E-11
	Up_Keywords: Disulfide bond	Disulfide bond	11	1.65E-08
	Up_Keywords: Signal	Signal	11	1.13E-07
	Up_Keywords: Polymorphism	Polymorphism	11	0.004699
2	Up_Keywords:	Homeobox	5	2.57E-08
	MF_GO:0043565	Sequence-specific DNA binding	5	8.77E-07
	Up_Keywords: DNA-binding	DNA-binding	5	9.82E-05
	Up_Keywords: Transcription regulation	Transcription regulation	5	1.64E-04
	Up_Keywords: Transcription	Transcription	5	1.84E-04
	Up_Keywords: Nucleus	Nucleus	5	0.004211
	CC_GO:0005634	Nucleus	5	0.007789
3	hsa04068	FoxO signaling pathway	3	0.001116
	hsa05200	Pathways in cancer	3	0.009398
4	Up_Keywords: Polymorphism	Polymorphism	7	0.040122
	Up_Keywords: Nucleus	Nucleus	6	0.005069
	GO:0005634	nucleus	6	0.010445
5	Up_Keywords: Coiled coil	Coiled coil	3	0.021754
	GO:0005248	voltage-gated sodium channel activity	2	0.001185
	GO:0001518	voltage-gated sodium channel complex	2	0.001536
6	MF_ GO:0005515	protein binding	7	0.019848
	CC_ GO:0005737	cytoplasm	6	0.008813
	CC_ GO:0005634	nucleus	6	0.010445

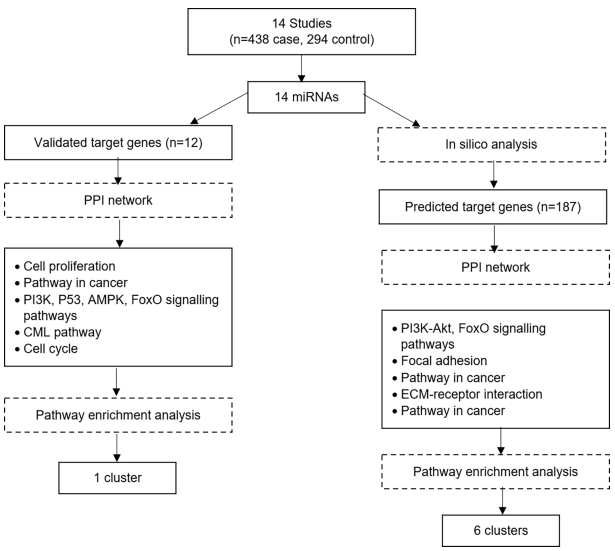

FIGURE 4 | Summary of the bioinformatical analysis findings.

protein-protein interaction network and modular analysis. One cluster was revealed from the first dataset. However, a second dataset was constructed and analysed to have a broader view of miRNA involvement in CML pathogenesis. The analysis of the second dataset involved the pooling of the miRNAs, followed by prediction of their target genes, gene's ontology analysis, signalling pathway enrichment analysis and finally, the protein-protein interaction network and modular analysis. Six significant clusters were revealed from the second dataset.

In this review, through integrated bioinformatical analysis, a cluster consisted of 12 nodes and 49 edges has been identified from the first dataset consisting of the reported target genes and downstream genes. All the 12 clustered proteins are enriched in the intracellular signalling through phosphorylation, and are involved in cancer pathogenesis. Seven of these proteins (MAPK8, CCND1, IRS1, MYC, BCL2, BAX, AKT1) are enriched in the cytosol and nucleus. Three proteins (PIK3CA, IRS2, BCL2L1) are enriched in the cytosol, and one protein is in the nucleus (GADD45A). MAPK8, CCND1, IRS1, GADD45A, MYC, BCL2, AKT1, IRS2, and BCL2L1 are phosphoproteins. Phosphoproteins are proteins that bind to the phosphate group that can be switched on or off. These proteins are essential in most cellular processes such as protein synthesis, cell division, signal transduction, cell growth, development, and ageing (32).

From the KEGG pathway, MAPK8, CCND1, PIK3CA, MYC, BCL2, BAX, AKT1, MMP9, and BCL2L1, are essential genes in cancer pathways that regulate cell cycle, proliferation, apoptosis, genomic instability, and block of differentiation. At the same time, MAPK8, CCND1, PIK3CA, IRS1, GADD45A, AKT1 and IRS2 are involved in FoxO signalling pathway that can affect the regulation of cell cycle, oxidative stress resistance and DNA repair.

The first dataset of those experimentally validated target genes shows a significant effect of miRNAs in CML pathogenesis. However, it is known that miRNA can target more than one target gene due to its seed sequence (33). Therefore, it is crucial to identify the other miRNA's target genes and their pathways involved. This review identified the other miRNA's target genes using four different in silico analyses. All the predicted target genes were then analysed using bioinformatical analysis. Six clusters were identified from the functional annotation clustering analysis of 187 proteins.

Cluster 1 from the second dataset consisted of 11 proteins, including COL3A1, ADAMTS2, SPARC, COL1A2, COL11A1, COL4A4, COL5A3, COL7A1, COL5A2, COL9A1, COL19A1. All these proteins are in the extracellular region and appear to be crucial for extracellular matrix organization. COL3A1, COL1A2, COL11A1, COL4A4, COL5A3, and COL5A2 are involved in ECM-receptor interaction pathways and focal adhesion pathways. This collagen genes family is essential as the component of tissues structure and can interact with cells *via* several receptor families and regulate cell's proliferation, differentiation and migration (34). Other than that, SPARC, another gene in cluster 1, is significantly downregulated in CML patients. In CML cells exposed to exogenous SPARC, the G0/G1 cell cycle arrest and reduced growth rate of the cells were reported (35). However, the mechanism involved in SPARC downregulation in CML is still unknown. Thus, in CML, inhibition of SPARC by miR-29b-3p could be one of the CML pathogenesis mechanisms and is worth investigating.

Cluster 2 consisted of five proteins, including PBX3, HOXA7, HOXB7, HOXB6, and HOXA5, enriched in the nucleus. These proteins have sequence-specific DNA binding and are essential in transcription regulation. Interestingly, PBX3 is an important co-factor for the HOXA gene family (36). The HOX family is a group of highly conserved genes in mammals and are crucial in regulating cell differentiation and proliferation (36). HOXA5 impairs myelopoiesis, causing blockage of hematopoietic stem cells differentiation. The downregulation of HOXA5 is commonly related to DNA methylation (37). Apart from DNA methylation, the current review also found that miR-196b-5p could also regulate HOXA5 expression in CML cells.

Cluster 3 consisted of FOXG1, AKT3, VEGFA dan FOXO1. From KEGG pathway analysis, FOXG1, AKT3, and FOXO1 are enriched in the FoxO signalling pathway, while AKT3, FOXO1, and VEGFA are important in the cancer pathway having essential roles in apoptosis, proliferation and angiogenesis. FOXO1 is a Forkhead box O (FoxO) family member. It plays a role in the regulation of differentiation and metabolism in tissues and organs. In CML, FOXO1 could increase the activation of CML cells (38). Thus, it will be an excellent move to inhibit FOXO1 in deactivating CML cells. In this review, it is suggested that miR-223-3p is a potential regulator of FOXO1 in CML.

Cluster 4 consisted of seven proteins, including WEE1, CCNE2, FBXW7, TDG, TET3, TET2, and TET1. Six proteins (WEE1, CCNE2, TDG, TET3, TET2, and TET1) are located in the nucleus, while four (TDG, TET3, TET2, TET1) are involved in DNA methylation. TET oncogene family that includes TET1, TET2, and TET3 plays a role in the DNA methylation process (39). Although there are no reports on the role of TET genes in CML pathogenesis, TET3 has been identified as a prognostic biomarker for acute myeloid leukaemia (AML) (40), suggesting its involvement in myeloproliferative pathogenesis. Furthermore, the WEE1 gene in the cluster has been linked to the cell cycle and identified as a critical mediator of cell fate in AML (41). Meanwhile, high WEE1 kinase expression in acute lymphoblastic leukaemia (ALL) has been identified as a poor prognostic factor that functions as a cancer-conserving oncogene which helps protect cancer cells from DNA damage (42). As a result of WEE1's participation in the cell cycle and DNA damage repair, the WEE1 kinase family was identified as one of the most promising targets in the DNA damage response (DDR) pathway (43).

Cluster 5 consisted of three proteins, including SCN9A, NAV1, and SCN2A. These proteins are involved in the voltage-gated sodium channel complex. Although there are still no CML studies on these genes, ion channel signalling mechanisms are known to be involved in cancer cells migration, invasion, and metastasis (44). Thus, it will be very informative to study the effect of ion channel signalling through SCN9A and SCN2A *via* miR-301-5p in CML cells.

Cluster 6 consisted of seven protein-binding proteins, including KITLG, SOCS1, AGO4, TRA2B, PTEN, SRSF7, and QKI. KITLG is localised in the cytoplasm, TRA2B is in the nucleus, while SOCS1, AGO4, PTEN, SRSF7, and QKI can be found in both intracellular regions. KITLG, AGO4, PTEN are involved in phosphatidylinositol-mediated signaling that is crucial in regulating cancer cells' survival, proliferation, invasion, and growth. SOCS1 plays an important role in regulating optimal JAK/STAT activity. However, regulation of SOCS1 *via* DNA methylation in CML patients is still uncertain as the findings are contradictory (45, 46). Thus, from the analysis, regulation of SOCS1 *via* miR-30a-5p in CML is suggested.

The studies included in this review only focused on their miRNA of interest and its few target genes. Therefore, this review are not able to rule out the entire networks of miRNAs and their target genes in CML. In silico analyses were done to improve the coverage of miRNA's target genes. However, further *in vitro* analysis and clinical studies need to be done to validate the predicted mechanisms. Nevertheless, this review added new insight into the involvement of miRNA in CML pathogenesis for future studies.

CONCLUSION

Pathogenesis of CML at the molecular level involves a wide range of mechanisms that are still undiscovered. In this study, the function of miRNAs was found to be significant in the development of CML. The miRNA's target genes are localised in the extracellular, cytosol and nucleus of CML cells. Thus, the importance of miRNAs cannot be denied as miRNAs are universally involved in various pathways that regulate genomic instability, proliferation, apoptosis, cell cycle, differentiation, and migration of CML cells. Therefore, from the identified miRNAs and their pathways involved in CML pathogenesis, potential new biomarkers for a better prognosis and new miRNA-based treatment for CML patients could be developed.

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

ME collected and/or assembled data and wrote the manuscript. SS wrote, reviewed, and edited the manuscript. NA confirm the authenticity of all raw data, proofread, edited and gave approval 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/fonc.2022.848199/full#supplementary-material

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Differential Expression of Steroid Hormone Receptors and Ten Eleven Translocation Proteins in Endometrial Cancer Cells

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Steroid hormones govern the complex, cyclic changes of the endometrium, predominantly through their receptors. An interplay between steroid hormones and epigenetic mechanisms controls the dynamic endometrial gene regulation. Abnormalities in expression of genes and enzymes associated with steroid hormone signaling, contribute to a disturbed hormonal equilibrium. Limited evidence suggests the involvement of TET (Ten Eleven Translocation)-mediated DNA hydroxymethylation in endometrial cancer, with some data on the use of TET1 as a potential prognostic and diagnostic biomarker, however the mechanisms guiding it and its regulation remains unexplored. This study aims to explore the changes in the expressions of TETs and steroid hormone receptors in response to estrogen and progesterone in endometrial cancer cells. Gene expression was examined using real-time PCR and protein expression was quantified using fluorescent western blotting in endometrial cancer cell lines (AN3 and RL95-2). Results indicate that TET1 and TET3 gene and protein expression was cell-specific in cancer cell-lines. Protein expression of TET1 was downregulated in AN3 cells, while TET1 and TET3 expressions were both upregulated in RL95-2 cells in response to estrogen-progesterone. Further, a decreased AR expression in AN3 cells and an increased ERα and ERβ protein expressions in RL95-2 cells was seen in response to estrogen-progesterone. PR gene and protein expression was absent from both cancer cell-lines. Overall, results imply that expressions of steroid hormones, steroid-hormone receptors and TETs are co-regulated in endometrial cancer-cells. Further studies are needed to interpret how these mechanisms fit in with DNMTs and DNA methylation in regulating endometrial biology. Understanding the role of TETs and hydroxymethylation in steroid hormone receptor regulation is crucial to comprehend how these mechanisms work together in a broader context of epigenetics in the endometrium and its pathologies.

Keywords: gene expression, steroid hormones and receptors, endometrial cancer cells, ten eleven translocation (TET proteins), DNA hydroxymethylation (5hmC)

BACKGROUND

A two-way communication between epigenetic mechanisms and steroid hormones is crucial for the healthy functioning of the endometrium. Estrogen and progesterone, secreted by the ovaries, execute their functions predominantly *via* steroid hormone receptors - estrogen receptor (ER) and progesterone receptor (PR). Transcriptional regulation of steroid hormone receptors in the endometrium is partly controlled by epigenetic factors like DNA methylation and hydroxymethylation (1–5). DNA methylation yields 5-methylcytosine (5mC), making for one of the most important forms of epigenetic modification in the mammalian DNA (6). However, the modification of DNA from 5C (5-Cytosine) to 5mC can be actively or passively reversed *via* the process of DNA de-methylation. The DNA de-methylation cascade consists of the initial oxidation of 5mC into 5-hydroxymethylcytosine (5hmC) followed by a series of additional oxidation steps (7, 8). 5hmC is identified as an independent epigenetic modification that can alter gene expression and might be important in epigenetic reprogramming (8). The active de-methylation process is catalysed by ten-eleven translocation (TET) enzymes, making them an essential component in epigenetic machinery. Dysregulation of TETs and subsequent 5hmC marks have been implicated in endometrial diseases such as endometrial cancer and endometriosis. (9, 10). Knockout study models have previously been used to establish the function of TETs in various tissues and cells including the maintenance of reproductive axis and epigenetic reprogramming (11–15). DNA methylation is known to be involved in maintaining successful steroid hormone signaling by regulating steroid hormone receptors (16). On the other hand, estrogen and progesterone can influence mRNA and protein expression of DNA Methyltransferases (DNMTs), thereby affecting methylation patterns (17–19).

In the normal endometrium, increasing estrogen levels during the proliferative phase, lead to an increase in the expression of estrogen (ER), progesterone (PR) and androgen receptors (AR) (20). This is followed by an antagonistic progesterone action which is mediated by the increased levels of progesterone receptors (21, 22). The interplay between estrogen and progesterone implies that while estrogen action aids in upregulating steroid receptors in the endometrium, progesterone action downregulates them (20). Since the maintenance of this steroid hormone equilibrium is essential to endometrial biology, abnormal regulation of steroid hormone receptor expression can contribute to endometrial pathologies (23–28). Previously, it has been suggested that TETs and DNMTs could potentially be inversely regulated by steroid hormones, with epithelial cells being more sensitive and responsive to steroid hormone treatments (29). This study is aimed at mimicking the hormonal influences seen during the menstrual cycle *in vitro*, to explore the mechanisms involved in the regulation of TETs and steroid hormone receptors in endometrial cancer cells. Four steroid hormone receptors– Estrogen receptors alpha (ERα) and beta (ERβ), Progesterone receptor (PR) and Androgen Receptor (AR) along with TETs were examined to assess the role of steroid hormones in their transcriptional and translational regulation.

Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; AR, Androgen Receptor; AREs, Androgen Responsive Elements; CS-FBS, Charcoal stripped FBS; C treatment, Control; DNMTs, DNA Methyltransferases; E treatment, Estradiol treatment; EP treatment, Estrogen + progesterone treatment; Era, Estrogen receptor alpha; ERβ, Estrogen receptor beta; ER, Estrogen receptors; ERRα, Estrogen related receptor alpha; PR, Progesterone receptor; TDG, thymine DNA glycosylase; TET, Ten-eleven translocation.

MATERIAL AND METHODS

Preparation and Treatment of Cell Lines

Endometrial adenocarcinoma cell lines, AN3 (ATCC® HTB-111™) and RL95-2 (ATCC® CRL-1671™) were used for this study. All cells were cultured either in phenol-free DMEM or RPMI medium, supplemented with 10% charcoal stripped FBS (CS-FBS) as well as 1% of penicillin-streptomycin antibiotic (ThermoFisher Scientific, USA). The cells were then cultured in a humidified atmosphere with 5% CO_2 at 37°C until confluent. Cells were then plated in twelve-well culture plates and upon 80% confluence, they were primed with 0.01μM of β-estradiol (E treatment) for 24h. Followed by the addition of progesterone (1μM) to the estrogen primed (EP treatment) wells for 24, 48 and 72h. Ethanol at a concentration of less than 0.01% was used as control (C treatment). The treatment solutions were prepared using commercially available powdered concentrates (Sigma-Aldrich, USA) and dissolved in analytical grade ethanol. The final concentrations were prepared in culture media and stored at -80°C until further use.

RNA Extraction

Trizol® reagent (Life Technologies, NZ) was used to extract Total RNA. 1ml of Trizol® was added per well and cells were detached using a cell scrapper. The cells were homogenized and treated according to the manufacturer's instructions. Using the protocol provided, chloroform (0.2ml/1ml of Trizol®) was added to the samples and vigorously shaken and incubated for 3minutes at room temperature. After a 15minute centrifugation (12000xg) at 4°C, isopropanol (0.5ml/1ml of Trizol®) was added to the aqueous phase and incubated for 20minutes on ice. Followed by another centrifugation under similar conditions, the RNA pellet was obtained and washed in 70% ethanol with additional 10minute centrifugations, twice. The pellet was air dried at room temperature and suspended in DEPC treated water. The concentration and quality of RNA was assessed using the NanoPhotometer® (Implen, Germany). An OD260/280 ratio of 1.8 to 2.0 was considered quality RNA.

Reverse Transcription and Quantitative RT-PCR

As directed by the manufacturer's instruction manual, 1μg of RNA was treated with 1μl of 10xDNase and DNAse Buffer each and made up to 10μl with DEPC-water. After a 15minute incubation at room temperature, 1μl of EDTA was added to each reaction tube and incubated at 65°C for 10minutes. Reverse transcription into single-stranded cDNA was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). According to the manufacturer's instructions, each tube was mixed with

Reverse Transcriptase Buffer, Random primers, Deoxynucleotide Mix and Reverse Transcriptase and made it up to a total reaction volume of 20µl. Using the BioRad DNA Engine® Peltier Thermal Cycler under the following conditions: 10minutes at 25°C, 120minutes at 37°C and 5minutes at 85°C, reverse transcription was performed. The resulting cDNA was diluted with nuclease-free water (1:10) and used for real-time PCR. PCR analysis was performed and conducted using QuantStudio (Applied Biosystems, USA) as previously described (29). Primers for *TET1, TET2, TET3, RPL13a, YWHAZ* and *RPLO* (**Table 1**) were obtained from Primer Bank (30–33). Primers for *ERα*, *ERβ* and *PGR* were the PrimeTime predesigned qPCR Assays (IDT) (**Table 1**). Primer (**Table 1**) for *AR* was obtained from a previously published study by Kamal et al. (34). Gene expression analysis was done using the comparative CT method (ΔΔCT method) (35). All the results were normalized to the geomeans of the three reference genes- *YWHAZ, RPL13a* and *RPLO* as described previously (29).

Protein Extraction

AN3 and RL95-2 cells were extracted from culture plates using RIPA lysis and extraction buffer (ThermoFisher Scientific, USA). According to the instructions provided, culture media was aspirated and 1ml/well of cold RIPA buffer was added to lyse the cells. Halt™ Protease Inhibitor Cocktail (EDTA-Free (100X)) (Thermo Fisher Scientific) was also added (20µL per 1mL of RIPA lysis buffer). The plate was then incubated on ice for 5minutes with intermittent swirling for uniform spreading of the buffer. The lysate was gathered using a cell scraper and transferred to a 2ml tube. The samples were then centrifuged at 14000xg for 15 minutes and the supernatant protein was collected and stored at -80°C for further analysis.

Fluorescent Western Blot

Western blot analysis was performed to evaluate the expression of TETs and steroid hormone receptor proteins post-treatment. Protein extracted from the cells were loaded on a 3-8% NuPage™ Tris Acetate gel (Invitrogen, USA) and transferred onto a 0.45 micron pored fluorescent polyvinyl difluoride membrane (Fl-PVDF), (Millipore, USA). The protocol for a wet transfer was followed according to the manufacturer's instructions using 20X NuPage™ Transfer Buffer (Invitrogen, USA). Once the proteins were transferred onto the membrane, it was stained and washed with Revert™ total protein stain and wash solution respectively, (Licor Biosciences, USA). The membrane was imaged at 700nm Odyssey® imaging system and blocked using Intercept Blocking Buffer (Licor Biosciences, USA) for an hour at room temperature. Primary antibodies were diluted according to **Table 2** in the blocking buffer. Following which, the membrane was incubated in primary antibody and left overnight at 4° C and conjugated with secondary antibodies the next day. The membrane was washed thrice using 1XTBST (Tris-buffered saline with Tween-20) with 5minute intervals and incubated with the secondary antibody for 1hr at room temperature. The membrane was washed, dried, and imaged for 10minutes at 800nm channel using Odyssey® imaging system (Licor Biosciences, USA). All the primary antibodies (**Table 2**) used for this experiment were from Thermo Fisher Scientific and the secondary antibodies were from Licor Biosciences - donkey anti-mouse (P/N: 926-32212) or goat anti-rabbit (Catalog# P/N: 926-32211) IRDye® 800CW depending on primary antibody reactivity. Protein expression for all samples were normalized to the total protein stain for each blot. Target protein bands were normalized against the total protein transferred per lane. Total protein signal (TPS) was used to calculate the proteins in each lane and the normalization factor. The formulas used for each calculation are below:

$$\text{Lane Normalization Factor} = \frac{TPS\ \ for\ \ each\ \ lane}{TPS\ \ from\ \ the\ \ lane\ \ with\ \ the\ \ highest\ \ TPS}$$

$$\text{Normalization Signal} = \frac{Target\ \ band\ \ signal}{Lane\ \ normalization\ \ factor}$$

The normalized signal for each sample was calculated to be used for relative quantitative comparison. The x-axis demonstrated the fold change that was normalized to the control and was plotted against the treatment stage (y-axis) for each sample.

Statistical Analysis

GraphPad Prism 8 (GraphPad Software, La Jolla, CA) and IBM SPSS version 27.0 (Armonk, NY) were used to analyze the data obtained. Statistical tests included one-way analysis of variance (ANOVA) and paired t-test to determine significance ($P<0.05$ was considered statistically significant; $P \leq 0.1$ was considered as approaching significance). All the Graphs were generated using GraphPad Prism 8 (GraphPad Software, CA).

TABLE 1 | Primer Sequences used for qRT-PCR.

Gene	Sense	Antisense
TET1	CAGAACCTAAACCACCCGTG	TGCTTCGTAGCGCCATTGTAA
TET2	GAGCAGGTCCTAATGTGGCAG	GCTCGCTCCCGCACCAA
TET3	TCCAGCAACTCCTAGAACTGAG	AGGCCGCTTGAATACTGACTG
ERα	CCCACTCAACAGCGTGTCTC	CGTCGATTATCTGAATTTGGCCT
ERβ	AGCACGGCTCCATATACATACC	TGGACCACTAAAGGAGAAAGGT
PGR	ACCCGCCCTATCTCAACTACC	AGGACACCATAATGACAGCCT
AR	AGGATGCTCTACTTCGCCCC	CTGGCTGTACATCCGGGAC
RPL13a	GCCCTACGACAAGAAAAAGCG	TACTTCCAGCCAACCTCGTGA
YWHAZ	CCGTTACTTGGCTGAGGTTG	CAGGCTTTCTCTGGGGAGTT
RPLO	AGAAACTGCTGCCTC ATATCCG	CCCCTGGAGATTTTA GTGGTGA

TABLE 2 | Details of primary antibodies used and their dilutions.

Primary Antibody	Host	Dilution	Catalogue Number
ERα	Mouse	1:500	MA514501
ERβ	Mouse	1:1000	PA1311
PR	Mouse	1:500	MA1410
AR	Mouse	1:200	MA513426
TET 1	Mouse	1:400	MA5-16312
TET 2	Rabbit	1:300	PA5-76801
TET 3	Rabbit	1:200	PA5-31860

RESULTS

Gene Expression of TETs and Steroid Hormone Receptors in AN3 Cell Lines in Response to Steroid Hormone Treatment

TET1 was significantly downregulated (p=0.0479) post the initial 24 hour estrogen treatment, followed by a significant increase in response to a combined estrogen-progesterone treatment for 24 hours (p=0.0361). Prolonged exposure to combined estrogen-progesterone for 48 and 72 hours resulted in a significant reduction of *TET1* gene expression (p=0.0302). *TET2* and *TET3* did not display any significant in response to any treatments, although there was a significant increase in *TET2* between combined estrogen-progesterone treatment from 24 to 48 hours (p=0.0276). No significant changes in steroid hormone receptor expression were observed in AN3 cells in response to any treatments.

Protein Expression of TETs and Steroid Hormone Receptors in AN3 Cell Lines in Response to Steroid Hormone Treatment

TET1, 2 and 3 proteins in AN3 cells exhibited no changes during estrogen only treatment as observed in **Figure 2**. However, differential expression was observed when treated with combined estrogen-progesterone for 24, 48 and 72 hours. TET1 protein expression displayed a decreasing trend when exposed to 72 hours of combined estrogen-progesterone treatment (p=0.1). Conversely, TET3 protein expression displayed a trend toward increasing upon 72 hours of estrogen-progesterone treatment (p=0.1). Furthermore, there was a trend towards an increase in TET2 protein expression in response to 24-hour estrogen-progesterone treatment, approaching significance (p=0.1). Protein expression for steroid hormone receptors revealed no significant changes in ERβ expression. However, AR protein expression was consistently downregulated during treatment with estrogen-progesterone for 24 (p=0.08), 48 (p=0.059) and 72 (p=0.09) hours (**Figure 3**). No bands for ERα, PRA and PRB proteins were not detectable by western blotting in AN3 cells under any treatments.

Gene Expression of TETs and Steroid Hormone Receptors in RL95-2 Cell Lines Upon Steroid Hormone Treatment

Gene expressions for all three *TETs* varied significantly across different treatments (p< 0.0001) in RL95-2 cells. However, no statistical significance was found between individual treatment groups in comparison to the control. Gene expression for *ERα, PR* or *AR* were not detected in RL95-2 cells. However, *ERβ* gene expression was prominent in hormone treated RL95-2 cells with significant changes between control and treatments (p<0.0001), as revealed by one way ANOVA with no significant differences between individual treatment groups (**Figure 4**).

Protein Expression of TETs and Steroid Hormone Receptors in RL95-2 Cell Lines Upon Steroid Hormone Treatment

Protein expression of TETs varied across different treatments as shown in **Figure 5**. TET1 (p=0.01) expression was significantly decreased and a similar trend towards a decrease in TET3 (p=0.1) expression was also seen in response to estrogen only treatment. There was a significant increase in TET3 (p=0.019) and a trend towards an increase in TET1 (p=0.1), when treated with estrogen-progesterone for 72 hours. In response to 48 hours of estrogen-progesterone treatment, a significant increase in TET3 (p=0.02) and a trend towards reduction in TET1 (p=0.1) expression was observed. TET2 protein expression was significantly upregulated during estrogen only treatment (*p=0.059). The protein expression of ERs revealed a differential and treatment dependent regulation as shown in **Figure 6**. There was a trend towards an increase in ERα protein (p=0.1) expression in response to 24 hours of estrogen-progesterone treatment, and ERβ expression (p=0.1) in response to estrogen only treatment which stayed consistent across treatments with significant increase seen in response to 72 hours of a combined estrogen-progesterone treatment (p=0.038). Very faint bands for AR were observed with no significant differences between treatments. PRA and PRB were not detected in RL95-2 cells even in response to treatment.

DISCUSSION

DNA methylation and hydroxymethylation are crucial components of the epigenetic machinery. The aim of this study was to evaluate the contribution of steroid hormones in the transcriptional and translational regulation of TETs and steroid hormone receptors in endometrial cancer cells. The results indicate that the gene and protein expressions of TETs and steroid hormone receptors and their response to steroid hormones is cell-specific and differ between AN3 and RL95 cells.

TET and Steroid Hormone Receptor Regulation in AN3 Cell Line

Endometrial pathologies such as endometrial cancer are steroid dependent disorders. Steroid hormones guide the fluctuating

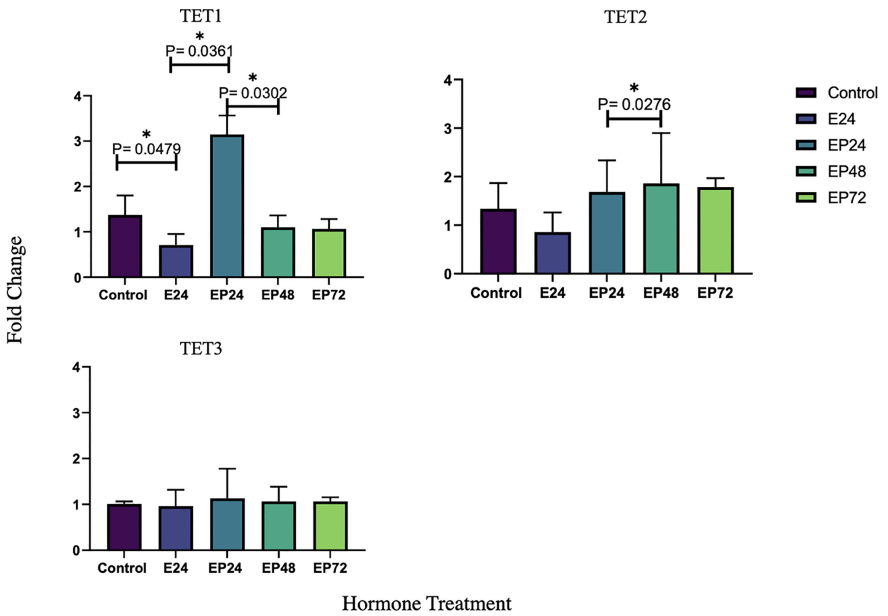

FIGURE 1 | Relative TET1 TET2 and TET3 mRNA expression in response to steroid hormone treatment in AN3 cells. The y-axis shows the fold change of mRNA levels following different treatments compared with control, all results corrected against geo-mean expressions of three reference genes - YWHAZ, RPLO and RPL13a. The x-axis shows different treatment groups. E24 = 24h Estrogen; EP24, EP48 and EP72 = both Estrogen + Progesterone for 24, 48 and 72h. Data are presented as mean ± SEM *p < 0.05. P ≤ 0.1 was considered as approaching significance. The experimental setup included three independent sets of cell culture experiments (n = 3) and triplicates of each sample for the RT-PCR.

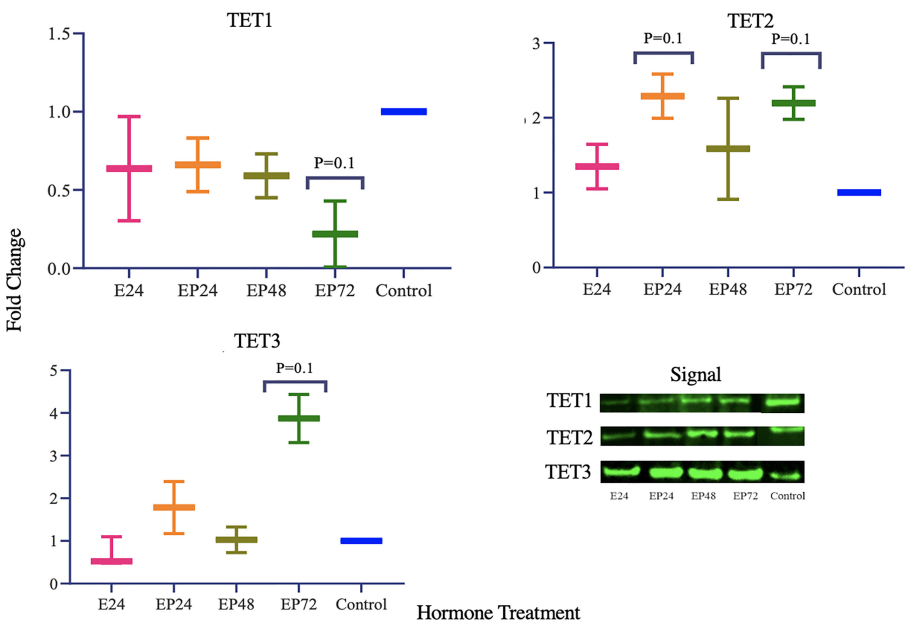

FIGURE 2 | TET protein expression in response to different steroid hormone treatments in AN3 cells. A representative blot image for the particular weight band is shown next to the graph. The y-axis shows the fold change of protein levels following different treatments compared to control and x-axis shows the different treatment groups. E24, 24h Estrogen; EP24, EP48 and EP72 = both Estrogen + Progesterone for 24, 48 and 72h. Data are presented as mean ± SEM, *p < 0.05 (P ≤ 0.1 is considered as approaching significance). The experimental setup included three independent sets of cell culture experiments (n = 3) with three technical replicates for each sample.

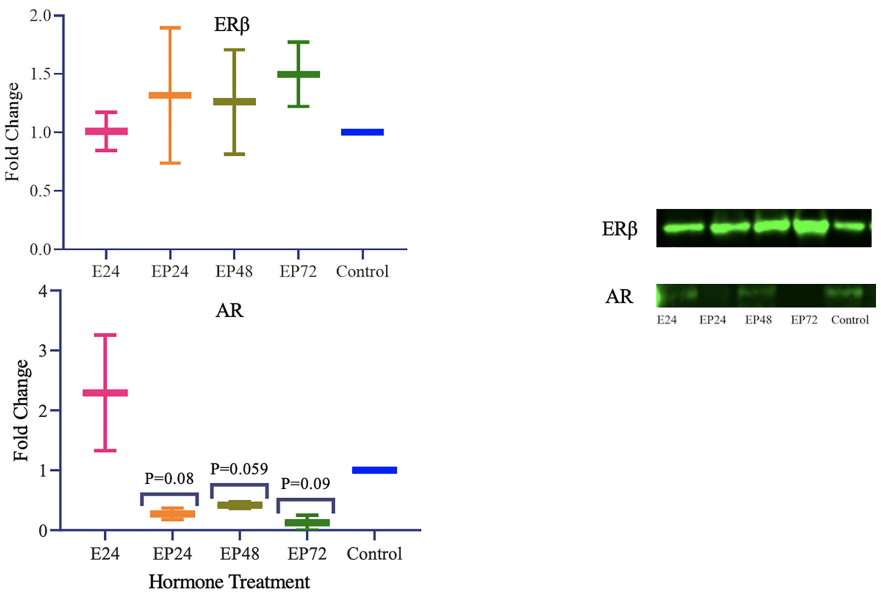

FIGURE 3 | Steroid hormone protein expression in response to different steroid hormone treatments in AN3 cells. A representative blot image for the particular weight band is shown next to the graph. The y-axis shows the fold change of protein levels following different treatments compared to control and x-axis shows the different treatment groups. E24 = 24h Estrogen; EP24, EP48 and EP72 = both Estrogen + Progesterone for 24, 48 and 72h. Data are presented as mean ± SEM. P ≤ 0.1 is considered as approaching significance. The experimental setup included three independent sets of cell culture experiments (n =3) with three technical replicates for each sample.

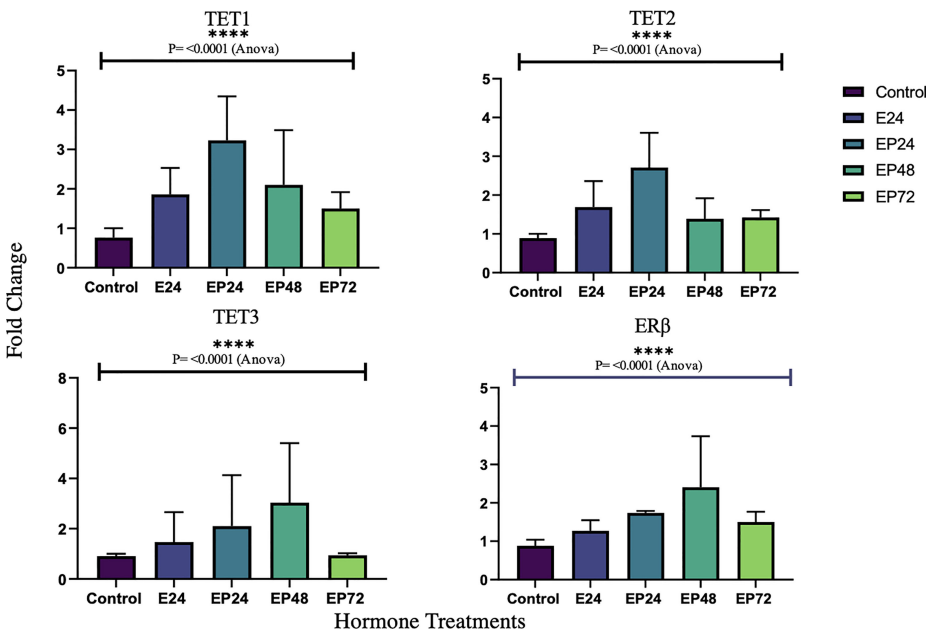

FIGURE 4 | Relative TET and ERb mRNA expression in response to different steroid hormone treatments in RL95-2 cells. The y-axis shows the fold change of mRNA levels following different treatments compared with control, all results corrected against geo-mean expressions of three reference genes - YWHAZ, RPLO and RPL13a. The x-axis shows different treatment groups E24 = 24h Estrogen; EP24, EP48 and EP72 = both Estrogen + Progesterone for 24, 48 and 72h. Data are presented as mean ± SEM; ***P < 0.001; P ≤ 0.1 was considered as approaching significance. One way ANOVA test revealed significant variations in TET and ERb expression across treatments (****P<0.0001). The experimental setup included three independent sets of cell culture experiments (n =3) and triplicates of each sample for the RT-PCR.

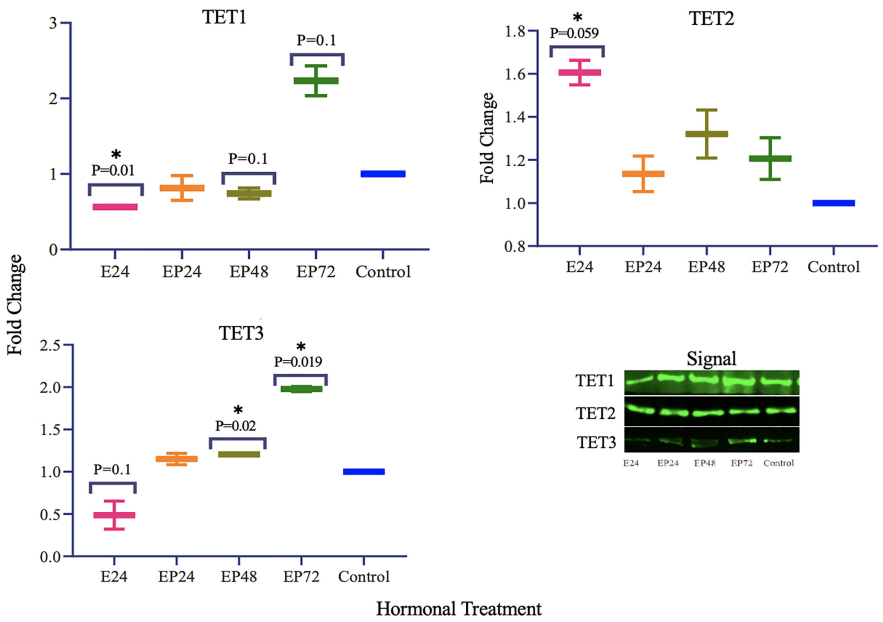

FIGURE 5 | TET protein expression in response to different steroid hormone treatments in RL95-2 cells. A representative blot image for the particular weight band is shown next to the graph. The y-axis shows the fold change of protein levels following different treatments compared to control and x-axis shows the different treatment groups. E24 = 24h Estrogen; EP24, EP48 and EP72 = both Estrogen + Progesterone for 24, 48 and 72h. Data are presented as mean ± SEM, *p < 0.05, P ≤ 0.1 is considered as approaching significance. The experimental setup included three independent sets of cell culture experiments (n =3) with three technical replicates for each sample.

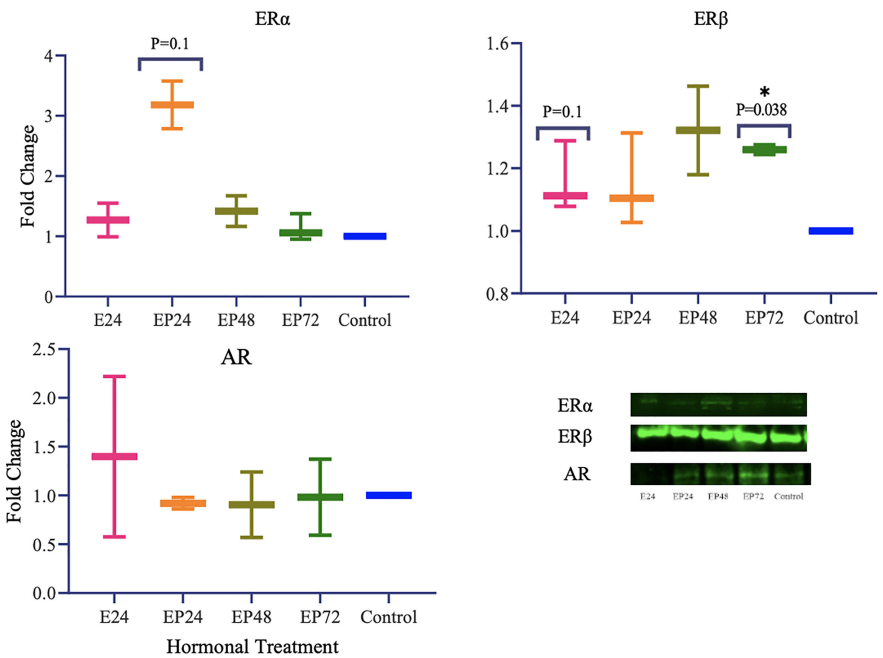

FIGURE 6 | Steroid hormone protein expression in response to different steroid hormone treatments in RL95-2 cells. A representative blot image for the particular weight band is shown next to the graph. The y-axis shows the fold change of protein levels following different treatments compared to control and x-axis shows the different treatment groups. E24 = 24h Estrogen; EP24, EP48 and EP72 = both Estrogen + Progesterone for 24, 48 and 72h. Data are presented as mean ± SEM, *p < 0.05, P ≤ 0.1 is considered as approaching significance. The experimental setup included three independent sets of cell culture experiments (n = 3) with three technical replicates for each sample.

epigenetic patterns, allowing genes to be expressed or repressed during the menstrual cycle (16). In AN3 cells, *TET1* transcription was significantly downregulated when exposed to estrogen for 24 hours. This was then marked by a significant increase when exposed to a combined estrogen-progesterone treatment for 24 hours. However, a further prolonged estrogen-progesterone treatment for 48 and 72 hours resulted in a significant decrease in *TET1* mRNA expression (**Figure 1**). *TET1* gene expression in AN3 cells, was responsive to the slightest change in treatment, which could indicate its sensitivity to subtle hormonal changes.

TET1 protein expression parallels the gene expression and is significantly downregulated when treated with estrogen-progesterone for 72 hours. According to the results of our previous study, *TET1* mRNA was upregulated during the mid-secretory phase in healthy endometrial tissues and in response to progesterone treatment in epithelial cells, *in vitro* (29). The decreased protein expression during 72 hours of estrogen-progesterone treatment, suggests a potential aberrant regulation of TET1 in AN3 cells. Data by other studies report similar findings with decreased TET1 mRNA and protein expression in endometrial cancer tissues compared to normal (9, 36). It has been suggested that overexpression of DNMT3a and DNMT3b contribute to hypermethylation of *ERα* and *PR*, subsequently silencing these genes in endometrial cancer (37). TETs mediate epigenetic alterations *via* DNA de-methylation, a process where they actively remove the methyl group, to activate gene expression (38). Downregulation of TET1 gene and protein expression, could be associated with the abnormal inactivation of ERα and PR seen in endometrial cancer tissues (37, 39, 40). While other studies have reported mRNA expression of *ERa* in AN3 cells at the basal level (41), neither gene nor protein expression of *ERa* or *PR* in AN3 cells in the present study. The discrepancy in the *ERa* gene expression in both the studies could be attributed to the differences in the treatment protocol used. The data obtained from this study indicates that the downregulation of TET1 in response to estrogen and progesterone could potentially be contributing to epigenetic deregulation and warrants the need for more studies to investigate its role in endometrial cancer.

TET2 has been previously implicated in various types of malignancies (11, 42–46). Data from the present study imply that TET2 protein expression is upregulated when exposed to a combined estrogen-progesterone treatment for 24 hours (**Figure 2**). Further, it is also seen that mRNA expression of *TET2* remains upregulated upon continued exposure to estrogen-progesterone treatment for 48 hours (**Figure 1**) in AN3 cells. *TET2* expression has been shown to be significantly reduced in severe endometrial cancer and cervical squamous cell carcinoma tissues compared to their normal counterparts (9, 47). So far to our knowledge, there are no studies that have evaluated any cell specific changes in relation to malignancy or hormonal treatment in endometrium. Our previous study reported an upregulation of *TET2* expression in non-estrogen primed, non-cancerous endometrial epithelial cells, in response to progesterone (29). Further studies are needed to fine tune the mechanisms by which TET2 might be deregulated in endometrial cancer.

While no hormonal effects on *TET3* mRNA expression were observed, TET3 protein was significantly increased during 72 hours of estrogen-progesterone treatment (**Figure 2**). This is in agreement with Cieselski et al., who also reported an increased *TET3* mRNA expression in endometrial cancer tissue biopsies (9). TET3 has been reported to be crucial in the maintenance of stem cell identity, DNA repair and overall genome stability in various tissues (48–51). Aberrations in stem cells have been implicated in the origin and progression of endometrial cancer (52, 53). Increased TET3 protein expression in cancer, could potentially indicate its involvement in abnormal stem cell regulation, contributing to progression, invasiveness and metastasis. Furthermore, reduced mRNA expression of *TET2* and *TET3* have been implicated in the induction of epithelial-mesenchymal transition in melanoma (54). This study, indicates an increased expression of TET2 and TET3 in the combined estrogen-progesterone treated samples in AN3 cells. The difference in results could be attributed to the type of cell and treatment protocols used. Collectively, it is implied that TET2 and TET3 could be involved in the differential regulation seen in cancers, however its exact association still needs to be explored further.

ER and PR have been extensively studied in endometrial pathologies such as cancer and endometriosis. AR, however, is a lesser explored steroid hormone receptor in endometrial biology. Our data imply that in AN3 cells, AR protein levels were significantly downregulated when treated with estrogen-progesterone together for 24, 48 and 72 hours (**Figure 3**). AR is a known anti-estrogen, which means that it has the ability to counteract the proliferative activity of estrogen (55). In normal epithelial cells, a differential and increased expression of *AR* is reported using immunohistochemistry during the secretory phase of the endometrium (56). Downregulation of AR levels are seen in the estrogen-progesterone treated samples, could be related to the decreased TET1 mRNA and protein expression. An association between TET1 and AR has also been suggested by Dhiman et al. (57). Their study reports that TET1, AR and thymine DNA glycosylase are co-recruited to the transcription start site of the Androgen Responsive Elements (AREs) to influence gene regulation in human prostate cells (57). Thus, suggesting that TET1 could be potentially involved in the transcriptional activation of AR in endometrial biology. Moreover, it has been suggested that AR suppresses tumor growth in ER positive breast malignancies (58). The findings of this study imply that the downregulation of *AR* and absence of *PR* gene expression in AN3 cells, could be contributing to the uncontrolled proliferation, seen in endometrial cancer cells. This study suggests that steroid hormones regulate the crosstalk between TETs and steroid hormone receptors in endometrial biology. Understanding this regulation more robustly in the endometrium, could help provide novel targets for therapeutic interventions for associated pathologies.

TET and Steroid Hormone Receptor Regulation in RL95-2 Cell Line

In RL95-2 cells, one way ANOVA analysis suggest a significant influence of hormones on TET mRNA expressions. However, no

significant differences between individual treatments were observed. Gene expression for *TET1*, *TET2 and TET3* were highest when exposed to combined estrogen-progesterone treatments for 24 hours and 48 hours (**Figure 4**). Protein data indicated a differential expression of TETs when exposed to different treatments. TET1 and TET3 levels were significantly increased in response to 72 hour combined estrogen-progesterone treatment (**Figure 5**). mRNA and Protein upregulation of TET1 is also demonstrated in another study suggesting that the hypoxic, chronic inflammatory environment seen in endometrial cancer, can up-regulate TET1 expression and induce its downstream gene transcription (59). Upon exposure to 24 hours of estrogen, TET1 and TET3 were significantly downregulated whereas TET2 was upregulated (**Figure 5**), implying a potential difference in the regulation and function of TETs. TET2 has been reported to serve as a co-activator of ERα by de-methylating and maintaining low CpG methylation levels in breast cancer cell lines (60). This could potentially explain the significant upregulation of ERα when treated with estrogen-progesterone for 24 hours (**Figure 6**). Additionally, the upregulation of ERβ could be correlated to the increased protein expression of TET1 and TET3 in response to 72 hours of estrogen-progesterone treatment. This finding suggests a possible interplay between TETs, regulated by hormones and influencing ERα and ERβ expression in RL95-2 cells.

Conflicting studies on the mRNA expression of *ERα* and *ERβ* have been reported in endometrial cancer. While some suggest lower *ERα* expression (37, 61), others report higher *ERα* expression in comparison to *ERβ* in endometrial cancer tissues (62, 63). The results of this study indicate a differential steroid hormone receptor regulation between RL95-2 and AN3 cells. RL95-2 cells have an increased expression of ERα and ERβ protein during treatments (**Figure 6**). Whereas in AN3 cells, no mRNA data for either were observed and only ERβ protein bands were seen with no statistically significant differences. The results of this study partially agrees with Sun *et al.*, who report significantly increased ERα mRNA and protein expression in AN3 and RL95-2 cells in comparison to other endometrial cancer cell lines (41). They also suggest an increased gene expression of estrogen related receptor alpha (*ERRα*), an orphan nuclear receptor known to mediate the effects of estrogen, in AN3 and RL95-2 cells (41). Therefore implying the need to study the involvement of orphan nuclear receptors in estrogen signaling and action, as well as understanding their association with TETs is imperative. This is also consistent with findings in other endometrial cancer cells that suggest, TET1 increases estrogen sensitivity by upregulating mRNA expression of orphan nuclear receptor - *GPER*, in ishikawa and HEC-1-A cells (64).

PR was not expressed in RL95-2 cells, either at the mRNA level or at the protein level, which is a finding reported in another study as well (65). AR however, was not expressed at the mRNA level but was seen at the protein level, however the differences were not significant (**Figure 6**). Previously, AR protein expression has been reported in endometrial carcinomas with conflicting data on the level of expression. While Sasaki et al., demonstrated hypermethylation mediated AR gene silencing, Ito et al., suggested increased AR expression in endometrial carcinoma tissues (56, 66). Recent data imply that AR positivity is seen in a subset of endometrial carcinomas and is expressed conversely to ERs (55). A similar correlation can be drawn from the findings of this study, wherein increased ERα and ERβ is associated with reduced AR protein expression in RL95-2 cells. Since AR is anti-proliferative, its use as a potential target for curbing uncontrollable cellular hypertrophy is implied however this might not be the case for all tumors and more studies on tumor endocrinology is needed. A better understanding of the steroid hormone regulation and epigenetic axis in female cancers could help in the development of targeted transcriptional endocrine therapies.

In this study, a degree of variation between mRNA and protein expression of TETs and steroid hormone receptors in both, AN3 and RL95-2 cells was observed. The correlation between transcription and translation is complex and depends on several biological and technical factors. It has been suggested that the physical properties of transcription, can alter the translation efficiency at various levels contributing to a discrepancy between mRNA and protein data (67). The other most important and highly variable factor, influencing mRNA-protein correlation is the individual half-lives of proteins (68). For instance, it has been reported that long term estrogen exposure, increases ERα half-life, maintaining protein stability and slowing rate of proteolysis, which could explain the presence of ERα in RL95-2, despite no mRNA expression being observed (69). Subsequently, post-translational and post transcriptional modifications and delayed synthesis between mRNA and protein, could also result in a poor mRNA-protein correlation (70, 71).

In summary, endometrial cancer is complex and involves abnormal steroid hormone signaling. This study evaluates steroid hormone regulation of TETs and steroid hormone receptors *in vitro* and also highlights the importance of evaluating different cancer cell lines independently, to understand the mechanisms of hormone action. It is proposed that differential protein expression of TETs during different hormonal treatments could be involved in the regulation of ERα and ERβ in RL95-2 and AN3 cells. The downregulation AR in AN3 cell line could be explored further as a potential target for hormone therapy. However, for a more comprehensive understanding of the association between TETs and steroid hormone receptors, additional studies including endometrial cancer tissues and primary cells, need to be undertaken. Overall, this study provides a preliminary account, indicating that TETs, steroid hormones and their receptors might be co-regulated to maintain hormone signaling in the endometrium. Future studies involving the assessment of 5-hmC levels and gene promoter sequencing might help in determining the epigenomic regulation of steroid hormone receptors in endometrial cancer cells more definitively.

The protocol used in this study, included a limited 24h of estrogen treatment prior to the addition of a combined estrogen and progesterone. This was done to mimic a snapshot of the molecular events that occur *in utero* during the early proliferative stage. The crucial estrogen priming process, enriches the endometrium with steroid hormone receptors preparing it for a successful progesterone action during the secretory phase. Due to the challenging nature of the tissue and complexity of the experiments, it was not possible to

include multiple time points for estrogen priming at this stage but is suggested in the scope for future studies.

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

VM: performed major experiments and primary contributor of manuscript writing and editing manuscript. PG: reviewed manuscript and minor experiments. LJ: reviewed manuscript and minor experiments. AP: conceived the idea, provided perceptive comments on drafts, and approved the content of the manuscript. All authors contributed to the article and approved the submitted version.

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Glycerophosphoinositol Promotes Apoptosis of Chronic Lymphocytic Leukemia Cells by Enhancing Bax Expression and Activation

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An imbalance in the expression of pro- and anti-apoptotic members of the Bcl-2 family of apoptosis-regulating proteins is one of the main biological features of CLL, highlighting these proteins as therapeutic targets for treatment of this malignancy. Indeed, the Bcl-2 inhibitor Venetoclax is currently used for both first-line treatment and treatment of relapsed or refractory CLL. An alternative avenue is the transcriptional modulation of Bcl-2 family members to tilt their balance towards apoptosis. Glycerophosphoinositol (GroPIns) is a biomolecule generated from membrane phosphoinositides by the enzymes phospholipase A_2 and lysolipase that pleiotropically affects key cellular functions. Mass-spectrometry analysis of GroPIns interactors recently highlighted the ability of GroPIns to bind to the non-receptor tyrosine phosphatase SHP-1, a known promoter of Bax expression, suggesting that GroPIns might correct the Bax expression defect in CLL cells, thereby promoting their apoptotic demise. To test this hypothesis, we cultured CLL cells in the presence of GroPIns, alone or in combination with drugs commonly used for treatment of CLL. We found that GroPIns alone increases Bax expression and apoptosis in CLL cells and enhances the pro-apoptotic activity of drugs used for CLL treatment in a SHP-1 dependent manner. Interestingly, among GroPIns interactors we found Bax itself. Short-term treatments of CLL cells with GroPIns induce Bax activation and translocation to the mitochondria. Moreover, GroPIns enhances the pro-apoptotic activity of Venetoclax and Fludarabine in CLL cells. These data provide evidence that GroPIns exploits two different pathways converging on Bax to promote apoptosis of leukemic cells and pave the way to new studies aimed at testing GroPIns in combination therapies for the treatment of CLL.

Keywords: CLL, apoptosis, Bax, glycerophosphoinositol, SHP-1

INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most common lymphoid malignancy in Western countries, is characterized by the accumulation of monoclonal $CD5^+$ B cells in peripheral blood, bone marrow and secondary lymphoid organs (1). Although the clinical course is highly variable, the most conserved feature of CLL is the extended survival of malignant B cells, which has been associated to defects in the apoptotic machinery (1, 2).

Alterations in the expression of pro-survival and pro-apoptotic members of the B-cell leukemia/lymphoma-2 (Bcl-2) family of apoptosis-regulating proteins is a hallmark of CLL and a key intrinsic factor underlying the longevity of CLL cells (1, 2). Increased expression of pro-survival members such as Bcl-2 and Mcl-1 (3, 4), concomitant with impaired expression of pro-apoptotic members such as Bax and Bak (5), tilts the finely regulated balance towards survival, leading to the accumulation of long-lived neoplastic cells that further acquire stroma-derived survival signals during their transit through secondary lymphoid organs (2, 6). It is therefore not surprising that restoring the Bcl-2 family balance has been pinpointed as strategy for overcoming the apoptosis defects of CLL cells, as witnessed by the recent approval of the Bcl-2 selective inhibitor Venetoclax for CLL treatment (7, 8). This effect is also elicited by chemotherapeutic drugs such as the fluorinated nucleotide analog Fludarabine, which affects the Bcl-2 family balance by indirectly promoting both expression and activation of Bax (9, 10). As opposed to Bcl-2, no drugs that specifically target Bax to enhance its expression or activation have been as yet developed (11).

Glycerophosphoinositols (GPIs) are water-soluble bioactive phospholipid derivatives of increasing interest as intracellular and paracrine mediators of eukaryotic cell functions. Generated from membrane phosphoinositides by the phospholipase $cPLA_2\alpha$, GPIs have diverse effects in a variety of cell types (12, 13). The most representative compound of the family is glycerophosphoinositol (GroPIns), a ubiquitous component of mammalian cells that participates in cell proliferation and survival in response to extracellular stimuli (14). When added exogenously, GroPIns elicits pharmacological effects relevant to both inflammatory responses and tumor spreading. In human blood monocytes GroPIns counteracts the LPS-induced proinflammatory and prothrombotic responses, inhibiting TLR4 signaling and leading to a decrease in the NF-κB-dependent transcription of inflammatory genes (15). GroPIns has also been recently found to reduce the invasive potential of melanoma cells through its ability to interact with and regulate the non-receptor tyrosine phosphatase Src homology region 2 domain-containing phosphatase-1 (SHP-1) (16, 17). GroPIns interaction with SHP-1 facilitates SHP-1 localization to invadopodia where it dephosphorylates cortactin, with subsequent impaired invadopodia function and hampered metastasis of melanoma cells both *in vitro* and *in vivo* (17).

Mainly expressed in hematopoietic and epithelial cells, the tyrosine phosphatase SHP-1 is a negative regulator of signaling pathways leading to cell proliferation, differentiation, survival and adhesion (18). Its dephosphorylating activity makes it a key regulator of cancer progression. Both expression and activity of SHP-1 are impaired in a number of cancer cell lines and tissues (19–21). Several pharmacological drugs used for cancer treatment enhance SHP-1 expression, which in turn downregulates aberrantly activated tyrosine kinase-dependent signaling pathways (22). The involvement of SHP-1 in cancer progression is also supported by evidence that SHP-1 promotes cancer cell apoptosis (23, 24) by enhancing the expression of Bax (23, 25). Although its expression levels are unaffected in CLL cells, SHP-1 activity is inhibited as a result of phosphorylation of the inhibitory residue Ser591 (26), making it an interesting molecular target for the treatment of this disease.

Here we asked whether GroPIns affects CLL cell apoptosis. We show that GroPIns exploits its SHP-1 modulating activity to promote CLL cell apoptosis by enhancing Bax expression. Moreover, we show that GroPIns directly interacts with Bax, rapidly promoting its activation and recruitment to the mitochondria. Hence GroPIns promotes CLL cell apoptosis by regulating the expression and activation of Bax through different pathways, highlighting the potential exploitability of this glycerophospholipid to overcome the apoptosis defects of CLL cells.

MATERIALS AND METHODS

Cells, Antibodies and Reagents

Peripheral blood samples were collected from 40 treatment-naive CLL patients. Diagnosis of CLL was made according to international workshop on CLL (iwCLL) 2008 criteria (27). The immunophenotypic analysis of lymphocytes obtained from peripheral blood of CLL patients was performed by flow cytometry. All patients expressed the typical phenotypic profile according to standard criteria for CLL diagnosis and were positive for CD19, CD5, CD23 and CD200. Flow cytometric plots of a representative CLL patient are shown in **Supplementary Figure 1**. Mutational *IGHV* status was assessed as reported (28). The main clinical features of CLL patients used in this study are listed in **Supplementary Table 1**. B cells from 24 buffy coats were used as healthy population controls. B cells were purified by negative selection using RosetteSep B-cell enrichment Cocktail (StemCell Technologies, Vancouver, Canada) followed by density gradient centrifugation on Lympholite (Cedarlane Laboratories, The Netherlands), as reported (29). Human HS-5 (30) stromal cells were used for co-culture experiments, as reported (31). Cells were maintained in RPMI (Roswell Park Memorial Institute)-1640 (Merck, #R8758) containing 7.5% Bovine Calf Serum (BCS) (HyClone, #SH30072.03). GroPIns was kindly provided by Euticals S.p.a (Lodi, Italy). GroPIns-Bio was obtained from Echelon Biosciences (Salt Lake City, UT, USA). NSC-87887 (Merck, #565851and Fludarabine (Merck, #F9813) were from Merck. Venetoclax was from Selleck Chemicals (#S8048). His-tagged Bax-α lacking 21 amino acids at the C-terminus (His-BaxΔTM) cloned in the pTrcHis vector (Invitrogen Srl) was a kind gift of Ingram

Iaccarino. This construct was expressed in *E. coli* BL21(DE3)/pLysS cells and purified as described (32).

Cell Treatments, Antibodies and Immunoblots

Treatments with 100 μM GroPIns, 35 μM Fludarabine, 3.5 nM Venetoclax or combination treatments were carried out at 37°C in RPMI 7.5% BCS for the indicated times. Control samples were treated with DMSO (Merck Millipore, #102952). Dose-response and time course experiments of CLL B cells treated with GroPIns are shown in **Supplementary Figure 2**. When required, cells were pretreated at 37°C for 20 min with 50 μM NSC-87887. Cells (5×10^6 cells/sample) were lysed in 1% (v/v) Triton X-100 in 20 mM Tris-HCl pH 8, 150 mM NaCl, in the presence of a cocktail of protease inhibitors (Calbiochem, #539134) and 0.2 mg/ml Na orthovanadate (Merck, #S6508), resolved by SDS-PAGE and transferred to nitrocellulose (GE Healthcare, #9004-70-0). Immunoblots were carried out using mouse anti-Bax (BD Biosciences, #610982), anti-penta-His (Life Technologies, #P21315) and anti-actin (Millipore, #MAB1501) primary antibodies. Secondary peroxidase-labeled anti-mouse antibodies were from Jackson Immuno-Research (#115-035-146). Labeled antibodies were detected using ECL kit (SuperSignal® West Pico Chemiluminescent Substrate, Thermo Scientific) and scanned immunoblots were quantified using the ImageJ software.

Intracellular Staining, Apoptosis, TMRM Assays and Flow Cytometry

Cells (2×10^5 cells/sample) were treated for 20 min in complete medium at 37°C as above, washed with PBS and fixed in 100 μl of fixation buffer (eBiosciences, #420801) for 15 minutes at RT. Cells were then washed with PBS added with 1% BSA (AppliChem PanReac, #A6588) and incubated with 10 μl permeabilization buffer (eBiosciences, #421008) containing either mouse anti-Bax (B-9) (Santa Cruz Biotechnology Inc., #sc-7480) or rabbit anti-phospho-SHP-1 Tyr564 (Cell Signaling, #D11G5) antibodies at RT for 1 h, washed twice in PBS 1% BSA and then incubated with 10 μl permeabilization buffer containing Alexa Fluor anti-mouse-488 (Thermo Fisher Scientific, #A11001) or anti-rabbit-488 (Thermo Fisher Scientific, #A11008) secondary antibodies for 45 min. After washing with PBS 1% BSA, cell pellets were resuspended in 200 μl PBS 1% BSA and subjected to flow cytometric analysis. Early apoptotic cells were quantified by flow cytometric analysis of 1×10^6 cells stained with FITC-labeled Annexin V (e-Bioscience, #88-8005-74) and Propidium iodide (PI, 20 μg/mL, Biotium, #40017). Mitochondrial membrane potential was measured using the fluorescent probe tetramethylrhodamine methyl ester (TMRM, Molecular Probes Europe BV). Cells (10^6 cells/sample) were suspended in 200 μl RPMI-1640 w/o phenol Red (Invitrogen srl) added with 25 mM Hepes pH 7.4 and 200 nM TMRM and incubated for 20 min at 37°C. Cells were then added with 500 ng/ml of the calcium ionophore A23187 (Sigma-Aldrich #C7522), incubated for 10 min at 37°C and subjected to flow cytometric analysis. Flow cytometry was carried out using a Guava Millipore cytometer as described (29). Data were analyzed using Flowjo (Tree Star, Inc.).

Co-Culture Experiments

Stromal cells were seeded on 96-well plates (1.5×10^5 cells/well) in complete culture medium and cultured to confluence. 2×10^5 cells/well CLL cells were added. Cells were co-cultured for 24 h at 37°C in the presence of either Venetoclax or DMSO. Wells were gently washed with RPMI to recover CLL cells, avoiding HS-5 cell detachment from the wells. Samples were stained with either CD19-FITC antibody (Biolegend, #392503) to identify the CLL cell population or with FITC-labeled Annexin V/Propidium iodide to evaluate early apoptotic cells, and analyzed by flow cytometry.

GroPIns-Bio Pull-Down Assay

GroPIns-Bio pull-down assays were previously described (16). Briefly, Raw 264.7 cells were centrifuged, washed with PBS and re-suspended in lysis buffer supplemented with a protease inhibitor cocktail (Complete Mini EDTA-free, Roche). The cell lysate was kept on a rotating wheel for 30 min at 4°C, centrifuged and the supernatant recovered, brought to a 0.2% (w/v) final concentration of Triton X-100, and dialyzed at 4°C. The cell extract was then precleared on 1 mg of uncoupled streptavidin-conjugated paramagnetic beads (Invitrogen Srl) on a rotating wheel, recovered and incubated with 1 mg of streptavidin-conjugated beads previously incubated with 2.5 nmoles of GroPIns-Bio or biotin in binding buffer (50 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM EDTA) supplemented with the protease inhibitor cocktail. Following incubation, the unbound materials were separated and the beads were washed with binding buffer. GroPIns-bound proteins were specifically eluted with 5 mM GroPIns. The elution was performed for 30 min at 4°C on a rotating wheel, eluted proteins were recovered, resuspended in SDS sample buffer and analyzed by SDS-PAGE. Protein bands were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS). For GroPIns-Bio pull-down assays with purified Bax, 100 ng of purified His-Bax were incubated for 2 h at 4°C with 0.5 mg of streptavidin-conjugated paramagnetic beads in the presence of 2.5 nmoles of biotin (Sigma-Aldrich, #B4501) or GroPIns-Bio in binding buffer plus protease inhibitors (Complete Mini EDTA-free, Roche). Following incubation, the unbound material was removed, and beads were washed with binding buffer. The beads with bound protein were boiled in 100 μl of SDS-sample buffer.

Immunofluorescence and Confocal Microscopy

Cells (1×10^5/sample) were cultured at 37°C in culture medium w/o BCS in the presence of 250 nM Mitotracker Orange (Invitrogen, Molecular Probes, #M7511) in the dark, then washed with PBS and treated for 20 min in culture medium w/o BCS at 37°C in the presence of 100 μM GroPIns, 35 μM Fludarabine or the combination of both. Diagnostic microscope slides were coated with polylysine (Sigma-Aldrich, #1274) and treated cells were allowed to adhere for 10 min. Slides were

immediately fixed in methanol (Carlo Erba, #412383) at -20°C for 10 min as described (33). Following fixation, samples were washed 5 min in PBS and incubated with anti-Bax (B-9) primary antibodies o/n at 4°C or 1 h at RT. After washing in PBS, samples were incubated for 1 h at RT with Alexa Fluor 488-labeled secondary antibodies. Confocal microscopy was carried out on a Zeiss LSM700 using a 63× objective, as reported (33). Images were processed with Zen 2009 image software (Carl Zeiss, Jena, Germany) and analyses were performed using ImageJ software (downloaded from http://www.embl-heidelberg.de/eamnet/).

RNA Isolation, Reverse Transcription and Real-Time Quantitative PCR

RNA was extracted and retrotranscribed as described (34). Real-time PCR was performed in triplicate on 96-well optical PCR plates (Sarstedt AG, Nümbrecht, Germany) using SSo FastTM EvaGreenR SuperMix and a CFX96 Real-Time system (Bio-Rad Laboratories, Waltham, MA). Results were processed and analyzed as described (34). Values are expressed as ΔΔCT relative to the housekeeping gene HPRT1. Primers used for real-time quantitative PCR amplification are listed in **Supplementary Table 2**.

Statistical Analyses

One-way ANOVA with *post-hoc* Tukey was used for experiments where multiple groups were compared. Mann-Whitney rank-sum tests were performed to determine the significance of the differences between two groups. Statistical analyses were performed using GraphPad Software (La Jolla, CA). P values <0.05 were considered significant.

Combination Index Calculation

The Combination index (Bliss index) was calculated according to the literature (35, 36). Briefly, CLL cells from 2 patients were mixed and plated into 96 well plates in 100 µl culture medium. GroPIns, Fludarabine and/or Venetoclax were added at different concentrations for 24 h, alone or in combination. Cell apoptosis was analyzed as above and the Combination index was calculated as in (35).

Study Approval

Written informed consent was received from CLL patients and healthy donors prior to inclusion in the study according to the Declaration of Helsinki. Experiments were approved by the local Ethics Committee.

RESULTS

GroPIns Has a Pro-Apoptotic Activity on CLL Cells Which Depends on SHP-1

The activity of the tyrosine phosphatase SHP-1, known to promote apoptosis (18, 25), has been shown to be impaired in CLL cells (26). Since GroPIns is a well-known regulator of SHP-1 in melanoma cells (17), we asked whether it promotes apoptosis of CLL cells through a SHP-1-dependent mechanism. B cells purified from peripheral blood of CLL patients were cultured for 24 h in the presence of 100 µM GroPIns and the percentage of early apoptotic Annexin V^+/PI^- cells was quantified by flow cytometry. B cells from healthy donors were used as control. GroPIns enhanced apoptosis of CLL cells (**Figures 1A, B**; **Supplementary Figure 2**). Apoptosis of healthy B cells was also enhanced by GroPIns, although at significantly lower levels compared to CLL cells (**Figure 1A**). The pro-apoptotic activity of GroPIns was partly reversed by the SHP-1-specific inhibitor NSC-87887 (**Figure 1B**), demonstrating that the pro-apoptotic activity of GroPIns relies on the tyrosine phosphatase activity of SHP-1.

The active form of SHP-1 is phosphorylated on tyrosine 564 (37). We hypothesized that, similar to melanoma cells (17), GroPIns interacts with and activates SHP-1 in CLL cells, thereby promoting their apoptosis. To test this hypothesis, B cells purified from peripheral blood of CLL patients and healthy controls were cultured in the presence of GroPIns and the active, phosphorylated form of SHP-1 was quantified by flow cytometry using a phospho-Y564-specific antibody (37). Consistent with previous reports (26), basal SHP-1 phosphorylation levels were significantly lower in CLL cells compared to healthy B cells (**Figures 1C, D**; **Supplementary Figure 3**). GroPIns enhanced SHP-1 phosphorylation (**Figures 1C, D**). These data suggest that GroPIns promotes CLL cell apoptosis by activating SHP-1. However, the fact that the enhancing effects of GroPIns on B cell apoptosis were only partially reversed by the SHP-1 inhibitor suggests that other, SHP-1-independent mechanisms may contribute to this function.

GroPIns Enhances the Expression of Bax in CLL Cells in a SHP-1-Dependent Manner

The apoptosis defects of CLL cells are caused in part by the decreased expression of the pro-apoptotic protein Bax (2). Since the phosphatase activity of SHP-1 has been causally linked to enhanced Bax expression and increased apoptosis in acute promyelocytic leukemia cells (25), we asked whether GroPIns promotes CLL cell apoptosis by upregulating Bax expression in a SHP-1-dependent manner. B cells purified from peripheral blood of CLL patients and healthy donors were cultured for 24 h in the presence of GroPIns. Bax expression was assessed by both immunoblot and qRT-PCR. Consistent with previous reports (2, 5), untreated CLL cells expressed lower Bax levels compared to healthy B cells (**Figures 2A–C**). GroPIns enhanced Bax expression in both CLL cells and healthy B cells (**Figures 2A–C**). Although the overall protein and mRNA amount of Bax was similar in healthy and CLL cells treated with GroPIns, the fold Bax expression, calculated as the ratio of Bax expression in treated versus untreated samples, was significantly higher in CLL cells compared to healthy B cells (**Figures 2D, E**). These results suggest a higher sensitivity of CLL cells to GroPIns compared to healthy B cells. NSC-87887 almost completely abolished the GroPIns-dependent Bax increase, demonstrating that the Bax-elevating activity of GroPIns depends on the phosphatase activity of SHP-1 (**Figure 2F**). Hence GroPIns

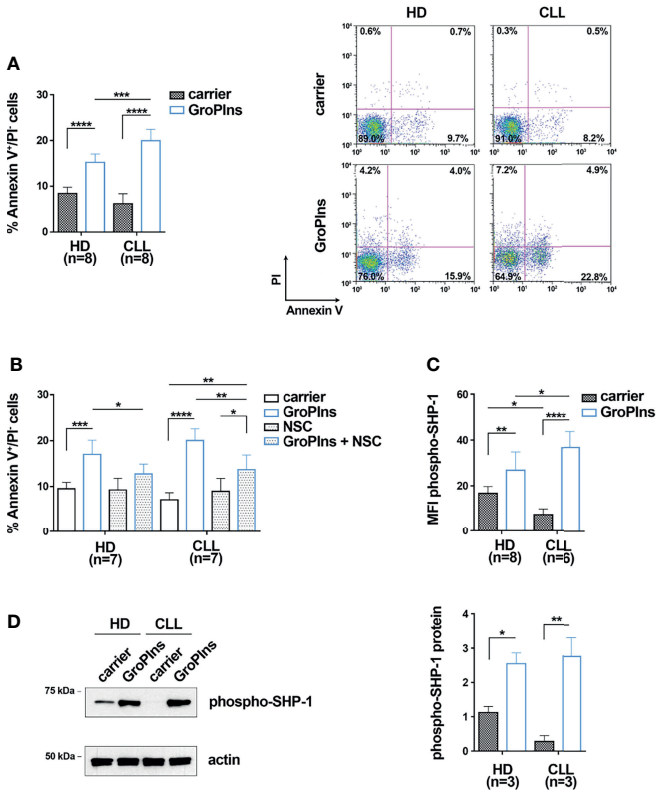

FIGURE 1 | GroPIns promotes CLL cell apoptosis in a SHP-1-dependent manner. **(A)** Flow cytometric analysis of the percentages of Annexin V^+/PI^- cells in B lymphocytes purified from peripheral blood of healthy donors (HD; n=8) and CLL patients (CLL; n=8). Samples were treated with either carrier or 100 μM GroPIns for 24 h at 37°C. Representative panels are shown on the right. **(B)** Flow cytometric analysis of the percentages of Annexin V^+/PI^- cells in B lymphocytes purified from peripheral blood of healthy donors (HD; n=7) and CLL patients (CLL; n=7). Samples were treated for 24 h at 37°C with either carrier or 100 μM GroPIns in the presence or absence of 50 μM NSC-87887 (NSC). **(C)** Flow cytometric analysis of phospho-SHP-1 in B lymphocytes purified from peripheral blood of healthy donors (HD; n=8) and CLL patients (CLL; n=6), treated with either carrier or 100 μM GroPIns for 30 min at 37°C. Data are expressed as MFI phospho-SHP-1 in live cells. **(D)** Immunoblot analysis with anti-phospho-SHP-1 antibodies of postnuclear supernatants of B lymphocytes purified from peripheral blood of healthy donors (HD; n=3) and CLL patients (CLL; n=3). Samples were treated with either carrier or 100 μM GroPIns for 30 min at 37°C. The stripped filters were reprobed with anti-actin antibodies. Molecular weights (kDa) are indicated on the left of the panel. The quantification of three independent experiments is shown on the right. Mean ± SD. Anova two-way test, Multiple Comparison. $p \leq 0.0001$, ****; $p \leq 0.001$, ***; $p \leq 0.01$, **; $p \leq 0.05$, *.

promotes CLL cell apoptosis by enhancing Bax expression in a SHP-1-dependent manner. Of note, GroPIns also decreased the mRNA expression of the pro-survival Bcl-2 family members Bcl-2, MCL-1 and B2CL1 in CLL cells (**Figure 2G**) in a SHP-1-dependent manner (**Supplementary Figure 4**). These data provide evidence that GroPIns profoundly shifts the Bcl-2 family balance toward apoptosis.

GroPIns Interacts With and Activates Bax in CLL Cells

We previously identified SHP-1 as a direct cellular target of GroPIns by pull-down assay coupled with liquid chromatography-tandem mass-spectrometry analysis (16). Among direct interactors of GroPIns (listed in **Table 1**) we also found Bax. We validated the direct binding of GroPIns with Bax in *in vitro* pull-down assays. The immunoblot analysis of Bax showed that purified recombinant Bax was specifically pulled-down by GroPIns-Bio-bound beads but not by control Biotin-bound beads, confirming that GroPIns directly binds Bax (**Figure 3A**).

Following pro-apoptotic stimulation, Bax undergoes a conformational change to become an active apoptosis promoter (9, 11). We assessed whether GroPIns promotes Bax activation. Purified healthy and CLL cells were treated with GroPIns for 20 min and Bax activation was assessed by flow cytometric analysis of cells stained with an anti-active Bax antibody that specifically recognizes the N-terminus of Bax which is exposed after the conformational change that accompanies Bax activation (9). The basal levels of Bax activation were significantly lower in CLL cells compared to healthy B cells (**Figures 3B, D**; **Supplementary Figure 3**). This was a consequence of the lower overall Bax levels, as assessed by normalizing the MFI of active Bax to the expression levels of Bax protein shown in **Figure 2B** (**Figure 3C**). GroPIns elicited Bax activation in CLL cells (**Figures 3B, C**). The fold Bax activation, calculated as the ratio of the MFI of active Bax in treated versus

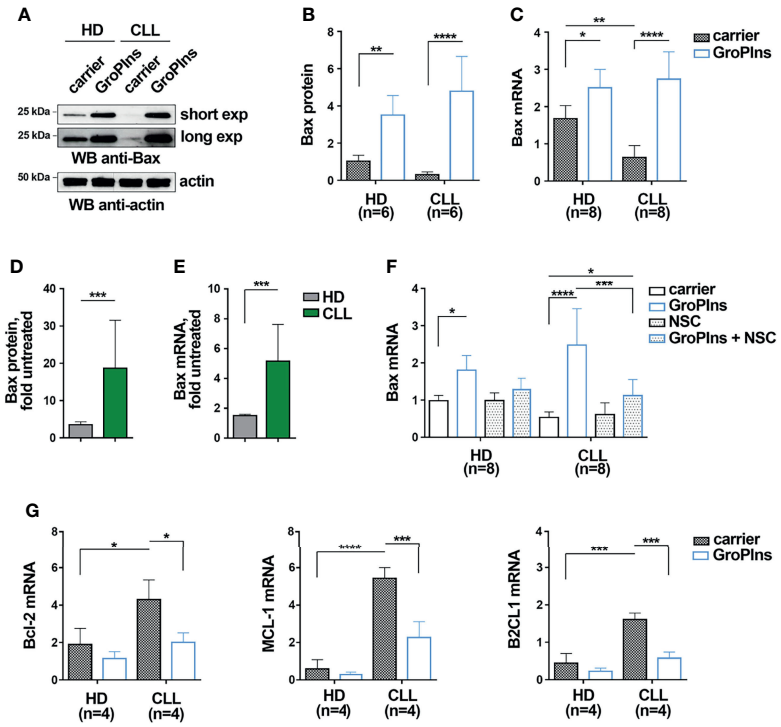

FIGURE 2 | GroPIns promotes Bax expression in CLL cells. **(A, B)** Immunoblot analysis with anti-Bax antibodies of postnuclear supernatants of B lymphocytes purified from peripheral blood of healthy donors (HD; n=6) and CLL patients (CLL; n=6). Samples were treated with either carrier or 100 μM GroPIns for 24 h at 37°C. The stripped filters were reprobed with anti-actin antibodies. Molecular weights (kDa) are indicated on the left of the panel. The quantification of eight independent experiments is shown in **(B, C)**. Quantitative RT-PCR analysis of Bax mRNA in B lymphocytes purified from peripheral blood of healthy donors (HD; n=8) and CLL patients (CLL; n=8), treated with either carrier or 100 μM GroPIns for 24 h at 37°C. The relative gene transcript abundance was determined on triplicate samples using the ddCt method and normalized to HPRT1. **(D, E)**). Fold protein **(D)** and mRNA **(E)** expression levels of Bax in samples from healthy donors and CLL patients. Data were calculated as fold Bax protein quantification of treated *vs* untreated samples shown in **(B, C)**. **(F)** Quantitative RT-PCR analysis of Bax mRNA in B lymphocytes purified from peripheral blood of healthy donors (HD; n=8) and CLL patients (CLL; n=7), treated for 24 h at 37°C with either carrier or 100 μM GroPIns in the presence or absence of 50 μM NSC-87887 (NSC). The relative gene transcript abundance was determined on triplicate samples using the ddCt method and normalized to HPRT1. **(G)** Quantitative RT-PCR analysis of Bcl-2, MCL-1 and B2CL1 mRNA in B lymphocytes purified from peripheral blood of healthy donors (HD; n=4) and CLL patients (CLL; n=4), treated with either carrier or 100 μM GroPIns for 24 h at 37°C. The relative gene transcript abundance was determined on triplicate samples using the ddCt method and normalized to HPRT1. Mean ± SD. **(B, C, F, G)**: Anova two-way test, Multiple Comparison. **(D, E)**: Mann Whitney Rank Sum Test. $p \leq 0.0001$, ****; $p \leq 0.001$, ***; $p \leq 0.01$, **; $p \leq 0.05$, *.

untreated samples, was significantly higher in CLL cells compared to healthy B cells (**Figure 3D**), further witnessing to a higher sensitivity of CLL cells to GroPIns compared to healthy B cells.

Active Bax translocates to the mitochondria (11). Immunofluorescence analysis of cells stained with anti-active Bax antibodies and Mitotracker Orange, a fluorescent probe that selectively stains mitochondria, showed that the colocalization of active Bax with mitochondria was significantly enhanced in both healthy and CLL cells treated for 20 min with GroPIns compared to untreated cells (**Figures 3E, F**). The fold active Bax/mitochondria co-localization was significantly higher in CLL cells compared to healthy B cells (**Figure 3G**), again demonstrating the higher sensitivity of leukemic cells to GroPIns.

Bax translocation to mitochondria leads to its oligomerization at the outer mitochondrial membrane, which in turn promotes mitochondrial depolarization (11). Purified healthy and CLL cells loaded with the fluorescent probe TMRM were treated for 4 h with GroPIns or with the calcium ionophore A23187, a potent inducer of apoptosis (38), and mitochondria depolarization was assessed by flow cytometric quantification of the percentage of $TMRM^{low}$ cells (**Supplementary Figure 5**). Mitochondrial depolarization was significantly enhanced in CLL cells treated with GroPIns when compared to untreated cells (**Figure 3H**). Of note, GroPIns elicited a slight, yet not significant increase in mitochondrial depolarization in healthy B cells (**Figure 3H**). These data demonstrate that GroPIns potently acts on CLL cells to restore apoptosis. The SHP-1 inhibitor NSC-87887 did not impair GroPIns-mediated Bax activation (**Figure 3I**), suggesting that GroPIns-mediated Bax activation does not require SHP-1. Collectively, these results support the existence of two unrelated pathways, of which one is SHP-1-dependent and one independent, converging on Bax and exploited by GroPIns to promote CLL cell apoptosis.

TABLE 1 | List of proteins identified from proteomic analysis.

Swiss-Prot Code	Protein name
O55143	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2
Q8CGC7	Bifunctional glutamate/proline–tRNA ligase
Q9JKR6	Hypoxia up-regulated protein 1
Q8BMJ2	Leucine–tRNA ligase, cytoplasmic
P70248	Unconventional myosin-If
Q64514	Tripeptidyl-peptidase 2
Q8K4Z5	Splicing factor 3A subunit 1
Q9EQK5	Major vault protein
Q60597	2-oxoglutarate dehydrogenase, mitochondrial
Q8BIJ6	Isoleucine–tRNA ligase, mitochondrial
Q9DBT5	AMP deaminase 2
Q61881	DNA replication licensing factor MCM7
Q9D0R2	Threonine–tRNA ligase 1, cytoplasmic
Q9JIK5	Nucleolar RNA helicase 2
Q9Z110	Delta-1-pyrroline-5-carboxylate synthetase
P26043	Radixin
Q80UM7	Mannosyl-oligosaccharide glucosidase
Q8BML9	Glutamine–tRNA ligase
Q8CHW4	Translation initiation factor eIF-2B subunit epsilon
Q8BNW9	Kelch repeat and BTB domain-containing protein 11
Q99MN1	Lysine–tRNA ligase
Q9WUA2	Phenylalanine–tRNA ligase beta subunit
P29351	Tyrosine-protein phosphatase non-receptor type 6 (Shp1)
P80316	T-complex protein 1 subunit epsilon
Q8BMF4	Dihydrolipoamide acetyltransferase PDH-E2
Q8BP47	Asparagine–tRNA ligase, cytoplasmic
Q91WQ3	Tyrosine–tRNA ligase, cytoplasmic
Q9DBG6	Dolichyl-diphosphooligosaccharide–protein glycosyltransferase subunit 2
Q61024	Asparagine synthetase
P09405	Nucleolin
Q61656	Probable ATP-dependent RNA helicase DDX5
P30416	Peptidyl-prolyl cis-trans isomerase FKBP4
Q99K87	Serine hydroxymethyltransferase, mitochondrial
P47738	Aldehyde dehydrogenase, mitochondrial
Q9Z0N1	Eukaryotic translation initiation factor 2 subunit 3
P80314	T-complex protein 1 subunit beta
P26443	Glutamate dehydrogenase 1, mitochondrial
Q9CZ44	NSFL1 cofactor p47
O88986	2-amino-3-ketobutyrate coenzyme A ligase, mitochondrial
Q922R8	Protein disulfide-isomerase A6
Q9DC69	NADH dehydrogenase 1 alpha subcomplex subunit 9
Q9DB05	Alpha-soluble NSF attachment protein
Q99LC5	Electron transfer flavoprotein subunit alpha, mitochondrial
Q64674	Spermidine synthase
Q9CR57	60S ribosomal protein L14
P35278	Ras-related protein Rab-5C
P84099	60S ribosomal protein L19
P20108	Thioredoxin-dependent peroxide reductase, mitochondrial
P61087	Ubiquitin-conjugating enzyme E2 K
P08030	Adenine phosphoribosyltransferase
P62821	Ras-related protein Rab-1A
Q9CZM2	60S ribosomal protein L15
Q9Z1B5	Mitotic spindle assembly checkpoint protein MAD2A
Q62159	Rho-related GTP-binding protein RhoC
P51410	60S ribosomal protein L9
Q9JM14	5'(3')-deoxyribonucleotidase, cytosolic type
P61028	Ras-related protein Rab-8B
P29391	Ferritin light chain 1
P53994	Ras-related protein Rab-2A
P70296	Phosphatidylethanolamine-binding protein 1
P19253	60S ribosomal protein L13a
P08030	Adenine phosphoribosyltransferase
P00375	Dihydrofolate reductase
O09167	60S ribosomal protein L21
Q07813	Apoptosis regulator BAX
Q9EQU5	Protein SET
P62301	40S ribosomal protein S13
P17742	Peptidyl-prolyl cis-trans isomerase A
P62281	40S ribosomal protein S11

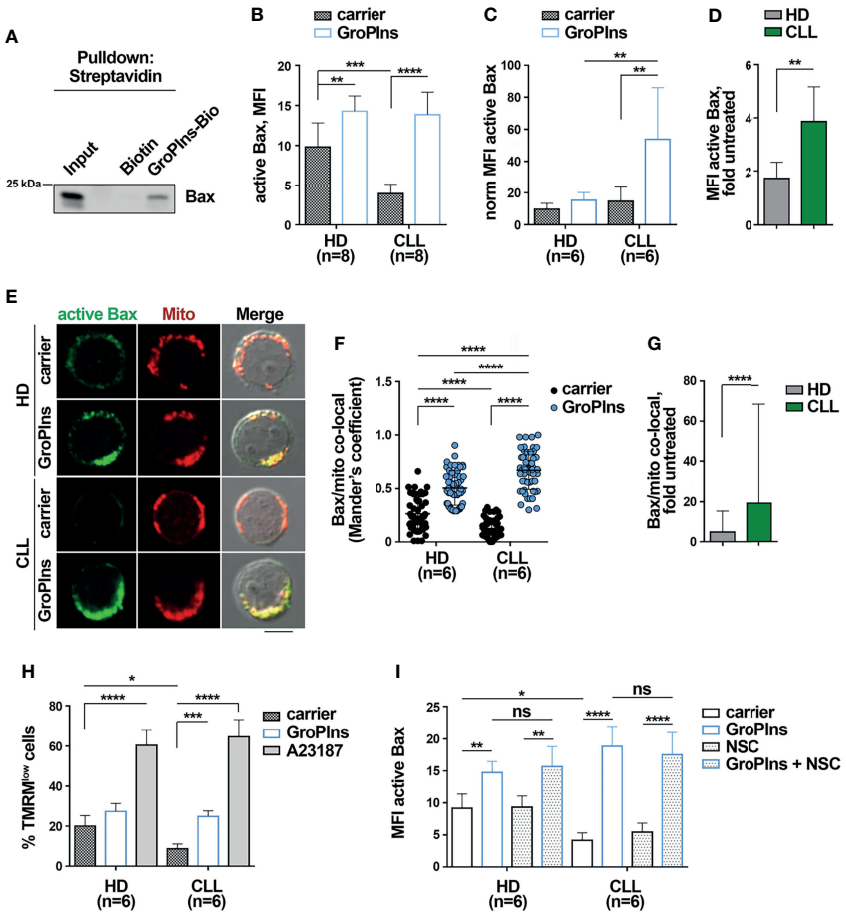

FIGURE 3 | GroPIns interacts with and activates Bax. **(A)** Representative pull-down of streptavidin-conjugated beads using Biotin or biotinylated GroPIns (GroPIns-Bio) with His-Bax. Eluted proteins were analyzed by immunoblot using anti-His antibodies. Molecular weights (kDa) are indicated on the left of the panel. **(B)** Flow cytometric analysis of active Bax in B lymphocytes purified from peripheral blood of healthy donors (HD; n=8) and CLL patients (CLL; n=8). Samples were treated for 20 min at 37°C with either carrier or 100 μM GroPIns. **(C)** The MFI of active Bax shown in panel **(B)** was normalized to Bax protein levels of untreated cells shown in **Figure 2B** (n=6). **(D)** Fold MFI active Bax in samples from healthy donors and CLL patients shown in panel **(C)**. Data were calculated as fold MFI of active Bax of treated vs. untreated samples. **(E)** Immunofluorescence analysis of active Bax (green) and mitochondria (Mitotracker) (red) in B lymphocytes purified from peripheral blood of healthy donors (HD; n=6) and CLL patients (CLL; n=6) treated for 20 min at 37°C with either carrier or 100 μM GroPIns. Immunofluorescence images were acquired on confocal microscope using 60 × objective. Representative immunofluorescence images are shown. Size bar, 5 μm. The quantification using Mander's coefficient of the weighted colocalization of active Bax with mitochondria in individual medial confocal sections is shown in **(F)**. **(G)** Fold active Bax/mitochondria co-localization in cells from healthy donors and CLL patients. Data were calculated as fold active Bax/mitochondria co-localization of treated *vs* untreated samples. **(H)** Flow cytometric analysis of the percentage of $TMRM^{low}$ cells in B lymphocytes purified from peripheral blood of healthy donors (HD; n=6) and CLL patients (CLL; n=6). Samples were treated for 4 h at 37°C with either carrier or 100 μM GroPIns or 500 ng/ml A23187. Stainings were performed in duplicate. **(I)** Flow cytometric analysis of active Bax in B lymphocytes purified from peripheral blood of healthy donors (HD; n=6) and CLL patients (CLL; n=6). Samples were treated for 20 min at 37°C with either carrier or 100 μM GroPIns in the presence or in the absence of NSC-87887. Mean ± SD. **(B, C, F, H, I)**: Anova two-way test, Multiple Comparison. **(D, G)**: Mann Whitney Rank Sum Test. $p \leq 0.0001$, ****; $p \leq 0.001$, ***; $p \leq 0.01$, **; $p \leq 0.05$, *; ns, not significant..

GroPIns Enhances the Pro-Apoptotic Effects of Venetoclax on CLL Cells

The Bcl-2 inhibitor Venetoclax promotes CLL cell apoptosis (39), and induces rapid and pronounced activation and mitochondrial translocation of Bax in cell lines of acute myeloid leukemia (40). We tested whether the combination of GroPIns with Venetoclax further enhances Venetoclax-induced CLL cell apoptosis. As shown in **Figure 4A**, the combination of GroPIns and Venetoclax enhanced apoptosis of leukemic cells compared to single treatments (**Figure 4A**), suggesting a synergic pro-apoptotic activity of GroPIns and Venetoclax in these cells. This was confirmed by analyzing Bax expression (**Figure 4B**) and activation (**Figure 4C**), which were enhanced in CLL cells subjected to combination treatments compared to single treatments (**Figures 4B, C**). Of note, while Venetoclax did not affect the expression of MCL-1 and B2CL1 in CLL cells, it led to a decrease in Bcl-2 expression to levels similar to GroPIns, which were further decreased in combination treatments (**Figure 4D**). The flow cytometric analysis of early apoptotic cells performed in CLL cells treated for 24 h with increasing concentrations of

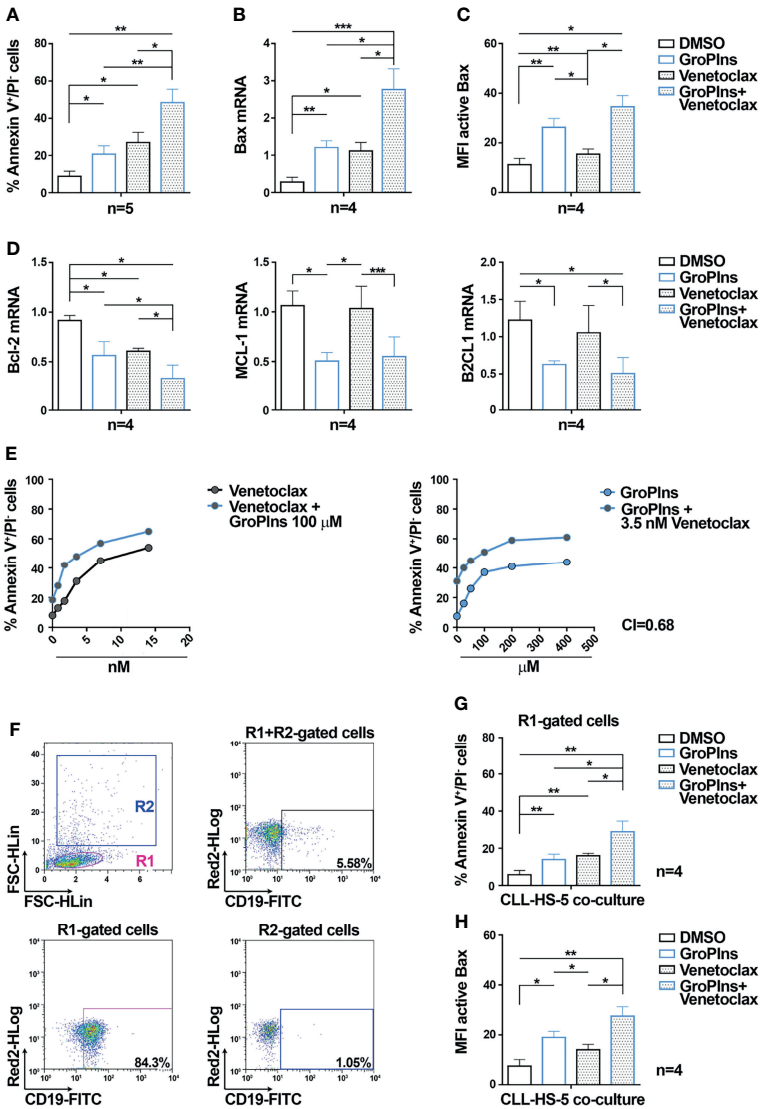

FIGURE 4 | GroPIns enhances the pro-apoptotic activity of Venetoclax in CLL cells. **(A)** Flow cytometric analysis of the percentages of Annexin V^+/PI^- cells in B lymphocytes purified from peripheral blood of CLL patients (CLL; n=5) treated with either 100 μM GroPIns or 3.5 nM Venetoclax or the combination of both for 24 h at 37°C. **(B)** Quantitative RT-PCR analysis of Bax mRNA in B lymphocytes purified from peripheral blood of CLL patients (CLL; n=4) and treated as in **(A)**. The relative gene transcript abundance was determined on triplicate samples using the ddCt method and normalized to HPRT1. **(C)** Flow cytometric analysis of active Bax in B lymphocytes purified from peripheral blood of CLL patients (CLL; n=4) and treated for 20 min at 37°C with either 100 μM GroPIns or 3.5 nM Venetoclax or the combination of both. **(D)** Quantitative RT-PCR analysis of Bcl-2, MCL-1 and B2CL1 mRNA in B lymphocytes purified from peripheral blood of healthy donors (HD; n=4) and CLL patients (CLL; n=4), treated as above. The relative gene transcript abundance was determined on triplicate samples using the ddCt method and normalized to HPRT1. **(E)** Flow cytometric analysis of the percentages of Annexin V^+/PI^- cells in B lymphocytes purified from peripheral blood of 2 CLL patients treated with either GroPIns or Venetoclax or with the combination of both at the indicated concentrations for 24 h at 37°C. The calculated Cooperation Index (CI) is indicated. **(F–H)** Flow cytometric analysis of the percentages of Annexin V^+/PI^- cells **(G)** and of Bax activation **(H)** in B lymphocytes purified from peripheral blood of CLL patients (CLL; n=4) co-cultured with HS-5 stromal cells for 24 h at 37°C in the presence of either 100 μM GroPIns or 3.5 nM Venetoclax or the combination of both. Analysis was carried out on R1-gated $CD19^+$ cells. The gating strategy is shown in **(F)**. Mean ± SD. Anova one-way test, Multiple Comparison. $p \leq 0.001$, ***; $p \leq 0.01$, **; $p \leq 0.05$, *.

GroPIns alone or in combination with Venetoclax showed a Combination Index (CI) below 1 (CI=0.68; **Figure 4E**), indicating a synergic cooperation between GroPIns and Venetoclax to promote CLL cell apoptosis.

Fludarabine, a chemotherapeutic drug used in the treatment of a small subset of CLL patients alone or in combination with other chemotherapeutic or immunomodulatory drugs, enhances Bax activation and expression and promotes apoptosis of CLL cells (9, 10). We tested whether, similar to Venetoclax, the combination of GroPIns with Fludarabine further enhances Fludarabine-induced CLL cell apoptosis. GroPIns enhanced Fludarabine-induced CLL cell apoptosis as well as Bax

activation, expression and translocation to mitochondria compared to single treatments (**Supplementary Figure 6A-F**). However, as opposed to Venetoclax, Fludarabine and GroPIns did not act in synergy to enhance CLL cell apoptosis, but rather showed independent effects (**Supplementary Figure 6G**).

The stromal microenvironment strongly contributes to protect CLL cells from apoptosis (6). We assessed the pro-apoptotic effect of GroPIns and Venetoclax, alone or in combination treatments, in CLL cells co-cultured for 24 h with the human stromal cell line HS-5 (29). As shown in **Figures 4F–H**, the combination of GroPIns and Venetoclax enhanced both apoptosis and Bax activation in leukemic cells co-cultured with HS-5 cells compared to single treatments (**Figure 4A**), albeit with less pronounced effects which are likely to be accounted for by the protective role of stromal cells on CLL cells.

These results demonstrate that GroPIns displays a pro-apoptotic activity also in the presence of drugs known to promote CLL cell apoptosis.

DISCUSSION

Apoptosis, which plays important roles in organism development and tissue homeostasis, becomes critical for the elimination of unwanted, damaged or infected cells (41). Insufficient apoptosis has been related to the onset and progression of cancer by extending tumor cell survival and promoting their resistance to treatment (42). A profound imbalance among Bcl-2 family members is a major factor in the apoptosis defects of CLL cells, which play a major role in leukemic cell accumulation in secondary lymphoid organs, where they are protected from chemotherapy (1, 2). The pro-survival protein Bcl-2, whose expression is frequently upregulated in CLL as a result of deletion of mir15-a/mir16-1, located at 13q14 and known to target BCL-2 mRNA (43), had long been viewed as a promising target for CLL therapy. In 2016 the selective Bcl-2 inhibitor Venetoclax, which acts as a BH3-mimetic to facilitate the activation of pro-apoptotic Bcl-2 family members, was approved for relapsed/refractory CLL (7). Since then, new combination therapy regimens have been approved for CLL treatment (8) and usually applied as first-line therapy. The use of chemoimmunotherapeutics such as Fludarabine, cyclophosphamide and rituximab has progressively decreased through the years as a consequence of the higher efficacy and better tolerability of targeted agents like Venetoclax. However, none of the recently introduced therapies appears to cure CLL, and some patients become resistant to Venetoclax due to the acquisition of Bcl-2 mutations.

Pro-apoptotic stimuli activate Bax, a major pro-apoptotic member of the Bcl-2 family, either directly or indirectly, leading to mitochondrial membrane permeabilization, release of the apoptotic factor cytochrome c and cancer cell death (11). The expression of Bax is profoundly impaired in CLL cells (2), which contributes to their apoptosis defects. A number of drugs currently in clinical use for the treatment of several types of cancer are known to indirectly enhance Bax expression and activation, including Fludarabine (9, 10) and Venetoclax (40). Here we demonstrate that GroPIns promotes CLL cell apoptosis by enhancing Bax expression. Moreover, GroPIns enhances the pro-apoptotic effects of both Venetoclax and Fludarabine, leading to higher levels of CLL cell apoptosis compared to single treatments. Interestingly, the activity of GroPIns and Venetoclax converge toward tilting the Bcl-2 family balance toward apoptosis, on the one hand by enhancing the expression and potentiating the activation of Bax, and on the other hand by decreasing the expression and inhibiting the activity of Bcl-2. Our findings highlight a potential new combinatorial strategy aimed at potentiating the pro-apoptotic activity of Venetoclax with a natural and well-tolerated compound, which could overcome potential resistance mechanisms to Venetoclax used as single agent (44).Several classes of small molecules have been identified in the last decade that selectively activate Bax to induce apoptosis, which demonstrated good *in vitro* but moderate *in vivo* anti-cancer activity (45, 46). The compound SMBA1 potently activates Bax and acts both *in vitro* and *in vivo* against lung cancer (47). New recently synthesized SMBA1 analogs show anti-proliferative activity against breast cancer (48). However, none of these molecules has been tested in CLL to date. In 2020 the small molecule BDA-366, a BH4-domain antagonist that kills both lung cancer and multiple myeloma cells, was tested for its therapeutic potential and mechanism of action in CLL and DLBCL. However, although BDA-366 displayed selective toxicity against both cell types, the underlying mechanism of Bax activation is as yet unknown (11, 49). Here we identified GroPIns as a naturally-occurring molecule provided with the intrinsic ability to bind and activate Bax. This makes of GroPIns an interesting pro-apoptotic molecule to be tested in malignancies characterized by hypoexpression or hypoactivation of Bax.

Along with an aberrant expression of anti-apoptotic molecules, CLL cells show high levels of intracellular phosphorylation mediated by the hyperactivation of several kinases downstream of the B-cell receptor, such as Lyn, Syk, Btk, PI3K, and AKT (50, 51). This condition is further sustained by an impairment in the expression or function of phosphatases. The expression of PTEN (52), CD45 (53), PTPROt (54), PHLPP1 (55, 56), PP2A (57), and SHIP1 (58) are significantly decreased in CLL cells, whereas PTPN22, which acts as a positive regulator of anti-apoptotic signals by hampering the negative regulation of B-cell receptor-dependent signaling pathways, is overexpressed (59). By contrast SHP-1, a tyrosine phosphatase that participates in signaling pathways regulating proliferation, survival and apoptosis of both hematopoietic and non-hematopoietic cells (18), is expressed in CLL cells at levels comparable to normal B cells (60) but is functionally dysregulated by mechanisms that are mediated by the Src family kinase Lyn (26), making this phosphatase an interesting target for activating-drug discovery.

Drugs able to promote phosphatase activity have been demonstrated to be effective in CLL. The novel SHIP-1 activator AQX-435 was demonstrated to be effective in the inhibition of anti-IgM-induced AKT phosphorylation, resulting in CLL cell apoptosis *in vitro* (61). Conversely, SHP-1 has proven to be an extremely challenging drug target, due both to the highly conserved and positively charged nature of its phosphatase active site, and to the

lack of either appropriate selectivity or membrane permeability of the majority of phosphatase inhibitors (62). We previously reported that in melanoma cells GroPIns interacts with SHP-1, promoting its recruitment to invadopodia where it dephosphorylates critical components of the actin polymerization pathways leading to matrix invasion, thereby counteracting metastasis (17). Here we added a tile to the puzzle by demonstrating that in CLL cells GroPIns enhances SHP-1 phosphorylation. Although the molecular mechanism underlying the GroPIns-dependent enhancement in SHP-1 phosphorylation remains unknown, we hypothesize that the interaction of GroPIns with SHP-1 might either stabilize SHP-1 in an active conformation, or alternatively promote its interaction with a specific kinase, thereby favoring SHP-1 phosphorylation. It is noteworthy that SHP-1 not only acts through dephosphorylation (18), but also promotes Bax expression (23, 25) through signaling pathways involving the MAP kinase p38 (25) and the transcription factor STAT3 (23). Our data show that, by promoting SHP-1 phosphorylation, GroPIns enhances Bax expression and CLL cell apoptosis. The existence of two distinct and independent pathways that, by taking advantage of the two GroPIns interactors SHP-1 and Bax, both converge to promote CLL cell apoptosis, contribute to enhance the activity of this compound. In this scenario GroPIns, *via* direct binding to and modulation of SHP-1 and Bax, could be an interesting tool to restore apoptosis in CLL cells.

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 authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Azienda Ospedaliera Universitaria di Padova, Padova Hospital and Azienda Ospedaliera Universitaria di Siena, Siena Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GB, ASV, VT, NC, FF, ADV, LT, DC, LP, and CB designed research and analyzed and interpreted data. GB, ASV, VT, NC, FF, ADV, and LP performed research. ASV, FF, ADV, LT, and DC contributed vital reagents. ASV, NC, FF, ADV, LT, DC, LP, and CB 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/fonc.2022.835290/full#supplementary-material

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CASE REPORT
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Case Report: A Novel Pathomechanism in PEComa by the Loss of Heterozygosity of *TP53*

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Since the introduction of next-generation sequencing, the frequency of germline pathogenic *TP53* variants and the number of cases with unusual clinical presentations have been increasing. This has led to the expansion of the classical Li–Fraumeni syndrome concept to a wider cancer predisposition syndrome designated as the Li–Fraumeni spectrum. Here, we present a case with a malignant, metastatic perivascular epithelioid cell tumor (PEComa) of the thigh muscle and a sinonasal carcinoma harboring a novel *TP53* germline splice mutation (NM_000546.5:c.97-2A>C). The classical presentation of LFS in the long-since deceased mother and the presence of a germline *TP53* variant in the proband suggested a possible familial *TP53*-related condition. Complex pathological, molecular, and clinical genetic analyses (whole exome sequencing of germline variants, multigene panel sequencing of tumor DNA, Sanger validation, an *in vitro* functional test on splicing effect, 3D protein modeling, p53 immunohistochemistry, and pedigree analysis) were performed. The *in vitro* characterization of the splice mutation supported the pathogenic effect that resulted in exon skipping. A locus-specific loss of heterozygosity in the PEComa but not in the sinonasal carcinoma was identified, suggesting the causative role of the splice mutation in the PEComa pathogenesis, because we excluded known pathogenetic pathways characteristic to PEComas (*TSC1/2*, *TFE3*, *RAD51B*). However, the second hit affecting *TP53* in the molecular pathogenesis of the sinonasal carcinoma was not identified. Although PEComa has been reported previously in two patients with Li–Fraumeni syndrome, to the best of our knowledge, this is the first report suggesting a relationship between the aberrant *TP53* variant and PEComa.

Keywords: Li–Fraumeni syndrome, heritable *TP53*-related cancer syndrome, *TP53*, p53, PEComa, germline mutation, Li-Fraumeni, Li-Fraumeni spectrum

INTRODUCTION

Germline pathogenic *TP53* variants are associated with Li–Fraumeni syndrome (LFS), which is a rare, autosomal-dominant, hereditary tumor syndrome (1). Five cancer types account for the majority of LFS tumors, which are called "LFS core" tumors: adrenocortical carcinoma, breast cancer, central nervous system tumors, osteosarcomas, and soft-tissue sarcomas. However, LFS patients have an increased risk of several additional cancers, such as leukemia, lymphoma, gastrointestinal cancers, and cancers of head and neck, kidney, larynx, lung, skin, ovary, pancreas, prostate, testis, and thyroid (2). Also, many cases of germline *TP53* pathogenic variants have been identified in children with cancers, or among adult females with breast cancers without a familial history of cancer. Hence the expansion of the LFS concept to a wider cancer predisposition syndrome: the terms "heritable *TP53*-related cancer (hTP53rc) syndrome" by the European Reference Network GENTURIS and "Li-Fraumeni spectrum" by Kratz et al. have been recently suggested (3, 4). Based on classic, familial cases, the cumulative cancer risk was initially given as 73%–100% by age 70, with risks close to 100% in women (5–7). However, based on population studies, and as a consequence of the increased availability for high-throughput testing, the overall cancer penetrance seems to be lower (3, 8). Still, based on a recent observational cohort study on cancer incidence, patterns, and genotype–phenotype associations, individuals with Li–Fraumeni syndrome had a nearly 24 times higher incidence of any cancer than the general population (9). Additionally, while the disease prevalence is not well established, the prevalence of the germline pathogenic *TP53* carrier status in the general population was recently estimated to be approximately 1:4,500 (8).

In clinical genetics, testing criteria for the *TP53* gene have been extensively discussed (3, 4), and for most tumors, based on the personal or family history suggestive of such a syndrome, germline testing is recommended (10). In addition, *TP53* pathogenic/likely pathogenic (P/LP) variants are commonly detected somatically, and it is the most frequently mutated gene in tumor tissues (11–13). Therefore, it has been recently recommended that when only somatic testing is performed and a P/LP variant is identified in the *TP53* gene, germline examination is indicated only when it is detected in sarcomas, breast cancer, or brain tumors (10).

In this current study, we report a peculiar case, where in the background of an unusual appearance of the Li–Fraumeni spectrum manifesting in a malignant perivascular epithelioid cell tumor (PEComa), a novel *TP53* pathogenic variant was identified. While PEComa was described in two previous case reports of Li–Fraumeni patients (14, 15), PEComa was the first manifestation of the disease in our patient. Our molecular genetic assays suggest a potential relationship between the pathogenic *TP53* variant and PEComa development.

METHODS

Immunohistochemistry

Immunohistochemical characterization was performed on a Ventana Benchmark autostainer (Roche Tissue Diagnostics, Oro Valley, AZ, USA) using the ultraView Universal DAB Detection Kit. Antibodies used (in alphabetical order) and vendors were as follows: ERG, H-Caldesmon, MelanA, and SOX10 (Ventana, Oro Valley, AZ, USA). Further antibodies CD34 (Dako-Agilent, Santa Clara, CA, USA, 1:200), Desmin (Dako-Agilent, 1:200), EMA (Dako-Agilent, 1:800), H3K27me3 (Cell Signaling, Danvers, MA, USA, 1:50), HHF35 (Dako-Agilent, 1:50), HMB45 (Dako-Agilent, 1:50), S100 (Dako-Agilent, 1:4000), SMA (Dako-Agilent, 1:100), STAT6 (Santa Cruz, Dallas, TX, USA, 1:100), Vim (Dako-Agilent, 1:100), and p53 (Dako-Agilent, 1:200) were used.

Fluorescent *In Situ* Hybridization

Fluorescent *in situ* hybridization (FISH) was performed using the ZytoLight® SPEC EWSR1/FLI1 TriCheck™ and ZytoLight® SPEC TFE3 Dual Color Break Apart Probe.

Genetic Analysis

Germline genetic analysis of the proband and family members was performed following an informed consent based on the ethical approval by the Scientific and Research Committee of the Medical Research Council of the Ministry of Health, Hungary (ETT-TUKEB 53720-4/2019/EÜIG).

Nucleic Acid Isolation From Peripheral Blood and From Tumor Tissue

DNA purification from peripheral blood and formalin-fixed paraffin-embedded (FFPE) tissues was performed using the Gentra Puregene Blood Kit (Cat No.: 158389, Qiagen, Hilden, Germany) and the Maxwell RSC DNA FFPE Kit on a Maxwell RSC Instrument (Cat. No.: S1450, Madison, WI, USA) as part of the routine molecular pathology diagnostic workflow. For RNA analysis, blood was collected in Tempus™ Blood RNA Tubes (Thermo Fisher Scientific, Waltham, MA, USA) and RNA extraction was performed by using the Tempus™ Spin RNA Isolation Kit. Nucleic acid quality and quantity were determined by a NanoDrop® 1000 Spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Waltham, MA, USA).

Whole Exome Sequencing From Peripheral Blood

Whole exome sequencing was done as previously reported, using a Twist Human Core Exome library preparation with a Twist mitochondrial panel (Cat. No.: 102026, Twist Bioscience, San Francisco, CA, USA) on a NovaSeq Illumina platform (Illumina, San Diego, CA, USA) with an average coverage of 100x (16). Data were analyzed by applying the Genome Analysis Toolkit (GATK) Germline short variant discovery (SNPs + Indels) algorithm. Annotation of coding variants was performed, following the American College of Medical Genetics and Genomics (ACMG) recommendations (17).

Sanger Validation, Site-Specific LOH Analysis, and RNA Splicing Effect Test

Sanger validation and site-specific LOH analysis were performed as previously reported (16). Primers used for validation were as follows: TP53_ex04_FOR 5′-CTGGTAAGGACAAGGGTTGG-3′; TP53_ex04_REV: 5′-GCCAGGCATTGAAGTCTCAT-3′,

and for LOH testing: TP53_int4ex4_F1: 5′-CTGGTAAGGACAAGGGTTGG-3′; TP53_int4ex4_F2: 5′-ACTTCCTGAAAACAACGTTCTG-3′; TP53_int4ex4_R1: 5′-TCATCTGGACCTGGGTCTTC-3′; TP53_int4ex4_R2: 5′-TCTGGACCTGGGTCTTCAGT-3′; TP53_int4ex4_R3: 5′-TCTGGGAGCTTCATCTGGAC-3′.

For testing the splicing effect, RNA extracted from whole blood was reverse transcribed using SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific, MA, USA). cDNA was then PCR-amplified with the following primers: TP53-C-e02_For: 5′-AGGAAACATTTTCAGACCTATGGA-3′, TP53-C-e06_Rev: 5′-CTGTCATCCAAATACTCCACACG-3′. PCR products were subjected to agarose gel electrophoresis next to controls and were then submitted for Sanger sequencing.

Multigene Panel Sequencing on FFPE Tumor DNA

Multigene panel sequencing of 161 genes related to personalized tumor therapy with Oncomine™ Comprehensive Assay v3M (Cat. No.: A35805, Thermo Fisher Scientific, Waltham, MA, USA) was performed as previously described on an Ion Torrent next-generation sequencing platform (Ion GeneStudio S5 System, Thermo Fisher Scientific, Waltham, MA, USA) (16). Data were analyzed using Oncomine Knowledge Reporter Software (Cat. No.: A34298, Thermo Fisher Scientific, Waltham, MA, USA).

Monogenic Mutation Analysis of Sinonasal Carcinoma

A real-time PCR test, a cobas 4800 KRAS Mutation test, and a *BRAF/NRAS* mutation test were used according to the manufacturer's instructions.

3D Protein Modeling

For protein modeling, prediction, and analysis, Phyre2 software was used to compare wild-type and variant amino acid sequences (18). For assessing the variant protein function and disorder prediction, the Phyre Investigator algorithm was applied.

Variant Classification

Specifications of the ACMG/AMP variant interpretation guidelines for germline *TP53* variants by Fortuno et al. were applied for variant classification (19). Accordingly, the ClinGen Sequence Variant Interpretation (SVI) Committee-approved decision tree (Abou Tayoun et al.) was used to determine the strength of PVS1 criteria, similarly to the *TP53*(NM_000546.5): c.97-1G>A variant (19, 20).

RESULTS

Case Report

A 90 × 60 × 115 mm soft tissue tumor was observed in the medial part of the right thigh of a 38-year-old, Caucasian male patient. In addition to the right-thigh tumor, soft-tissue MRI and thoraco-abdominal and pelvic CT revealed three nodules in the chest that appeared suspicious for metastatic processes (15-mm nodule in the right-lobe S10; 102-mm nodule in the left-side S6 segment and a 10 × 15 mm nodule subcarinal). Following the surgical removal of the thigh soft-tissue tumor, which was diagnosed as a grade III myxofibrosarcoma, chemotherapy (6 series of EPI-ADM, parallel Lartruvo treatment from the second series) was started (**Table 1**).

As the pulmonary nodules moderately regressed following chemotherapy, pulmonary surgery was performed to remove residual right-lobe nodules. Histology showed necrotizing granulomatous inflammation.

Three months later, the patient observed bloody rhinorrhea. Upon CT scanning, soft-tissue densities were observed in the sinonasal tract. After endoscopic surgery, intestinal-type sinonasal carcinoma showing typical histology and immunophenotype was diagnosed. Postoperative radiotherapy resulted in complete regression of the sinonasal tumor.

Ten months later, a control examination showed a right-lung nodule: therefore, a right lower lobectomy was performed. Pathological investigation revealed a cellular tumor showing a prominent perivascular arrangement. Tumor cells were pleomorphic with epithelioid or spindle-shaped character, and they had clear or abundant granular eosinophilic cytoplasm. Extensive necrotic areas and a very high mitotic rate (77/10HPF) were observed (**Figure 1**). Upon immunohistochemistry, tumor cells showed diffuse vimentin positivity. In the clear cell areas, tumor cells showed diffuse HMB45 positivity (**Figure 1A**). Focal but strong HMB45, desmin, H-Caldesmon, and smooth-muscle-actin expression were seen in the spindle cell areas (**Figure 1A**). Labeling for S100, SOX10, MelanA, cytokeratin (AE1–AE3), EMA, HHF35, CD34, STAT6, H3K27me3, and ERG was negative. Tumor cells were almost completely negative for p53 immunohistochemistry. Only scattered pleomorphic cells showed weak p53 expression. *EWSR1* and *TFE3* fluorescent *in situ* hybridization showed no rearrangement of the examined genes. The final diagnosis was metastasis of a malignant PEComa (grade III). In light of the histopathological results of the pulmonary lesion, the histological findings of the thigh tumor, which was originally diagnosed as a myxofibrosarcoma by another institute, were reevaluated by a specialist soft-tissue pathologist. Although the thigh tumor showed focal myxoid areas, probably resulting in the original diagnosis of myxofibrosarcoma, morphologically it was a similar mixture of epithelioid and spindle cells, as seen in the pulmonary lesion. Since only a limited panel of immuno histochemistry was performed at the time of the primary diagnosis, further immunohistochemistry including muscle markers and HMB45 was performed, which showed the same positive reaction as in the lung tumor (**Figure 1B**). As the thigh tumor showed a similar morphology and immunophenotype, it was reclassified as a primary PEComa, and the pulmonary tumor was considered as its metastasis.

Following lobectomy, a control CT was negative. Twelve months after the lung surgery, the patient appeared to be tumor free and is under close clinical follow-up (**Table 1**).

Molecular and Clinical Genetic Findings

We had only a limited amount of tissue from the sinonasal carcinoma, which was unfortunately not sufficient for multigene

TABLE 1 | Timeline of the patient history.

Date	Event
25 June 2018	Ultrasound confirmation of a lump on the right thigh (90 × 60 × 115 mm inhomogeneous, vascularized, cystic lesion)
3 July 2018	MRI of the thigh identified a 87 × 79 × 120 mm lesion
5 July 2018	Chest, abdominal, pelvic CT for staging identified 3 lesions suspected as metastasis in the lung (right lobe S10 segment -15 mm, S6 - 102 mm, subcarinal 10 × 15 mm)
11 July 2018	Surgical removal of the thigh lesion. Histological diagnosis: myxofibrosarcoma grade III. Following surgery, chemotherapy was started (6 cycles epiADM, from the second cycle with additional Latruvo treatment)
5 October 2018	Control MRI of the thigh: no tumor/recurrence was found
15 November 2018	Chest, abdominal, pelvic CT: lung nodules were regressed (right lobe S10 segment -12 mm, S6 - 6 mm, subcarinal 11 × 7 mm).
4 December 2018	Consultation of thoracic surgery: radiation therapy of the lung and mediastinal nodules are recommended. Right S10 and subcarinal nodules can be removed by minimal invasive approach.
20 January 2019	Video-assisted thoracoscopic surgery, VATS
21 February 2019	Histology of the lung nodules and lymph nodes: No malignancy can be detected. Necrotizing granulomatous inflammation.
3 March 2020	The patient observed nasal congestion in the right nostril along with bloody rhinorrhea
5 May 2020	Endoscopic Surgery (following head and face CT & MRI), histology: adenocarcinoma, intestinal type
9 June 2020–16 July 2020	Radiochemoterapy (tumor bed irradiation with 54 Gy, along with cisplatin and 5FU chemotherapy)
9 September 2020	Control MRI of the skull and neck; CT of the skull and rhinobasis. Postoperative radiotherapy resulted in complete regression of the sinonasal lesion. No residual or recurrent tumor can be detected
4 October 2020	Control chest CT scan revealed a 27-mm nodule in the S10 mediastinal segment of the right lobe. Consultation for thoracic surgery recommended removal.
29 October 2020	PET/CT scan identified FDG uptake in a soliter nodule in the right lower lobe nodule, suggesting a metastasis in the lung.
16 November 2020	Thoracic surgery: right lobectomy
27 November 2020	Histology: I. metastasis of a malignant PEComa (grade III) in the right lobe; II: lymph nodes are tumor free
14 December 2020	Control MRI of the skull and neck; CT of the skull and rhinobasis: no residual or recurrent tumor can be detected
7 January 2021	Tumor board recommended close follow up
7 April 2021	Control MRI of the skull and neck; CT of the skull and rhinobasis: no residual or recurrent tumor can be detected
17 June 2021–14 July 2021	Control whole body MRI (abdomen, pelvis and thigh), and skull and neck MRI and spine & chest MRI: no residual or recurrent tumor can be detected
17 November 2020–24 November 2020	Control MRI of the skull, chest, abdomen, and thighs indicated no residual or recurrent tumor

analysis. Monogenic analysis using COBAS kits showed no evidence of *KRAS*, *NRAS*, or *BRAF* mutation.

Multigene panel (161 genes) sequencing was performed in the malignant PEComa and identified a *TP53*(NM_000546.5):c.97-2A>C variant with 66.21% allele frequency (variant allele frequency, VAF), but no other therapy-predictive pathogenic variant or gene fusion was detected. As VAF of the *TP53* variant suggested a potential germline presence, the patient was referred for genetic consultation and molecular genetic analysis in our department. During the consultation and pedigree analysis, Li–Fraumeni core tumors in the long-since deceased mother of the proband were identified (osteosarcoma at age 14; breast cancer at the age of 33 and ovarian cancer at the age of 35). Based on the available information, no other relative was affected (**Figure 2**).

We performed targeted Sanger sequencing of the identified variant and proved the germline presence of the *TP53* (NM_000546.5):c.97-2A>C variant (**Figure 3A**). Exome sequencing was also performed but was not able to identify other pathogenic variants in the compulsory gene list report or in potential hereditary cancer genes. Comparing the germline and the somatic (tumor types) variants, a partial locus-specific loss of heterozygosity (normalized reduction of the reference allele quantity was 0.3 relative to the variant allele) was observed in the PEComa, whereas no LOH was identified in the sinonasal carcinoma (**Figures 3A, B**). Additionally, *in vitro* RNA testing proved whole exon 4 skipping due to the *TP53*(NM_000546.5):c.97-2A>C variant (**Figure 3A**).

While this exon skipping does not lead to a frame shift, it results in a loss of 93 amino acids (from amino acids 32 to 125) at the protein level. Based on protein modeling, the variant protein was predicted to have a different 3D structure (**Figure 4**). Additionally, 40.8% of the lost amino acids were predicted as "disordered" in the 3D structure, meaning that the change or loss might lead to damaged protein structure/function. This was also in line with the p53 immunohistological finding.

Based on the molecular and clinical findings, the *TP53*-specific ACMG classification of *TP53*(NM_000546.5):c.97-2A>C in this proband is Class 5, "pathogenic," because it affects a splice site (PVS1_strong), is not found in gnomAD exomes or genomes (PM2_supporting), and matches computational predictions (PP3_mooderate). Furthermore, our additional evidence supports its pathogenicity: i) exon-skipping using an *in vitro* functional test and ii) the negative *TP53* immunohistochemistry on the tumor tissue.

DISCUSSION

We identified the *TP53*(NM_000546.5):c.97-2A>C variant as a novel, germline pathogenic alteration in the background a thigh-muscle PEComa. This variant, to the best of our knowledge, has not been previously reported in the literature. In the ClinVar database, a single submitter reported a different variant at the same localization (accession: VCV000246337.1; NM_000546.5: c.97-2A>G), but the molecular *in vitro* characterization of this variant has not been performed. Additionally, another variant affecting the same splice site at a different localization, NM_000546.5:c.97-1G>A, was identified in a patient meeting

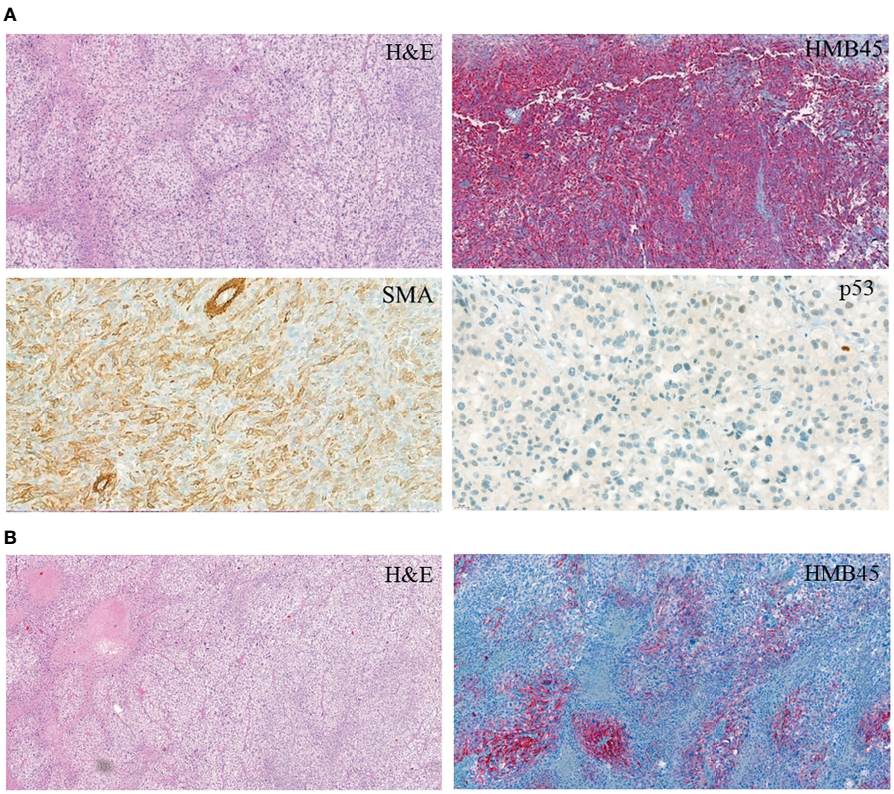

FIGURE 1 | Immunohistochemistry of the PEComa tissue. **(A)** PEComa metastasis in the lung. **(B)** Primary tumor.

Chompret criteria and was found to cause abnormal splicing upon functional assay analysis (21, 22). We proved that the newly identified NM_000546.5:c.97-2A>C variant led to exon 4 skipping, potentially resulting in a different p53 protein structure that would be predicted to have decreased stability. This is supported by p53 immunohistochemistry, where tumor cells were predominantly negative, and only scattered, focal positivity could be seen in pleomorphic cells.

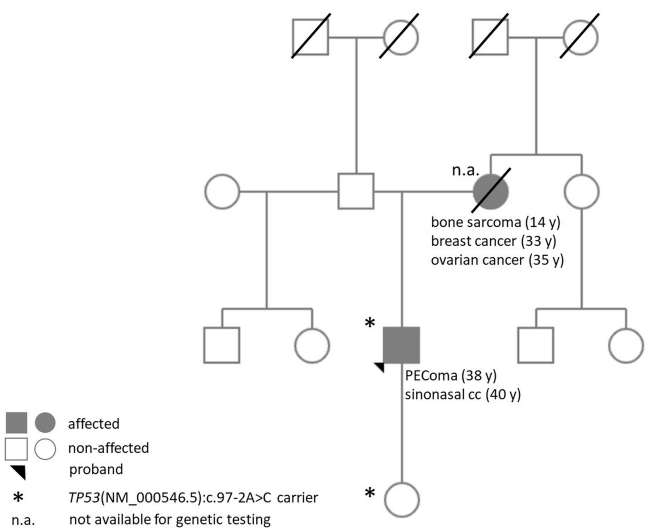

FIGURE 2 | Pedigree of the proband.

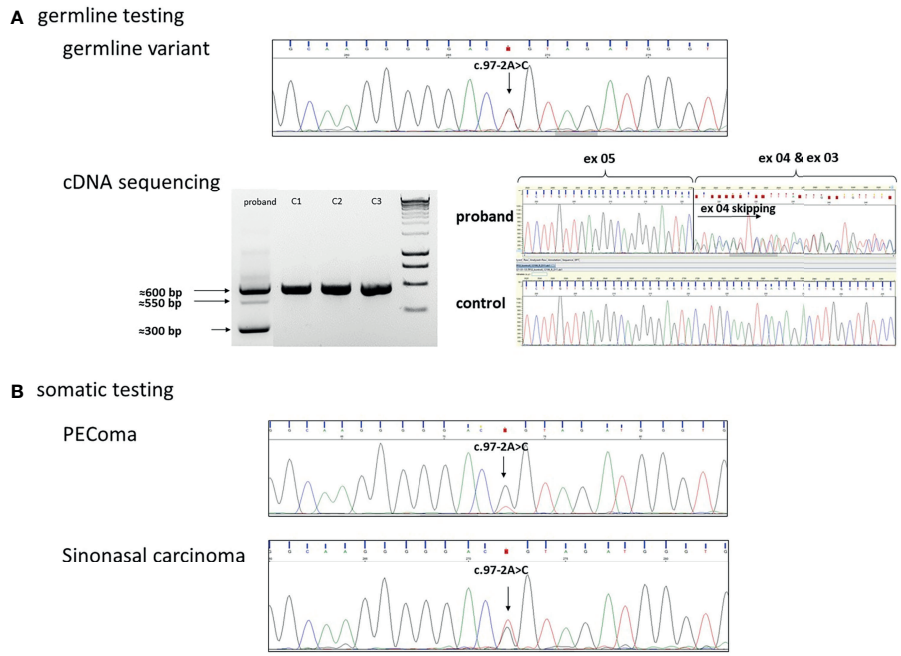

FIGURE 3 | **(A)** Germline heterozygous *TP53*(NM_000546.5):c.97-2A>C variant in the DNA isolated from blood. cDNA sequencing identified exon 4 skipping, accordingly. On the electrophoresis gel, the 600-bp PCR product indicates the wild type, the 550-bp PCR product indicates a heteroduplex, and the 300-bp PCR product was confirmed as a skipped exon 4 transcript following Sanger sequencing. (C1, C2, C3 were used as controls). **(B)** Sanger sequencing in the PEComa tissue sample indicated the loss of the wild-type (wt) allele in the tumor: loss of heterozygosity (LOH) was detected. In sinonasal carcinoma, wild-type and variant alleles are presented in ~50%–50%: no LOH was detected.

PEComas (perivascular epithelioid cell tumors) are rare, mesenchymal tumors of uncertain malignant potential, as recurrences may occur years after the initial diagnosis. Malignant metastasizing PEComas are very rare (23). The differential diagnosis can include carcinomas, smooth muscle tumors, and adipocytic neoplasms (23). Our case (first diagnosed as myxofibrosarcoma of the muscle) highlights the difficulties in the pathological diagnosis of malignant PEComa. Regarding PEComa pathogenesis, alterations in two, or recently three, main pathways have been described. Most commonly, a loss of function in the tuberous sclerosis complex subunit 1, *TSC1* (~27%) or *TSC2* (~73%), has been observed due to deletion or

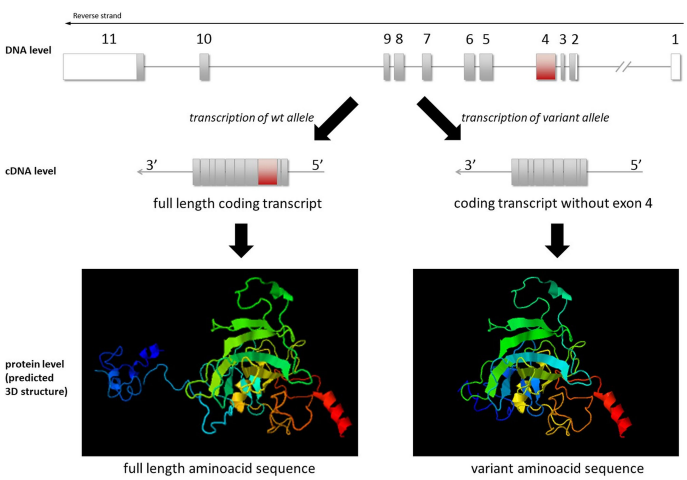

FIGURE 4 | Illustration of *TP53*(NM_000546.5) exon 4 skipping at the DNA, cDNA, and protein levels. 3D modeling of the wild-type and variant protein indicated different structures (visualized by JSmol).

pathogenic missense variants, leading to activated mTOR signaling and increased cell growth (23, 24). *TSC1* or *TSC2* inactivation can appear somatically, or in individuals already harboring a germline *TSC1/2* mutation. In both cases, mTOR inhibition can be a potential therapeutic option. The other main molecular feature behind PEComa pathogenesis (in approximately 23% of cases) is the rearrangement affecting *TFE3* (transcription factor binding to IGHM enhancer 3), which is implicated in cell differentiation (23, 24). This has a significant clinical importance, as these tumors might be non-responsive to mTOR inhibition. Lately, rearrangements of *RAD51B* in uterine PEComas have also been identified (23). Similar to other tumors, somatic *TP53* mutations have been described in PEComas, and they are potentially linked to malignancy (24–27).

Most PEComas are sporadic, and only a small subset is associated with the hereditary condition TSC. Recently, our group also identified a *PTCH1* mutation in a patient with bilateral intra-abdominal PEComas suffering from Gorlin-Goltz syndrome (16). PEComas have been reported in only two Li–Fraumeni cases in the literature to date (14, 15). Contrary to these two examples, in our case the PEComa was the first manifestation in the LFS proband. In our case, we could not detect *TSC1* or *TSC2* sequence- or copy-number variants, either in the germline or somatically. The causative role of *RAD51B* was also excluded by multigene panel sequencing, copy number analysis, and fusion analysis. We did not detect *TFE3* rearrangement by FISH analysis, which further reduces the likelihood of a causative role of the *TFE3* pathway in the pathogenesis. However, we identified a site-specific LOH in the PEComa tissue regarding the novel *TP53* pathogenic variant. The normal allele was lost in favor of the non-functional allele harboring the pathogenic variant, and this was supported by the immunohistochemical findings. This suggested a role for the defective *TP53* pathway in the PEComa pathogenesis, which is also reported to be associated with the malignant, metastatic form of this tumor type in this patient.

While a sinonasal carcinoma can be part of the Li–Fraumeni spectrum, we were not able to identify the second hit affecting *TP53* that causes the tumor development.

As *TP53* pathogenic variants contribute to cancer proliferation and metastasis, targeting the signaling pathways that become altered by p53 mutation seems to be an attractive strategy (28). Whereas in the clinical practice there is currently no such drug available, several agents are under investigation in clinical trials (28). The prognostic and predictive role of *TP53* pathogenic variants has been intensively investigated and reported in somatic settings (29). Currently, there are no special recommendations for treatment of the Li–Fraumeni spectrum; indeed, there are reports of treatment (chemo- and radiotherapy) failure (30). While the primary goal is always the treatment of the actual malignant disease, the radiation (both diagnostic and therapeutic) exposure should be minimized, as subsequent primary tumors, particularly within the radiotherapy field, often develop after the exposure (3). Therefore, avoiding radiotherapy when possible and instead using preferably non-genotoxic chemotherapies are recommended by recent guidelines (3).

The genetic counseling of patients carrying pathogenic *TP53* variants is essential. Following international and national guidelines, the patients have to be informed of the disease, the risk of tumor development and localization, the potential options related to surveillance, and the screening of first-degree or at-risk relatives (3, 9). Accordingly, pre- and posttest genetic counseling and family screening were performed in our PEComa patient.

CONCLUSION

We identified a novel *TP53* splice variant in an attenuated LFS patient manifesting with a malignant PEComa of unusual appearance. This rare, unexpected phenotype of the patient highlights the importance of the introduction of the Li–Fraumeni spectrum instead of the classic LFS concept. Additionally, using complex molecular genetic assays, we demonstrated the pathogenic role of a novel *TP53* germline variant in the development of the PEComa. This may help with the interpretation of this variant in other patients identified in the future.

DATA AVAILABILITY STATEMENT

The original datasets presented in this study are available in a publicly accessible repository: NCBI SRA database under accession number PRJNA815946.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Scientific and Research Committee of the Medical Research Council of the Ministry of Health, Hungary (ETT-TUKEB 53720-4/2019/EÜIG). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Conceptualization, JL, HB, and AP. Germline genetic tests, AB, HB. Regular and molecular pathology tests, MS, ET. Genetic interpretation, HB. Writing—original draft preparation, HB. Writing—review and editing, AP, JL. Visualization, HB. Supervision, AP and JL. Project administration, HB. Funding acquisition, AP and HB. All authors contributed to the article and approved the submitted version.

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We are grateful for the proband agreeing to participate in this study. He read the manuscript and agreed to this submission, and we received his answer on January 2, 2022. The patient is regularly assessed by JL and is currently symptom-free. The patient is also in contact with the Hungarian Li-Fraumeni Advocacy Group.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.849004/full#supplementary-material

Supplementary Table | Germline variants detected by exome sequencing (mean allele frequency (MAF) <0.01). All data is available at GeneBank under Submission # 2537264.

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Breast Cancer Patients With Positive Apical or Infraclavicular/Ipsilateral Supraclavicular Lymph Nodes Should Be Excluded in the Application of the Lymph Node Ratio System

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Aim: Increasing studies have demonstrated lymph node ratio (LNR) to be an accurate prognostic indicator in breast cancer and an alternative to pN staging; however, the AJCC-TNM staging system classified apical or infraclavicular/ipsilateral supraclavicular lymph node-positive (APN(+)) patients with a worse prognosis as the pN3 stage. Until now, different reports on LNR in breast cancer have ignored this possibility. Consequently, it is necessary to discuss the role of APN(+) patients in the LNR system to obtain a precise LNR that predicts the prognosis accurately.

Materials and Methods: We collected data on 10,120 breast cancer patients, including 3,936 lymph node-positive patients (3,283 APN(–) and 653 APN(+) patients), who visited our hospital from 2007 to 2012. Then we applied X-tile analysis to calculate cut-off values and conduct survival analysis and multivariate analysis to evaluate patients' prognosis.

Results: We confirmed that some APN(+) patients were mis-subgrouped according to previously reported LNR, indicating that APN(+) patients should be excluded in the application of LNR to predict prognosis. Then we applied X-tile analysis to calculate two cut-off values (0.15 and 0.34) for LNR-APN(–) patients and conducted survival analysis and found that LNR-APN(–) staging was superior to pN staging in predicting the prognosis of APN(–) breast cancer patients.

Conclusion: From this study, we conclude that excluding APN(+) patients is the most necessary condition for effective implementation of the LNR system. LNR-APN(–) staging could be a more comprehensive approach in predicting prognosis and guiding clinicians to provide accurate and appropriate treatment.

Keywords: breast cancer, cut-off values, lymph node ratio, prognosis, pN stage

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INTRODUCTION

The latest American Joint Committee on Cancer (AJCC) staging system recommends that pathologists evaluate the prognosis of patients by pN stage (Greene, 2002; Singletary et al., 2002). However, this classification only considered the number of positive lymph nodes and did not take the total number of lymph nodes into account. In recent years, emerging researchers have proposed lymph node ratio (LNR), the number of involved positive lymph nodes divided by the total number of lymph nodes examined, to be a better prognostic indicator than absolute lymph node number (Woodward et al., 2006; Vinh-Hung et al., 2009; Yang et al., 2017).

Remarkably, the WHO classification of breast tumors and AJCC demonstrated that patients with apical or infraclavicular/ipsilateral supraclavicular lymph node metastasis should be classified into the pN3 stage according to the traditional pN staging system, regardless of the status of lower-level metastatic lymph nodes, which were considered to exhibit a poor prognosis (Güven et al., 2007; Mary et al., 2009; Shalaka et al., 2019). Until now, reports on LNR in breast cancer from different research groups did not focus on the impact of APN(+) on the LNR system (Vinh-Hung et al., 2009; Kim et al., 2011; Duraker et al., 2013; Wu et al., 2013).

In order to illustrate the role of APN(+) in the LNR system and obtain the most precise LNR, we collected data on 10,120 patients diagnosed with breast cancer from 2007 to 2012 in our hospital (Tianjin Medical University Cancer Institute and Hospital). A total of 3,936 patients had positive lymph nodes, including 3,283 APN(−) and 653 APN(+) patients. We found that APN(+) patients had a significantly worse prognosis than APN(−) breast cancer patients in the same group according to previously reported LNR, indicating that APN(+) patients should be excluded in the application of the LNR system to predict prognosis. Then, we applied X-tile analysis to the data on the cohort of APN(−) patients to calculate two cut-off values (0.15 and 0.34) based on overall survival of these patients and defined the group as LNR-APN(−). Survival analysis further revealed that LNR-APN(−) staging was superior to pN staging in predicting the prognosis of APN(−) breast cancer patients.

MATERIALS AND METHODS

Ethical Statement and Patient Selection

A total of 10,120 patients were diagnosed with breast cancer, including 6,184 patients with negative axillary lymph nodes and 3,936 patients with positive axillary lymph nodes, from January 2007 to December 2012 according to data from the archives of the Department of Breast Cancer Pathology and Research Laboratory, Tianjin Medical University Cancer Institute and Hospital. Patients with positive lymph nodes were further classified into APN(−) (3,283 patients) and APN(+) (653 patients). This study was approved by the Institutional Ethics Committee of Tianjin Medical University Cancer Institute and Hospital (bc2017019), and each participant signed an informed consent document.

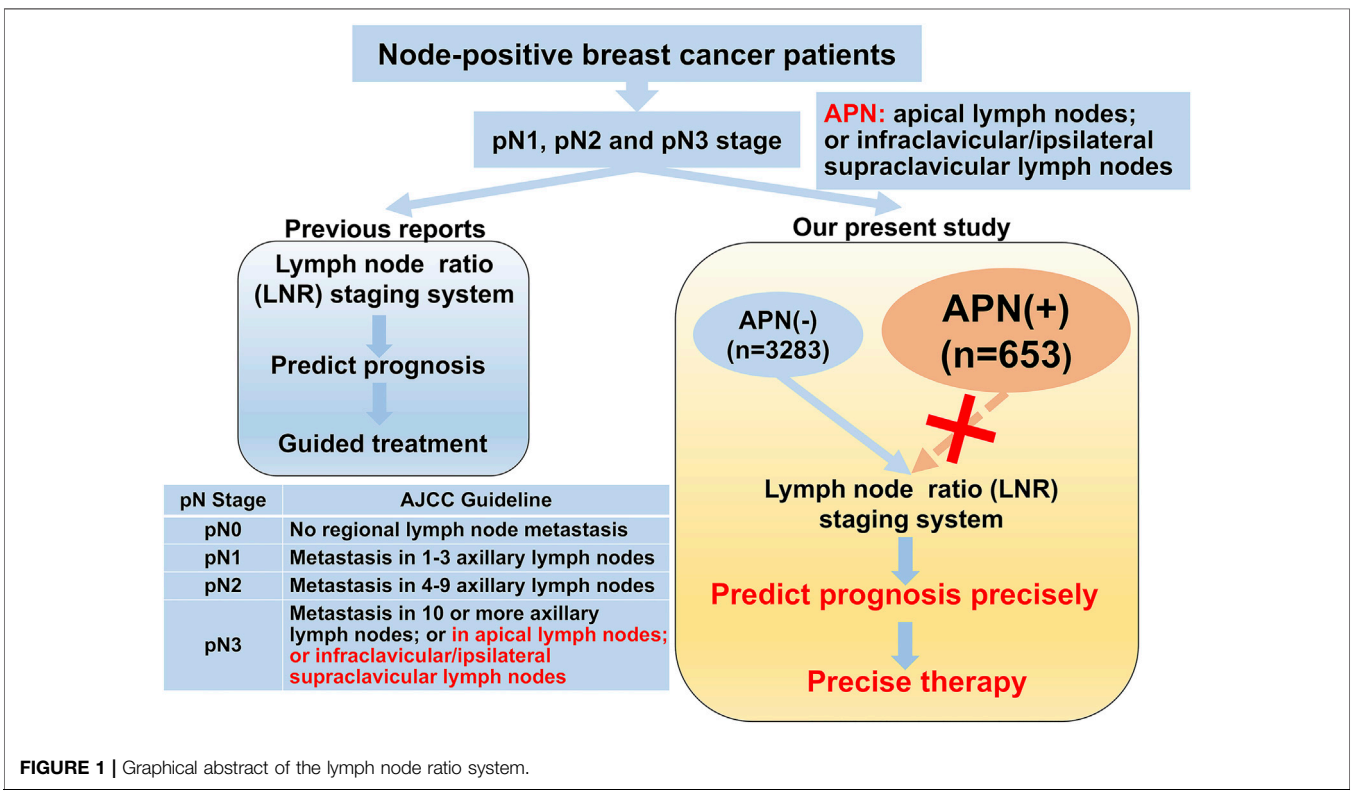

FIGURE 1 | Graphical abstract of the lymph node ratio system.

All patients who underwent axillary lymph node dissection and received radical mastectomy or modified radical mastectomy were selected. Surgical specimens were then prepared for histological analysis: the specimens were fixed in 10% formaldehyde, and 2-μm sections were taken every 1.5 mm. Two experienced pathologists evaluated the status of the lymph nodes based on the World Health Organization histological classification of breast tumors. Metastatic nests >0.2 mm in diameter were scored as lymph node-positive metastases. After surgery, all patients were administered adjuvant chemotherapy and/or radiotherapy and/or endocrine therapy according to the National Comprehensive Cancer Network (NCCN) guidelines. Patients with multisource tumor, bilateral breast cancer, and loss to follow-up were excluded. We defined loss of follow-up as patients lost to follow up after being discharged from the hospital. Lumpectomy is the common treatment for early-stage breast cancer; most of these patients who underwent sentinel lymph node biopsy (SLNB) usually have a small number of lymph nodes. So, patients who received lumpectomy were excluded from the study. Information recorded for each patient included age at diagnosis, year of surgery, histologic features of the tumor, lymph node status, and survival. The median follow-up period was 81 (range 1–149) months.

Cut-Off Values of LNR-APN(–) Staging

Positive lymph nodes identified on histopathological examination were classified according to the eighth edition of the AJCC staging system into three stages: pN1 (one to three positive lymph nodes), pN2 (four to nine positive lymph nodes), and pN3 (more than nine positive lymph nodes and at least one positive apical or infraclavicular/ipsilateral supraclavicular lymph node). LNR was calculated by the number of positive lymph nodes/total lymph nodes examined in node-positive patients. We excluded APN(+) breast cancer patients and obtained the optimal cut-off values of LNR-APN(−) staging by using the X-tile plots (X-tile software 3.6.1, Yale University, New Haven, CT, United States) in terms of overall survival. X-tile is a bioinformatics tool for biomarker assessment and outcome-based cut-point optimization (Robert et al., 2004). The X-tile plot shows the robustness of the relationship between LNR-APN(−) and patient outcome *via* construction of a two-dimensional projection of every possible subpopulation. Chi-square values were calculated for every possible division of LNR-APN(−), and the program selected the optimal division of LNR-APN (−) by choosing the highest chi-square value. The interval between the given set of divisions was 0.01. Therefore, the X-tile program divided the entire cohort into three subgroups based on the ratio of positive lymph nodes, which were LNR1-APN(−) (<0.15), LNR2-APN(−) (0.15–0.34), and LNR3-APN(−) (>0.34).

SEER Database

We collected information on female breast cancer patients diagnosed between 1 January 2010 and 31 December 2012 from the SEER (Surveillance, Epidemiology, and End Results) database. Patients diagnosed with breast cancer before 2010 were excluded from this study because of unavailability of HER2 data.

TABLE 1 | Clinicopathologic characteristics of breast cancer patients in the TCIH database (*n* = 10,120).

Characteristic	Number of patients (*n* = 10,120)	%
Age (years)		
<50	4,636	45.8
≥50	5,484	54.2
Histopathologic type		
Invasive ductal	7,500	74.1
Invasive micropapillary	335	3.3
Invasive lobular	249	2.5
Mucinous	136	1.3
Other types	1,900	18.8
Histological grade		
I	985	9.7
II	6,150	60.8
III	1,221	12.1
Unknown	1,764	17.4
Estrogen receptor[a]		
Negative	3,034	34.3
Positive	5,802	65.7
Progesterone receptor[a]		
Negative	3,525	39.9
Positive	5,302	60.1
HER2 expression[a]		
0 and 1+	6,282	71.3
2+	1,733	19.7
3+	790	9.0
pT stage		
pT1	4,905	48.5
pT2	4,753	47.0
pT3	386	3.8
pT4	76	0.7
Number of lymph nodes removed		
1–3	42	0.4
4–9	223	2.2
≥10	9,855	97.4
pN stage		
pN0	6,184	61.1
pN1	2,213	21.9
pN2	804	7.9
pN3	919	9.1

TCIH, Tianjin Medical University Cancer Institute and Hospital.
[a]*Some data missing.*

A total of 10,163 patients who met the following criteria were included: breast cancer as the primary cancer, unilateral breast cancer, received radical mastectomy or modified radical mastectomy, one or more involved lymph nodes, one or more positive lymph nodes, and known tumor size.

Statistical Analysis

Overall survival (OS) and disease-free survival (DFS) were the main endpoints of this trial. The follow-up interval for OS and DFS was calculated in months. OS was defined as the time between the date of diagnosis and the date of death from any cause or the date of last follow-up. DFS was defined as the time from the date of diagnosis to the date of the first locoregional recurrence or/and distant metastasis, or the last follow-up date. OS and DFS curves were estimated using the Kaplan–Meier method and compared by the log-rank test, and the chi-square test was used to compare differences between groups. The

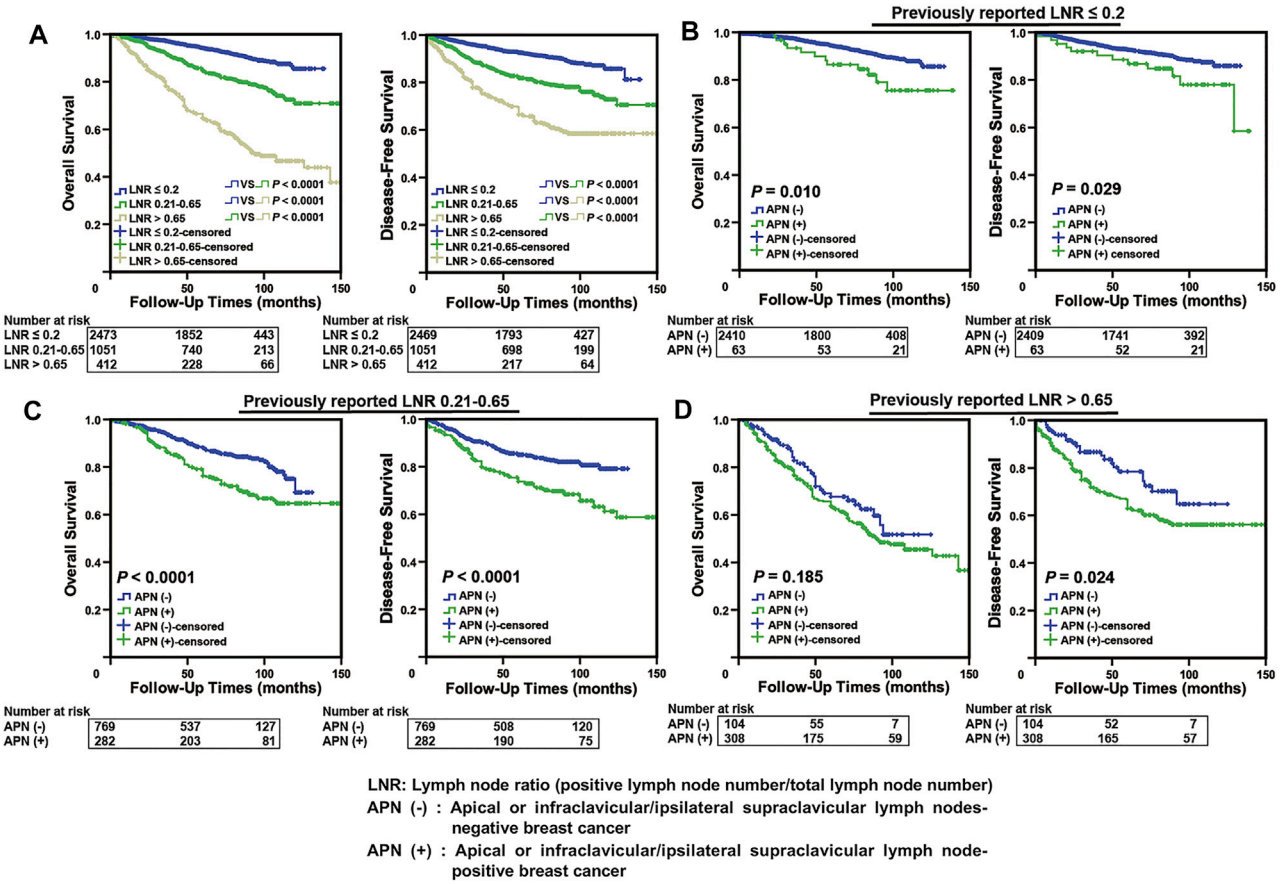

FIGURE 2 | Some APN(+) patients with poor prognosis were mis-subgrouped in the low LNR stage using the LNR system. **(A)** Kaplan–Meier analysis in our breast cancer cohort according to previously reported LNR (n = 3,936). **(B)** Comparison of Kaplan–Meier curves of APN(–) and APN(+) breast cancer patients based on previously reported LNR ≤ 0.2 (n = 2,473, OS: p = 0.010, DFS: p = 0.029). **(C)** Comparison of Kaplan–Meier curves of APN(–) and APN(+) breast cancer patients based on previously reported LNR 0.21–0.65 (n = 1,051, OS: p < 0.0001, DFS: p < 0.0001). **(D)** Comparison of Kaplan–Meier curves of APN(–) and APN(+) breast cancer patients based on previously reported LNR > 0.65 (n = 412, DFS: p = 0.024).

independent prognostic effect of LNR-APN(–) was investigated using the Cox regression analysis, adjusting for age at diagnosis, histological grade, pT stage, and pN stage. Hazard ratios (HRs) along with 95% confidence intervals (95% CIs) were calculated. Two-tailed p values of less than 0.05 were considered to be statistically significant. All statistical analyses were performed using the SPSS version 26.0 software package for Windows (IBM SPSS Statistics, Chicago, IL, United States).

RESULTS

Patients and Characteristics

The graphical abstract is shown in **Figure 1**. A total of 10,120 patients were diagnosed with breast cancer from 2007 to 2012 in Tianjin Medical University Cancer Institute and Hospital, and the clinicopathologic characteristics of the breast cancer patients are summarized in **Table 1**. Of the 10,120 breast cancer patients, 6,184 (61.1%) and 3,936 (38.9%) patients were node-negative and node-positive, respectively. The mean number of dissected lymph nodes was 23.1. Based on the eighth edition of the AJCC staging system, 2,213 patients were classified as pN1 (21.9%), 804 patients as pN2 (7.9%), and 919 patients as pN3 (9.1%). The median follow-up time for all 10,120 patients was 81 (range 1–149) months. We also present the detailed description of abbreviations in **Supplementary Table S1**.

Some APN(+) Patients With Poor Prognosis Were Mis-Subgrouped Into Low LNR Stage Using the LNR System

We applied the Kaplan–Meier survival analysis to our cohort based on the representative previously reported LNR (cut-off values: 0.2 and 0.65) (Vinh-Hung et al., 2009; Wu et al., 2013; Wu et al., 2015; Quintyne et al., 2017) and found that there was a significant difference in survival among different groups (p < 0.0001, **Figure 2A**). In the subgroup analysis, APN(+) patients were found to have a significantly worse prognosis than APN(–) patients in the LNR1 (LNR ≤ 0.2) and LNR2 (LNR 0.21–0.65) groups (p < 0.05, **Figures 2B,C**). In the LNR3 (LNR > 0.65)

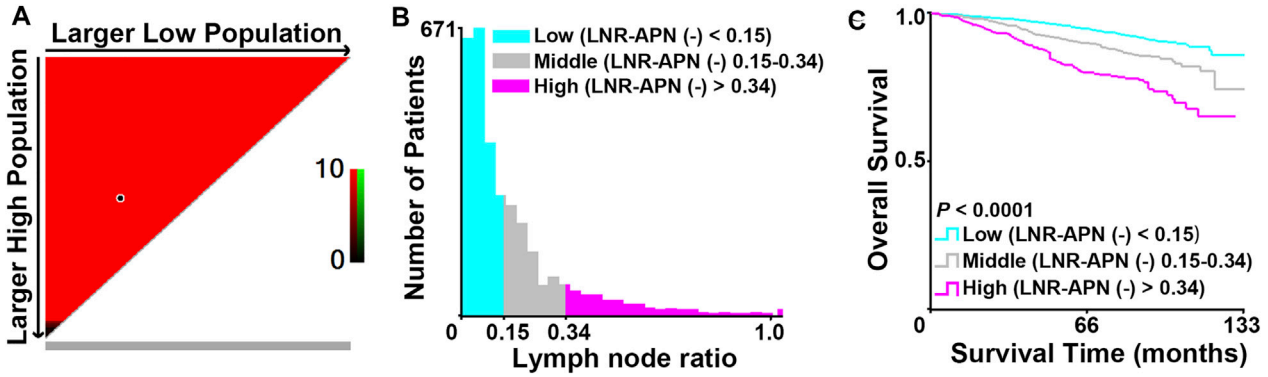

FIGURE 3 | Identification of the optimal cut-off values (0.15 and 0.34) for LNR-APN(–) by X-tile analysis in APN(–) patients with positive lymph nodes among 10,120 breast cancer patients. **(A)** Red indicates a negative association. *X*-axis demonstrates all potential cut-off values from low to high (left to right), defined as larger low population. *Y*-axis demonstrates cut-off values from high to low (top to bottom), defined as larger high population. **(B)** Histogram of the entire cohort divided into three subgroups according to the optimal cut-off values of 0.15 and 0.34. **(C)** Kaplan–Meier curves showing the division of overall survival according to the cut-off values of 0.15 and 0.34 (n = 3,283, p < 0.0001).

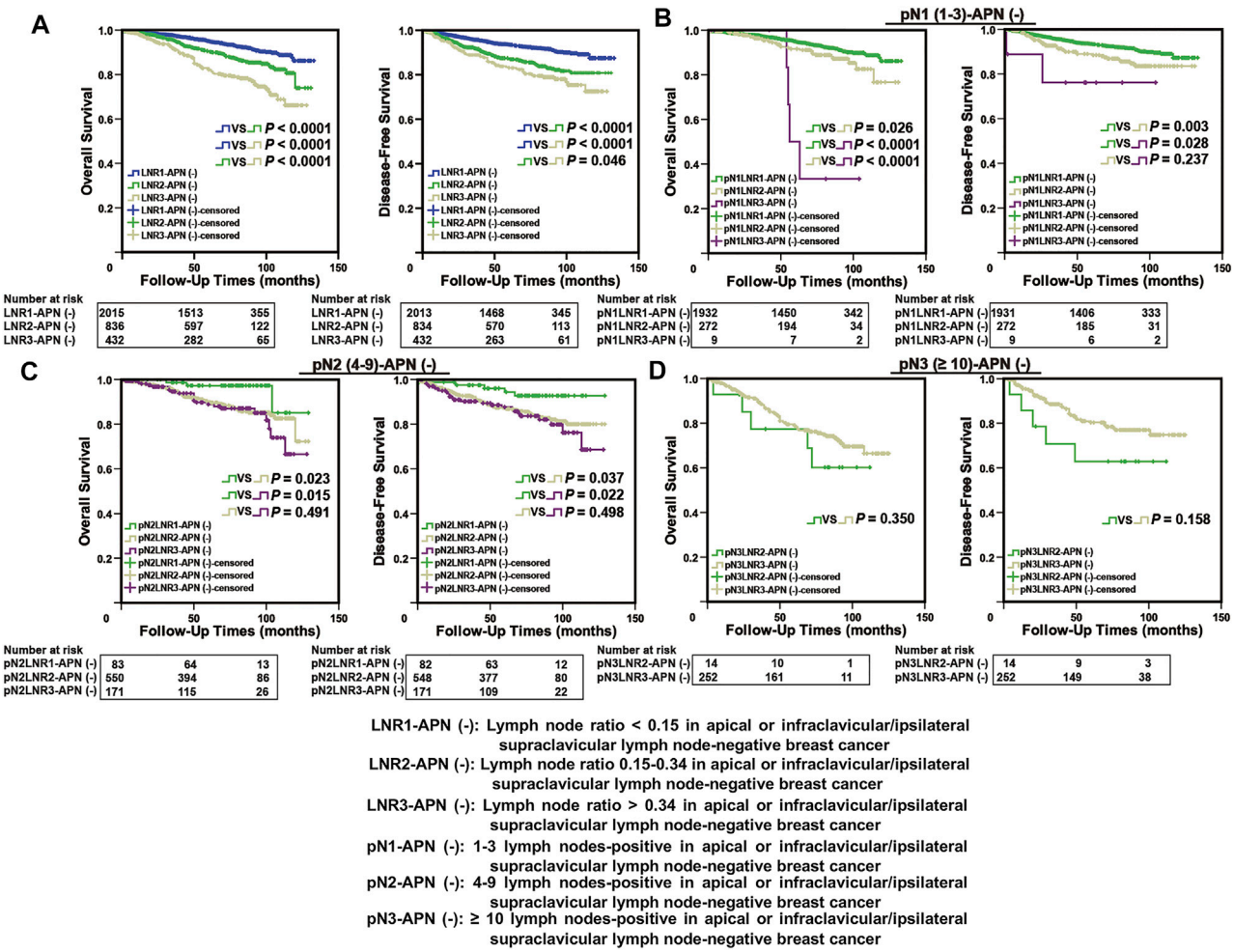

FIGURE 4 | LNR-APN(–) could accurately predict the prognosis of APN(–) breast cancer patients. Kaplan–Meier analysis of **(A)** LNR-APN(–) breast cancer patients (n = 3,283), **(B)** pN1-LNR-APN(–) breast cancer patients (n = 2,213), **(C)** pN2-LNR-APN(–) breast cancer patients (n = 804), and **(D)** pN3-LNR-APN(–) breast cancer patients (n = 266).

TABLE 2 | Overall survival multivariable analysis of APN(–) patients among 10,120 breast cancer patients.

Variable	HR	95% CI	*p* value
Age (years)			
<50	1	Reference	
≥50	1.378	1.085–1.749	0.009**
Histological grade			
I	1	Reference	
II	1.159	0.630–2.131	0.635
III	0.958	0.493–1.859	0.898
Estrogen receptor[a]			
Negative	1	Reference	
Positive	0.764	0.561–1.040	0.087
Progesterone receptor[a]			
Negative	1	Reference	
Positive	0.808	0.601–1.086	0.158
HER2 expression[a]			
0 and 1+	1	Reference	
2+	1.467	1.119–1.922	0.006**
3+	1.176	0.789–1.753	0.426
pT stage			
pT1	1	Reference	
pT2	1.699	1.269–2.276	<0.0001***
pT3	2.406	1.564–3.702	<0.0001***
pT4	6.413	3.677–11.185	<0.0001***
pN-APN(–)			
pN1-APN(–)	1	Reference	
pN2, 3-APN(–)	1.040	0.742–1.458	0.818
LNR-APN(–)			
LNR1-APN(–)	1	Reference	
LNR2, 3-APN(–)	2.006	1.424–2.826	<0.0001***

pN2, 3-APN(–): pN2-APN(–) and pN3-APN(–).
LNR2, 3-APN(–): LNR2-APN(–) and LNR3-APN(–).
***p < 0.01.*
****p < 0.001, Cox regression analysis.*
[a]Some data missing.

group, there was no difference in OS between APN(+) and APN(−) breast cancer patients, but a significant difference was noted in DFS, considering the poor prognosis within this group (**Figure 2D**). These results indicated that some APN(+) patients have been mis-subgrouped using the LNR system.

Identification of the Optimal Cut-Off Values (0.15 and 0.34) for LNR-APN(–) Staging by X-Tile Analysis in APN(–) Patients With Positive Lymph Nodes Among 10,120 Breast Cancer Patients

In order to obtain the precise LNR, we focused on 3,283 APN(−) patients with positive lymph nodes from the 10,120 breast cancer patients and applied X-tile analysis to calculate two cut-off values (0.15 and 0.34) based on the OS of these patients (**Figures 3A–C**).

LNR-APN(–) Staging Could Accurately Predict the Prognosis of APN(–) Breast Cancer Patients

The APN(−) breast cancer patients were classified into three groups based on the cut-off values and defined as LNR1-APN(−) (LNR > 0 and <0.15; $n = 2015$), LNR2-APN(−) (LNR ≥ 0.15 and ≤0.34; $n = 836$), and LNR3-APN(−) (LNR > 0.34; $n = 432$), which represented 52.1%, 24.0%, and 23.9% of patients in this study cohort, respectively. The groups categorized by LNR-APN(−) yielded a significant difference between the OS and DFS curves ($p < 0.0001$, **Figure 4A**). Consequently, LNR-APN(−) staging could predict the prognosis of breast cancer patients accurately.

Next, we divided pN1 breast cancer patients ($n = 2,213$) into three groups, namely, pN1-LNR1-APN(−), pN1-LNR2-APN(−), and pN1-LNR3-APN(−); pN2 breast cancer patients ($n = 804$) into pN2-LNR1-APN(−), pN2-LNR2-APN(−), and pN2-LNR3-APN(−); and pN3 breast cancer patients ($n = 266$) into pN3-LNR2-APN(−) and pN3-LNR3-APN(−). Survival analysis between different subgroups revealed that the pN1-LNR2-APN(−) and pN1-LNR3-APN(−) groups had a significantly worse prognosis than pN1-LNR1-APN(−) ($p < 0.05$, **Figure 4B**), and the pN2-LNR1-APN(−) group had a significantly better prognosis than the pN2-LNR2-APN(−) and pN2-LNR3-APN(−) groups ($p < 0.05$, **Figure 4C**). Moreover, pN1-LNR2-APN(−) and pN1-LNR3-APN(−) patients had a significantly worse prognosis than patients with pN1 stage, and pN2-LNR1-APN(−) patients had a better prognosis than patients with pN2 stage; however, there was no significant difference between LNR3-APN(−) and pN3 groups (**Figure 4D**; **Supplementary Figure S1**).

Multivariate analysis revealed LNR-APN(−) to be a better prognostic predictor of OS than pN-APN(−) in breast cancer by using the Cox proportional hazard regression model ($p < 0.05$). LNR2,3-APN(−) (LNR2-APN(−) and LNR3-APN(−)) breast cancer patients had a significantly worse OS than LNR1-APN(−) patients (HR = 1.843, $p < 0.0001$, **Table 2**).

Verify the Accuracy of the LNR-APN(–) System Using the SEER Database

To further verify the accuracy of the LNR-APN(−) system in different clinical databases, we fixed our attention on the SEER database, which comprised 10,163 breast cancer patients. The clinicopathologic characteristics of the breast cancer patients are summarized in **Supplementary Table S2**. As information on the pathological features of the lymph nodes was unavailable in the SEER database, pN3 patients were excluded from further analysis. As expected, the groups categorized by LNR-APN(−) yielded a significant difference between the OS curves ($p < 0.0001$, **Figure 5A**). Moreover, pN1-LNR2-APN(−) and pN1-LNR3-APN(−) patients had a significantly worse prognosis than pN1-LNR1-APN(−) patients ($p < 0.05$, **Figure 5B**); pN2-LNR1-APN(−) and pN2-LNR2-APN(−) patients had a better prognosis than pN2-LNR3-APN(−) patients ($p < 0.05$, **Figure 5C**). The aforementioned results indicate that LNR-APN(−) could predict the prognosis of patients included in the SEER database.

Neither the Published Cut-Off Values (0.2 and 0.65) nor Our Cut-Off Values (0.15 and 0.34) Could Accurately Predict the Prognosis of APN(+) Patients

We applied both the published cut-off values (0.2 and 0.65) and our cut-off values (0.15 and 0.34) to APN(+) patients, and the results indicated that none of them could accurately predict the

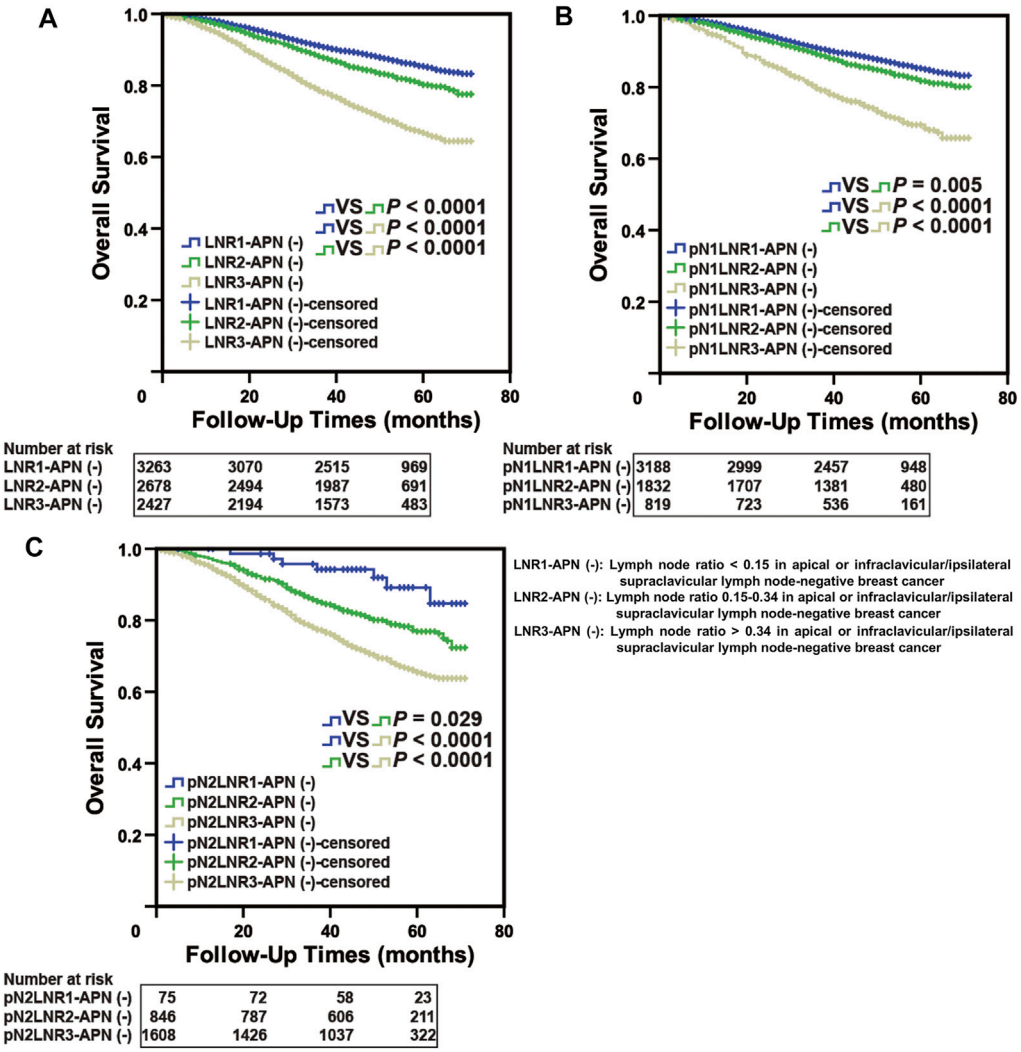

FIGURE 5 | LNR-APN(–) could accurately predict the prognosis of pN1 and pN2 stage breast cancer patients in the SEER database. Kaplan–Meier analysis of **(A)** LNR-APN(–) breast cancer patients (n = 8,380), **(B)** pN1-LNR-APN(–) breast cancer patients (n = 5,846), and **(C)** pN2-LNR-APN(–) breast cancer patients (n = 2,534).

prognosis of APN(+) patients. In the previously published system (0.2 and 0.65), there was no statistical difference in OS or DFS between LNR1-APN(+) and LNR2-APN(−) patients (OS: p = 0.842, DFS: p = 0.921) and between LNR2-APN(+) and LNR3-APN(−) patients (OS: p = 0.085, DFS: p = 0.636) (**Figures 6A,B**). There was also no difference in OS or DFS between LNR1-APN(+) and LNR2-APN(−) (OS: p = 0.402, DFS: p = 0.351) or LNR2-APN(+) patients (OS: p = 0.484, DFS: p = 0.955) in our system (0.15 and 0.34) (**Figures 6C,D**).

DISCUSSION

The current AJCC-TNM staging system classifies the pN stage based on only the number of positive lymph nodes. Over the past decades, increasing studies have suggested that the LNR system could be an accurate prognostic indicator in breast cancer, and LNR could be considered as an alternative to pN staging (Ahn et al., 2011; Ataseven et al., 2015; Chen et al., 2015; Solak et al., 2015; Cho et al., 2018). However, the AJCC-TNM staging system classified APN(+) breast cancer patients with a worse prognosis into the pN3 stage regardless of the lower-level lymph node metastasis state (Greene, 2002; Singletary et al., 2002). This point indicated a possibility that pN3-APN(+) patients with a small number of positive lymph nodes could be misclassified as low LNR stage. Until now, reports on LNR in breast cancer from different research groups have not mentioned this possibility using the LNR system (Dings et al., 2013; Yu et al., 2015; He et al., 2017; Ayşegül and Mehmet, 2020). Our results indicated that APN(+) patients had a significantly worse prognosis than APN(−) patients in the LNR1 (LNR ≤ 0.2) and LNR2 (LNR 0.21–0.65) groups, which strongly suggests that APN(+) patients should be excluded in the LNR system. In our study, we focused on 3,283 APN(−) patients with positive lymph nodes from among

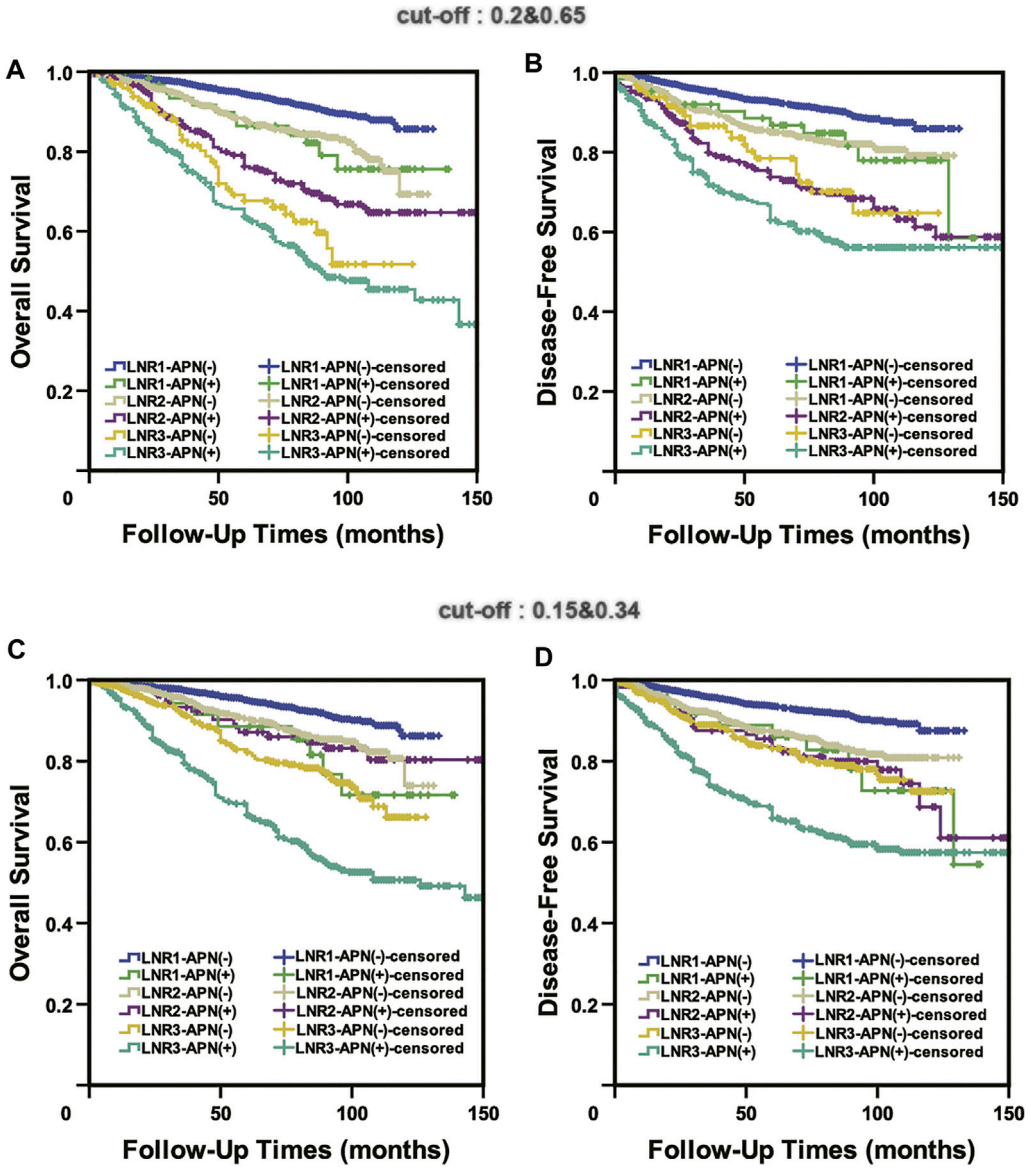

FIGURE 6 | Neither the published cut-off values (0.2 and 0.65) **(A,B)** nor our cut-off values (0.15 and 0.34) **(C,D)** could accurately predict the prognosis of APN(+) patients.

10,120 breast cancer patients and applied X-tile analysis to calculate two cut-off values (0.15 and 0.34) based on the OS of these patients. Using these cut-off values, we classified our patients into LNR1-APN(−) (LNR > 0 and <0.15), LNR2-APN(−) (LNR ≥ 0.15 and ≤0.34), and LNR3-APN(−) (LNR > 0.34). We found that the LNR-APN(−) system could distinguish pN1-LNR2-APN(−) and pN1-LNR3-APN(−) patients with a significantly worse prognosis from pN1-LNR1-APN(−) patients to avoid inadequate treatment and could also distinguish pN2-LNR1-APN(−) patients with a significantly better prognosis from pN2-LNR2-APN(−) patients to avoid overtreatment, but had no role in identifying pN3 and LNR3-APN(−) patients. The study by Yu et al. (2015) suggested that LNR could be a significant prognostic factor in pN3 breast cancer patients. However, the study did not consider pN3 patients with or without APN(+) and did not compare the prognosis of subgroups of pN3 patients categorized by LNR with that of pN1 and pN2 patients. Therefore, the authors could not find the difference in the prognosis of subgroups of pN3 patients distinguished by LNR from that of pN1 and pN2 patients.

In our study, we applied the LNR system to APN(+) patients and compared their prognosis with that of other patients, and the results indicated neither the published cut-off values (0.2 and 0.65) nor our cut-off values (0.15 and 0.34) can accurately predict the prognosis of APN(+) patients. Despite the ethnic heterogeneity, the prognostic effect of LNR-APN (−) was

successfully validated in another independent cohort from the SEER database. Due to the unavailability of data on the pathological features of the lymph nodes in the SEER database, pN3 patients were excluded from further analysis in this study. These results indicated that the LNR-APN(−) system could predict the prognosis of APN(−) patients accurately, and it may be a more comprehensive and valuable supplement to the previously reported LNR (Oven Ustaalioglu et al., 2010; Vinh-Hung et al., 2010; Xiao et al., 2013; Tonellotto et al., 2019). In the future, a comprehensive consideration of LNR and N staging may be a better choice when clinicians evaluate lymph node status in breast cancer patients.

Our cohort size of 10,120 breast cancer patients including 3,936 patients with positive lymph nodes, which comprise 3,283 APN(−) patients, is a large sample size, much larger than the sample size in comparable reports (Vinh-Hung et al., 2009; Danko et al., 2010; Li et al., 2012; Saxena et al., 2012; Liao et al., 2015), which makes our analysis more credible and representative. Moreover, uniform pathologic examination of the lymph node samples by a single institution ensures that similar surgical and pathologic procedures were performed. An additional advantage is a longer follow-up duration with a median of 81 months, which suggests that our data have a greater ability to predict the prognostic value of the variables being studied (Wang et al., 2012; Kim et al., 2016; Tsai et al., 2016; Wang et al., 2017). However, the retrospective nature of this study could have introduced bias in terms of patient and treatment selection. For this retrospective study to be meaningful, the baseline of patients such as sex, age, basic disease, and treatment cannot be considered. Individual differences exist objectively in any research and cannot be overcome one by one. It is an inherent disadvantage faced by any research. The way to minimize errors caused by treatment is to increase the sample size. Our study applied a breast cancer cohort of 10,120, which is a very large cohort size, even reaching the top of the international level. The cohort is large enough to ignore the errors caused by treatment. In addition, statistical analysis based on the Cox proportional hazards model showed that sample size has a significant impact on the results. To solve this problem, we should try more statistical methods or apply our cut-off values to another database for further validation.

CONCLUSION

Our present study revealed that excluding APN(+) patients is the most necessary supplement to LNR and that LNR-APN(−) staging should be a more comprehensive approach in predicting prognosis and guiding clinicians to provide accurate and appropriate treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion 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 Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FG and YM had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: FG and YM. Acquisition, analysis, and interpretation of data: FG, LF, ZW, WC, HZ, XL, YZ, and ZG. Drafting of the manuscript: ZW and WC. Critical revision of the manuscript for important intellectual content: FG and YM. Statistical analysis: FG, ZW, and WC. Administrative, technical, and material support: FG and YM. Study supervision: FG and YM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2022.784920/full#supplementary-material

Supplementary Figure S1 | LNR-APN(–) could predict the prognosis of breast cancer patients more accurately than pN staging. Kaplan–Meier analysis of **(A)** LNR-APN(–) breast cancer patients (n = 3283), **(B)** pN1-LNR-APN(–) breast cancer patients (n = 2213), **(C)** pN2-LNR-APN(–) breast cancer patients (n = 804), and **(D)** pN3-LNR-APN(–) breast cancer patients (n = 266).

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CASE REPORT
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Case Report: Short-Term Response to First-Line Crizotinib Monotherapy in a Metastatic Lung Adenocarcinoma Patient Harboring a Novel *TPR-ROS1* Fusion

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ROS1-rearranged patients account for 1-2% of non-small cell lung cancer (NSCLC) cases. Approximately 10 fusion partners have been discovered, while clinical practice is actively generating knowledge of new ones and their therapeutic responses. Herein, we report a patient with stage IV NSCLC that harbored a novel *TPR-ROS1* fusion, which demonstrated a rapid but short partial response to first line crizotinib and primary resistance to subsequent ceritinib. Computed tomography detected a pulmonary nodule in a 53-year-old woman who presented with persistent cough. Histopathologic and molecular examination of the tissue biopsy indicated a poorly differentiated adenocarcinoma staining negative for PD-L1 but harbored a novel translocated promoter region (*TPR*)-*ROS1* (T4:R35) gene fusion. Frontline crizotinib monotherapy elicited a rapid partial response after 1 month, although the disease progressed another 2 months later. After another 3 months of continued crizotinib treatment, the patient manifested newly emerged and enlarged lung and brain lesions. Genomic profiling still identified *TPR-ROS1* as the only aberration, while a lymph node biopsy indicated PD-L1 immunopositivity. The patient was then treated with ceritinib and progressed within 1 month. She was started on chemotherapy with pemetrexed plus carboplatin and has achieved rapid partial response as of the latest follow-up. In summary, we provided clinical evidence of a novel *TPR-ROS1* fusion and its roles as an oncogenic driver in metastatic NSCLC. To the best of our knowledge, ours is the first case to report this fusion in NSCLC. This case was characterized by a rapid yet short-term response to first line crizotinib and primary resistance to subsequent ceritinib, while no known genetic resistance mechanism was identified and other mechanisms including histologic transformation were unlikely. Future research is needed to unveil the resistance mechanism and formulate effective treatment strategies.

Keywords: non-small cell lung cancer (NSCLC), ROS1, translocated promoter region (TPR), crizotinib, ceritinib

INTRODUCTION

Chromosomal rearrangements leading to fusion genes that encode a chimeric protein with aberrantly elevated ROS1 kinase activity represent an established oncogenic driver in non-small cell lung cancer (NSCLC). *ROS1*-positive patients account for 1-2% of NSCLC cases (1). Multiple fusion partners have been reported for *ROS1* rearrangement, the most common of which being *CD74*, followed by *SDC4*, *EZR*, and *SLC34A2* (2). Due to structural similarity, several tyrosine kinase inhibitors (TKIs) targeting anaplastic lymphoma kinase (ALK) or neurotrophin receptor tyrosine kinase (NTRK), such as crizotinib and entrectinib, have shown remarkable clinical efficacy and are currently recommended as first- or second-line therapy for *ROS1*-positive NSCLC (3). In a phase II clinical trial of 127 East Asian patients treated with crizotinib, median progression-free survival (PFS) was 10.2 and 18.8 months in patients with and without baseline central nervous system (CNS) metastasis, respectively (4). Studies of patients after progression on these TKIs have shed light on a handful of resistance mechanisms. For crizotinib, Gainor et al. found *ROS1* resistance mutations in 53% specimens from 16 patients (5), and McCoach et al. proposed *KIT* and β-catenin mutations and HER2-mediated signaling as off-targeted mechanisms (6). Meanwhile, new fusion partners and therapeutic properties are actively discovered in the clinic, such as a recent report of a *NPM1-ROS1* fusion (7). Herein, we report a patient with stage IV NSCLC that harbored a novel *TPR-ROS1* fusion and achieved rapid but short partial response to first line crizotinib monotherapy.

CASE PRESENTATION

A 53-year-old woman presented with persistent cough in April 2021. Past medical history was not remarkable, although the patient's mother had lung cancer. Chest computed tomography (CT) scans detected a left lower lobe (LLL) mass and enlarged mediastinal and hilar lymph node (LN). Carcinoembryonic antigen (CEA) level was 41.6 ng/ml. A neoplasm in the left lower trachea was found on bronchoscopy, and biopsy of the neoplasm revealed a poorly differentiated adenocarcinoma (**Figure 1A**). On immunohistochemistry, the tumor stained positively for TTF1 (+++), Napsin A (+), CK7 (++), E-cadherin (++), Ki67 (50%), and negatively for P40, CD68, and PD-L1. Cancer cells were also found in biopsies of the right paratracheal, subcarinal, and mediastinal LNs. Additionally, brain magnetic resonance imaging (MRI) and enhanced CT showed a left cerebral frontal lobe mass and lesions in the T5 and L1 vertebrae. The patient was diagnosed with stage IV NSCLC (T2N3M1c). Next-generation sequencing analysis of tumor

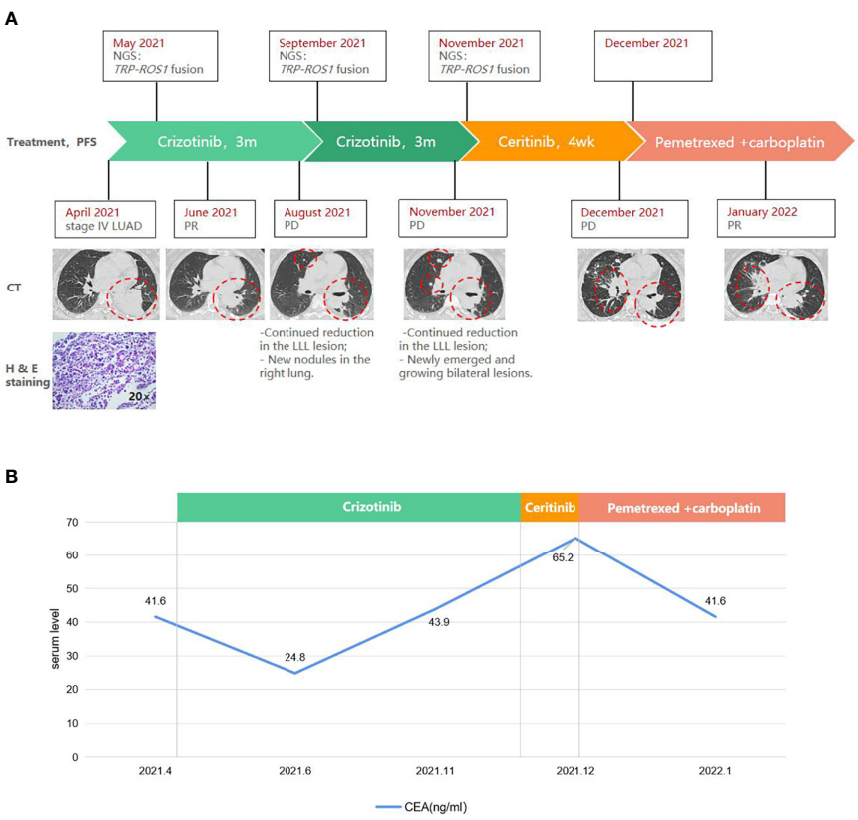

FIGURE 1 | A schematic diagram of the course of management highlighting **(A)** radiographic, histopathologic, and molecular findings, and **(B)** carcinoembryonic antigen (CEA) levels at key time points. Red circles indicate the target lesion. CT, computed tomography. H & E, hematoxylin and eosin. LLL, left upper lobe. LN, lymph node. LUAD, lung adenocarcinoma. Met, metastasis. PD, progressive disease. PR, partial response. *TPR*, translocated promoter region.

tissue and blood samples with a 168-gene panel (Burning Rock, Guangzhou, China) are as previously described (8, 9). A novel translocated promoter region (TPR)-ROS1 (T4:R35) gene fusion was detected from both samples (**Figure 2**).

Frontline treatment with crizotinib (250 mg bid) started in May and after 1 month elicited a rapid response consistent with partial response (PR) per RECIST v1.1 guidelines, manifested as a 46% reduction (59×53 mm to 31×20 mm) of the LLL mass (**Figure 1A**). CEA level also lowered to 24.8 ng/ml (**Figure 1B**). Follow-up CT in August found newly emerged right lung nodules despite continued reduction of the target lesion (25×14 mm; **Figure 1A**). Molecular testing with blood revealed similar results as baseline, with TPR-ROS1 fusion as the only alteration. As the patient was asymptomatic, crizotinib was continued. Follow-up in November showed continued reduction of the original LLL lesion (27×13 mm) but enlargement of other bilateral lung lesions and the right supraclavicular LN on CT and enlarged brain lesions on MRI, which were consistent with progressive disease (**Figure 1A**). CEA level also rose to 43.9 ng/ml. A biopsy of the right supraclavicular LN revealed poorly differentiated adenocarcinoma with immunoreactivity to PD-L1 (combined positive score 60%+). Genomic profiling of this biopsy again identified *TPR-ROS1* as the only aberration. The patient was subsequently started on ceritinib (450 mg qd) but did not appear to respond, as follow-up CT one month later indicated growing and new bilateral lung lesions and enlarged supraclavicular LN and brain metastasis, accompanied by continued rise in CEA level (65.2 ng/ml; **Figure 1**). She is now receiving a combination of pemetrexed and carboplatin and has achieved PR (sum of target lesions 46.5 mm to 31.0 mm). There was also a minor drop in CEA level (**Figure 1B**).

DISCUSSION

Approximately 10 genes have been reported as upstream fusion partners with *ROS1* in NSCLC (2, 7). In this case report, we provided clinical evidence of a new one. Moreover, evidence supported this novel *TPR-ROS1* (T4:R35) fusion as an oncogenic driver. The putative gene product retained the intact ROS1 kinase domain (**Figure 2**). Also, this rearrangement was identified with targeted sequencing using a moderately sizable panel (**Supplementary Table S1**) as the sole genomic abnormality prior to any treatment and after progression on crizotinib and on ceritinib. *TPR-ROS1* fusion was recently identified in a patient with lipofibromatosis, a rare pediatric soft tissue tumor (10). More interestingly, *TPR* is also known to partner with other

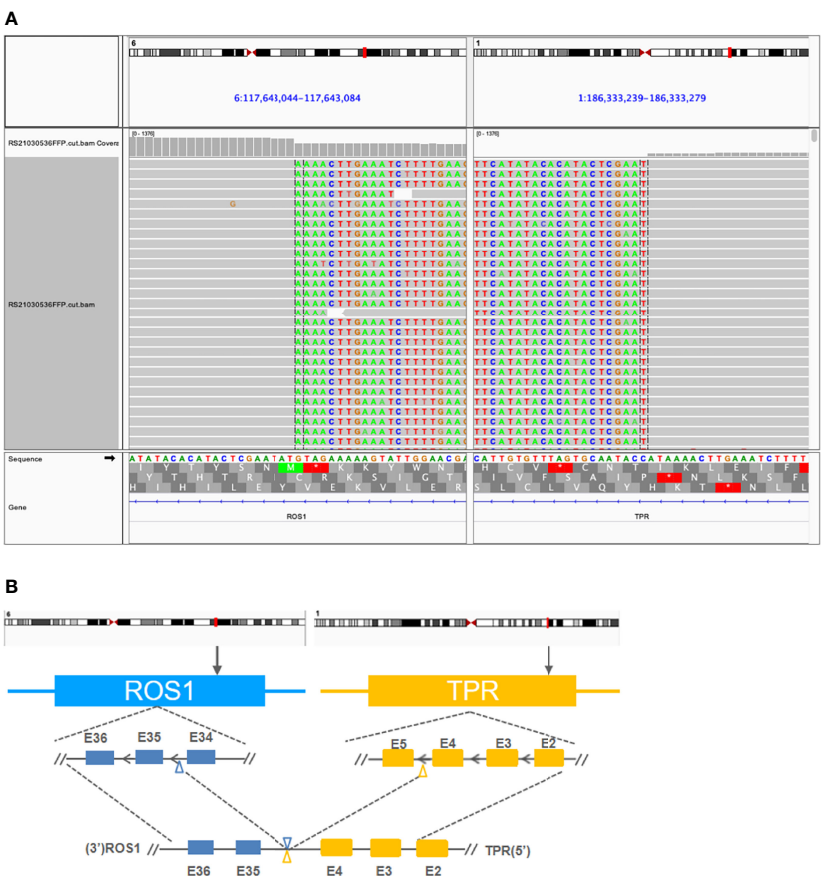

FIGURE 2 | Detection of a novel *TPR-ROS1* (T4:R35) gene rearrangement using next-generation sequencing. **(A)** Identification of a *TPR-ROS1* gene fusion. **(B)** Structural illustration of the resultant putative chimeric protein. *TPR*, translocated promoter region.

driver genes in NSCLC. *MET* was originally identified as a proto-oncogene after molecular cloning of *TPR-MET* from chemically transformed osteosarcoma cell lines (11). Choi et al. identified *TPR-ALK* in a 60-year-old male Korean smoker who underwent lobectomy. He then received adjuvant chemotherapy with vinorelbine and cisplatin and displayed no evidence of disease as of an 18-month follow-up (12). *TPR-NTRK1* fusions have also been reported in thyroid carcinoma (13), pancreatic cancer (14), and spindle cell neoplasm, a mesenchymal tumor (15). Along with reports of *TPR-RAF* and *TPR-FGFR1*, these findings highlight *TPR* as a promiscuous fusion partner with pivotal kinases in cancer biology, although there is a dearth of knowledge regarding how patients carrying these rearrangements responded to TKI treatment.

Another noteworthy aspect of our case is the rapid progressions on crizotinib and on ceritinib. After initial PR at one month since treatment initiation, the disease progressed another two months later, leading to a PFS of 3 months. In addition to reduced inhibitory potency compared with next-generation ROS1 inhibitors, progression on crizotinib results from acquisition of resistance mechanism and/or development of CNS disease (1), which are not uncommon in *ROS1*-positive NSCLC. Patil et al. reported that CNS was the first and sole site of progression in 47% (9/19) of *ROS1*-rearranged stage IV patients (16). On the other hand, the disease did not respond to ceritinib, which unlike crizotinib, demonstrates remarkable CNS penetration. While it was possible that the patient experienced progression on first line crizotinib because of limited intracranial activity, our findings suggested existence of unidentified mechanisms driving resistance to ceritinib. Liu et al. recently reported upregulation of PD-L1 in bronchial epithelial cells after expression of *ROS1* fusion protein, which was also modulated by MEK-ERK signaling in crizotinib-resistant *ROS1*-rearragned NSCLC cells (17). It is therefore interesting to study the role of MEK-ERK signaling in mediating therapeutic resistance in our case and the efficacy of MEK inhibitors, which is the goal of our ongoing cell model experiments.

In summary, we provided clinical evidence of a novel *TPR-ROS1* fusion and its role as an oncogenic driver in metastatic NSCLC. This case was characterized by a rapid yet short-term response to first line crizotinib and primary resistance to subsequent ceritinib, while no known genetic resistance mechanism was identified and histologic transformation was unlikely. We found upregulated PD-L1 in a metastatic lesion compared with the primary after progression on crizotinib, suggesting PD-L1 increases as a potential resistance mechanism, although the possibility of inter-tumoral heterogeneity in PD-L1 expression is there. Possible mechanisms include MEK-ERK signaling, which has been reported *in vitro*, and warrant further mechanistic and clinical investigations.

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 Ethics Committee for Human Research of The Second Affiliated Hospital of Zhejiang University School of Medicine. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the patient for publication of this case report and any accompanying images in an anonymized manner.

AUTHOR CONTRIBUTIONS

PW conceived of and designed the study. SW, MH, YY, and XH collected and analyzed the data. SW and PW wrote the manuscript. BL provided pathological analysis. LD provided valuable intellectual input to the manuscript and provided administrative supervision. All authors approved the final version of the manuscript and are accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.862008/full#supplementary-material

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Cholesterol and Its Derivatives: Multifaceted Players in Breast Cancer Progression

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Cholesterol is an essential lipid primarily synthesized in the liver through the mevalonate pathway. Besides being a precursor of steroid hormones, bile acid, and vitamin D, it is an essential structural component of cell membranes, is enriched in membrane lipid rafts, and plays a key role in intracellular signal transduction. The lipid homeostasis is finely regulated end appears to be impaired in several types of tumors, including breast cancer. In this review, we will analyse the multifaceted roles of cholesterol and its derivatives in breast cancer progression. As an example of the bivalent role of cholesterol in the cell membrane of cancer cells, on the one hand, it reduces membrane fluidity, which has been associated with a more aggressive tumor phenotype in terms of cell motility and migration, leading to metastasis formation. On the other hand, it makes the membrane less permeable to small water-soluble molecules that would otherwise freely cross, resulting in a loss of chemotherapeutics permeability. Regarding cholesterol derivatives, a lower vitamin D is associated with an increased risk of breast cancer, while steroid hormones, coupled with the overexpression of their receptors, play a crucial role in breast cancer progression. Despite the role of cholesterol and derivatives molecules in breast cancer development is still controversial, the use of cholesterol targeting drugs like statins and zoledronic acid appears as a challenging promising tool for breast cancer treatment.

Keywords: breast cancer, cancer metabolism, cholesterol, mevalonate (MVA) pathway, cholesterol metabolism, statins, breast cancer therapy

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INTRODUCTION

Breast cancer (BC) is estimated to account for one-third of all new cancer diagnoses in American females in 2022. Despite a 1% decrease annually in mortality during the 2013-2019 timeframe, the estimated death for BC in females is 15% among all types of cancer, thus representing the second leading cause of cancer death among women (1). Molecularly, it is possible to subdivide BC into four main subtypes: Luminal BC are positive for the expression of steroid hormone receptors, the estrogen receptor (EsR) and progesterone receptor (PR), and they can be further characterized in Luminal A (EsR+, PR+, HER2-) and Luminal B (EsR+, PR+, HER2+). HER2+ BCs overexpress the

HER2/ERBB2 oncogene and include both the Luminal B and the HER2+, EsR-, PR- patients. In contrast, Basal-like or Triple-Negative BC (TNBC) lacks both the hormonal receptors and the HER2 receptor (2), which represents a major obstacle for therapeutic intervention in this aggressive BC subtype.

Several epidemiological and genetic studies have tried to determine whether levels of circulating lipids are associated with risks of various cancers, including BC. Dietary cholesterol represents a significant risk factor for BC, as suggested by a comprehensive meta-analysis study (3) and genetically elevated plasma high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels appear to be associated with increased BC risk (4). However, additional studies are required to address the putative causal relationship between BC and cholesterol, with the goal to develop potential therapeutic strategies aimed at altering the cholesterol-mediated effect on BC risk.

Metabolic reprogramming has been extensively proved to be a key cancer hallmark (5); indeed, tumor cells exhibit metabolic abnormalities required to satisfy their growth and survival needs (6). Compared to more investigated metabolic phenotypes and metabolites such as glucose in the Warburg effect (7, 8), the contribution of cholesterol in cancer is still controversial (9, 10). To date, it is well known that frequently altered oncogenes and tumor suppressors in BC, like the PI3K and p53, affect cholesterol homeostasis in a variety of tumors (11–13). Interestingly, several BC samples showed increased expression of proteins involved in endogenous cholesterol synthesis, which occurs through the mevalonate (MVA) pathway (14). Moreover, BC cells display aberrant cholesterol uptake at mitochondrial levels *via* increased expression of STAR and STARD3 proteins, essential for regulating cholesterol import to the mitochondria, that, in turn, impinge on proliferation, metastasis, and survival (9, 15). Indeed, STARD3 is overexpressed in BC patients, where it is frequently co-amplified with HER2; high STARD3 levels correlate with a poor prognosis and lower response to Trastuzumab (16), a monoclonal antibody that targets the HER2 receptor (17). These data suggest a central role of mitochondria in such metabolic reprogramming.

The up regulation of cholesterol metabolism in BC cells depicts a scenario in which cholesterol and its derivatives may play a crucial role in sustaining tumor growth, hence numerous clinical trials have tried to investigate the effect of drugs able to reduce circulating cholesterol, like statins, in several cancer types. Notably, the use of cholesterol-lowering drugs in preventing or curbing BC progression has revealed controversial results (11, 18) and the ongoing clinical trials will provide a clearer view on their beneficial role. By using robust and routinely available techniques both the luminal and basal breast cancer phenotypes have shown to contain distinct subgroups and therefore to be heterogeneous (19). The single cell-based approaches to depict the BC intratumor heterogeneity, will also help in defining the co-existence of different clones in a given tumor, may help characterize distinct metabolic phenotypes and drug responses (20, 21). Nevertheless, statins treatment is a safe approach in lowering cholesterol levels (22) and the hormone dependency of BC appears to be the most promising predictive marker of response to statin treatments, probably due to the precursor role of cholesterol in steroid hormones production (11). This review will focus on the cholesterol homeostasis aberrations in BC and the relevance of MVA pathway inhibitors in BC therapy.

Cholesterol Homeostasis

Cholesterol is a lipid molecule crucial for the viability of mammalian cells. It is involved in the synthesis of steroid hormones (23), bile acids (24) and oxysterols (25), and its localization in cell membranes is critical in dictating membrane integrity and fluidity (26). Cellular cholesterol results from *de novo* cholesterol synthesis and dietary intake with an estimated ratio of 70:30 (27) (**Figure 1**). The synthesis, uptake, efflux, and cholesterol conversion is tightly regulated intracellularly (28). Cholesterol is primarily synthesized in the liver and transported to other tissues through the bloodstream as an LDL-bound form. Exogenous cholesterol is mainly derived from LDL, and thanks to the LDL receptor (LDLR)-mediated endocytosis, LDLs are up-taken and stored in the early endosome (28). In the late endosome, thanks to the lipase activity, LDL undergo hydrolysis, and the derived cholesterol arrives either directly to the plasma membrane (PM) or to the endoplasmic reticulum (ER) (29), where it becomes available for esterification (30). The exit of cholesterol from late endosomes critically depends on the two cholesterol-binding proteins, NPC1 and NPC2 (29, 31, 32).

In addition to dietary intake, in nucleated cells, nearly 30 enzymatic reactions led to the polymerization of acetyl-CoA into cholesterol through the MVA pathway (18, 27). The intracellular cholesterol pool generated by the MVA pathway is controlled by two rate-limiting enzymes: 3-hydroxy-3methylglutaryl-CoA (HMG-CoA) reductase (HMGCR) and squalene epoxidase (SQLE) (27). Indeed, the homeostasis of intracellular cholesterol metabolism is mainly controlled through the transcriptional regulation of the HMGCR coding gene by the Sterol Regulatory Element-Binding Proteins (SREBPs) transcription factors, mainly by the SREBP-2 isoform in the liver. Whenever cholesterol levels at the ER membrane are high, cholesterol itself can bind the sterol sensing domain of the SCAP chaperones, while oxysterols such as 25-hydroxycholesterol can bind the INSIG chaperones at the ER membrane. INSIG and SCAP bind each other and retain SREBPs at the ER membrane (33–35). In case of low cholesterol level, INSIG is degraded, and the SCAP/SREBP2 complex can be packed into COPII-coated vesicles and targeted to the Golgi where SREBP can be proteolytically cleaved by site-1 protease and site-2 protease (S1P and S2P) (35, 36). The N-terminal domain of SREBP resulting from cleavage can enter the nucleus, bind to sterol responsive elements (SREs) and act as transcription factors, increasing the expression of LDLR, HMGCR, and SQLE, thus enhancing cholesterol synthesis and uptake (23, 36).

Cholesterol homeostasis does not rely only on its endogenous synthesis or uptake from the diet; indeed, cholesterol is heavily transported between subcellular membranes, and such trafficking may be the result of vesicular transport, membrane contact sites, or sterol transfer proteins (27). Additionally, cholesterol

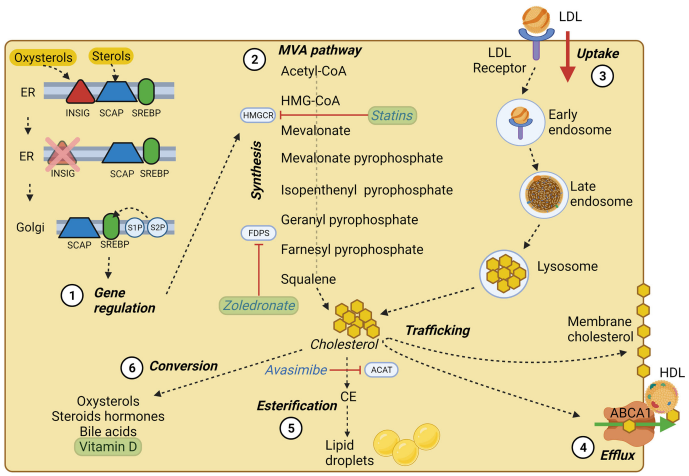

FIGURE 1 | Cholesterol homeostasis main processes. (1) SREBP processing at the ER membrane and Golgi apparatus; in high cholesterol condition, SREBP is retained at the ER membrane by INSIG and SCAP, which sense oxysterols and sterols, respectively. At low cholesterol condition, SREBP can be transported to the Golgi apparatus and cleaved by S1P and S2P proteases. Cleaved SREBP can enter the nucleus and trigger the transcription of crucial MVA pathway genes. (2) Main steps of cholesterol synthesis through the MVA pathway, of which HMGCR represents the rate-limiting enzyme. (3) LDL-cholesterol intake *via* LDL-receptor mediated endocytosis. (4) Cholesterol efflux by ABCA1 transporter, which employs ATP molecules to deliver cholesterol and lipids on apoA-I, triggering the assembly of nascent HDL. (5) ACAT enzyme mediated cholesterol esterification to fatty acids tightly packaged and stored in the core of intracellular lipid droplets, which represent a ready storage of lipids that can be used without investing energy in biosynthesis. (6) Cholesterol conversion in its main derivatives, some of which may play a role in BC pathology and progression. The main cholesterol homeostasis inhibitors and their targets are underlined in blue, while drugs and substances used in clinical trials (see **Table 1**) are shown in green. Created with BioRender.com.

molecules can be esterified to fatty acid chains within the ER by the acyl-CoA cholesterol acyltransferase (ACAT) and stored into lipid droplets (37) (**Figure 1**).

The Impact of Circulating Cholesterol in BC

The scientific community has for a long time attempted to elucidate the relationship between BC development and serum cholesterol in terms of association and causality. A plethora of investigations conducted in humans has interrogated the link between BC, LDL and HDL. Some authors have reported that high LDL levels are associated with increased BC risk (38) and are predictive of poor prognosis (39). Nevertheless, additional studies showed no association between LDL and BC risk (40–42). Concerning the prognostic value of HDL, some evidence suggests an association between low HDL and BC risk (43), especially in premenopausal women (41, 44, 45). Moreover, a retrospective study found that decreased HDL levels in pre-operative patients had a significant association with worse overall survival (46). However, others suggest that low HDL is associated with an increased risk of postmenopausal breast carcinogenesis (47).

Overall, different studies have generated contrasting results, possibly due to the multifactorial etiology of BC, its heterogeneity, and the differences in the design of the studies (48, 49). Because of the discrepancies that have emerged from clinical investigations, it is crucial to understand the potential mechanisms underlying the role of lipoproteins in BC leveraging on both animal and *in vitro* studies.

The MVA Pathway Aberrations in BC

The MVA pathway is crucial in cell viability, not only due to cholesterol synthesis, but also because the metabolites generated through such anabolic pathway represent potential building blocks to meet the high proliferative requirements of cancer cells (11). The intracellular levels of MVA metabolites, as previously cited, are tightly controlled mainly by SREBP proteins and corresponding sterol regulatory elements (SREs). SREBPs activities can get integrated into cellular signaling pathways from growth factors and some of them are known to play a major and driver role in tumorigenesis. Among them, the PI3K-AKT signaling pathway triggered by the epidermal growth factor receptor (EGFR), is the most altered one in cancer (50). PI3K phosphorylates AKT which in turn can induce the activation of the mechanistic target of rapamycin complex 1 (mTORC1) *via* inhibition of TSC1-2 (51). Upregulation of SREBPs, caused by the PI3K/Akt signaling and mTORC1, have been associated to cancer (52) and several inhibitors of SREBPs, that are under clinical studies, proved to reduce the tumour growth in various tumor types, including BC (53).

Mutations to the catalytic α subunit of PI3K (PIK3CA) are found in 40% of Luminal A breast tumors (54). In breast epithelial cells, expression of oncogenic PI3K correlates with induced *de novo* lipogenesis *via* AKT and mTORC1 (55). Moreover, mTORC1 signaling was shown to increase RNA and protein levels of SREBP targets in primary human breast cancer samples (55). Activated AKT promotes SREBPs released from the ER by decreasing the sterol binding ability of INSIG

chaperones at the ER membrane in human hepatocellular carcinoma (HCC) (56) in this model, the downstream effector of AKT, the phosphoenolpyruvate carboxykinase 1 (PCK1), once activated, can phosphorylate and promote the proteasomal degradation of INSIG, thus leading to increased SREBP maturation (56). Interestingly, PCK1 was shown to be upregulated in BC samples and to play a key role in tumor metastasis (57). Regarding mTORC1, it has been shown that its signaling may enhance SREBP maturation through the phosphorylation and activation of the downstream effector ribosomal S6 kinase 1, *via* an unknown mechanism (58). Highlighting the relevance of the mTORC1 signaling in MVA pathway aberrations and in BC, one of the downstream effectors of S6K, the ribosomal S6 is, indeed, highly phosphorylated in BC samples (55).

Interestingly, mTORC1 signaling protects BC cells from ferroptosis a cell death caused by the iron-dependent accumulation of lipid reactive oxygen species (59), by increasing SREBP1. In HER2+ cell lines bearing constant activation of PI3K-AKT-mTORC1 axis, the genetic ablation of a SREBP1 gene (SREBF1) decreased primarily the lipid synthesis-related gene SCD1, while pharmacological inhibition of SCD1, sensitized BC cells to ferroptosis (60). The antioxidant role of SCD1 is not new (61) and the mechanistic explanation may come from the role of SCD1 in producing monounsaturated fatty acid (MUFAs) (62). MUFAs can decrease lipid peroxidation sensitivity and, therefore ferroptosis, by displacing the more easily oxidized polyunsaturated fatty acids (PUFAs) from the cell membrane (63). Interestingly, SCD1 is enriched in almost all tumor tissues with a greater enrichment of SCD1 in BC compared to other tumours and to their non-neoplastic counterparts (64).

Additionally, mTORC1 may promote the chromatin accessibility of SREBPs by inhibiting Lipin 1, a phosphatidic acid phosphatase (65). Taken together, the constant activation of the PI3K-AKT-mTORC1 axis increases SREBPs translocation in the nucleus and its stabilization onto chromatin to boost the MVA pathway and increase apoptosis resistance. MYC is another well-known oncogene that is highly mutated in BC (66). MYC can interact with SREBPs and promote cell dedifferentiation (67). Notably, the SREBP2-dependent increase in cholesterol synthesis is associated with stemness maintenance and proliferation in intestinal stem cells (ISC); indeed, despite the mechanism has not been elucidated, abnormalities in phospholipid bilayer caused by the absence or inhibition of the phospholipid-remodeling enzyme LPCAT3, increases SREBP-2 nuclear activation and intestinal stem cell growth (68). Such results highlight a putative link between phospholipid content, cholesterol synthesis and stemness. As a matter of fact, stemness is a cell state that appears to be widely spread in TNBCs (69).

In 80% of TNBC cases, the tumor suppressor p53 is mutated (70). p53 null cells and mice were found capable of increasing the MVA pathway *via* inhibition of the retrograde transport of cholesterol from the PM to the endoplasmic reticulum controlled by the cholesterol transporter ABCA1 (71, 72). Mechanistically, decreased cholesterol transport from the PM to the ER results in increased maturation of SREBPs.

In BC, evidence of molecular mechanisms responsible for increased cholesterol biosynthesis is fewer than in other tumor types. The oncogenic players known to boost the MVA pathway in these tumors are also crucial in BC, where they may play similar roles. Indeed PI3K, p53 and MYC are known to modulate the MVA pathway in different tumor models and belong to the ten most frequently mutated genes in BC (73), strongly suggesting that BC cells may exploit them to upregulate cholesterol synthesis and fulfil their proliferative requirements. Interestingly, many studies identified HMGCR and SREBPs as prognostic markers in BC; in a cohort of 82 BC patients, high levels of SREBP-1 are associated with metastatic features and poor survival (74). Also, SREBP-1 knockdown negatively influences the migration and invasion of BC cells (74).

On the other hand, clinical data regarding the predictive value of HMGCR are much more controversial. Since the fact that high HMGCR expression is associated with better clinical outcomes (75, 76), is still debatable (77), further studies on larger cohorts may define a clearer scenario on the prognostic value of HMGCR.

THE ROLES OF MEMBRANE CHOLESTEROL

Cholesterol is an essential constituent of membranes, where it accounts for about 25% of total lipids (78, 79). Cholesterol plays a pivotal role in modulating PM integrity and intracellular signal transduction by interacting with specific proteins and several phospholipids and sphingolipids (80, 81). The cholesterol molecule contains a small polar hydroxyl group, a rigid steroid ring, and a flexible hydrocarbon tail. Due to its unique structure and biophysical properties, cholesterol is well-suited to pack its bulky sterol ring against the fatty acyl chains of phospholipids, leading to increased packing density and cohesion of adjacent lipids, therefore shifting from the lipid membrane liquid-crystalline state to a more ordered state (82). Alteration in the motional freedom of lipids and proteins in the PM is a major trait of cancer cells that may affect various biological processes such as the response to chemotherapeutic drugs (83–85) the activity of membrane receptors (86–88), cell motility and metastasis (89–92).

In addition to providing integrity of cell membranes, cholesterol is the major lipid component of specific membrane microdomains, named lipid rafts that range between 10 to 200 nm in size and are known to compartmentalize various cellular processes. The lipid raft concept was proposed in 1997 by Simons and Ikonen (93). It defines the lipid rafts as a dynamic clustering of sphingolipid and cholesterol within the PM that can selectively recruit and concentrate proteins while excluding others, creating a specialized membrane environment that functions as a platform for receptor trafficking and signal transduction (94). The consensus within the context of cancer cells is that lipid rafts contribute to the positive modulation of signal transmission implicated in diverse cancer cell processes, such as cell

adhesion, migration, invasion, metastasis, and angiogenesis (95–97).

Increasing cholesterol levels in the PM may affect the permeability of certain metabolites and drugs, including anticancer agents (98, 99). Recently, Rivel and coworkers studied the permeation of the chemotherapeutic drug cisplatin through PM models. In this context, the increase in relative cholesterol concentration in the range of 0% to 33% induced the stiffening of lipid tails, leading to decreased drug permeability by one order of magnitude (98). Importantly, BC cells that are resistant to doxorubicin exhibit higher levels of sphingomyelin and cholesterol in the cell membrane and an increased lipid packing density than the corresponding doxorubicin-sensitive cells (100). Another study demonstrated how reducing membrane cholesterol content in BC cells could increase the efficacy of tamoxifen treatment by improving its membrane permeability (101). Therefore, the reduced drug permeability driven by increased membrane cholesterol levels may represent a strategy for cancer cells to induce drug resistance. Moreover, it is worthy of note that PM cholesterol might provoke specific conformational changes in ATP-binding cassette (ABC) transporters that are involved in multidrug efflux, potentially modulating their activity, as discussed below (83).

Researchers have paid interest in the role of cholesterol in the modulation of cancer cell migration. Overall, the general idea is that altering cholesterol abundance in cancer cells would likely affect cellular architecture and signal transduction, thus, interfering with the migratory ability of cells. It is widely recognized that lower levels of cholesterol in the plasma membrane enhance membrane fluidity and therefore favor cancer cell migration, which might eventually promote dissemination (91, 102, 103). In support of this idea, a research work from Zhao and colleagues highlighted how membrane fluidity is causally correlated with metastatic capacity *in vivo* and that many antimetastatic drugs function by inhibiting fluidity of cancer cells (103). Besides inducing membrane rigidity, cholesterol has been indirectly implicated in cell migration by affecting the stability and localization of specific proteins into lipid rafts (102, 104, 105). For instance, the presence of the transmembrane glycoprotein cluster of differentiation 44 (CD44) to lipid rafts impairs the interaction of CD44 with its migratory binding partner ezrin, leading to inhibition of BC cell migration (104, 105). In line with the anti-migration role of membrane cholesterol, another study reported that repressing cholesterol abundance in the cell membrane activates TGF-β receptor signaling, promoting metastasis of BC (102). In this work, the authors showed that mild depletion of membrane cholesterol by using low dosages (0.3 mM in MDA-MB-231 cells) of the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD) led to increased cell migration and hypothesized that further cholesterol reduction might negatively influence cell survival pathways rather than promoting migratory ability of cancer cells. However, a recent study highlighted that disrupting lipid rafts in TNBC cells by using MβCD at a concentration of 0.1 mM for 48 hours is sufficient to determine up to 20% of cytotoxicity (106, 107).

On the other hand, many studies support the positive role of cholesterol-rich lipid rafts in cancer progression, since disrupting lipid rafts by using MβCD can effectively promote cancer cell death in several types of cancer cells, including BC (97, 101, 108, 109). Among the lipid-raft associated proteins whose signaling pathways contribute to more aggressive and invasive behavior of BC cells are the ion channels SK3 and Orai1 (110), the GPI-anchored cell membrane receptor uPAR, the matrix metallopeptidase protein MMP-9 (111), and the glycoprotein Muc-1 (112). Remarkably, disruption of lipid rafts by treating cells with MβCD inhibits the formation of Caveolin-1-dependent invadopodia during BC cell invasion (113, 114). In a recent study, cholesterol was found to promote the maintenance of surface levels of HER2. In this context, reducing cholesterol levels in the PM leads to the endocytic degradation of HER2, synergizing with the tyrosine kinase inhibitors to curb HER2-positive BC growth (86).

Plasma-Membrane Cholesterol, Cholesterol Efflux and ABC Transporters

The increased amount of cholesterol incorporated in plasma-membrane also determines an increased rigidity of the membrane detergent-resistant membrane (DRM) domains and lipid rafts, which are rich in the ABC transporters -as ABCB1 (also known as P-glycoprotein, Pgp), ABCC1 (multidrug resistance-related protein 1, MRP1) and ABCG2 (BC resistance protein, BCRP), involved in the efflux of multiple chemotherapeutic drugs (115) in different tumors, including BC (116). A rigid membrane forces the transporters to assume a conformation that grants the highest catalytic capacity (83). Not only the increased endogenous synthesis (116), but also the increased uptake of LDL (8) is a typical feature of chemoresistant cells. This feature has been exploited to find an Achille's heel to overcome drug resistance, by producing LDL-masked doxorubicin that acts as a Trojan horse to deliver the drugs within the cells (117).

The increased rigidity is not the only mechanism by which cholesterol causes chemoresistance. Indeed, oxisterols activate the transcription factors SREBP1 which cooperates with HIF-1α in up-regulating ABCB1 (118), and Liver X receptorβ (LXRβ), that increases both ABCB1 and ABCG2 at transcriptional level in ovarian cancer cells (83). Although part of conserved mechanisms, the activation of specific transcription factors is tumor specific: indeed, in TNBC and EsR-negative BC patients, LXRα, a second isoform that can be activated by oxysterols, is associated with the high expression of ABCB1 (119). Moreover, the upstream metabolites farnesyl pyrophosphate (FFP) and geranylgeranyl pyrophosphate (GGPP), synthesized in the MVA pathways, activate the signalling pathways Ras/ERK1/2/HIF-1α and RhoA/ROCK/HIF-1α, up-regulating ABCB1 and determining resistance to doxorubicin in BC (116).

Collectively, these observations sustain the direct correlation between chemoresistance and high endogenous synthesis of cholesterol, supported by the review of Tissue Cancer Genome Atlas (TCGA) (9) and on Gene Ontology (120) and Ingenuity Pathway (121)-based analysis.

Cholesterol is mainly effluxed by another ABC transporter, ABCA1, which delivers cholesterol and lipids on apoA-I and triggers the assembly of nascent HDL (**Figure 1**), followed by the efflux of cholesterol by ABCG1 and its delivery on apoE (122). Sporadic observations correlated ABCA1 to pro-tumor (103) or tumor suppressive (123) functions in cancer, and the pathways involved in ABCA1 regulation in cancer cells are still poorly explored. In dendritic cells, the high cholesterol synthesis, associated with an increased ER stress induced by cholesterol accumulation, and the inhibition of PI3K/Akt/mTOR axis, which constitutively blocks LXRα, regulate the expression of ABCA1 (124). Given the presence of aberrant activation of PI3K and Akt, caused by oncogenic mutations, we cannot exclude that this mechanism is also important in regulating ABCA1 expression and cholesterol efflux from BC cells. Together with cholesterol, ABCA1 also effluxes another isoprenoid metabolite of the MVA pathway, the isopentenyl pyrophosphate (IPP). IPP is a strong endogenous activator of Vγ9Vδ2 T-cells (125), a T-cell subset that plays a key role in anti-tumor immunity and is considered a good prognostic factor when present in the bulk of solid tumors (126). The ABCA1/apoAI system is now regarded as a useful tool to increase the activation of the host immune system Vγ9Vδ2 T-cells (127–129). Upregulating this system, by oxysterols activating LXRα, could represent a safe and effective way to boost the anti-tumor immune-response against BC tumors, where the presence of a cytotoxic T-cells infiltrate is usually associated with better prognosis and better response to the immunogenic cell death elicited by neoadjuvant or adjuvant chemotherapy (130–132).

CHOLESTEROL ESTERIFICATION AND FATTY ACIDS STORAGE IN LIPID DROPLETS

Cholesterol esterification to fatty acids tightly packaged in the core of intracellular lipid droplets or circulating lipoproteins is a well-assessed mechanism for storage and transport of cholesterol molecules, also used to prevent cellular toxicity caused by the excess of free cholesterol (133).

Lecithin-cholesterol acyltransferase (LCAT) is a glycoprotein synthesized by the liver and secreted in the plasma. The LCAT enzyme is responsible for the synthesis of cholesterol esters in plasma and, together with cholesteryl ester transfer protein (CETP), plays a critical role in the maturation of high-density lipoproteins (HDL), helping to determine their composition, structure, metabolism and plasma concentration (134).

At an intracellular level, cholesterol esterification is accomplished by two sterol O-acyltransferase enzymes: Acetyl-CoA Acetyltransferase 1 (ACAT1), which is widely distributed in all tissues, and ACAT2, which is preferentially expressed in the liver and the intestine. Both enzymes play a key role in cellular cholesterol homeostasis, using long-chain fatty acyl-coenzyme A as the fatty acyl donor to convert cholesterol to cholesteryl esters (CE) in the cytoplasm, leading to lipid droplets formation. Their main function is to avoid cell toxicity due to an excessive accumulation of free cholesterol in cell membranes (135). However, ACAT is highly expressed in some tumors, and its expression is reported to be activated by several factors such as IFN-γ, TNF, and insulin, but not by cholesterol and fatty acids, which are indeed able to mediate ACAT2 proteasomal degradation through reactive oxygen species (ROS) induction (18).

Lipid metabolism gene expression resulted to be also impaired in BC cells in comparison to the regular surrounding tissues (136, 137). In fact, a high ACAT expression leads to a faster recovery of BC cells proliferation upon nutrients deprivation. TNBC cells have been observed to have an enhanced CE synthesis and storage. The inhibition of ACAT1 reduces LDL-induced both proliferation and migration in these cells (138, 139).

Proliferating BC cells, in fact, need a constant lipid supply, which can derive both from a *de novo* synthesis or exogenous cholesterol and fatty acid uptake from plasmatic LDL, leading to increased storage in cytoplasmic lipid droplets (139). The lipid-accumulation represents a lower energy-consuming strategy, as lipid droplets represent a ready storage of lipids which can be used without investing energy in biosynthesis.

Some *in vivo* studies showed a correlation between intratumor CE accumulation and Ki-67, a well-known marker of tumor cell proliferation, poor patient survival, and higher risk of relapse. Furthermore, there is some evidence of a causal relationship between CE and BC. Exogenous and endogenous CE can increase mammary tumor growth and ACAT1 may be a potential target for the treatment of BC (140).

CE accumulated in lipid droplets have been correlated also with resistance to chemotherapy. After an acute exposure to doxorubicin, chemoresistant clones of TNBC increased both mitochondria, induced by the peroxisome proliferator activated receptor α (PPARA) and γ (PPARG) proteins, and lipid droplets. Overall, these changes shift the metabolism toward oxidative phosphorylation (OXPHOS), supported by the accumulation of fatty acids in the CE of LD (141) and antagonized by perilipin 4 (PLIN4). Interestingly, high intratumor levels of PPARA, PPARG and PLIN4, and consequently of CE and lipid droplets, are new biomarkers predicting resistance to neoadjuvant chemotherapy in TNBC (141).

CHOLESTEROL DERIVATIVES AND THEIR ROLES IN BC: (HYDR)OXY STEROLS, STEROID HORMONES AND VITAMIN D

Not only cholesterol, but also its derivatives, may play a role in BC pathology and progression. Here, among all, we report the following:

(Hydr)oxy Sterols

Oxysterols are cholesterol metabolites that can be synthesized through oxidation by both enzymatic reactions and radical processes. They are involved in several cellular functions and physiological processes, such as the modulation of membrane fluidity and cholesterol metabolism and transport, but also in BC

pathology and progression (142). Moreover, oxysterols have been described to be LXR-specific ligands. Some oxysterols are implicated in tumor formation (143), as recent data put in correlation their plasma levels in BC patients with clinical data (144), while others are considered anti-tumor agents (49).

For instance, 25-hydroxycholesterol (25-HC) and 27-hydroxycholesterol (27-HC) have been shown to enhance EsR expression in estrogen-deprived BC cell lines, suggesting that oxysterol are able to substitute estrogen in receptors activation and can play a potential role in resistance to the therapy (142, 145). In fact, 25-HC and 27-HC have been associated with resistance to aromatase inhibitors, which block estrogen synthesis but do not affect EsR expression. Indeed, BC patients treated with aromatase inhibitors had significantly increased plasma levels of 27-HC and (even if more moderately) 25-HC after treatment (146), supporting the potential role of 25-HC and 27-HC level and therapy outcome of patients (142). Accordingly, 25-HC, has been found elevated in the circulation of BC patients who have relapsed compared to those with primary disease (146).

In particular, 27-HC is produced by the cytochrome P450 27A1 (CYP27A1) enzyme, of particular interest in BC. It is, in fact, highly expressed among patients with high tumor grade, i.e., with less differentiated tumor cells (142, 147), and some *in vivo* experiments indicate that it could be a potential target for BC treatment (148). Accordingly, it has been reported that high levels of CYP7B1, a cytochrome p450 enzyme responsible for the catabolism of 27-HC, are associated with better survival outcomes in mice (49, 147).

Moreover, upon 27-HC exposition, BC cells showed increased proliferation and growth (18, 142). Additionally, 27-HC promotes BC cells migration and metastasis by affecting tumor microenvironment (18) through the recruitment of immune suppressive neutrophils in the metastatic niche (149). Consistently with these data, Moresco and colleagues demonstrated that oxysterols depletion reprograms the tumor microenvironment favoring the control of breast tumors and metastasis formation (150).

Overall, these data suggest that oxysterols could be potential targets for BC therapy.

Steroid Hormones

Cholesterol is also an important precursor of steroid hormones, many of which have clinical relevance (151). Steroid hormones are the products of steroidogenesis, a process that takes place in the mitochondria and smooth ER starting from cholesterol, which is mainly taken from LDL (152). Cholesterol is metabolized down a number of enzymatic pathways and converted to the 21-, 19-, and 18-carbon steroid hormones. Steroidogenesis starts with the transport of cholesterol into the mitochondria. This passage is controlled by the steroidogenic acute regulatory protein (StAR) (153). Subsequently, cholesterol is converted by the mitochondrial side-chain cleavage enzyme complex into pregnenolone. Pregnenolone, from which the other entire steroid hormones derive, is metabolized by several enzymes, leading to progesterone or androstenedione formation by 17-hydroxylase/17, 20-lyase enzyme. Androstenedione is further transformed into other androgens or estrogens (152).

Steroid hormones can be grouped into five categories: glucocorticoids, mineralocorticoids, androgens, estrogens and progestogens. Due to their lipophilic nature, steroid hormones cannot be stored in intracellular vesicles. As a consequence of their easy diffusion, they are synthesized as precursors and rapidly converted into active hormones when needed upon stimulation of the parent cell (151).

Of particular interest in the BC context are the ovarian hormones progesterone and estrogen, which are involved in tumor aetiology, progression and treatment. It is well assessed that a large percentage of BC are hormone-dependent, where cancer cells take advantage of local or systemic estrogens for sustaining their growth (154). In recent studies, androgens (in particular 11-oxygenated androgens) and glucocorticoids have been identified as biomarkers of BC risk, especially in women with a family history of BC, despite being much less studied (155).

The signalling events downstream hormone receptors include the direct or indirect modulation of gene expression, post-transcriptional regulation by miRNAs and signal transduction factors. Moreover, it has been described that these players act on BC stem cells (154, 156).

Furthermore, it is reported that prolonged exposure to ovarian hormones and progestin correlates with a BC risk, while progesterone and EsR are targets for advanced tumor therapy (154, 157). In fact, hormonal therapy is mandatory for all patients with hormone receptor-positive BC (158). This therapy aims to prevent estrogens stimulation of signalling pathways in cancer cells and can be performed through different strategies, including estrogens biosynthesis blockage or estrogens action through the use of agonist, antagonist or both (158).

Moreover, a close relationship between estrogen/testosterone metabolism and the MVA pathway in BC has been demonstrated. In particular, recent studies have shown that 17β estradiol and testosterone play key roles in rising MVA pathway enzymes, impacting on RAS proteins prenylation and farnesylation in several tumors, including breast and prostate cancer (159).

Taken together, this common evidence indicates that steroid hormones play an essential role in the development and classification of BC since they are commonly associated with risk and aetiology. In addition, they are potential targets for diagnostic tools (160) and BC treatment (152).

Vitamin D

Another interesting cholesterol derivative with hormonal activity, is vitamin D. Vitamin D3 is a fat-soluble vitamin whose biosynthesis takes place in skin cells and involves the irradiation of 7-dehydrocholesterol (a cholesterol precursor in the MVA) by ultraviolet (UV) radiation. It is influenced by several factors such as the availability of 7-dehydrocholesterol and atmosphere condition, skin pigmentation and age (161).The newly synthesized vitamin D3 is further hydroxylated in the liver, by the enzyme 25-hydroxylase, to 25-hydroxyvitamin D or

calcitriol, the active hormonal form of vitamin D. Once released in the extracellular space, Vitamin D3 binds to the vitamin-D binding protein, which shuttles Vitamin D through the bloodstream, and finally interacts with its receptor (VDR), which is ubiquitously expressed (162). Vitamin D can also derive from the diet, and it is an essential player in many physiological processes, including bone metabolism, cell growth and calcium and phosphorus absorption. On the other hand, pleiotropic effects of vitamin D such as anti-inflammatory and anti-neoplastic properties, are still under study (163). In particular, preclinical studies underlined that the vitamin D system has onco-protective functions, hindering several cellular processes such as differentiation, regulation of inflammation, apoptosis, proliferation, invasion and angiogenesis and metastasis formation (164).

As a matter of fact, vitamin D deficiency is one of the most common health problems worldwide (165, 166) and is a risk factor for several diseases, including metabolic syndrome (167), cardiovascular disease and cancer (162, 168). Interestingly, the first link between vitamin D and cholesterol has been described by Li et al., who demonstrated that vitamin D deficiency could enhance the amount of serum cholesterol by lowering the vitamin D receptor activity, leading to an increased cholesterol biosynthesis in the liver (168). These data appear to be consistent with another study performed by Jiang and colleagues that reported a link between vitamin D deficiency and dyslipidaemia. In particular, they described an inverse correlation between vitamin D and LDL cholesterol/triglycerides levels, while they demonstrated a positive association with the HDL cholesterol level (169).

The relationship between vitamin D and BC has been extensively studied and its role in tumor progression is well assessed (170, 171). In particular, it has been described an association between the impaired vitamin D and VDR molecular pathway and tumorigenesis in breast tissues (172), while VDR levels inversely correlates with a most aggressive tumor phenotype. Hence, VDR is considered a favourable prognostic factor and associated with a lower risk of BC death, supporting the protective anticancer role of vitamin D (164, 173, 174). Consequently, preclinical, clinical and epidemiological studies have established that vitamin D deficiency is a risk factor for BC development (175, 176).

Interestingly, calcitriol exhibited antiproliferative effects in BC cell cultures and delayed tumor growth in animal models of BC through different mechanisms (177). In particular, due to its anti-inflammatory activity and ability to suppress estrogen biosynthesis by down-regulating ERα expression, its potential therapeutic utility has been suggested in combination with other drugs in EsR+ BC patients (177).

Furthermore, recent studies speculate about vitamin D inducing molecular mechanisms able to reverse drug resistance in several tumors, including BC. Thus, many authors suggest using calcitriol in combination with anti-cancer drugs to potentiate BC therapy (178, 179).

Numerous randomised clinical trials attempted to define the efficacy of vitamin D supplementation in BC outcomes (Tab. 1). However, despite the promising results from observational studies, none of these trials could confirm reduced cancer-related mortality among cancer patients (180).

CLASSIC MVA PATHWAY INHIBITORS AND BC THERAPY

Statins

Dysregulation of the MVA pathway is a relevant lipid reprogramming often observed in BC. Several trials and epidemiologic studies support an inverse correlation between the use of MVA inhibitors, such as statins, and mortality rate in BC (181). Statin class of drugs has been largely used to lower blood cholesterol levels, by inhibiting the core HMGCR enzyme of the MVA pathway, in particular for cardiovascular diseases treatments. During the last years, several epidemiologic and clinical research studies underlined their beneficial role in concomitant diseases such as BC, even though an exact mechanism in this context is not yet fully understood (182, 183).

Despite some studies suggesting no close association between statin use and BC risk (184, 185), recent evidence showed a link between statin use and reduced recurrence and disease-specific mortality in BC patients, with an improved BC prognosis and survival (186–188).

Interestingly, Beckwitt and colleagues in their work demonstrated that statins are able to interfere with metastatic cascade and suppress metastatic BC outgrowth, suggesting that this class of drugs could be a potential long term adjuvant in order to prevent dormant BC micro-metastasis, which are responsible for the majority of BC deaths (189).

Moreover, a positive correlation between statins treatment and some clinical benefits in TNBC was observed in women starting statins therapy within one year after the diagnosis (190).

In particular, recent preclinical data describe an impact for Atorvastatin in favouring chemotherapy effects in TNBC, suggesting its possible use in conjunction with metastatic chemotherapy to reduce TNBC cancer progression (191).

Taken together, these data indicate a general protective role for statins in the treatment of BC in combination with standard therapy, although completed clinical trials have provided controversial results (**Table 1**). Ongoing and future interventional studies will give a better understanding concerning the safety and the efficacy of these compounds.

Zoledronate

Another MVA pathway inhibitor is zoledronate (or zoledronic acid - ZA). It is a potent and long-acting bisphosphonate drug in clinical use. It acts by blocking the farnesyl pyrophosphate synthase (FPPS) in the MVA pathway, thereby inhibiting the synthesis of cholesterol and isoprenoid lipids required for prenylation of signalling proteins (192). Clinical practice guidelines recommend the use of ZA for the treatment of early BC in post-menopausal women (193, 194), since it improves osteoclast bone resorption for the treatment of hypercalcemia of malignancies and management of bone metastasis (195)

(**Table 1**). Interestingly, its potential effects in reducing cancer, cardiovascular diseases and mortality could be more important than its skeletal actions (196, 197).

However, recent evidence has shown that ZA is able to modulate signaling pathways involved in apoptosis and that could be beneficial to be used together with letrozole to treat EsR-positive BC patients (198).

Interestingly, ZA involvement in immunomodulation of tumor microenvironment has also been described. In fact, Ubellacker et al. demonstrated that a single relevant dose of ZA is able to generate BC suppressive bone marrow cells, which could concur in a reduction of breast tumor development and progression (199). Moreover, ZA seems to explicate an anti-tumor activity enhancing the proliferation, migration, and immunosuppressive function of T-regulatory cells (Tregs) by affecting Tregs interaction with BC cells and synergistically acting with cytokine or IDO inhibitors leading to enhanced anti-tumor immunity (200).

Another benefit of ZA treatment is overcoming BC cells chemo-resistance due to the induction and activation of apoptosis pathway. In fact, BC stem cells, considered mainly responsible for tumor recurrence and drug resistance, decrease their viability in a dose- time-dependent manner upon ZA exposition (201). In correlation with these data, Jia and colleagues described ZA inhibition on ERK/HIF pathway leading to a higher drug sensitization in EsR-positive BC (202).

However, some data demonstrated that ZA does not increase disease-free survival, despite improving the pathologic complete response, thus might not being sufficient to ameliorate post-menopausal patient outcomes in HER2-negative BC (203).

Nevertheless, ZA is the object of clinical studies with other types of bisphosphonates (204).

Taken together, the available data indicate a general protective effect of MVA pathway inhibition with drugs in BC (**Table 1**). Despite any case and effect needing to be individually evaluated, it could be an interesting adjuvant tool in BC therapy.

CONCLUSION AND OUTLOOK

From the above data, a complex picture on the role of cholesterol and its derivatives in BC is emerging. The enzymes that control the various steps leading to cholesterol or derivatives synthesis and the protein involved in trafficking towards the membrane or in the uptake from the circulation are all involved in cholesterol homeostasis and can be affected by cell transformation. At the same time, they look promising as targets for antitumor drugs.

As stated in **Table 1**, current clinical trials indicate that the MVA pathway inhibition with specific drugs like statins and ZA,

TABLE 1 | A list of completed interventional studies with published results that assess the beneficial role of cholesterol-lowering drugs and vitamin D in BC patients.

Target	Drug	Objectives	Results	Phase	NCT Number and References
HMGCR	Simvastatin	Identification of biomarkers modulated by simvastatin in women at increased risk of a new BC	Reduction of circulating estrone sulfate No changes in mammographic density (MD)	II	NCT00334542 (205);
		Investigating concurrent anastrozole and simvastatin treatment in post-menopausal women	Simvastatin does not compromise the activity of anastrozole	II	NCT00354640 (206);
	Lovastatin	Lovastatin effect on women with a high inherited BC risk	No significant biomarkers modulation	II	NCT00285857 (207);
	Fluvastatin	Evaluating biomarkers changes	Decreased proliferation and increased apoptosis markers	II	NCT00416403 (208);
Farnesyl Diphosphate Synthase	Zoledronic Acid	Investigating the effects on bone marrow micrometastases	Reduced abundance of disseminated tumor cells	II	NCT00295867 (209);
		Effect of ZA in combination with Letrozole in post-menopausal BC patients	Improved disease-free survival Preserved bone mineral density	III	NCT00171340 (210);
		Investigating the effect of ZA in combination with chemotherapy and/or hormone therapy	Adjuvant ZA reduced the risk of fractures	III	NCT00072020 (211);
			Improved disease-free survival in pre-menopausal patients with early-stage BC taking anastrozole or tamoxifen	III	NCT00295646 (212);
		Assess the efficacy and safety	Therapeutic effect maintained at reduced dosing frequency	III	NCT00375427 (213);
			No significant differences in disease-free survival or overall survivor Improved the bone mineral density	II	NCT00213980 (214);
		Assess the efficacy and safety in combination with Dasatinib	Combination well tolerated Indication of clinical benefit for HR-positive patients	II	NCT00566618 (214);
Vitamin D Receptor	Vitamin D	Evaluate changes in BC biomarkers	No significant changes in MD	III	NCT01224678 (215);
				I/II	NCT00976339; NCT00859651 (216);

is protective in BC. In addition to statins and ZA, some new cholesterol metabolic molecules have recently emerged as promising drug targets for cancer treatment (18). An example comes from targeting the cholesterol esters through inhibition of ACAT1 with the potent inhibitor avasimibe. In melanoma, in the immune response to cancer, avasimibe promotes TCR aggregation and immune synapse formation in CD8+ T cells by elevating the cholesterol content of the PM, thus enhancing the killing effect of CD8+ T cells (18). Avasimibe has been proven to have a good human safety profile in previous clinical trials in the treatment of atherosclerosis (127). Therefore, targeting ACAT1 by avasimibe may be a safe and effective method to disrupt cholesterol metabolic homeostasis in cancer treatment, as it has begun to be explored in recent preclinical BC studies (217) and it would be interesting to evaluate its effects in BC clinical practice.

It is also very important to underline those synergistic effects of low doses of cholesterol inhibitors, statins or ZA, together with low doses of chemotherapy drugs, might reach the target of increased efficacy and decreased adverse effects and resistance. Therefore, at least preclinical experiments are required to set the optimal range of treatments in BC mouse syngeneic models, in which both the tumor and the tumor microenvironment with the complex immune repertoire can be explored.

BC heterogeneity and the complex cellular architecture plays a key role in drug responsiveness and resistance to therapy that are the major challenges in BC treatment of aggressive tumors, like the TNBC, and are responsible for tumor relapse. However, deciphering the neoplastic subtypes and their spatial organization is still challenging. Nowadays in addition to panels of protein biomarkers useful for classifying clinical phenotypes of breast cancer (19), the progress in single-nucleus RNA sequencing will allow the identification of cell populations and of their spatial distribution in breast cancer tissues with costs that will become more and more accessible. This could be performed in parallel with metabolomics analysis of cell populations. Data coming from these experiments will allow tracing the clonal evolution of cells that are more addicted to the MVA pathways in the tumor. Finally, coupling innovative combinatorial therapies, chemotherapy and inhibitors of the cholesterol pathways, with the analysis at a single cell level will highlight in a given BC specific different clones, which may contribute to metabolic phenotype and drug response.

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

GC, DN, AP, and CR searched for current literature on the topic and wrote the manuscript. GC and AP designed the figure. CR and PD reviewed the manuscript and finalized it for publication. All authors contributed to the article and approved the submitted version.

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Trends in Women's Leadership of Oncology Clinical Trials

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It has been widely reported that women are underrepresented in leadership positions within academic medicine. This study aimed to assess trends in women representation as principal investigators (PIs) in oncology clinical trials and to characterize trends in women's leadership in such trials conducted between 1999 and 2019. The gender of 39,240 PIs leading clinical trials was determined using the gender prediction software Genderize.io. In total, 11,516 (27.7%) women served as PIs. Over the past 20 years, an annual increase of 0.65% in women PIs was observed. Analysis by geographic distribution revealed higher women representation among PIs in North America and Europe compared to Asia. Industry-funded trials were associated with lower women PI representation than academic-funded trials (31.4% vs. 18.8%, p<0.001). Also, women PIs were found to be underrepresented in late-phase as compared to early-phase studies (27.9%, 25.7%, 21.6%, and 22.4% in phase I, II, III, and IV, respectively; Cochran-Armitage test for trend, p<0.001). Furthermore, an association was found between the PI's gender and enrolment of female subjects (50% vs. 43% female participants led by women vs men PIs, respectively, p<0.001). Taken together, while the gender gap in women's leadership in oncology trials has been steadily closing, prominent inequalities remain in non-Western countries, advanced study phases, industry-funded trials and appear to be linked to a gender gap in patient accrual. These observations can serve for the development of strategies to increase women's representation and to monitor progress toward gender equality in PIs of cancer clinical trials.

Keywords: women representation, women's leadership, gender gap, oncology clinical trials, principal investigators

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INTRODUCTION

Over the past few decades, women have made substantial gains in participation in the medical profession. As of 2020, women represented 34% of practicing physicians and 50.5% of medical students within the United States (AAMC 2019 Physician Specialty Data Report). However, the underrepresentation of women remains prevalent in science and medicine. A growing body of

literature has shown an achievement gap between men and women faculty in research practices, career advancement, leadership opportunities, financial compensation, and scientific recognition (1–6).

In oncology, women are estimated to represent 36% of the workforce (7) but account for only one-fifth of full professors and one-third of department leaders (8). Despite positive trends, the percentage of women among authors in major oncology journals remains low (20-30%), lagging behind the proportions serving as oncology faculty (9, 10). In addition, women represented ~40% of invited speakers in oncology international congresses and one-third of board members of oncology societies (11, 12).

Clinical trials are the backbone of evidence-based medicine and promote informed clinical decision-making. They require infrastructure, advanced research training, and massive funding, and take years from planning to completion. Serving as a principal investigator (PI) in a clinical trial confers recognition among peers and at international oncology meetings, and can result in academic promotion. Therefore, being a PI is a major milestone in an oncologist's career.

In this study, we aimed to assess women's representation as PIs in oncology clinical trials, characterize trends, and determine factors associated with women leadership.

MATERIALS AND METHODS

Study Selection

Study record data were downloaded from ClinicalTrials.gov in extensible markup language (XML) format on October 24, 2019. This search yielded 320,210 trials conducted between January 1999 and October 2019 (**Figure 1**). Trial data, including ClinicalTrials.gov identifier, year of submission, investigator names, investigator role, study phase, study type, sponsorship, affiliation, Medical Subject Headings (MeSH) term, and the number of male and female participants in the study, were abstracted. The analysis was restricted to oncology trials by including studies matching the MeSH term "Neoplasms". Studies with empty or ambiguous PI names were excluded, and only investigators with an official role assigned as "Principal Investigator" were included in the analyses.

The study and reporting followed Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (13).

Determination of Principal Investigator Gender

First names were subjected to basic processing to remove extra spaces, ambiguous characters, and prefixes such as doctor (e.g., Dr.) or professor (e.g., Prof.). The PI gender, treated for the purpose of this analysis as binary (i.e., woman or man), was predicted using the validated software Genderize.io (https://genderize.io). For each name, the software returns a predicted gender and a probability. The standard threshold of 60% was used to assign the gender, as has been implemented in previous works (14–16). Names predicted with a lower probability, for which prediction failed, or which were ambiguous were marked as not applicable.

Geocoding

Google Maps API through the R package mapsapi version 0.5.0 was used to locate the country of the PI. Since a given trial may be led by more than one investigator and the documented country in the study records is not necessarily the country of the PI, the affiliation of the investigators was used for geocoding (e.g., Department of Family Medicine, University of Michigan). Countries with fewer than 30 studies were excluded from the analysis. Countries were classified as low/lower-middle, upper-middle and high-income based on their World Bank Classification.

Statistical Analysis

All analyses were conducted in R version 4.0.3 (R Project for Statistical Computing). Odds ratios (OR) were estimated by logistic regression using the R package glm. The Cochran–Armitage trend test was used to estimate the association between representation of women PIs over time and study phases using the R package CATT. The association between the genders of the PI and participants was evaluated using the Wilcoxon rank-sum test. Two-sided p values <0.05 were considered statistically significant.

Ethical Approval

Institutional review board approval was waived because no human data were included, and publicly available information was used.

RESULTS

The online system ClinicalTrials.gov is a web-based resource that provides access to summary information about ongoing and completed clinical studies. Out of 320,210 trials registered in ClinicalTrials.gov between 1999 and 2019, we identified 36,002 unique cancer clinical trials led by 41,648 PIs (**Figure 1**). The gender of 39,240 (94.2%) investigators could be determined. In total, 11,516 (27.7%) women served as PIs in cancer clinical trials, compared to 27,724 (66.6%) men. Categorizing by cancer disease site found low women leadership rates for hepatobiliary (17.4%), urinary tract (17.5%), prostate (18.2%) and gastroesophageal (19.3%) cancer trials and higher rates for breast (45.4%), gynecologic (39.5%), sarcoma (32.4%), central nervous system (31.9%) and endocrine (30.2%) cancer clinical trials (**Figure 2**).

A significant association was found between the clinical trial phase and proportions of women PIs, where late phases were led by fewer women compared to early phases – 27.9%, 25.7%, 21.6%, 22.4% in phase I, II, III, and IV, respectively (Cochran-Armitage test for trend, $p<0.001$, **Figure 3**). Observational trials had more women PIs than interventional trials (29.8% vs. 27.2%; OR 1.27, 95% CI, 1.27 – 1.35; $P<0.001$). This disparity was most apparent in phase III clinical trials (Phase III vs observational

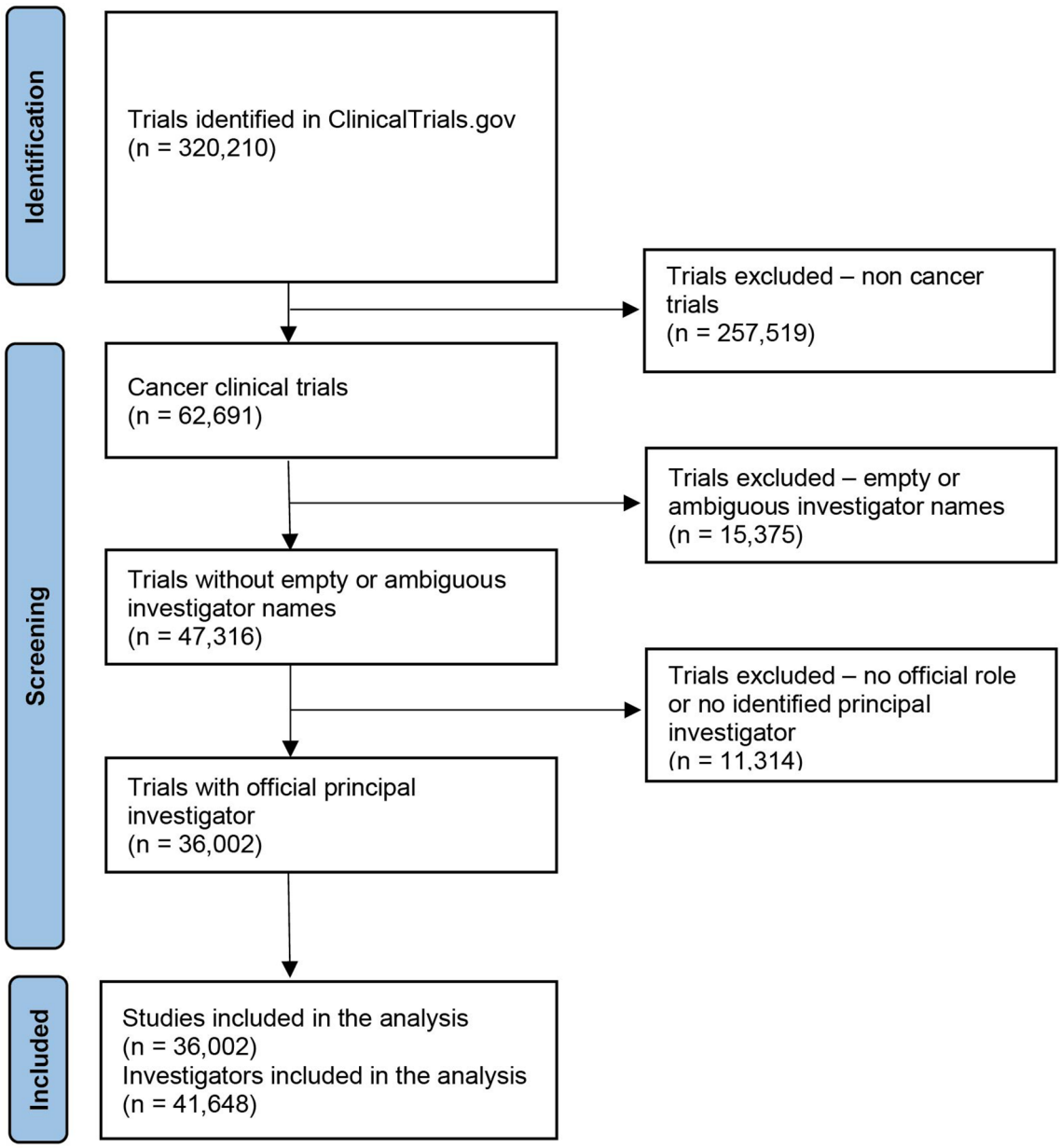

FIGURE 1 | Flow chart of trial screening and eligibility.

studies, OR 1.54, 95% CI, 1.40 – 1.70; p<0.001). In addition, a significant relationship between study sponsorship and the gender of the PI was observed, where fewer clinical trials led by women were funded by the industry (18.8%) as compared with those funded by the NIH (31.4%; OR 0.53, 95% CI, 0.48 – 0.60; p<0.001) or US federal agencies (34.8%; OR 0.46, 95% CI, 0.32 – 0.66; p<0.001) (**Figure 4**). Over time, there was a substantial increase in women's leadership of clinical trials from 17.5% in 1999 to 30.6% in 2019 (5-year interval: 17.5%, 22.1%, 25.6%, 28.9%, 30.6%), representing an average annual increase of 0.65% (**Figure 4**). A Cochran-Armitage trend test found this growth of women PI's proportion to represent a steady and significant increase over time (p=0.001). Women's leadership increased in both industry and academic-funded trials (**Figure 4B**).

Analysis by geographic distribution revealed higher women representation among PIs in North America (30.7%) and Europe (23.8%) compared to Asia (15.5%), although the rates of women PIs varied across European countries (**Figure 5** and **Supplementary Table 1**). For example, Denmark (39.3%), Sweden (31.1%) and France (28.6%) had higher women representation than Germany (14.2%), Italy (21.3%) and

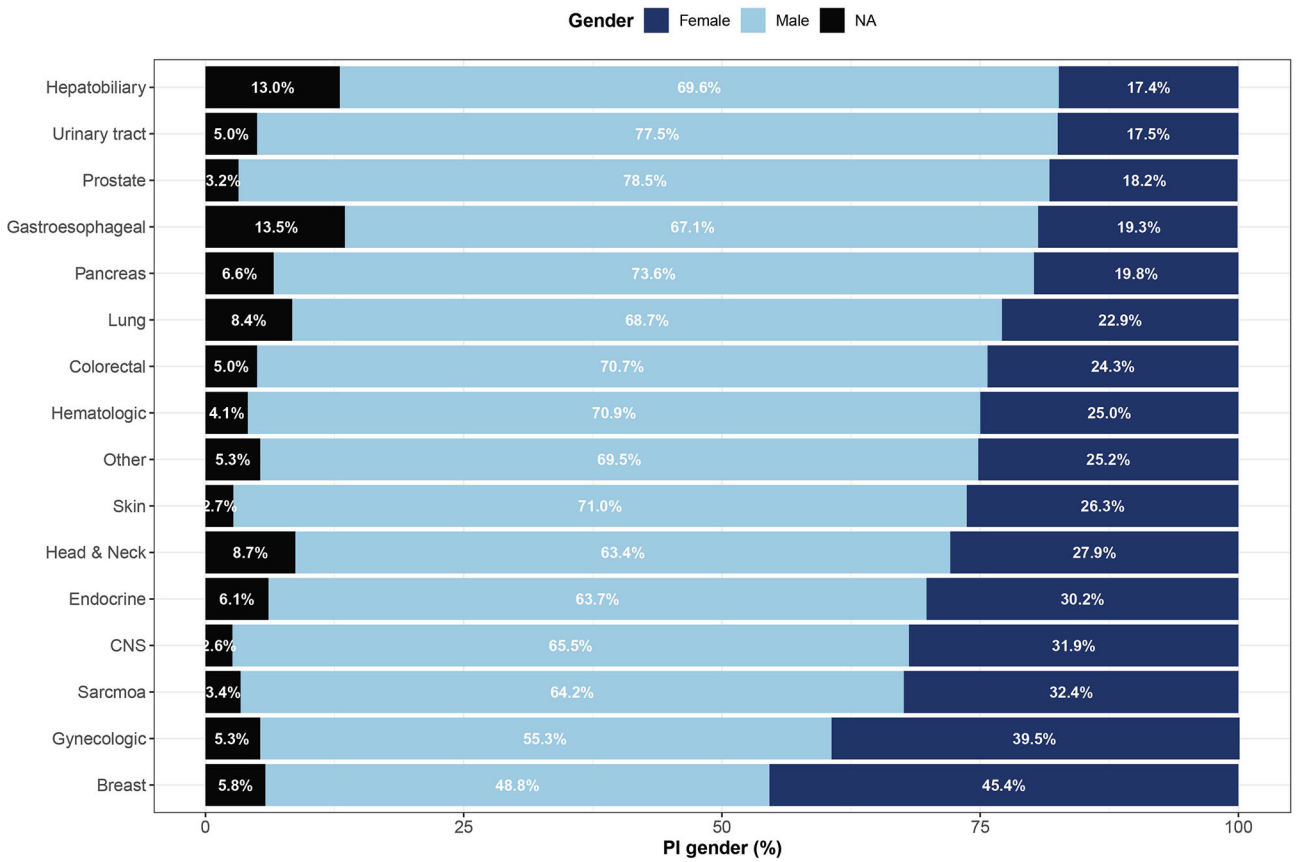

FIGURE 2 | Representation of women among lead investigators of oncology clinical trials by cancer site. Shown are gender proportions of principal investigators leading studies of 16 cancer sites. Names with low gender prediction scores or names for which gender could not be determined are marked as not applicable (NA). CNS, central nervous system.

Austria (15.5%). A comparison according to the level of resources showed higher representation of women PIs among high-income countries compared with middle-income countries (28.6% vs 20.5%).

It was previously shown that women are underrepresented as study participants in clinical trials (17). We found that men leading clinical trials were less likely to recruit women participants, whereas women leading clinical trials tended to recruit more women participants (50% vs. 43%, female participants led by women vs men PIs p<0.001) (**Figure 6**). This observation remained statistically significant even after excluding gender-specific diseases such as breast, prostate, and gynecologic malignancies (44% vs. 41% p-value 0.013) (**Figure 6**). Of note, only 1,749 (4.9%) studies reported the number of women and men participants in the trial.

DISCUSSION

This study of gender representation in cancer clinical trials found that while men lead the majority of clinical trials, women representation among PIs is growing. Women's leadership of clinical trials is more prevalent in Western countries, early-phase trials, and nationally sponsored studies. In addition, clinical trials led by women PIs have a greater representation of female study participants.

It was previously shown that women are a minority among first authors in cancer-related publications, oncology faculty members, subjects of phase III randomized clinical trials, invited speakers, and board members of oncology societies (7, 11, 12, 18). To the best of our knowledge, this is the first study to comprehensively evaluate women's representation among registered cancer clinical trials and trends in women leadership. Interestingly, similar findings were found in other fields as well (19, 20) and may represent a more general phenomenon.

Multiple factors may underlie women's underrepresentation as PIs. First, women remain a minority in many medical fields. For example, the urologic oncology workforce is primarily comprised of men (21), and women radiation oncologists in genitourinary cancer are a minority (22). This gender gap might affect the observed lower representation of women PIs. Moreover, previous publications have demonstrated marked

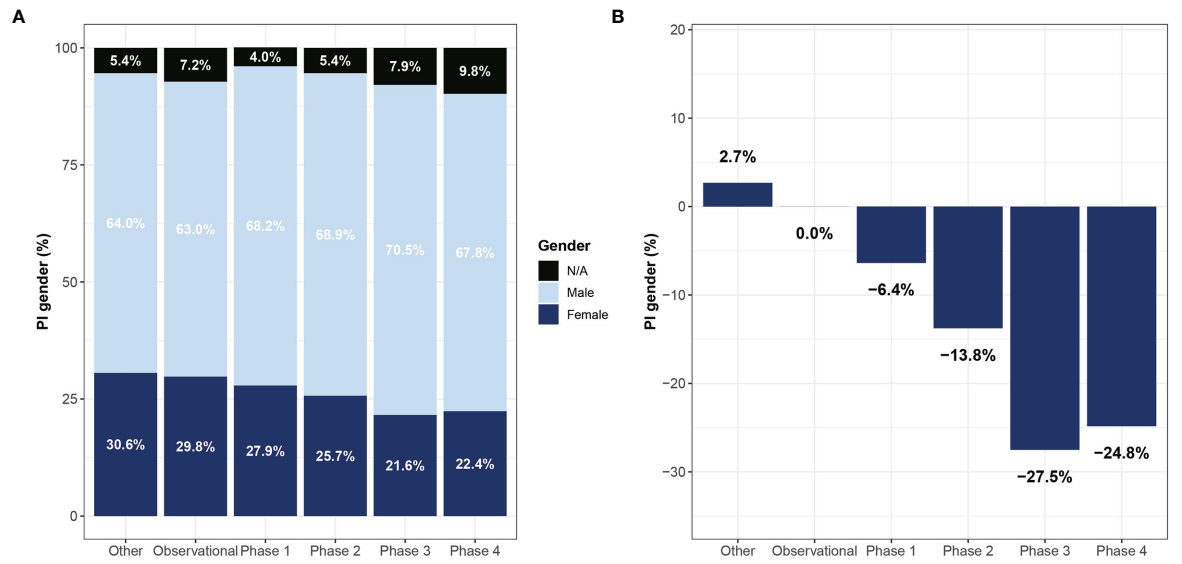

FIGURE 3 | Representation of women lead investigators in oncology clinical trials by study type. Shown are gender proportions of principal investigators leading trials of different study phases **(A)** and the reduction in proportions relative to observational studies **(B)**. Names with low gender prediction score or names who which gender could not be determined are marked as not applicable (NA).

disparity in the proportion of women in high academic positions (7), board members of oncology societies (11) and as authors in major oncology journals (23). Women comprise 31% of department chairs in medical oncology, 11.7% in radiation oncology and 3.8% in surgical oncology (7). In addition, major oncology societies (ESMO, ASCO) have low percentages of women occupying board position (14-25%). As the oncology field progresses towards gender equality in career development (12), better representation for women as PIs is anticipated.

It will be of great interest to follow the gender gap in clinical trial leadership as the proportion of women leaders increases.

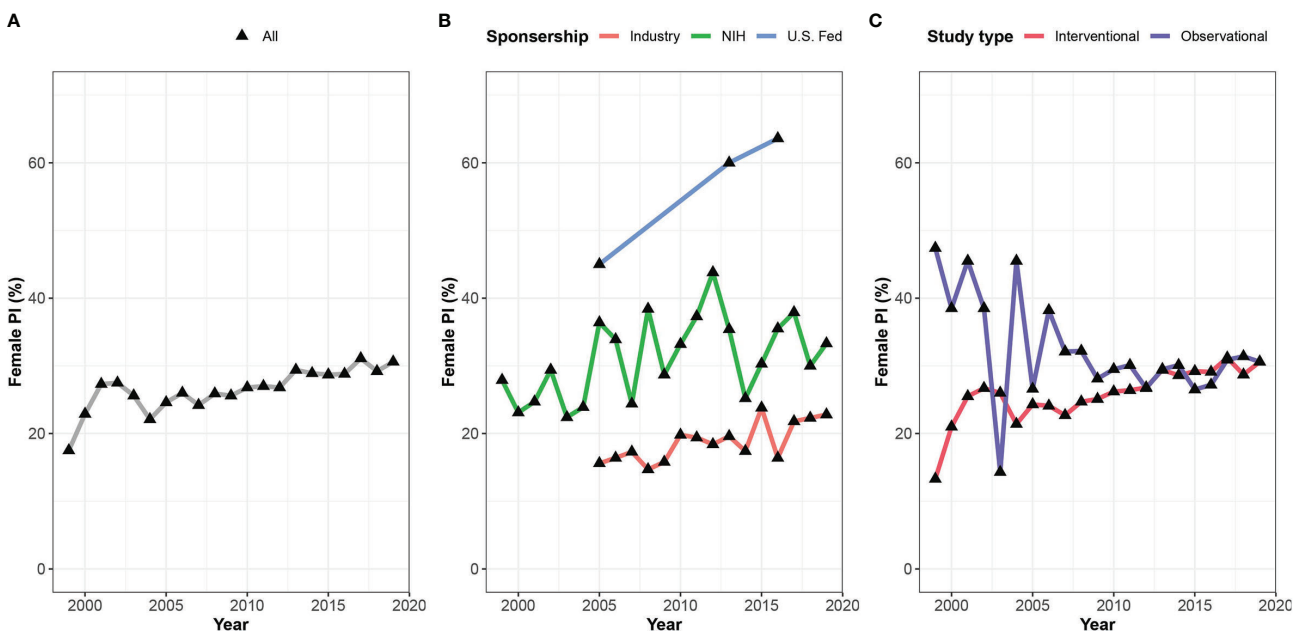

FIGURE 4 | Gender gap among lead investigators in oncology clinical trials over time. Shown are proportions of women leading oncology trials between 1999 and 2019 for **(A)** all included studies, **(B)** studies stratified by sponsorship, and **(C)** type of study.

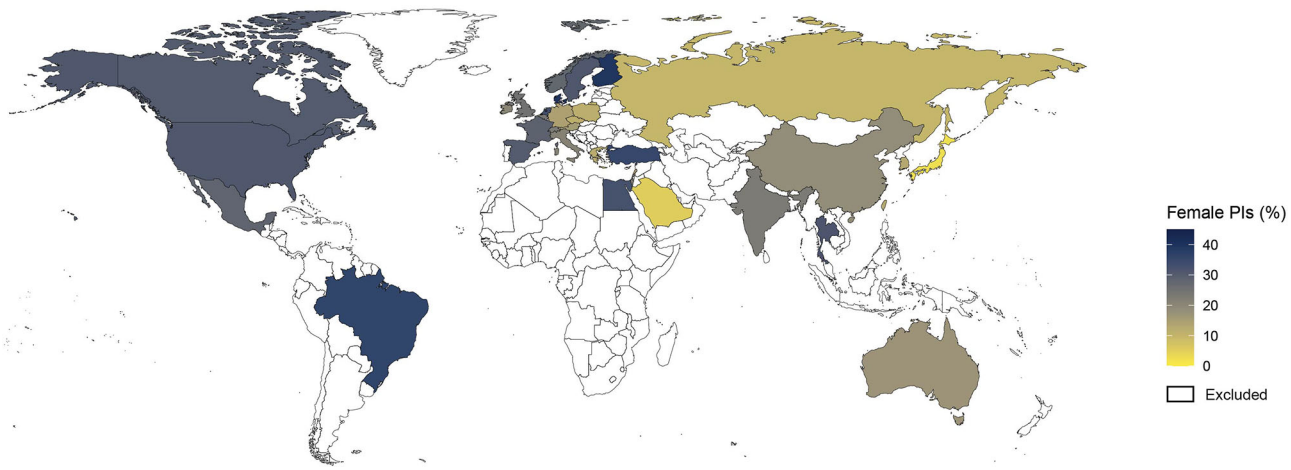

FIGURE 5 | Map of the gender gap among lead investigators of cancer clinical trials. Shown is the distribution of proportions of women principal investigators (PIs) by country. Countries with less than 30 clinical trials are colored in white and were excluded from the analysis.

The proportion of women PIs in industry-funded trials was significantly lower than in governmental-funded trials. An earlier study found that 75% of the physicians who had financial relationships with biomedical companies were men (24). Similar results were reported specifically for radiation oncologists (25).

The observed gender disparities in industry-funded trials are in line with gender discrimination and inequality in the general and health workforce (26). We also examined the relationship between the investigator's gender and women enrollment. Our results demonstrated that clinical trials led by women had higher female subject enrollment. This observation is in accordance with previous studies (27, 28), and supports the notion that reducing the gender gap in women leadership may assist closing the gender gap in recruitment.

The strengths of this study include the longitudinal and comprehensive evaluation of gender representation of PIs in

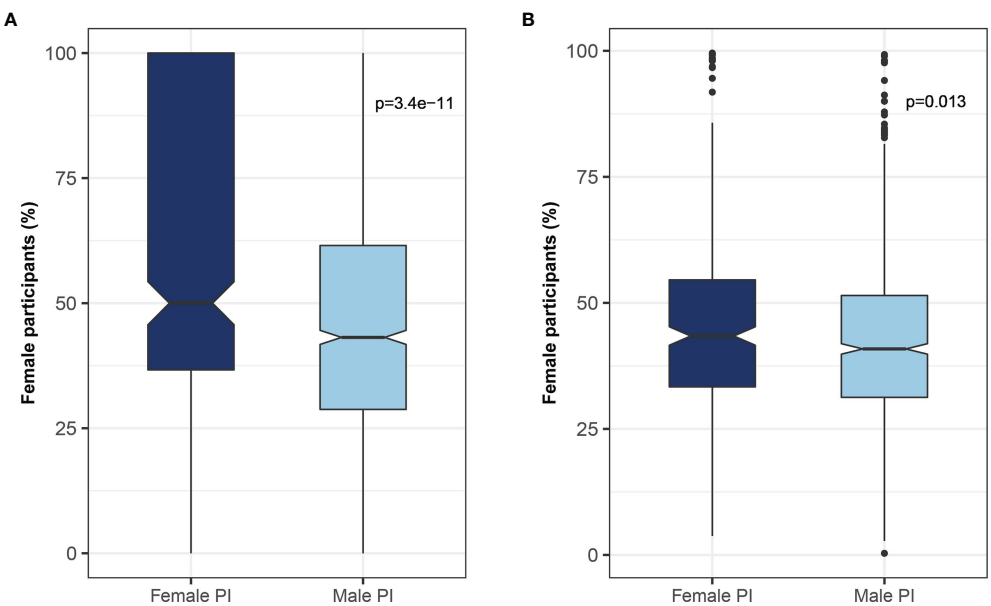

FIGURE 6 | Relationship between the gender of the investigator and trial participants. The association between the gender of the lead investigator and the proportion of female participants enrolled in the trial for **(A)** all studies, and **(B)** studies excluding gender-specific malignancies (e.g., uterine, ovary, and prostate).

cancer clinical trials. Evaluation of factors associated with gender representation including time trends, study phase, oncology field, sponsorship, and gender of study participants provides a broader prospective on the PIs gender gap. Several limitations of this study warrant mention. First, gender was assumed to be binary (male and female) as in previous studies. The study used validated methods to determine PI's gender, but misclassifications may have occurred. Manual validation of the prediction performance in several countries was performed by random sampling of the predicted genders of names. In addition, this analysis did not account for the proportion of women oncologists in each country and their academic rank. Further, only a small number of oncology trials contained information about the number of participants for each gender. Finally, the observational nature of the study precluded inference of causal relationships.

In conclusion, the present work shed light on trends in women's leadership in cancer clinical trials over the past two decades. While women comprise a growing proportion of PIs in cancer clinical trials, they remain in the minority. Our findings show significant differences between oncology fields, geographical regions, study phases, and funding agencies. The presented results are important for developing practices and strategies to promote gender equality in the leadership of clinical trials in oncology.

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

Concept and design: IW, DB, TM. Acquisition, analysis, or interpretation of data: DB, IW, AD, AM, ES, DS, NT, SS, GM, TM. Drafting of the manuscript: IW, TM. Statistical analysis: DB, TM. Supervision: TM. 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/fonc.2022.885275/full#supplementary-material

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REVIEW
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Cross-Resistance Among Sequential Cancer Therapeutics: An Emerging Issue

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Over the past two decades, cancer treatment has benefited from having a significant increase in the number of targeted drugs approved by the United States Food and Drug Administration. With the introduction of targeted therapy, a great shift towards a new era has taken place that is characterized by reduced cytotoxicity and improved clinical outcomes compared to traditional chemotherapeutic drugs. At present, targeted therapies and other systemic anti-cancer therapies available (immunotherapy, cytotoxic, endocrine therapies and others) are used alone or in combination in different settings (neoadjuvant, adjuvant, and metastatic). As a result, it is not uncommon for patients affected by an advanced malignancy to receive subsequent anti-cancer therapies. In this challenging complexity of cancer treatment, the clinical pathways of real-life patients are often not as direct as predicted by standard guidelines and clinical trials, and cross-resistance among sequential anti-cancer therapies represents an emerging issue. In this review, we summarize the main cross-resistance events described in the diverse tumor types and provide insight into the molecular mechanisms involved in this process. We also discuss the current challenges and provide perspectives for the research and development of strategies to overcome cross-resistance and proceed towards a personalized approach.

Keywords: targeted-therapy, cancer therapeutics resistance, cross-resistance, sequential therapeutics, personalized oncology

INTRODUCTION

The history of targeted cancer therapy started in the 1970s with the approval of tamoxifen, the first selective estrogen receptor (ER) modulator (1). At the beginning of the '80s, advances in molecular biology allowed to identify new molecular targets involved in neoplastic transformation and progression. These discoveries sparked a revolution in cancer therapy, at the time mainly based on combination chemotherapy regimens, that culminated in the development of targeted monoclonal antibodies (mAbs) and selective protein kinase small molecule inhibitors (PKIs) (2). Following the development of

hybridoma technology by George Köhler and Cèsar Milstein in 1975 (3), who were awarded a Nobel prize for their discoveries in 1984, several attempts to develop murine mAbs against myelo- and lympho-proliferative diseases and lymphomas did not give the expected results (4, 5). In 1986 the United States (U.S.) Food and Drug Administration (FDA) approved the first therapeutic mAb, muromonab-CD3, which was to be used as an immunosuppressive for prevention of transplant rejection (6). At the beginning of the '90s, growing scientific and industrial interest in developing targeted drugs ushered us into an era characterized by the approval of an increasing number of MAbs and PKIs. The first tyrosine-kinase inhibitor (TKI), Imatinib mesylate, directed towards the fusion protein BCR-ABL, obtained approval by the FDA in 2001 (7). Since then, more than 70 PKIs have been introduced (8), and 100 mAbs have been approved by April 2021, with GlaxoSmithKline's Programmed cell Death protein 1 (PD1) blocker dostarlimab (9). More recently, checkpoint inhibitory mAbs and chimeric antigen-specific receptor (CAR)-transfected T-cells (CAR-T cells) have also had impact in the oncology field (10).

Targeted cancer therapy has provided huge benefits in terms of improved response and survival rates as well as reduced side effects compared to traditional chemotherapy. However, one of the greatest drawbacks to all currently available cancer therapies is the emergence of drug resistance leading to tumor progression (11). For this reason, many patients affected by advanced malignancy receive sequential anti-cancer therapies, which may include chemotherapy, immunotherapy, targeted therapy, endocrine therapy, or a combination of them. The complexity requires strict criteria to define and enumerate the sequential lines of therapy uniformly across solid malignancies (12). From a mechanistic point of view, recent high-throughput sequencing studies and quantitative modeling approaches have revealed extensive intratumor heterogeneity and highly dynamic tumor clonal evolution under the selective pressure exerted by drug treatments (13–15). It is therefore easy to anticipate that the evolutionary trajectories imposed by drugs may intersect through subsequent lines of treatment in unpredictable ways. In this scenario, the probability that cross-resistance emerges between sequential treatments increases with a higher number of therapeutic possibilities. Unfortunately, the current adoption of sequential lines of therapy according to guidelines is a strategy that does not consider cross-resistance as well as the possible development of new targetable vulnerabilities (16).

In this review, we summarize the main known events of cross-resistance and the molecular mechanisms involved. We also provide an overview of real-world data (RWD) as a tool to address the complexity of cancer therapy, and the possible strategies to adopt in an attempt to overcome or prevent cross-resistance.

CROSS-RESISTANCE AMONG CANCER THERAPEUTICS

Cross-resistance occurs when acquired resistance induced by a drug treatment results in resistance to other drugs (**Figure 1**). It may occur in the sequential administration of agents with overlapping working mechanisms, such as receptor tyrosine kinase erbB-2 (HER2)-targeting agents trastuzumab+pertuzumab and trastuzumab-emtansine (T-DM1) in breast cancer (BC). In this case, T-DM1 second line treatment might have reduced efficacy. In a more complex scenario, the characterization of tumor evolution in terms of clonal selection during therapy has revealed that under prolonged drug exposure, cancer cells enter a drug-tolerant state known as drug tolerant persister cells (DTPCs) (17). At this stage, the activation of heterogeneous mechanisms of drug resistance causes these subclones to expand and generate stable resistant cell populations (17–19). The sensitivity of these populations to subsequent drugs is difficult to predict unless biomarkers will be defined to represent specific collateral trajectories. The main events of cross-resistance described thus far for the different types of targeted therapies are reported below.

Chemotherapeutic Drugs

The use of cytotoxic/cytostatic chemotherapy was the first approach adopted in the treatment of tumors. However, the effectiveness of these drugs was often limited by the emergence of multiple drug resistance (MDR) (20) which determined cross-resistance to diverse structurally and functionally unrelated chemotherapeutic agents.

Although cancer cells develop various mechanisms to escape chemotherapy, drug transporters belonging to ATP-binding cassette (ABC) family are the main players implicated in MDR. These ATP-dependent efflux pumps actively remove drugs from cancer cells (21). Glycoprotein P (P-gp) is the most relevant ABC drug transporter. It is encoded by the *multidrug resistance protein 1* gene (*MDR1, ABCB1*) and overexpressed in over 50% of cancers with a MDR phenotype (22). P-gp overexpression has been implicated in resistance to approximately 20 different cytotoxic drugs including doxorubicin, paclitaxel and related taxane drugs (23). Many anticancer drugs have been reported to induce the up-regulation of Forkhead Box O3 (FOXO3A), a transcription factor closely implicated in MDR, that in turn enhances *ABCB1* transcription and P-gp expression (24).

Other ABC family members involved in MDR include Breast Cancer Resistance Protein (BCRP; also known as mitoxantrone resistance protein, MXR), and multidrug resistance-associated proteins (MRPs) (25). BCRP (encoded by the *ABCG2* gene) is the second most relevant drug transporter. Its overexpression has been described in many cancers including breast and ovarian and is associated with resistance to mitoxantrone and topotecan (26, 27). MRPs include MRP1 and MRP2 (also known as MDR-related protein 1 and MDR-related protein 2) encoded respectively by the *ABCC1* and *ABCC2* genes (21, 25, 28). The drug resistance spectra of MRP1 is similar to that of P-gp except for taxanes, while MRP2 confers resistance to MRP1 substrates and cisplatin, one of the most frequently used drugs in cancer therapy (23, 26).

DNA damage repair (DDR) genes have been implicated in the cross-resistance among chemotherapeutic drugs. In multiple mouse models of NSCLC, prolonged cisplatin treatment promoted the emergence of resistant tumors that were cross-resistant to platinum analogs. These cisplatin-resistant tumors showed enhanced DNA repair capacity due to elevated levels of

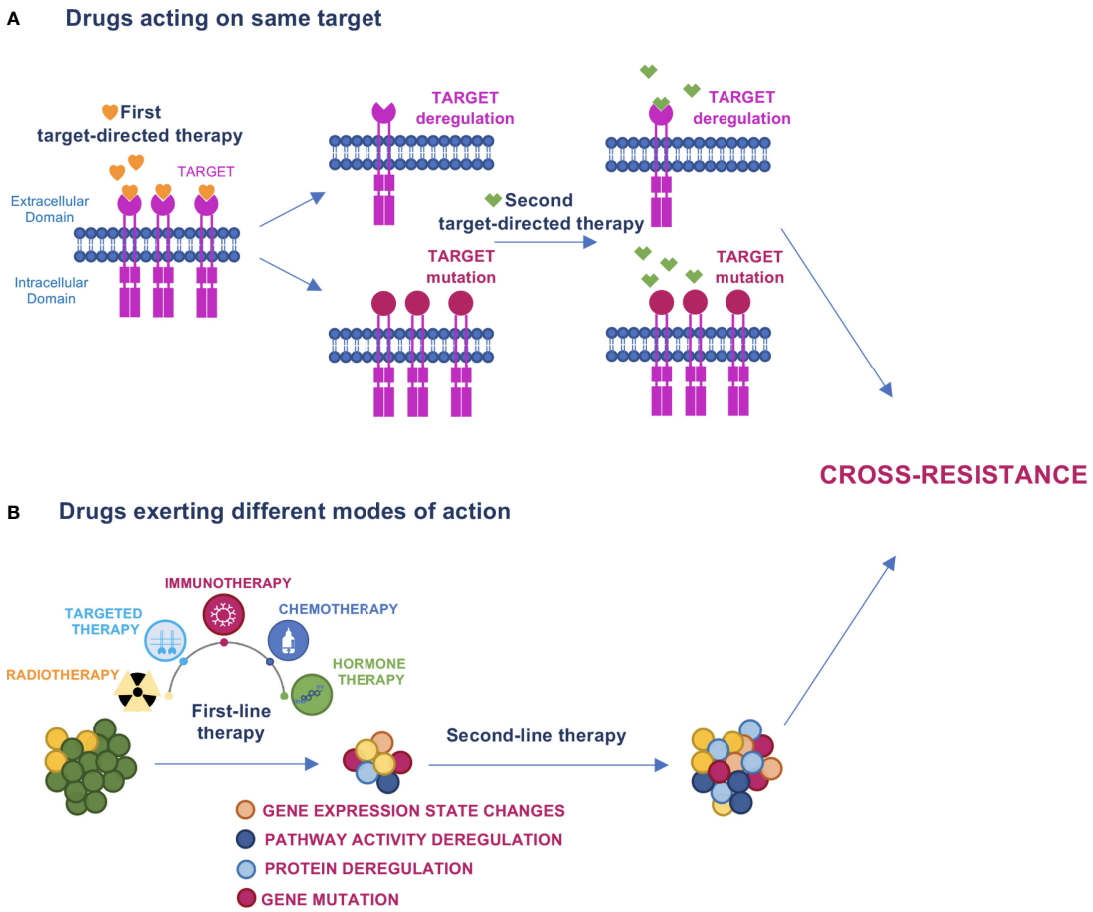

FIGURE 1 | Models of cross–resistance. **(A)** When drugs acting on the same target are sequentially administered, the first drug can induce target deregulation or mutation that causes escape from therapy and cross–resistance to the subsequent drug. **(B)** Cancer therapy promotes evolutionary dynamics fostering mutations, protein or pathway activity deregulations, and changes in gene expression states that can determine cross–resistance to the next treatment.

multiple DDR-related genes (29). In support of these findings, the DNA repair capacity measured in peripheral lymphocytes is an independent predictor of survival for non-small cell lung cancer (NSCLC) patients treated with platinum-based chemotherapy (30) and the inhibition of DNA repair kinases could also prevent doxorubicin resistance in BC cells (31). Furthermore, DDR pathways can be enhanced in cancer cells providing a survival advantage after chemotherapy (32).

HER2- and Estrogen Receptor-Targeted Therapies

HER2 is a member of the Epidermal Growth Factor Receptor (EGFR) family of receptor tyrosine kinases. HER2 amplification and/or overexpression have been described in BC (20% of cases) and in a variety of other solid tumors, including gastric cancer (GC, 20%), biliary tract cancer (BTC, 20%), bladder cancer (BlC, 12.5%), colorectal cancer (CRC, 5%) and NSCLC (2.5%) (33). Although HER2 is an established therapeutic target in a subset of women with BC, the early HER2-targeted therapies have not proven to be as effective in HER2-positive (HER2+) GC or other solid tumors.

Since the approval of trastuzumab, the first anti-HER2 agent (34) for BC treatment in 1998, an array of other anti-HER2 agents, such as pertuzumab, lapatinib, T-DM1, and trastuzumab-Deruxtecan (T-Dxd) mAb-drug conjugates (ADCs) and others have been approved, significantly improving the outcome of BC patients (**Figure 2**). In addition, a widening arsenal of novel HER2-targeting drugs are under development (35). Anti-HER2 treatments are administered in neoadjuvant, adjuvant, and advanced settings of BC patients. However, there is a growing body of evidence suggesting that HER2-targeted treatment may significantly influence the loss/reduction of HER2 expression (36–47). Mittendorf and colleagues have described the loss of HER2 amplification in residual disease in 32% of BC patients treated with neoadjuvant trastuzumab in combination with anthracyclines and taxanes, as this change is associated with poor recurrence-free survival (43). In a retrospective cohort study involving 21,755 Japanese BC patients, loss of HER2 was observed in 20.4% following neoadjuvant trastuzumab (44). In the advanced setting, Ignatov and colleagues have shown that loss of HER2 is associated with previous HER2-targeted treatment and reduced disease-free survival. Interestingly, a change in HER2 expression was observed in

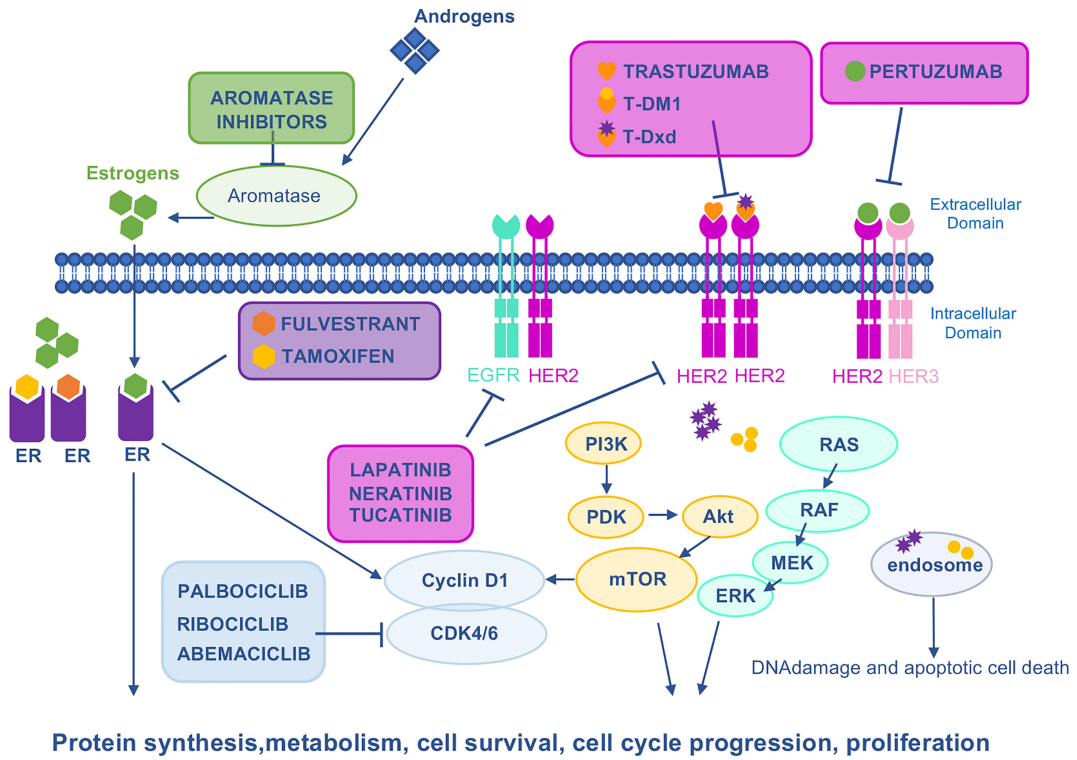

FIGURE 2 | Mechanism of action of HER2–, ER–, and CDK4/6–targeted drugs. HER family RTKs (EGFR, HER2, and HER3) activate several oncogenic signaling pathways such as Ras/Raf/MEK/ERK or PI3K/Akt/mTOR to stimulate growth and proliferation. Direct HER2 inhibitors include trastuzumab and the conjugates of trastuzumab with DM1 (T–DM1) or Dxd (T–Dxd). In the case of drug–antibody conjugates, upon binding of trastuzumab to HER2, the payload is internalized by endocytosis to induce DNA damage. Pertuzumab mAb binds HER2, preventing homodimerization and heterodimerization with other family members, especially HER3. Lapatinib is a EGFR/HER2 TKI that attenuates cell proliferation, cell–cycle regulation, and downstream pathways. Tucatinib is a selective HER2 TKI with minimal inhibition of EGFR. Neratinib is a pan–HER irreversible TKI. ER is a transcription factor which, under estrogen stimulation, is recruited on the promoter of its target genes to induce cell proliferation. Aromatase inhibitors prevent the aromatase–dependent conversion of androgens to estrogens, whereas fulvestrant and tamoxifen are both anti–estrogens that counteract the effects of estrogen by directly binding to the ER. CDK4 and CDK6 form complexes with CyclinD1 to stimulate proliferation. Palbociclib, ribociclib, and abemaciclib are CDK4/6 small molecule inhibitors. CDK 4/6, cyclin–dependent kinases 4/6; DM1, derivative of maytansine 1; Dxd, deruxtecan; EGFR, epidermal growth factor receptor; ER, estrogen receptor; ERK, extracellular–signal regulated kinase; HER2, human epidermal growth factor 2; HER3, human epidermal growth factor 3; MEK, mitogen–activated protein kinase kinase; mTOR, mammalian target of rapamycin; PDK, phosphatidylinositol–dependent kinase; PI3K, phosphoinositide 3–kinase; RAF, rapidly accelerated fibrosarcoma; RAS, RAS proto–oncogene.

47.3% of trastuzumab-treated patients and in 63.2% of trastuzumab plus pertuzumab-treated ones (46). In concordance, reduced T-DM1 efficacy has been described in HER2+ advanced BC patients previously treated with dual HER2 blockade by trastuzumab plus pertuzumab combination as compared to trastuzumab alone (47–51). At the molecular level, a marked reduction of HER2 expression on cell membrane and HER2 nuclear translocation have been shown to account for cross-resistance between trastuzumab plus pertuzumab and T-DM1 (47). In agreement with reduced expression of HER2 on trastuzumab plus pertuzumab rather than its loss, T-Dxd showed a remarkable improvement in progression-free survival (PFS) vs T-DM1 in second-line treatment for previously treated BC patients (preliminary results from DESTINY Breast 03 trial, The Asco Post, posted 9/19/21). This striking result is probably due to the unique linker-payload system of T-Dxd, that contributes to its preclinical efficacy against tumors with low HER2 expression (52).

Another possible explanation for cross-resistance among subsequent HER2-targeted drugs is represented by the clonal evolution under the selective pressure of treatments. In this case, based on tumor heterogeneity, trastuzumab or other HER2-targeting drugs preferentially eradicate HER2+ clonal populations selecting the HER2-negative ones, that in turn emerge and drive tumor progression (41, 42, 44).

Similar cross-resistance has been reported between other HER2-targeting agents. Neratinib is an irreversible HER2 TKI approved for adjuvant treatment of HER2+/estrogen receptor-positive (ER+) early BC following adjuvant-trastuzumab-based therapy, and, in combination with capecitabine, for HER2+ metastatic BC patients who have received two or more prior anti-HER2-based regimens in the metastatic setting. Evidence from a pre-clinical model of neratinib-resistant BC cell lines indicates cross-resistance to trastuzumab and lapatinib. This cross-resistance is bi-directional, as lapatinib- and

trastuzumab-resistant cells are also cross-resistant to neratinib (53). In agreement, in phase II studies, drug-naïve patients responded better to neratinib than patients previously treated with trastuzumab (54) or with lapatinib (55).

Although the incidence of HER2+ disease in patients with GC is similar to that observed in patients with BC, the success rate achieved in BC with several HER2-targeted therapies has not yet been observed in GC. This might be explained by biological differences among these tumor types, such as the pattern of expression of HER2, or the higher degree of intratumoral heterogeneity of HER2 expression in GC compared to BC (56). Nevertheless, based on data from the ToGA trial, the combination of chemotherapy plus trastuzumab represents the standard of care for first-line treatment of HER2+ advanced GC (57). By contrast, HER2-targeted ADCs explored in the second-line setting showed promising results; in January 2021, based on the robust data from DESTINY-Gastric01 phase II trial, the U.S. FDA approved T-Dxd ADC for patients with metastatic GC who have received a prior trastuzumab-based regimen (58). Although the introduction of T-Dxd has represented an important step forward, the benefit in this setting was much higher in patients with a HER2 score of 3+ on immunohistochemical analysis (IHC), while a lower response rate was observed in patients with a 2+ score with positive results on *in situ* hybridization (58% vs 29%) (58, 59). It is worth noting that in this study the HER2 status was evaluated using archival tissue specimens and thus the HER2 status immediately prior to T-Dxd administration had not been investigated. Indeed, similarly to what has been described in BC, loss of HER2 expression after trastuzumab treatment has been reported in patients with HER2+ advanced GC (60–63).

Another mechanism of cross-resistance among HER-2 targeted therapies involves the emergence of the *HER2 L755S* variant after therapy. L755S is an activating mutation of *HER2* accounting for 60% of *HER2* mutations found in metastatic BCs (64). Recent studies have described the emergence of *HER2 L755S* under the pressure of lapatinib and trastuzumab that results in cross-resistance to other single agents or combination HER2-targeted therapy, both in the pre-clinical and patient-derived models (65, 66). Similarly, no significant response to trastuzumab has been observed in HER2+ metastatic BC patients whose tumors harbor *HER2* mutations (67). In supporting the association of *HER2* mutations with trastuzumab resistance, the frequency of acquired *HER2* mutations in patients with advanced BC after trastuzumab treatment is much higher compared to patients with early-stage tumors, and an enrichment of *HER2* mutations in metastatic lesions from patients undergoing adjuvant trastuzumab has been reported (64, 68).

HER2 mutations account for cross-resistance also in HER2 non-amplified BC patients. In BC patients, about 70% of *HER2* mutations have been found in metastatic ER+/HER2 non-amplified tumors, suggesting that the emergence of *HER2* mutations may represent a mechanism of acquired resistance to endocrine therapy (69). In line with this, Nayar and colleagues described the appearance of *HER2* mutations in metastatic lesions from eight ER+ BC patients under the selective pressure of ER-directed aromatase inhibitors, tamoxifen, or fulvestrant. An *in vitro* analysis showed that *HER2* mutations confer estrogen independence and resistance to tamoxifen, fulvestrant, and to the Cycline Dependent Kinase 4 (CDK4)/ Cycline Dependent Kinase 6 (CDK6) inhibitor palbociclib, which was overcome by combining ER-therapy with the HER2-inhibitor neratinib (70). Overall, these data indicate that acquired *HER2* mutations account for cross resistance in *i*) HER2+ BC patients treated with HER2-targeting agents, where they are potentially useful biomarkers of trastuzumab/lapatinib resistance in subsequent lines of treatments; *ii*) HER2- BC patients treated with endocrine therapy.

Table 1 summarizes the cross-resistance events described between sequential HER2-targeted therapies and ER-targeted therapies and between ER-targeted agents and the CDK4/ CDK6 inhibitor Palbociclib.

CD4/6 Inhibitors

The clinical management of ER+ BC (mainly Luminal A and Luminal B) includes endocrine therapy (ER downregulators, selective ER modulators, and aromatase inhibitors) as primary treatment, albeit luminal B tumors are mainly treated with chemotherapy due to lower sensitivity to endocrine therapy (71). However, resistance to endocrine therapy has been shown to be dependent on the Cyclin D-CDK4/6 pathway (72). On this basis, three CDK4/6 inhibitors, namely palbociclib (73), ribociclib (74), and abemaciclib (75) have been FDA approved in combination with endocrine therapy for the first- or second-line treatment of ER+ HER2- advanced BC (**Figure 2**). In an *in vitro* model of ER+ HER2- BC cell lines, cross-resistance among different CDKis has been reported, but not between CDK inhibition and chemotherapeutic agents (76) (**Table 2**). Loss or dysregulation of Retinoblastoma-associated Protein 1 (RB1) have been demonstrated to emerge under selective pressure from CDK4/6 inhibitors potentially conferring therapeutic resistance (77, 78). Whether continuing a CDK4/6 inhibitor beyond progression may prove to be an effective strategy is currently being tested by several ongoing phase I and II trials (MAINTAIN NCT02632045, PACE NCT03147287, NCT01857193, NCT 02871791, and TRINITI-1 NCT 02732119).

Recently, clinical cross-resistance mediated by PTEN loss has been shown between CDK4/6 inhibitors and alpelisib, an alpha-specific PI3K inhibitor (PI3Ki) recently approved for the treatment of PIK3CA-mutated ER+ advanced BC that progressed on previous endocrine therapy (79, 80) (**Table 2**). Costa and colleagues demonstrated that loss of Phosphatase and Tensin Homolog (PTEN) promotes translocation of p27 outside the nucleus by raising AKT activity, which in turn increases CDK4/6 activity, ultimately overcoming the blockade of CDK4/6. PTEN loss had been shown to cause resistance to PhosphatidylInositol 3-Kinase (PI3K) inhibition in previous studies (81, 82).

EGFR-Targeted Therapies

EGFR overexpression has been reported in diverse tumor types including head and neck, ovarian and cervical cancers, Bladder Cancer and CRC, where it has been associated with poor

TABLE 1 | Cross–resistance in HER2– and ER–targeted therapies.

Previous agent	Subsequent agent	Ref.	Type of study	Proposed mechanism	Supporting literature
Trastuzumab +pertuzumab	T–DM1	*Bon G*, *J Exp Clin Cancer Res 2020* (47) *Vici*, *Oncotarget 2017* (48) *Noda–Narita S, Breast Cancer 2019* (50) *Pizzuti L*, *Ther Adv Med Oncol 2021* (51)	*In vitro* BT474 and SkBr3 BC resistant cell model + Observational Observational Observational Observational	Reduction of membrane HER2 expression; HER2 nuclear translocation	*Burstein HJ, J Clin Oncol 2003* (36) *Pectasides D, Anticancer Res 2006* (37) *Hurley J, J Clin Oncol 2006* (38) *Harris LN, Clin Cancer Res 2007* (39) *van de Ven S, Cancer Treat Rev 2011* (40) *Niikura N, Ann Oncol 2016* (41) *Mittendorf EA, Clin Cancer Res 2009* (43) *Gahlaut R, Eur J Cancer 2016* (45) *Ignatov T, Breast Cancer Res and Treat 2019* (46) *Pietrantonio F, Int J Cancer 2016* (60) *Saeki H, Eur J Cancer 2018* (61) *Seo S, Gastric Cancer 2019* (62) *Kijima T, Anticancer Res 2020* (63)
Neratinib	Trastuzumab Lapatinib	*Breslin S*, *British J Cancer 2017* (53)	*In vitro* cell model	Increased cytochrome CYP3A4 activity *bidirectional*	*Burstein HJ, J Clin Oncol 2010* (54) *Awada A, Ann Oncol* (55)
Lapatinib	Trastuzumab	*Cocco E*, *Sci Signal 2018* (66)	*In vitro* BT474 and SkBr3 BC resistant cell model + patient analysis	Emergence of HER2 L755S mutation *bidirectional*	*Zuo WJ, Clin. Cancer Res 2016* (64) *Xu X, Clin Cancer Res 2017* (65) *Boulbes DR, Mol Oncol 2015* (67) *Yi Z, Breast Cancer 2020* (68)
Aromatase inhibitors Tamoxifen Fulvestrant	Tamoxifen Fulvestrant Palcociclib	*Nayar U*, *Nat genetics 2019* (70)	*In vitro HER2*–mutated T47D and MCF7 BC cell model + patient analysis	Emergence of HER2 L755S, V777L, L869A, and S653C mutation	*Croessmann S, Clin Cancer Res 2019* (69)

TABLE 2 | Cross–resistance in CDK4/6–targeted therapies.

Previous agent	Subsequent agent	Ref.	Type of study	Proposed mechanism	Supporting literature
Palbociclib	Abemaciclib	Ogata R, *Breast Cancer* 2021 (70)	*In vitro* MCF7 and KPL4 BC resistant cell model	Downregulated retinoblastoma protein RB. Hypothethical	*Condorelli R, Ann Oncol. 2018* (71) *Pandey K, Int J Cancer 2019* (78)
Ribociclib	Alpelisib	*Costa C, Cancer Discov 2020* (72)	Patient analysis + CRISPR PTEN KO T47D and MCF7 BC cell and mouse model	Loss of PTEN, that results in p27 exclusion from the nucleus and increased activation of CDK2 and CDK4	Razavi P, *Nat Cancer* 2020 (73) Juric D, Nature 2015 (74)

outcomes and prognosis (83). Furthermore, driver *EGFR* activating mutations are common in NSCLC (84) and occur in 3% of CRC (85). For these reasons EGFR became a popular therapeutic target; both EGFR-targeted mAbs and TKIs demonstrated efficacy in large phase III clinical trials and were approved for treating lung, colorectal and head and neck cancers.

EGFR-specific first-generation (gefitinib and erlotinib) or second-generation (afatinib and dacomitinib) TKIs were developed for treatment of patients with metastatic, *EGFR*-mutated NSCLC (86). Given that up to 60% of patients progressing on TKIs acquire the secondary *EGFR T790M* mutation (87), the third generation irreversible EGFR TKI osimertinib was developed which demonstrated clinical activity in *T790M* patients who had progressed on previous TKIs (**Figure 3**). Recently, based on results from the FLAURA trial showing OS benefit over first-generation TKIs, upfront use of osimertinib became the standard of care (88).

Concomitantly with the introduction of osimertinib in the clinical practice, cross-resistance has been reported between gefitinib and irreversible EGFR-TKIs in human lung cancer cells (89). (**Table 3**) Mechanistically, in a gefitinib-resistant cell model, *Kelch Like ECH Associated Protein 1* (*KEAP1)* gene mutation disrupts the KEAP1-Nuclear factor erythroid 2-Related Factor 2 (NRF2) oncogenic signaling pathway leading to constitutive activation of NRF2, cell proliferation, and resistance to gefitinib as well as cross-resistance to afatinib and osimertinib. Somatic mutations in the *NFE2L2* (encoding NRF2) and *KEAP1* genes have been described in 23% of patients with lung adenocarcinoma (LAC) (84) and are usually mutually exclusive. Mutations in the KEAP1-NRF2 pathway have been associated with worse clinical outcomes and earlier disease progression to chemotherapy in LAC patients (90). More importantly, the emergence of KEAP1 loss/NRF2 activation has been reported as a mechanism of acquired resistance to

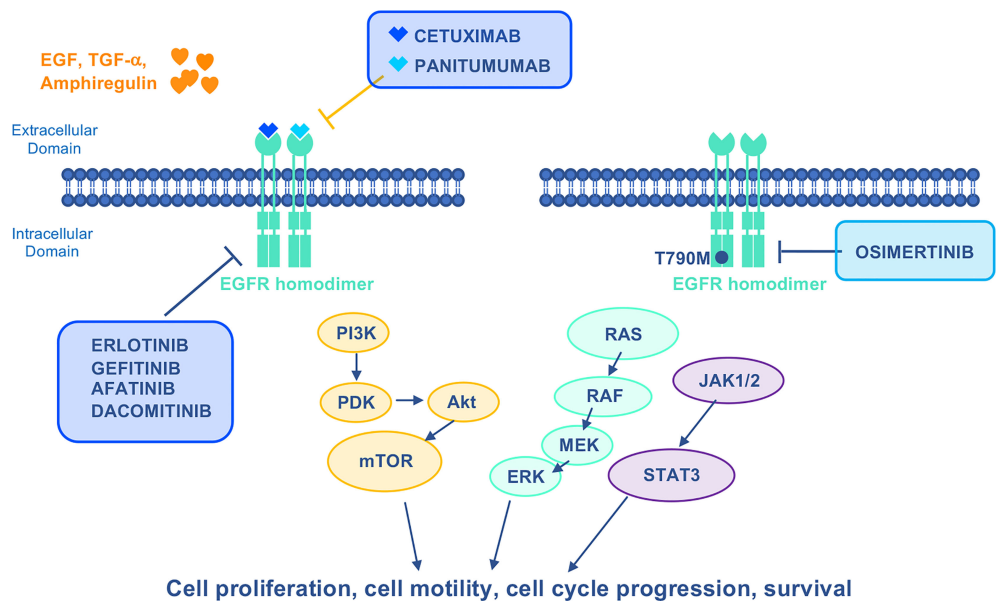

FIGURE 3 | Mechanism of action of EGFR–targeted drugs. EGFR activates Ras/Raf/MEK/ERK, PI3K/Akt/mTOR, and JACK1/2/STAT3 oncogenic signaling pathways to stimulate growth and proliferation. Cetuximab and Panitumumab are mAbs that specifically inhibit EGFR. First–generation reversible (gefitinib and erlotinib) and second–generation irreversible (afatinib and dacomitinib) TKIs were developed to target mutant EGFR. The third generation irreversible TKI Osimertinib is highly selective for EGFR– activating mutations as well as the EGFR T790M mutation. EGF, epidermal growth factor; JACK1/2, janus kinases 1/2; STAT3, signal transducer and activator of transcription 3; TGF–α, transforming growth factor alpha.

TABLE 3 | Cross–resistance in EGFR–targeted therapies.

Previous agent	Subsequent agent	Ref.	Type of study	Proposed mechanism	Supporting literature
Gefitinib	Afatinib Osimertinib	*Park SH, FASEB J. 2018* (89)	*In vitro* HCC827 NSCLC resistant cell model + *In vivo* resistant–NSCLC mouse model	*KEAP1* mutation leading to constitutive activation of NRF2	*Krall EB, Elife 2017* (91) *Yamadori T, Oncogene 2012* (92) *Yu HA, Clin Cancer Res 2018* (93) *Hellyer JA, Lung Cancer 2019* (94) *Foggetti G, Cancer Discov 2021* (95)
Cetuximab	Panitumumab	*Arena S, Clin Cancer Res. 2015* (98)	*In vitro* CRC resistant cell model and *EGFR*–mutated CRC cell model + patient analysis	Emergence of *EGFR* S464L, G465R, and I491M mutations	*Van Emburgh BO, Nat Commun 2016* (99) *Misale S, Cancer Discov 2014* (100) *Montagut C, Nature Med 2012* (101)
Cetuximab Panitumumab	Cetuximab Panitumumab	*Diaz LA Jr, Nature 2012* (102)	Patients' sera analysis	Emergence of *KRAS* mutations, indirect evidence. Presumably bidirectional	*Peeters M, Eur J Cancer 2015* (105) *De Roock W, Lancet Oncol 2015* (106)
		Misale S, Nature 2012 (103)	*In vitro* DiFi and Lim1215 CRC resistant cell model+ patient analysis		
		Van Cutsem E, J Clin Oncol 2011 (104)	Phase III clinical trial		

EGFR-TKIs in *EGFR*-mutated LAC cells (91, 92) and patients (93). Furthermore, patients with *KEAP1-NFE2L2*-mutant tumors have shorter recurrence-free interval on treatment with EGFR TKI (94) and KEAP1 inactivation reduces the sensitivity of EGFR-driven tumors to osimertinib in an EGFR-driven Trp53-deficient LAC mouse model (95). Overall, these results suggest the involvement of *KEAP1-NFE2L2* genetic alterations in cross-resistance occurring between first-generation and third-generation irreversible EGFR TKIs, that has been shown to be overcome with the introduction of osimertinib as first-line treatment. The post-osimertinib treatment options for EGFR-mutated NSCLC including innovative drugs or combination therapies are under investigation in ongoing clinical trials (96).

Cetuximab and panitumumab EGFR-targeted mAbs have been approved in combination with chemotherapy for the first-line treatment of Kirsten RAt Sarcoma (KRAS) wt CRC (**Figure 3**). They can also be administered as monotherapy upon progression following prior chemotherapeutic regimens. Despite clinical benefits obtained in CRC by combining EGFR-targeted mAbs and chemotherapy, this has been shown to last 8-10 months due to drug resistance (97). Multiple *EGFR* and *RAS* mutations were among the mechanisms of resistance reported (98, 99). *EGFR* acquired mutations preferentially occur in the extracellular domain, which impair antibody-binding (100). Among the different specific mutations identified in cetuximab-resistant CRC patients, some proved to be permissive for panitumumab binding, whereas others determined cross-resistance (98, 101). The emergence of *RAS* mutations induced by anti-EGFR therapies has been reported in approximately 50% of patients with RASwt CRC and is responsible for acquired resistance to cetuximab (102, 103) (**Table 3**). *RAS* mutations can result in constitutive activation of RAS-associated signaling that renders anti-EGFR therapies ineffective for CRC. Consistent with this, the predictive role of *RAS* mutations in the clinical responses of CRC to anti-EGFR therapies has been demonstrated in several pivotal studies (104, 106).

Androgen Receptor-Targeted Therapies

Prostate cancer (PC) is the most common cancer in men and is dependent on the Androgen Receptor (AR) signaling for its growth and progression (107). For this reason, androgen deprivation represents the gold standard first-line treatment for PC patients. Progression is due to transition from a hormone sensitive stage to castration resistant disease (CRPC) (108). Over the past decade, multiple treatment options have demonstrated clinical efficacy in metastatic hormone sensitive PC (mHSPC), non-metastatic CRPC (nmCRPC) and metastatic CRPC (mCRPC) (109). The development of novel, highly potent AR signaling inhibitors (ARSIs) such as enzalutamide and abiraterone acetate (FDA approved in 2012, and 2018 respectively) (**Figure 4**) has represented a major step towards more efficient inhibition of AR signaling and conferred survival benefit in mCRPC and nmCRPC patients (110). Taxanes represent the other class of current treatments for CRPC.

More recently, ARSIs have also been approved in hormone-sensitive disease (111–113). With the adoption of ARSIs in early disease, cross-resistance to sequential ARSI treatment has rapidly emerged as a limitation in the sequential use of AR-targeted therapies (110), however the optimal sequence of available ARSIs and taxane-based chemotherapy have not yet been defined (114). Data from pre-clinical models of abiraterone acetate- and enzalutamide-resistant CRPC confirmed cross-resistance among ARSIs (115, 116) and showed cross-resistance between ARSIs and docetaxel but not carbazitaxel (117, 118) (**Table 4**).

Mechanistically, cross-resistance among enzalutamide and abiraterone acetate is mainly caused by the re-activation of AR pathway by the emergence of AR constitutively active splice variants. Zhao and colleagues demonstrated the involvement of the AR splice-variant 7 (AR-V7) and identified a Aldo-Keto Reductase family 1 member C3 (AKR1C3)/AR-V7 axis, in which AKR1C3 plays a dual function: first, it catalyzes androgen synthesis; second, it binds AR-V7 promoting its stabilization (116, 119). These data indicate that the AKR1C3/AR-V7 axis plays critical roles in cross-resistance between enzalutamide and

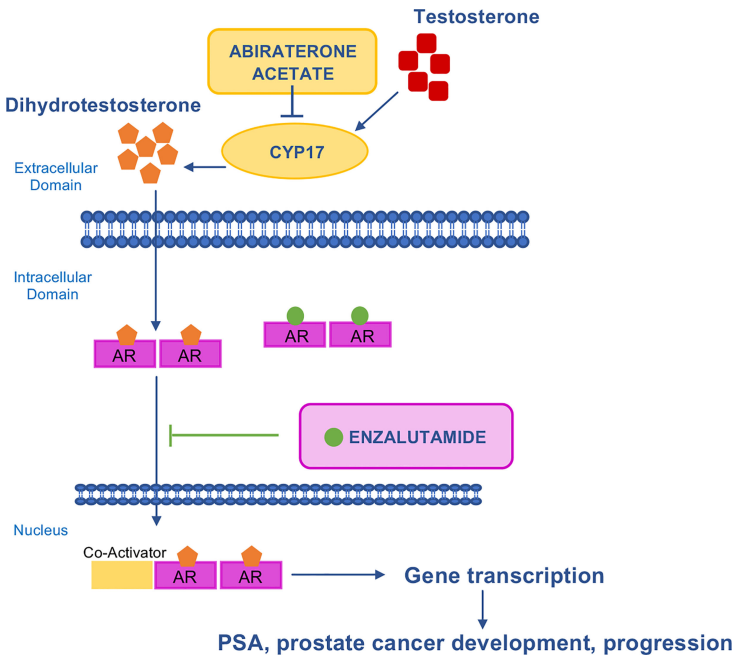

FIGURE 4 | Mechanism of action of AR–targeted drugs. AR is a transcription factor that is activated by androgenic hormones binding. Upon activation, AR translocates into the nucleus where it activates the transcription of genes involved in cancer development and progression. Abiraterone acetate inhibits CYP17, the enzyme responsible for the conversion of testosterone to dihydrotestosterone. Enzalutamide is a potent, competitive binder of androgens at the AR. It prevents the translocation of the AR from the cytoplasm to the nucleus. AR, androgen receptor; CYP17, 17 α–hydroxilase/C17,20–lyase; PSA, prostate–specific antigen.

TABLE 4 | Cross–resistance in AR–targeted therapies.

Previous agent	Subsequent agent	Ref.	Type of study	Proposed mechanism	Supporting literature
Enzalutamide	Abiraterone	*Lombard AP, Mol Cancer Ther 2018* (115)	*In vitro* CRPC resistant cell model	Emergence of constitutively active AR variants Bidirectional	*Liu C, Mol Cancer Ther 2019* (119) *Antonarakis ES, J Clin Oncol 2017* (121)
Enzalutamide Abiraterone	Apalutamide Darolutamide	*Zhao J, Mol Cancer Ther 2020* (116)	*In vitro* CRPC resistant cell model	Activation of the axis AKR1C3/AR–V7 constitutively active variant	*Guo Z, Cancer Res 2009* (123) *Azad AA, Clin Cancer Res 2015* (124) *Joseph JD, Cancer Discov 2013* (125) *Antonarakis ES, N Engl J Med 2014* (129)
Enzalutamide	Docetaxel	*van Soest RJ, Eur J Cancer 2013* (117)	*In vitro* PC346C resistant and HEP3B PC cell model	Overlapping mechanism of action (inhibition of AR nuclear translocation)	*Mezynski J, Ann Oncol 2012* (126) *Schweizer MT, Eur Urol 2014* (127) *van Soest RJ Eur. Urol 2015* (128)

abiraterone acetate. In addition, patients treated with enzalutamide or abiraterone acetate showed inferior OS and PFS if they were AR-V7 positive rather than AR-V7 negative (120, 121). On the other hand, AR splice variants do not affect sensitivity to chemotherapy: similar overall survival (OS) and PFS were observed in AR-V7 positive and negative patients receiving taxanes (122). Accordingly, AR alterations including gene aberrations and constitutively active splice variants arising from prolonged ARSIs treatment have been widely implicated in the development of resistance to ARSIs (110, 116, 120, 123–125).

The efficacy of chemotherapy after ARSIs treatment has been investigated in multiple retrospective studies. Overall, clinical evidence showed reduced efficacy of docetaxel in CRPC patients previously treated with enzalutamide or abiraterone acetate (126, 127). Mechanistically, inhibition of AR nuclear translocation may be implicated in cross-resistance as a common mechanism of action of AR-targeting agents and docetaxel (117). Conversely, cabazitaxel efficacy is not affected by prior ARSIs treatment (128).

Moreover, based on clinical evidence, it is widely recognized that enzalutamide administration after abiraterone acetate is of

greater clinical benefit than *vice versa* (129, 130), whereas the CARD trial showed that switching to taxane chemotherapy is preferred after ARSI failure (130).

MAPK Inhibitors

Genetic alterations affecting the RAS-RAF-MEK-ERK (Mitogen-Activated Protein Kinase, MAPK) pathway occur in approximately 40% of all human cancers. Mutations in the proto-oncogene *BRAF* and *RAS* family genes (*KRAS* and *NRAS*) are quite frequent in melanoma, CRC, anaplastic thyroid cancer (ATC) and LAC, whilst alterations affecting genes encoding MEK and ERK have rarely been identified (131, 132). For these reasons, targeting of the aberrantly activated MAPK pathway is one of the most explored therapeutic approaches in cancer. Among different neoplasms, melanoma mostly benefited from MAPK-targeted therapy. However, despite the survival advantages observed with BRAF-targeted drugs versus chemotherapy, many melanoma patients progressed within 6-7 months (133, 134), mainly due to ERK re-activation (135). Based on clinical evidence from large clinical trials (136–138), the current therapeutic strategy combines BRAF and MEK inhibition, including three FDA approved combinations for the treatment of metastatic BRAF-mut melanoma: dabrafenib plus trametinib, vemurafenib plus cobimetinib, and encorafenib plus binimetinib (**Figure 5**). Moreover, dabrafenib plus trametinib combination has been approved for the treatment of metastatic *BRAF*-mutated NSCLC and metastatic/unresectable *BRAF*-mutated ATC.

More recently, immunotherapies with checkpoint blockade Abs directed against PD-1 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) have revolutionized the treatment of patients with metastatic cancer including melanoma (139) (**Figure 5**). Even though the optimal sequence of targeted therapy and immunotherapy for the treatment of patients with *BRAF*-mutated melanoma is still under investigation in clinical trials [DREAMseq (NCT02224781) and SECOMBIT (NCT02631447)], currently the American Society of Clinical Oncology and the European Society of Medical Oncology guidelines recommend both therapies as first-line treatment for metastatic melanoma (140, 141). Due to the lack of mechanistic knowledge indicating the best first-line therapy to adopt, many centers treat these patients with targeted therapy first, and then switch them to immunotherapy on progression. However, patients who relapse on MAPK inhibition show a lower overall response rate (ORR) to immunotherapy compared with MAPKi naïve patients (142–144). In line with this, melanomas with acquired resistance to MAPK inhibitors show CD8 T-cell deficiency/exhaustion and loss of antigen presentation functions, which suggests cross-resistance to anti-PD1/Programmed Death-Ligand 1 (PD-L1) immunotherapy (145–147). More recently, a cancer cell-instructed, immunosuppressive tumor microenvironment lacking functional CD103$^+$ dendritic cells that preclude an effective T cell response has been described in melanoma patients and mouse models (148). This mechanism is involved in the cross-resistance between MAPK inhibitors and subsequent immunotherapies (**Tables 4**, **5**). Mechanistically,

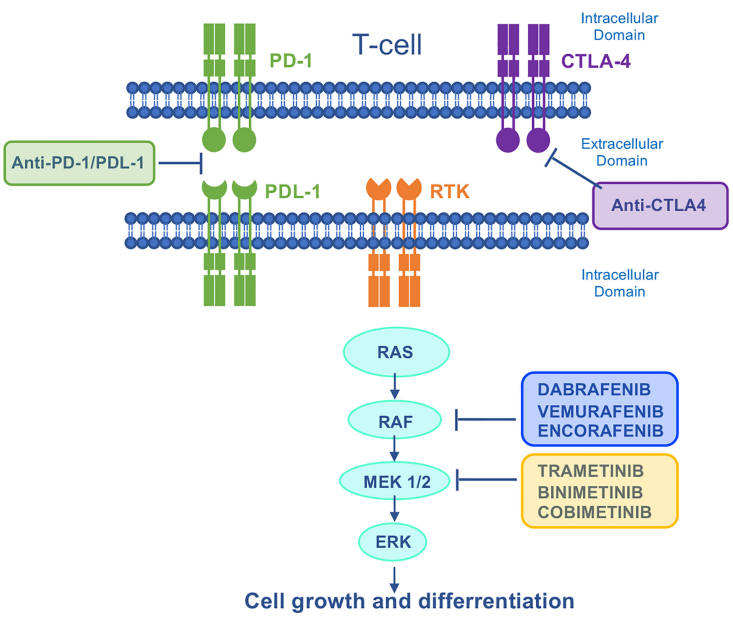

FIGURE 5 | Mechanism of action of MAPK–targeted drugs and immunotherapies. The Ras/Raf/MEK/ERK signaling pathway is activated by several upstream receptor tyrosine kinases. Dabrafenib, vemurafenib and encorafenib are specific BRAF–inhibitors used in the treatment of BRAF–mutant melanoma. In a strategy to vertically target the MAPK signaling pathway, they are used in combination with trametinib, cobimetinib, and binimetinib respectively. Immune checkpoint blockade inhibits the negative regulation of T cell activation, thereby unleashing antitumor T–cell responses. CTLA4, cytotoxic T–lymphocyte antigen 4; PD1, programmed cell death protein 1; PDL–1, programmed death ligand 1; RTK, receptor tyrosine kinase.

TABLE 5 | Cross–resistance in MAPK–targeted therapies.

Previous agent	Subsequent agent	Ref.	Type of study	Proposed mechanism	Supporting literature
Dabrafenib Dabrafenib +trametinib	Anti–PD1 Anti–CTLA4	*Haas L, Nat Cancer 2021* (148)	RAFi and RAFi/MEKi resistant melanoma mouse model + Patient analysis	Reprogramming of MAPK transcriptional output driving immunosuppressive microenvironment that lacks functional $CD103^+$ dendritic cells	*Ackerman A, Cancer 2014* (142) *Johnson DB, J. Immunother 2017* (143) *Tétu P, Eur J Cancer 2018* (144) *Mason R, Pigment Cell Melanoma Res 2019* (145) *Hugo W, Cell 2015* (146) *Pieper N, Oncoimmunology 2018* (147)
Vemurafenib	Dacarbazine	*Erdmann S, Sci Rep 2019* (149)	Patient–derived resistant melanoma cell model	Reactivation of MAPK pathway and enhanced activation of PI3K/AKT signalling	
Dabrafenib Vemurafenib	Radiotherapy	*Shannan B, Eur J Cancer 2019* (151)	Patient–derived melanoma cell model + Observational	Enrichment of H3K4 demethylase JAR–ID1B/KDM5B, that regulates the transcription of genes favoring cell survival	

patients displaying MAPK re-activation who progress on dual BRAF/MEKi, also exhibits an enhanced transcriptional output driving immune evasion.

Another noteworthy cross-resistance event between unrelated drugs that deserves mention, has been reported between the BRAF inhibitor vemurafenib and dacarbazine chemotherapeutic in a patient-derived *BRAF*-mutated melanoma cell model (149) (**Tables 4**, **5**). In this case, dacarbazine-resistant cells re-activate the MAPK pathway by autocrine IL-8 cytokine, thereby sustaining cross-resistance to vemurafenib. By contrast, desensitization of vemurafenib-resistant cells to dacarbazine is mediated by enhanced AKT serine/threonine kinase signaling.

Brain metastases affect approximately 50% of stage IV melanoma patients requiring the combination of MAPK inhibition or immunotherapy with radiotherapy protocols (150). Cross-resistance between combined MAPK inhibition and radiotherapy has also been observed (**Tables 4**, **5**), but the extent may vary depending on the treatment sequence (151). Shannan and colleagues reported a higher rate of tumor relapse in preclinical cell models that were first treated with BRAF inhibition followed by radiotherapy compared to the reverse sequence. At the molecular level, the histone H3K4 demethylase JARID1B/KDM5B is more frequently upregulated following BRAF inhibition and predicts cross-resistance towards radiotherapy.

REAL-WORLD DATA AS A TOOL TO IDENTIFY CROSS-RESISTANCE

It is increasingly evident that, due to the recent rapid drug development, pivotal clinical trials might not have explored the full spectrum of the cancer population. A significant proportion of cancer patients cannot be enrolled in clinical trials due to stringent exclusion criteria, even though they are still treated in clinical practice (152). Conversely, patients enrolled in clinical trials exploring (for instance) a second-line treatment could not have necessarily received current first-line treatments. Consequently, there is an unmet medical need for additional clinical practice information when choosing the optimal sequence of new anticancer agents. RWD can potentially address this knowledge gap by providing a good deal of information concerning specific drug scheduling.

RWD is referred to data collected from sources outside of conventional research settings, including electronic health records, administrative claims, tumor registries, daily clinical routine (153), and information related to disease status, treatments and their sequence, safety, concomitant medications, comorbidities, or to cancer patient population not extensively enclosed in randomized clinical trials (RCTs). As such, RWD is gaining increasing interest for the potential to provide additional evidence that can complement and support the data from RCTs.

RWD has significantly contributed to highlighting many cross-resistance events. These include the reduction in T-DM1 activity observed in BC patients previously treated with dual HER2 blockade by pertuzumab plus trastuzumab, as discussed in detail above. Not only did observational studies in the real-world setting (47–51) highlight cross-resistance but they also revealed another critical issue: due to concomitant approval of pertuzumab and T-DM1, none of the patients enrolled in the EMILIA and Th3resa trials (T-DM1 registrative studies) had previously received pertuzumab. Consequently, at the time of T-DM1 approval, clinical data on its efficacy in pertuzumab-pretreated patients was lacking.

Cross-resistance among ARSIs and between ARSIs and taxane-based chemotherapy in PC have been extensively addressed in this review. Recently, a systematic review (154) has explored optimal treatment sequencing of abiraterone acetate and enzalutamide in chemotherapy-naïve mCRPC patients. The analysis was conducted with RWD from 17 observational studies and showed a favorable trend in outcomes and cost effectiveness for the sequence abiraterone acetate-enzalutamide compared to enzalutamide-abiraterone acetate.

In addition, RWD contributed in highlighting cross-resistance between ARSIs enzalutamide and abiraterone acetate (155) and suggested a reduced efficacy of sequential ARSI treatment in chemotherapy pretreated patients.

Another relevant contribution deriving from RWD was the demonstration of lower efficacy of immunotherapy in BRAF-mutated metastatic melanoma patients relapsing on MAPK inhibition compared with MAPKi naïve patients (144).

Finally, the real-world experience in *EGFR*-mutated NSCLC demonstrated that sequential afatinib and osimertinib was beneficial in prolonging the chemotherapy-free interval in patients with T790M acquired resistance (156).

One challenging and unresolved issue relates to patients affected by advanced hepatocarcinoma (HCC). The first-line treatment of these patients is represented by the combination of anti-VEGF bevacizumb and anti-PD-L1 atezolizumab mAbs, that showed significant OS benefit over the multikinase inhibitor (MKI) sorafenib previously used in this setting, which was thereby approved by the FDA in 2020 (157). Data on efficacy and safety were subsequently confirmed by RWD analyses (158). Many therapeutic options are available for the second-line treatment of these patients, including a MKI, mAbs, or anti-PD1 agents such as cabozantinib, ramucirumab, nivolumab with or without the anti-CTLA4 ipilimumab (159). At present, the optimal second-line treatment has not yet been defined. An observational retrospective study reported comparable efficacy of second-line sorafenib and lenvatinib (160). Data from the real-world setting could help define the optimal sequence of treatments.

STRATEGIES TO OVERCOME CROSS-RESISTANCE

Molecular Re-Evaluation of Recurrences as a Strategy to Refine Clinical Trials Design

The loss of target on HER2-targeted therapy is a widely recognized issue that has been discussed above for both BC (36–47) and GC (60–62). Provided that the knowledge of the underlying mechanisms is of paramount relevance, this evidence offers the opportunity to reconsider the strategies behind the design of RCTs. Indeed, many of these studies investigate the efficacy of new therapeutic approaches in metastatic/recurring patients stratified based on the molecular features of primary tumors. Interpreting the results generated from these trials could lead to sub-optimal clinical decision-making.

An emblematic example of this is the failure of the randomized phase II study WJOG7112G (T-ACT). The aim of the study was to explore the efficacy of paclitaxel with or without trastuzumab in 99 patients with HER2+ advanced GC who had disease progression after first-line chemotherapy with trastuzumab. Median PFS and OS were not significantly different between the two groups. In this case, loss of HER2 has been reported as a possible explanation for failure. Indeed, when HER2 status was re-evaluated in tumor biopsy specimens from 16 patients following disease progression, HER2 loss was observed in 11 patients (69%) (161).

In the specific case of HER2-targeted therapy, re-evaluating the HER2 status at the time of disease progression would be required (43). Supporting this, an ongoing phase II, open-label, single arm trial aimed at evaluating the efficacy and safety of T-Dxd in Western GC patients progressed with a trastuzumab-containing regimen (DESTINY-Gastric02, NCT04014075) required patients to be re-tested for HER2 positivity before being treated with T-Dxd.

The Darwinian selection hypothesis assumes that cancer therapy selects pre-existing mutant cells that overtake the bulk cell population. However, this is a simplified mechanism that does not account for therapy resistance alone. In a more complicated scenario, genetic alterations and changes in the gene expression state often emerge under the selective pressure exerted by the therapy itself, fueling the increasingly aggressive behavior of recurring tumors (162).

On this basis, a molecular re-evaluation of patient recurrences is of paramount importance in order to identify subsets of patients to be included in RCTs where unfortunately re-biopsy is not feasible in most cases. Recently, minimally invasive liquid biopsy for the selection of patients for targeted therapies has demonstrated equivalent clinical utility to that of invasive tumor tissue testing (163). The analyses of cell-free tumor DNA (ctDNA) allows a much more rapid identification of actionable mutations compared to tissue profiling. Specifically, the ctDNA analysis has been exploited to show the acquisition of specific mutations on emerging resistance to targeted therapy (164). Currently, a few FDA diagnostic tests have been developed to provide tumor mutation profiling on NSCLC, BC, and ovarian cancer. These tests have been used to select patients for targeted therapy in the advanced setting. At the moment, global efforts aim to obtain standardized procedures for liquid biopsy tests in order to allow their rapid implementation into clinical practice. The future use of this high-potential tool will rapidly help match patients for clinical trials as well as for proper clinical decision-making.

Identification of Collateral Sensitivities

The emergence of evolutionary dynamics (165, 166) and nongenetic reprogramming of TME (167) in therapy resistance provide a field of action for possible subsequent therapy. Interestingly, available pre-clinical and clinical evidence indicate cases of collateral sensitivities that are novel, exploiting vulnerabilities emerging concurrently with therapy resistance.

In the current scenario where most patients are still treated with traditional chemotherapy, several cases of collateral sensitivities between chemotherapeutic agents have been reported. Pre-clinical and clinical evidence suggest that cisplatin resistance can result in sensitivity to paclitaxel, and vice-versa (168, 169). Despite the underlying mechanism remaining unknown, combining the two drugs has been proven to be effective in lung, ovarian, skin, breast, and head and neck tumors (170). Similarly, vinblastine-resistant cell lines are sensitive to paclitaxel, and vice-versa (171). In this case, the two drugs exert opposing mechanisms of action (vinblastine destabilizes microtubles while paclitaxel stabilizes microtubles); resistance can stem from stabilizing (vinblastine) or destabilizing (paclitaxel) mutations in α- and β- tubulin.

In the context of targeted therapies, the first collateral sensitivity network was provided by Dhawan and colleagues in 2017. In an attempt to characterize collateral sensitivities to several TKIs in Anaplastic Lymphome Kinase (ALK)-positive

NSCLC, they found that cell lines resistant to first-line TKIs are often sensitized to the chemotherapeutic drugs etoposide and pemetrexed (172). More recently, the same authors showed that resistance to chemotherapy in Ewing's sarcoma cell lines is associated with sensitivity to the histone demethylase 1 inhibitor SP-2509 (173).

These findings have fueled further exploration in pre-clinical models, consequently expanding our knowledge in this field. Melanoma cells which developed resistance to MAPKi showed enhanced susceptibility to platinum-based drugs such as cisplatin and carboplatin, that is inversely correlated with the expression level of the p53 family member TAp73. Mechanistically, low TAp73 expression level results in reduced efficacy of the nuclear excision repair system and enhanced sensitivity towards platinum-based cytostatic agents (174). Similarly, resistance to BRAF/MEK inhibitors is associated with increased levels of reactive oxygen species and enhanced efficacy of the histone deacetylase (HDAC) inhibitor vorinostat in resistant cell and mouse models, as well as in patients (175). Accordingly, a pilot study in patients demonstrated that treating BRAF inhibitor-resistant melanoma patients with HDAC inhibitors killed the drug-resistant cell population (175).

In EGFR-mutant LUAD cells, acquired resistance in response to EGFR inhibitors requires Aurora Kinase A activity, and is therefore associated with increased sensitivity to Aurora kinase inhibitors (176).

In the context of BC, HER2 mutations, resulting in cross-resistance between HER2-targeted therapies, are associated with higher efficacy of some irreversible HER2 TKIs such as neratinib and pyrotinib both in HER2-amplified (65, 66) and HER2 non-amplified (177, 178) BC. In a panel of 115 cancer cell lines, neratinib was the most effective against HER2-mutant cell lines among HER2-targeted TKIs (179). The phase II SUMMIT trial concluded that neratinib in combination with fulvestrant is clinically active in heavily pretreated HER2-mutant HR+ BC patients (180). Thus, HER2 mutations might be predictor of benefit from Neratinib TKi. By employing a cell-model and 3D *ex vivo* organotypic culture model, Singh and colleagues showed that a high level of the detoxifying enzyme Sulfotransferase Family 1A Member 1 (SULT1A1) confers resistance to Tamoxifen and collateral sensitivity to the anticancer compounds with SULT1A1-dependent activity RITA (Reactivation of p53 and Induction of Tumor Cell Apoptosis), aminoflavone (AF), and oncrasin-1 (ONC-1) (181).

In pancreatic ductal adenocarcinoma (PDAC) patient-derived organoids, chemotherapy-induced vulnerabilities were investigated that highlighted increased sensitivity to MEK inhibition, driven by tumor plasticity in response to chemotherapy regimen FOLFIRINOX (combination therapy with Folinic Acid, fluorouracil, irinotecan, and oxaliplatin) (182). In this case, therapeutic vulnerabilities were identified by unbiased drug screening experiments and did not seem to be associated with a specific genetic marker. This is a significant indication that molecular deregulations alone may not account for collateral sensitivities, and an additional functional layer is needed for precision oncology. Similarly, some of these studies suggested the involvement of rapidly changing gene expression regulations in the response to drugs rather than providing specific mechanisms for collateral sensitivities.

On the other hand, it is worth considering that our current knowledge of potentially therapeutically targetable dependencies is still limited and recurrently mutated genes account for this burden only partially (183). New emerging categories of cancer targets that include cell-autonomous and tumor microenvironment (TME)-mediated targets, are likely to result in the development of novel targeted agents and thereby novel therapeutic options in the near future.

In this scenario, identifying predictive biomarkers to stratify patients who would likely benefit from cancer therapies is currently an active field of investigation. In this regard, it is expected that many categories of drug-induced deregulation may be considered, spanning from genetic/epigenetic deregulations to nonmutational, functional alterations.

Investigation of Rational Mechanistic-Based Cancer Treatment Regimens

One strategy used to overcome resistance to targeted therapies is represented by combination therapy simultaneously blocking parallel or alternative pathways activated in cancer cells. However, due to the complexity of signaling networks, efficient screening for effective targeted combination therapies is a challenging issue, which is further complicated by the need to address clinically relevant doses and dosing schedules that can impact the emergence and evolution of resistance.

Mathematical modeling represents a reasonable tool for testing clinically relevant drug combinations prior to investment in clinical trials. Branching process models had been used to study resistance to chemotherapy in tumor cell populations as early as in the 1980s (184). Since then, other groups exploited mathematical modeling to characterize drug resistance and investigate potential effective schedules in order to minimize the development of acquired resistance (185, 186). More recently, a computational modeling platform and software package have been developed for identifying optimum dosing for combination treatments of oncogene-driven cancers (187).

In addition, refining doses and scheduling in combination therapy is of paramount importance in order to reduce the emergence of resistance and cross-resistance. Currently, some rational combination strategies are under investigation which have the potential to reach this goal, thereby improving cancer therapy.

One of these strategies is represented by multiple low-dose treatment. So far, the vast majority of novel cancer drugs are developed as single agent therapies and are delivered to patients at a maximum tolerated dose. In case of drug combinations, it is generally believed that each drug should be used according to the same criteria. However, recent available evidence indicates that multiple low-dose treatment can be effective: in EGFR-mutant lung cancer, vertical targeting of EGFR signaling pathway with three or four drugs can be effective even when the drugs are used at 20% of the single agent concentration (188). Similarly, dual RAF/ERK low-dose was effective in KRAS-mutant cancers (189). In the specific case of vertical targeting of multiple nodes of a signaling pathway, the adoption of a low-dose regimen reduces the selective pressure on these nodes and the eventual emergence of resistance mutation.

Sequential drug treatment is conceptually based on the induction of a major vulnerability by the first drug, that is

targeted by a second drug to kill tumor cells. According to this principle, sequential, but not simultaneous, treatment of triple-negative BC cells with EGFR inhibitors and DNA-damaging drugs results in efficient cell killing (190). In metastatic BC patients, pretreatment with cisplatin and doxorubicin resulted in enhanced responses to anti PD-1 therapy (191). Also, sequential drug treatment for combination immunotherapies is supported by preclinical data (192).

Parallel to studies of drug scheduling, drug holidays, or metronomic therapy, has also been proposed as a strategy to limit the development of resistance in cancer treatment (193, 194). It is conceptually based on the principle that upon removal of therapy, cancer cells do not need to develop advantageous adaptations that drive resistance. From a molecular point of view, this effect can be achieved by reversible adaptation (194) or mutation–independent phenotypical variations (195). In preclinical models of melanoma, intermittent dosing with BRAF inhibitors results in delayed emergence of resistance as compared to continuous dosing (196). However, conflicting results derived from clinical data indicating that intermittent dosing is inferior to continuous administration, highlighted that careful attention must be paid when translating dosing and treatment schedules from preclinical models to humans (197).

Overall, these efforts are intended to lay a solid mechanistic basis for drug combination regimens and avoid clinical trials investigating combination treatments without a rational basis.

CONCLUDING REMARKS

The emergence of drug resistance has proven to be a major obstacle from the first available cancer chemotherapies available right up to the latest, rapidly developing targeted therapies. Next–generation sequencing and computational data analysis approaches have revealed that genomic instability sustains tumor heterogeneity which allows human cancers to escape from therapies and develop resistance. An increasing number of therapeutic possibilities available entails further levels of complexity and cross–resistance to secondary or subsequent therapies can occur, impacting on patient outcomes and survival rates.

The emergence of cross–resistance among drugs acting on a shared target may occur. In response to the first specific agent, threatened cancer cells acquire deregulation or mutation to the target guaranteeing not only escape from therapy, but also cross–resistance to a secondary drug acting on the same target. The reversible/irreversible nature of target deregulation deserves further investigation. It has been reported that the time interval between consecutive HER2–targeted therapies in BC may play a key role in cross–resistance, as HER2 downregulation is associated with a shorter interval between the last HER2–targeted agent administered and the time of HER2 assessment (45). At the moment, we do not know whether a reversible loss of HER2 may be induced by HER2–targeting agents and to what extent the reversible (internalization/nuclear translocation) and irreversible (clonal selection) loss of HER2 could impact the efficacy of subsequent therapy.

Many cross–resistance events have been reported between therapies that exert different modes of action. Highly representative of current practice is the recent cross–resistance reported between dual BRAF/MEK inhibition and subsequent immunotherapies. One fundamental point with this finding is the acquisition of cross–resistance during MAPKi treatment, questioning once again the hypothesis of clonal selection of resistant cells pre–existing before therapy.

It is critical to decipher the underlying mechanism(s) of cross–resistance in order to overcome it. To this aim, a powerful tool is represented by recent studies that exploit complex preclinical cell models including not only primary tumor cells, but also cells from fibroblastic, vascular, and immune compartments. These models resemble the tumor heterogeneity and the contribution of TME and immune compartments to cross–resistance dynamics which are typically observed *in vivo* (198, 199) and therefore represent an ideal tool for investigating new vulnerabilities.

Accordingly, the conceptual design behind RCTs needs to swiftly and adequately incorporate the growing knowledge of cancer evolution in response to therapy. Experience from past RCTs indicates an urgent need to reconsider the molecular landscape of recurring tumors and exploit newly acquired targetable vulnerabilities for making more effective therapeutic decisions.

AUTHOR CONTRIBUTIONS

GB, RL, PV, and FL were involved in manuscript drafting. GB, SS, and MM–S revised the manuscript. All authors contributed to the article and approved the submitted version.

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Uterine perivascular epithelioid tumors (PEComas) with lung metastasis showed good responses to mTOR and VEGFR inhibitors: A case report

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Perivascular epithelioid cell tumors (PEComas) are extremely rare mesenchymal neoplasms for which the uterus is the most common site. The prognosis of malignant PEComa is poor as it is characterized by resistance to classical chemotherapies. Both mTOR inhibitors and VEGFR inhibitors exhibited clinical utility in treating malignant PEComas, but the combination of these two regimens has rarely been reported. In the present case, a uterine PEComa patient developed lung and bone metastases after the failure of chemotherapies and derived benefit from the combination regimen of an mTOR inhibitor (everolimus) and a VEGFR inhibitor (apatinib), achieving a 15-month progression-free survival. Targeted NGS revealed *TP53* and *TSC2* mutations in the patient's primary uterine tumors and plasma ctDNA at disease progression. Plasma ctDNA clearance was consistent with a radiologic partial response determined by RECIST 1.1 and a reduction of neuron-specific enolase (NSE) and cancer antigen 125 (CA125) levels. Thus, we provided clinical evidence supporting the administration of combined therapy of mTOR and VEGFR inhibitors to metastatic uterine PEComa patients and highlighted the application of serial plasma ctDNA profiling for dynamic disease monitoring.

KEYWORDS

uterine PEComa, everolimus, apatinib, *TSC2*, lung metastasis

Introduction

Perivascular epithelioid cell tumors (PEComas) are rare mesenchymal neoplasms containing epithelioid cells with a perivascular distribution and are characterized by immunophenotypic features of smooth muscle and melanocytic differentiation (1). The main members of PEComas include angiomyolipoma (AML) and pulmonary lymphangioleiomyomatosis (LAM), which are often characterized by a benign clinical course and are observed at high frequencies in patients with tuberous sclerosis complex (TSC). While the presence of aggressive PEComas is usually accompanied by locally invasive recurrences or distant metastases, a gynecologic-specific algorithm has been proposed to classify malignant PEComas, including the following atypical features: size ≥5 cm, high-grade atypia, mitoses >1/50 high-power fields (HPF), necrosis, and vascular invasion (2).

Studies of the genetic changes in PEComas revealed a high incidence of *TSC1* or *TSC2* alterations, which constitutively activated the mTOR pathway and promoted translational initiation and cell growth (3, 4). mTOR inhibitors, including sirolimus, everolimus, and temsirolimus, were used in malignant PEComa patients with clinical benefits (5–7). At present, multiple clinical trials are actively investigating the clinical benefit of mTOR inhibitors including everolimus and sirolimus in advanced solid tumors with inactivating *TSC1* or *TSC2* mutation (NCT02352844, NCT02201212, and NCT05103358). In addition, *TFE3* rearrangements were reported in some cases with wild-type *TSC1/2*, indicating that alternative pathways of tumorigenesis exist and that alternative treatment strategies are needed (8, 9). Other gene alterations such as *ATRX* mutations, *RB1* deletions, and the amplification of *FGFR3*, *NTRK1*, and *ERBB3* were also detected by targeted massively parallel sequencing (10). Except a clinical trial evaluating the benefit of erdafitinib in patients carrying *FGFR3* gene amplification, more effort needs to be made to develop effective therapy targeting other mentioned genomic aberrations.

The management of malignant PEComas is challenging, and systemic chemotherapy has shown little efficacy in retrospective studies (11–13). The response to VEGFR inhibitors has also been suboptimal, with very low objective response rates (ORR) (8.3%) (11). Data on the combination of mTOR and VEGFR inhibitors for the treatment of malignant PEComas are limited, but one case reported a remarkable response for the treatment of a uterine PEComa patient with kidney and lung metastases using sirolimus and sorafenib (14). Herein, we present a malignant uterine PEComa patient who developed lung and bone metastases after the failure of chemotherapy but responded well to the combined therapy of mTOR and VEGFR inhibitors, with a 15-month progression-free survival (PFS).

Case presentation

A 47-year-old woman without a genetic family history or past diseases presented with lower abdominal pain in September 2017. The color Doppler ultrasound revealed a mass in the right side of the uterus, which was surgically removed (Figure 1A). Immunohistochemistry (IHC) examinations of the resected tumor tissues were positive for melanoma antigen (Melan-A, 90%) and negative for human melanoma black (HMB45), smooth muscle actin (SMA), S-100, desmin, Myo-D1, synaptophysin (Syn), and creatine kinase (CK). Ki67 labeling in the tumor cells was 20% (Figure 1B). Necrosis and vascular invasion were observed. Based on the histological and IHC results, the patient was diagnosed with stage Ib malignant uterine PEComa, without metastasis. Four cycles of postoperative adjuvant chemotherapy with epirubicin (90 mg on d1) and cyclophosphamide (2 g on d1–4) were administrated, but obvious side effects with grade 3 myelosuppression were observed. In March 2018, the patient received intraperitoneal chemotherapy with cisplatin (80 mg) and sodium bicarbonate (150 ml), but the disease progressed rapidly with the development of lung and bone metastases within 2 months (Figure 1C). The levels of neuron-specific enolase (NSE) and cancer antigen 125 (CA125) were 38.52 and 21.2 U/ml, respectively (Figure 2). To identify a more efficient therapeutic strategy, freshly collected plasma and formalin-fixed, paraffin-embedded (FFPE) primary uterine tumor tissues were subjected to targeted next-generation sequencing (NGS) of over 400 cancer-related genes (Supplementary Methods). As shown in Table 1, the plasma ctDNA exhibited *TP53* (R273P) and *TSC2* (P1497H) mutations, compared to the primary tumor sample, while copy number variants of *ZNF703*, *FGFR3*, *FLT4*, and *RB1* were only detected in the primary tumor. A combined treatment of apatinib (250 mg, once a day) and the mTOR inhibitor everolimus (10 mg, once a day) was administrated in May 2018. Plasma ctDNA sequencing was performed every 3 months until progressive disease (PD), as well as measurements of NSE and CA125. The patient achieved a partial response (PR) after 3 and 6 months of apatinib and everolimus treatment, after which the plasma ctDNA was still tested negative for genomic alterations and the levels of NSE and CA125 were dramatically decreased (Figure 2). Stable disease (SD) was observed in February and May 2019, with the positive detection of ctDNA alterations in plasma samples; however, the allele frequencies (AFs) were relatively low (Table 1). Additionally, the levels of NSE and CA125 were slightly but continuously increased after 9 and 12 months of combined treatment (Figure 2). After 15 months of apatinib and everolimus treatment, the disease progressed with the detection of high-AF *TP53* and *TSC2* mutations, as well as an acquired *ARID1B* (G169R) mutation (Table 1). The NSE and CA125 levels were also dramatically elevated. Grade 1–2 nausea

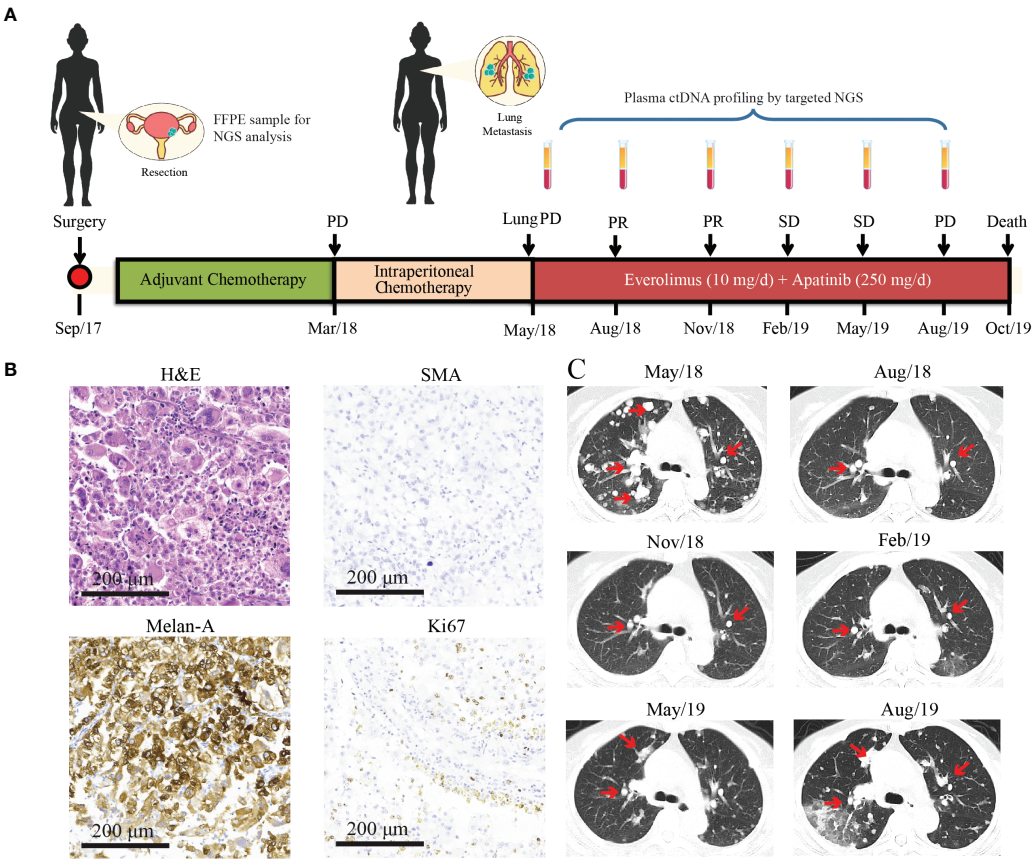

FIGURE 1
Treatment history and clinical information of the presented case. **(A)** The medical history of the presented case is shown with information about treatment timeline, response evaluation, and sample collection timepoints. During the combination treatment with everolimus and apatinib, plasma ctDNA sequencing was performed every 3 months along with treatment response evaluation as indicated by the arrowheads. **(B)** H&E staining and immunohistochemical (IHC) examinations (×200) of the primary uterine PEComa which was negative for the SMA marker and positive for Melan-A (90%). The Ki67 index is 20%. **(C)** CT images of lung metastases during everolimus and apatinib treatment. Lesions are indicated by the red arrows. PR, partial response; SD, stable disease; PD, progressive disease; NGS, next-generation sequencing; PEComa, perivascular epithelioid cell tumors; ctDNA, circulating tumor DNA; FFPE, formalin-fixed, paraffin-embedded; H&E, hematoxylin and eosin; SMA, smooth muscle actin.

and rash were reported during the combined treatment, and the patient died of a respiratory failure in October 2019.

Discussion

PEComas are rare, and the metastatic sites of malignant PEComas usually include the gastrointestinal tract, lung, retroperitoneum, uterus, and somatic soft tissues (15, 16). The uterus is the most common site of PEComas, but the uterine PEComa presented in this case was negative for the HMB45 marker, which is extremely rare. HMB45 is considered as the most reliable IHC marker for identifying PEComas, with over 95% exhibiting a positive expression (17). However, this case revealed an HMB45-negative profile, suggesting that the diagnosis of PEComas should be based on histological and IHC examinations.

Radical resection is the primary treatment option for uterine PEComas, as they are typically resistant to radiation and chemotherapy. A retrospective study (11) showed an ORR of 13% for anthracycline-based chemotherapy in advanced PEComa patients whose median PFS was 3.2 months. Similarly, that study (11) also showed that the ORR and median PFS in a gemcitabine-based chemotherapy subgroup were 20% and 3.4 months, respectively. In the current case, neither postoperative adjuvant chemotherapy nor intraperitoneal chemotherapy provided optimal outcomes.

Considering the frequent detection of *TSC1/2* loss-of-function alterations as causing the activation of the mTOR signaling pathway (18), treatment with mTOR inhibitors exhibited clinical benefits to malignant PEComa patients,

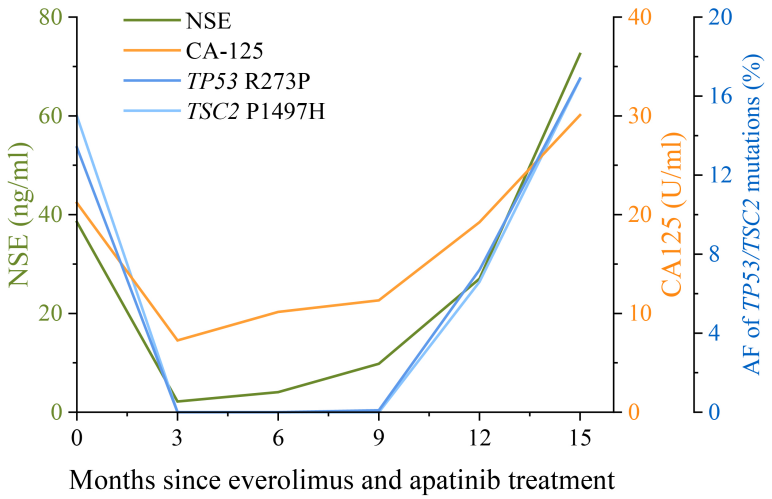

FIGURE 2
Changes in NSE and CA125 levels, and the allele frequencies (AFs) of *TP53* and *TSC2* mutations during apatinib and everolimus treatment. The levels of the lung cancer biomarkers NSE (neuron-specific enolase) and CA125 (cancer antigen 125) in serum examined every 3 months are shown by the green and orange lines, respectively. Plasma ctDNA sequencing was also performed every 3 months during apatinib and everolimus treatment. The AFs of *TP53* R273P (blue) and *TSC2* P1497H (light blue) mutations are shown by the dark blue and light blue lines, respectively. The units of NSE, CA125, and AF were indicated by the different y-axes.

which were first reported in 2010 (6). Subsequently, the application of mTOR inhibitors in patients with malignant PEComas was demonstrated in additional studies. The efficacy of mTOR inhibitors was better than that of classical chemotherapies, with an ORR of 41% and a 9-month median PFS (11, 19). In the current case, targeted NGS detected a *TSC2* P1497H mutation in the primary uterine PEComa and the plasma ctDNA collected after the occurrence of metastases. Although the clinical significance of this missense mutation remains unknown, we hypothesized that the *TSC2* P1497H mutation might affect the function of TSC2 and further activate the mTOR signaling pathway as the patient benefited from everolimus treatment. However, additional clinical data are needed to support this single-case observation.

Antiangiogenic VEGFR inhibitors also exhibited clinical responses in PEComa patients, but mainly in stabilizing disease in patients with malignant PEComas (ORR = 8.3%, median PFS = 5.4 months) (11, 20). The combination of the VEGFR inhibitor, sorafenib, with the mTOR inhibitor, sirolimus, led to a complete response in a uterine PEComa case reported in 2016; however, the patient's molecular features were not discussed in the study (14). In the current case, the combined use of the VEGFR inhibitor, apatinib, and the mTOR inhibitor, everolimus, led to the best PR (PFS = 15 months).

TABLE 1 The allele frequencies of genetic alterations detected by targeted NGS in the primary PEComa tumor and serial plasma ctDNA.

Gene	Alteration	Primary PEComa (FFPE)	Plasma ctDNA(months since everolimus and apatinib treatment)					
			0	3	6	9	12	15
TP53	R273P	30.27%	13.41%	–	–	0.10%	7.20%	16.89%
TSC2	P1497H	30.88%	15%	–	–	–	6.62%	16.87%
ZNF703	CNV	2.9-fold	–	–	–	–	–	–
FGFR3	CNV	2.6-fold	–	–	–	–	–	–
FLT4	CNV	2.5-fold	–	–	–	–	–	–
RB1	CNV	single-copy loss	–	–	–	–	–	–
ATRX	T1545fs	–	9.10%	–	–	–	5.44%	12.83%
ARID1B	G169R	–	–	–	–	–	–	54.83%

FFPE, formalin-fixed, paraffin-embedded; "-", not detected; CNV, copy number variant; ctDNA, circulating tumor DNA
the allele frequency (AF) of the ctDNA mutation was 0.5%. As the TP53 (R273P) mutation was detected in the primary tissue and the first plasma sample had a high AF, a 0.1% mutation AF is reported.

In the present case, we also demonstrated the utility of NGS for treatment decision making and response monitoring. Besides the common *TP53* and *TSC2* mutations, amplification of *ZNF703*, *FLT4*, and *FGFR3* was also detected in the primary uterine tumor. The overexpression of ZNF703 was reported to activate the Akt/mTOR signaling pathway in breast cancer cells (21). The consequence of *ZNF703* amplification in PEComas remains to be determined, but it might also contribute to the response to everolimus in this case. *FGFR3* is a predictive biomarker for use of erdafitinib in patients, but no effective therapies target other mentioned genomic aberrations in the presented case. After the failure of chemotherapy, the plasma ctDNA exhibited an *ATRX* frameshift mutation. Additionally, at the time of progression on the combined therapy (everolimus + apatinib), another *ARID1B* mutation was detected. These acquired mutations may inspire the investigations of the resistance to chemotherapy and mTOR inhibitors in PEComa patients, although no studies have reported an association between these acquired mutations and the specific treatments. The differences in genetic alterations between primary and metastatic samples also suggested tumor evolution, which may assist in changing therapeutic strategies. In addition, we also found that serial ctDNA profiling during treatment could forecast disease progression earlier than CT scanning (22). The increase in the mutational AF of plasma ctDNA was observed prior to image-confirmed progression and also displayed a similar trend as the changes in NSE and CA125 levels. NSE is a reliable tumor marker in several cancers, especially in patients with neuroblastoma or small cell lung cancer (23). Similarly, CA-125 is widely used to identify early signs of ovarian cancer (24). Thus, the changes in NSE and CA-125 levels can also assist with disease monitoring in uterine PEComa patients with lung metastases.

The limitation of the single-case presentation in this study should also be noted. Thus, the efficacy and the side effects of the combined treatment with mTOR and VEGFR inhibitors must be further evaluated in larger cohorts. The missense mutation of *TSC2* (P1497H) in this case might be a potential target of mTOR inhibitors; however, additional preclinical studies and additional clinical evidence are needed.

Conclusion

In summary, we reported a patient with a rare uterine PEComa who harbored a *TSC2* P1497H mutation and received a combined treatment with apatinib and everolimus after chemotherapy failed. The patient's metastatic lung lesions were stable for 15 months, and serial plasma ctDNA profiling and profiling using the serum tumor markers, NSE and CA125, facilitated disease monitoring. This case detailed a reliable treatment option for rare uterine PEComas with distant metastases and highlighted the importance of longitudinal ctDNA profiling during 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.

Ethics statement

This research was approved by the Ethics Committee of The Second Hospital of Dalian Medical University (Approval ID: DMU2021139). Written informed consent to publish the clinical details and images were obtained from the patient.

Author contributions

All authors contributed to data analysis and drafting or revising of the manuscript. All authors agreed on the journal to which the article is submitted, provided final approval of the manuscript version to be published, and agreed to be accountable for all aspects of the study.

Conflict of interest

Authors EP, PY, TW, YM, and QO are employed by Nanjing Geneseeq Technology Inc.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2022.797275/full#supplementary-material

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Cyclin-dependent kinase inhibitors in malignant hematopoiesis

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The cell-cycle is a tightly orchestrated process where sequential steps guarantee cellular growth linked to a correct DNA replication. The entire cell division is controlled by cyclin-dependent kinases (CDKs). CDK activation is balanced by the activating cyclins and CDK inhibitors whose correct expression, accumulation and degradation schedule the time-flow through the cell cycle phases. Dysregulation of the cell cycle regulatory proteins causes the loss of a controlled cell division and is inevitably linked to neoplastic transformation. Due to their function as cell-cycle brakes, CDK inhibitors are considered as tumor suppressors. The CDK inhibitors $p16^{INK4a}$ and $p15^{INK4b}$ are among the most frequently altered genes in cancer, including hematopoietic malignancies. Aberrant cell cycle regulation in hematopoietic stem cells (HSCs) bears severe consequences on hematopoiesis and provokes hematological disorders with a broad array of symptoms. In this review, we focus on the importance and prevalence of deregulated CDK inhibitors in hematological malignancies.

KEYWORDS

cyclin-dependent kinase inhibitors, hematopoiesis, hematopoietic diseases, INK4 family, Cip/Kip family

1 Introduction

Cell-cycle progression is a fundamental biological process which requires tight regulation to guarantee a correct cell division. Perturbations of cell cycle components may provoke an uncontrolled cell proliferation. Dysregulated G1-S transition is a common feature of tumor development and associated with genetic alterations of key regulators of the cell-cycle machinery (1). Based on their function as a cell cycle brake, CDK inhibitors (CKIs) mainly act as tumor suppressors and are frequently deactivated in human neoplasia (2–4).

2 CKIs regulate the cell cycle

Cyclin-dependent kinases (CDKs), their activating cyclins and CDK inhibitors guide cells through the cell cycle (Figure 1). Distinct cyclins are periodically produced and assemble to cyclin-CDK complexes that drive the specific cell-cycle steps, from G1 to M phase. Fine tuning is achieved by inhibitory phosphorylation or binding of CDK inhibitory subunits (CKIs) (5–7).

Cyclin-dependent kinase 4 (CDK4) and CDK6 are closely related serine/threonine kinases responsible for driving cells through the G1 phase. Mitogenic signals induce transcription of D-type cyclins (D1, D2 and D3). Their association with CDK4 and CDK6 leads to kinase activation and phosphorylation of the retinoblastoma protein (Rb) (8). CDK-dependent Rb phosphorylation releases Rb from E2F transcription factors and induces transcription of E2F target genes required for S-phase entry (9). G1-S transition is then initiated by CDK2-cyclin E/A complexes, which are active during the entire S-phase (10–12). CDK1 activity is low during G1/S transition but raises during G2-M phase, controlling the initiation of mitosis (13, 14).

CDK-cyclin activity is counterbalanced by members of the two CDK inhibitor families, the INK4 family and the Cip/Kip family (8). $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ are the members of the INK4 family and are specific for CDK4 and CDK6 (15). In response to anti-proliferative signals, INK4 proteins are transcribed and bind CDK4 and CDK6 causing a conformational change which reduces their affinity for D-type cyclins (16).

The Cip/Kip family consists of $p21^{Cip1/Waf}$, $p27^{Kip1}$ and $p57^{Kip2}$. In contrast to INK4 proteins,

Cip/Kip proteins have the ability to bind CDK4/6-cyclin D and CDK-cyclin A/B/E complexes (8, 16–19). $p21^{Cip1/Waf}$ and $p27^{Kip1}$ are described to have a dual function in cell cycle regulation. Whereas they mainly inhibit CDK-cyclin activity they have been reported to also enhance the assembly of CDK4/6-cyclin D complexes, resulting in a proliferative advantage for the cell (18, 20, 21).

When present at low levels, $p21^{Cip1/Waf}$ preferentially binds to CDK4/6-cyclin D complexes, facilitating complex formation, nuclear localization and cell-cycle progression. In response to DNA damage and p53 stimulation, $p21^{Cip1/Waf}$ accumulates at high levels in a cell and provokes a robust cell cycle arrest by inhibiting CDK2- cyclin E-A complexes (8, 22–25). The mechanism behind these observations is given by *in vitro* experiments showing that changes in $p21^{Cip1/Waf}$ stoichiometry reflect the conversion of active to inactive cyclin-CDK complexes. Active complexes contain a single $p21^{Cip1/Waf}$ molecule, while two molecules are required for complex inhibition (26, 27).

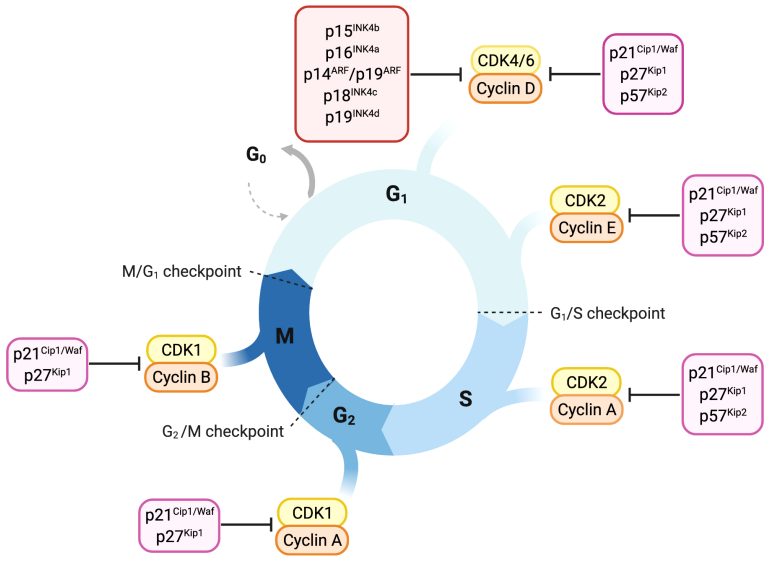

FIGURE 1
Overview of cell-cycle control and its main regulators. Progression through cell cycle phases is governed by different CDK-cyclin complexes and the respective cyclin-dependent kinase inhibitors. Members of the INK4 family, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, specifically bind and inhibit CDK4/6-cyclin D complexes promoting cell cycle arrest in the G1 phase. The Cip/Kip proteins including $p21^{Cip1/Waf}$, $p27^{kip1}$ and $p57^{Kip2}$, play their role as cell-cycle inhibitors by counteracting a broader spectrum of CDK-cyclin complexes. $p21^{Cip1/Waf}$, $p27^{kip1}$ and $p57^{Kip2}$ restrain cell-cycle both during early and late G1 phase by binding either CDK4/6-cyclin D or CDK2-cyclin E complexes. Later in the cell-cycle, they can bind and inhibit CDK2-cyclin A complex, thus imposing a brake during the S-phase. $p21^{Cip1/Waf}$ and $p27^{kip1}$ are able to delay entry in the M phase by inhibiting CDK1-cyclin A complex and thereby prevent the progression through mitosis counteracting CDK1-cyclin B complex.

This double-faced role has been described also for $p27^{Kip1}$. On the one hand, $p27^{Kip1}$ binds to the conserved cyclin box residues thus promoting the subsequent complex formation between $p27^{Kip1}$-cyclin A and CDK2. Upon complex formation, $p27^{Kip1}$ induces a distortion on the CDK2 N-terminal lobe in proximity of CDK2 catalytic site, thereby preventing ATP binding. On the other hand, phosphorylated $p27^{Kip1}$ binds to CDK4 leading to a remodeling of the ATP site and results in increased RB phosphorylation. Data suggest a similar mechanism for $p21^{Cip1/Waf}$ activating CDK4 *via* phosphorylation sites (28).

$p57^{Kip2}$ mainly functions during G1-S and G2-M transitions where it blocks any CDK-cyclin complexes. No cell cycle activating mechanisms have been described yet.

The Cip/Kip members, $p57^{Kip2}$ and $p21^{Cip1/Waf}$ are major players in cellular stress responses, where they balance the induction of cell cycle arrest, apoptosis and senescence (29). $p21^{Cip1/Waf}$ has a unique role as it mediates cell cycle arrest downstream of the tumor suppressor p53 (22). A variety of cellular stresses, such as DNA damage and oncogene activation, stimulate p53 expression, which in turn transactivates its targets including the pro-apoptotic genes Bax, PUMA and Noxa as well as $p21^{Cip1/Waf}$ (30–32). Therefore, $p21^{Cip1/Waf}$ might be an exploitable candidate for therapeutic intervention in p53 mutated tumors.

3 CKIs in hematopoietic stem cells

Under homeostatic conditions, hematopoietic stem cells (HSCs) reside in the hypoxic bone marrow niche in a quiescent state (33–35). When needed, HSCs rapidly enter the cell cycle to replenish peripheral hematopoiesis. Self-renewal and differentiation are tightly balanced to maintain the stem cell pool while giving rise to hematopoietic progenitors, which ultimately differentiate into mature blood cells (35, 36). The delicate balance between quiescence and proliferation in HSCs requires a strictly controlled cell cycle progression.

Cyclin dependent kinase inhibitors (CKIs) represent a major break for cell cycle entry and the prevention of uncontrolled proliferation. Several studies started to unravel the impact of CKIs in HSCs (37–40).

$p16^{INK4a}$ is encoded by exons 1α, 2 and 3 of the *INK4a* locus (Figure 2). A different transcript derived from the same locus, encoded by the exons 1β, 2 and 3, encodes for the protein p19ARF (Figure 2) which has the capacity to block the cell cycle progression at the G1 and G2 phase (41–43). Thus, the INK4a locus represents a master growth regulator through its capacity to interface with both proliferation (Rb pathway *via* p16INK4a) and apoptosis (p53 pathway *via* p19ARF) (4, 44).

The transcriptional repressor Bmi-1 is part of the Polycomb group and it is present at high levels in HSCs (45–47). Bmi-1 represses the *INK4a* locus, thus limiting $p16^{INK4a}$ and $p19^{ARF}$ expression (39, 48). Bmi-1 deficiency impairs HSCs self-renewal as it increases $p16^{INK4a}$ and $p19^{ARF}$ levels thereby leading to proliferative arrest and cell death (39). Mice lacking $p16^{INK4a}$ do not show any dramatic effect on hematopoiesis, which could be explained by the reported low $p16^{INK4a}$ expression in normal HSCs (49, 50).

$p16^{INK4a}$ expression increases in HSCs with aging and this is associated with lower HSC numbers. $p16^{INK4a}$ inhibition

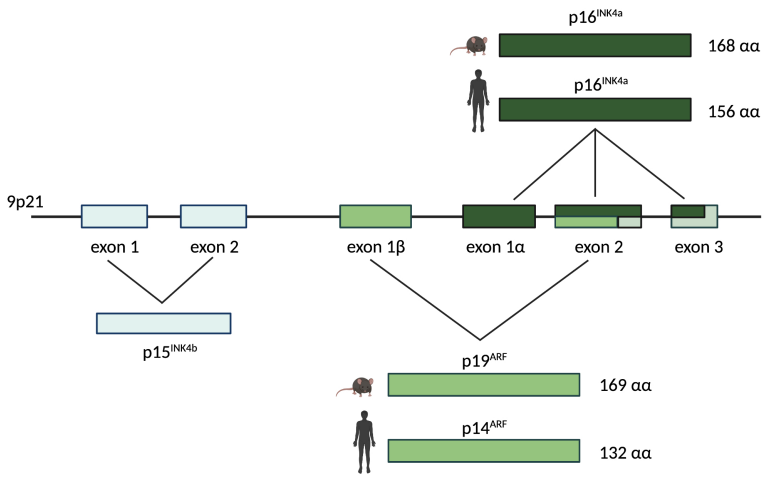

FIGURE 2
The human/murine *INK4a/ARF* locus. The *INK4a/ARF* locus resides on chromosome 9p21 and encodes for two different proteins in human and mouse: $p16^{INK4a}$ and $p14^{ARF}$ (named $p19^{ARF}$ in mouse). The *INK4a* gene is represented by exons 1α, 2, and 3 and it encodes for $p16^{INK4a}$, a 168 amino acids protein in mouse and a 156 amino acids protein in human. The *ARF* gene is composed by exons 1β, 2, and 3. It encodes for $p19^{ARF}$ in mouse (169 amino acids) and for $p14^{ARF}$ in human (132 amino acids). Upstream of the *INK4a* and *ARF* genes on the same chromosome, exons 1 and 2 represent the *INK4b* gene encoding for $p15^{INK4b}$.

counteracts the reduced HSC maintenance associated with aging, improves their repopulation ability and mitigates apoptosis (51).

The role of $p16^{INK4a}$ and $p19^{ARF}$ for the regulation of hematopoietic progenitor cells becomes evident in mice harboring a targeted deletion of the *INK4a* locus that eliminates both proteins. Young $p16^{INK4a-/-}/p19^{ARF-/-}$ mice show extramedullary hematopoiesis in the spleen with a high proportion of lymphoblasts and megakaryocytes in the red pulp and proliferative expansion of the white pulp. Aging aggravates this phenomenon and extends extramedullary hematopoiesis to nonlymphoid organs (49).

Among the CKIs, $p18^{INK4c}$ is the most powerful player and cell cycle inhibitor involved in murine HSC self-renewal (40, 52). $p18^{INK4c}$ deficient mice show HSCs with enhanced self-renewal ability which leads to the expansion of the HSC pool. This is also evident in serial transplantation experiments where $p18^{INK4c}$ deletion allows for an advanced HSC repopulation ability (40, 53).

Information on $p15^{INK4b}$ and $p19^{INK4d}$ in regulating HSC function is scarce. Characterization of the hematopoietic stem and progenitor cells of $p15^{INK4b}$ deficient mice revealed an increased frequency in common myeloid progenitors, but no alterations in the HSC compartment (54, 55).

The need to get first insights into the role of $p19^{INK4d}$ in HSCs leads to the characterization of the hematopoietic system of mice lacking $p19^{INK4d}$. Knockout mice do not reveal any defect under homeostatic conditions (56). However, *in vitro* studies highlight the involvement of $p19^{INK4d}$ in megakaryopoiesis, where it regulates the endomitotic cell cycle arrest coupled to terminal differentiation (57).

Moreover, $p19^{INK4d}$ effects become evident when HSCs are exposed to genotoxic stress. In this context, $p19^{INK4d}$ is required to maintain HSCs in a quiescent state, protecting them from apoptosis as genotoxic substances act during the S-phase (58).

The p53 induced CKI $p21^{Cip1/Waf}$ also regulates effects upon stress. Bone marrow transplantation experiments, using cells derived from mice after 2 Gy irradiation show that $p21^{Cip1/Waf}$ deficiency leads to a significantly reduced repopulation ability (37, 59).

In contrast, $p27^{Kip1}$ knock-out mice lack any perturbations in HSC number, self – renewal ability or cell-cycle state. The role of $p27^{Kip1}$ is restricted to more committed progenitor cells where its deletion increases proliferation and the pool size of $Sca1^{+}Lin^{+}$ cells (38).

In quiescent HSCs $p57^{Kip2}$ dominates as major CKI, where it is expressed at high levels. $p57^{Kip2}$ deficiency reduces the HSC population, compromises the maintenance of quiescence and impairs repopulation capacity (60).

In summary this led us to conclude that CKIs have distinct essential roles in hematopoietic stem and progenitor cells that are only partially understood. Whereas Cip/Kip proteins are predominantly involved in stress responses, INK proteins dominate in the control of hemostatic conditions.

4 Alterations in CKIs

In human cancers the *INK4a-ARF-INK4b* locus at chromosome 9p21 is one of the most frequently mutated and epigenetically silenced sites (61–63). This locus encodes for the cyclin dependent kinase inhibitors p16INK4a and p15INK4b and for the tumor suppressor protein $p14^{ARF}$ ($p19^{ARF}$ in the mouse), which is induced upon p53 activation (Figure 2) (64, 65). Many solid tumors including melanoma, pancreatic adenocarcinomas, esophageal and non-small cell lung carcinoma, harbor mutations in the $p16^{INK4a}$ and $p15^{INK4b}$ genes. In hematological malignancies $p16^{INK4a}$ and $p15^{INK4b}$ are frequently deleted e.g. in chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) (66–70).

$p18^{INK4c}$ and $p19^{INK4d}$, mapped on chromosome 1p32 and 19p13.2 respectively (71, 72), are involved in the development of a more distinct set of tumors. Somatic mutations of $p18^{INK4c}$ are associated with medullary thyroid carcinoma, hepatocellular carcinoma and breast cancer (73–75). Only little information is available regarding the role of $p19^{INK4d}$ in human malignancies; frame shift mutations and rearrangements in the $p19^{INK4d}$ gene have been documented in osteosarcoma (76), while its loss or downregulation have been detected in hepatocellular carcinoma (77) and testicular germ cell tumors (78).

The deletion of the Cip/Kip proteins in mice leads to an increased development of malignancies (79–81), underlining their main role as tumor suppressors. Contradictorily, in some tumor types Cip/Kip proteins also display an oncogenic activity when relocated to the cytoplasm (82–84).

Low $p27^{Kip1}$ levels are associated with more aggressiveness and poor prognosis in several human cancers (85–87). Control of $p27^{Kip1}$ levels involves a nuclear to cytoplasmic redistribution which is regulated by phosphorylation sites on distinct residues. Mitogenic signals induce $p27^{Kip1}$ phosphorylation on Ser10, inducing nuclear export (88, 89), while phosphorylation on Thr198, mediated by PKB/Akt, promotes $p27^{Kip1}$ association with 14-3-3 proteins and its transport to the cytoplasm (90).

Whereas nuclear $p27^{Kip1}$ inhibits cell proliferation and suppresses tumor formation, cytoplasmatic $p27^{Kip1}$ is involved in cytoskeleton rearrangement and contributes to cell migration (82, 89) and may promote metastasis. In some hematologic malignancies (91–93) and carcinomas (such as breast, esophagus, cervix and uterus tumors) (94–98), a positive association of cytoplasmic $p27^{Kip1}$ levels with a poor clinical outcome has been reported.

$p21^{Cip1/Waf}$ acts as a tumor suppressor in breast, colorectal, gastric, ovarian and oral cancers. Similar to $p27^{Kip1}$ it may display oncogenic activities when retained in the cytoplasm.

$p21^{Cip1/Waf}$ cytoplasmic accumulation is caused by phosphorylation at Thr145 by activated AKT1 (99). Through the association with proteins involved in the apoptotic process, cytoplasmatic $p21^{Cip1/Waf}$ mediates their inhibition, thus exhibiting anti-apoptotic effects. As such, cytoplasmic $p21^{Cip1/Waf}$ is indicative for aggressiveness and poor survival in prostate, cervical, breast and squamous cell carcinomas (100).

In contrast, the role of $p57^{Kip2}$ is limited at being a tumor suppressor, as there is so far no evidence of an oncogenic role so far (101–104).

Given the extensive knowledge regarding the role of CDK inhibitors in tumor biology there is increasing interest in exploiting them as potential target for cancer treatments. Here we review and discuss the importance they play in hematopoietic malignancies.

5 CKIs in hematologic malignancies

Hematologic malignancies consist of a spectrum of malignant neoplasms that affect bone marrow, blood and lymph nodes and originate from the uncontrolled proliferation of hematopoietic cells. They are driven by genetic and epigenetic aberrations, which can be exploited for diagnosis and therapeutic decisions. The dominant alterations of CKIs are reviewed below and illustrated in Figures 3, 4.

5.1 INK4 proteins in leukemia and lymphoma

5.1.1 $p16^{INK4a}$ and $p15^{INK4b}$

The *CDKN2A/B* locus encodes for $p16^{INK4a}$, $p14^{ARF}$ ($p19^{ARF}$ in mice) and $p15^{INK4b}$. This locus is affected by deletion, mutation or promoter hyper-methylation (62, 63) and frequently altered in patients with hematologic malignancies (4, 105, 106). The design of mouse strains with single or multiple targeted disruptions of the $p16^{INK4a}$, $p19^{ARF}$ and $p15^{INK4b}$ loci shed light on their distinct roles.

$p19^{ARF-/-}$ mice spontaneously develop a variety of tumors already by the age of 2 months. Analysis of diseased mice shows

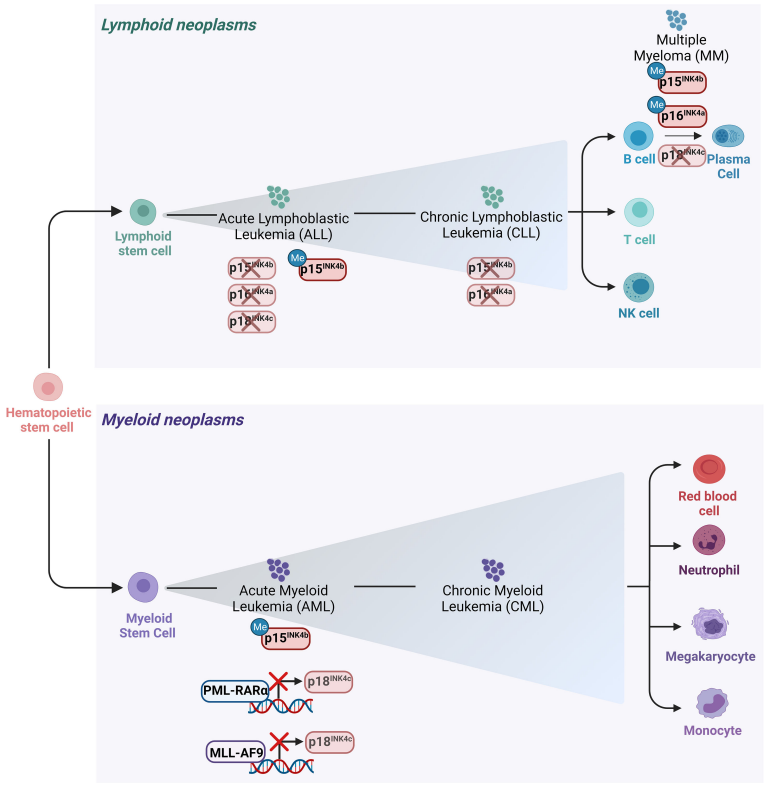

FIGURE 3
Main alterations of the INK4 proteins in leukemia and lymphomas. Schematic representation of the hematopoietic tree and main alterations affecting the INK4 proteins in different hematopoietic malignancies. Deletion of $p15^{INK4b}$ and $p16^{INK4a}$ together with their 5′ CpG islands hypermethylation in their promoter regions are the most frequent modes of $p15^{INK4b}$ and of $p16^{INK4a}$ inactivation in various subtypes of hematopoietic neoplasms including ALL and CLL. Deletion of $p18^{INK4c}$ has been rarely observed in ALL, whereas it is frequently deleted in MM. $p18^{INK4c}$ is subjected to a transcriptional repression imposed by the oncofusion protein PML-RARα in APL blasts and it is similarly downregulated by MLL-AF9 in cell lines derived from AML patients.

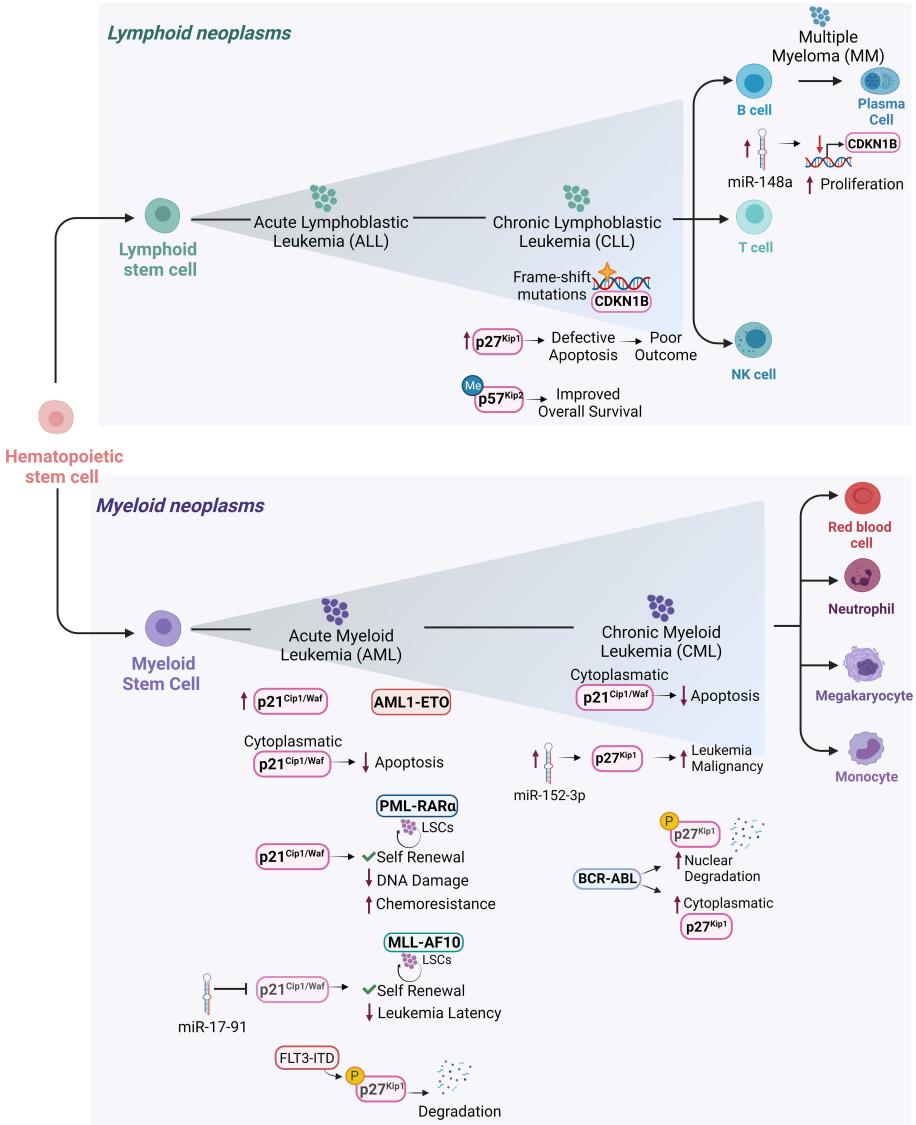

FIGURE 4
Cip/Kip proteins main deregulations and functions in different hematopoietic malignancies. Schematic representation of the hematopoietic tree and main functions exerted by Cip/Kip proteins in different hematopoietic malignancies. Increased $p21^{Cip1/Waf}$ levels have been reported in AML1-ETO positive AML patients, where it is believed to support LSCs maintenance and self-renewal ability. $p21^{Cip1/Waf}$ anti-apoptotic functions associated with its cytoplasmatic localization have been observed in AML blasts and in cell lines derived from human CML in blast crisis. In PML-RARα LSCs, *p21*$^{Cip1/Waf}$ expression maintains self-renewal of LSCs and limits DNA damage, thus protecting them from functional exhaustion and conferring chemoresistance. In MLL-AF10 induced AML, *p21*$^{Cip1/Waf}$ suppression mediated by miR-17-91 leads to decreased leukemia latency. Elevated $p27^{Kip1}$ levels in B-CLL where they confer protection against apoptosis, are associated with poor outcome. In hairy cell leukemia, a form of B-CLL, *CDKN1B* gene encoding for $p27^{Kip1}$ is the second most common altered gene by frame shift mutations. In MM, higher miR-148a levels correlate with decreased *CDKN1B* expression leading to sustained proliferation. In CML, overexpression of miR-152-3p targets $p27^{Kip1}$ and promotes leukemia malignancy. In AML, $p27^{Kip1}$ is subjected to FLT3-ITD phosphorylation (pY88- p27Kip1) which mediates $p27^{Kip1}$ degradation. BCR-ABL1^{+} CML can promote degradation of nuclear $p27^{Kip1}$ and to increased cytoplasmatic $p27^{Kip1}$, thus compromising $p27^{Kip1}$ tumor suppressor activity and promoting leukemic cell survival. *p57*^{Kip2} gene has been frequently found methylated in diffuse large B-cell lymphoma patients, where the low-risk group it is associated with a more favorable overall survival.

that T cell lymphoma is the second most common tumor type (107, 108). In line, *p19*$^{ARF-/-}$ newborn mice exposed either to X-ray or to γ-irradiation develop anaplastic T cell lymphoma (107, 108). In an acute lymphoblastic leukemia (ALL) model, the loss of *p19*^{ARF} initiates a more aggressive disease BCR-ABL1+ transformation. In this model, *p19*^{ARF} deletion also confers resistance to the kinase inhibitor imatinib (109). These data suggest a specific role for $p19^{ARF}$ in the lymphoid lineage.

Therefore, it would be interesting to analyze if $p19^{ARF}$ could serve as a marker for prognosis and therapeutic outcome.

Homozygous deletion of $p16^{INK4a}$ is not associated with an increased spontaneous cancer development. Of note, the concomitant heterozygous loss of $p19^{ARF}$ in $p16^{INK4a-/-}$ animals increases tumorigenesis and provokes the development of a wide spectrum of malignancies, including lymphoma (110). Importantly, the spontaneous tumors originating from mice harboring the heterozygous loss of $p19^{ARF}$ and $p16^{INK4a}$ homozygous deletion, retain the second $p19^{ARF}$ allele. However, the observed increased tumorigenesis in $p16^{INK4a-/-}$ mice upon heterozygous $p19^{ARF}$ loss underlines the cooperation of the two tumor suppressors.

Young mice show spontaneous tumorigenesis and a higher sensitivity to carcinogenic treatments, especially B cell lymphoma (49).

$p15^{INK4b-/-}$ mice show lymphoproliferative disorders including lymphoid hyperplasia in the spleen and formation of secondary follicles in lymph nodes but rarely develop lymphoma. This suggests that $p15^{INK4b}$ controls homeostasis of the hematopoietic compartment, rather than acting as a tumor suppressor (111).

Although $p15^{INK4b}$ and $p16^{INK4a}$ function as repressors of the cell cycle, in view of the phenotypes shown by the mouse models described above, they seem to have roles in different contexts. $p15^{INK4b}$ is mainly responsible for homeostasis and $p16^{INK4a}$, together with $p19^{ARF}$, is more involved in regulating the response to oncogenic stress. This suggests that $p16^{INK4a}$ might function as a sensor of oncogenic signals thus representing a safeguard against neoplasia.

$CDK4^{R24C}/CDK6^{R31C}$ double knock-in mice have been used to address the importance of INK4 inhibitors in regulating CDK4 and CDK6. INK4 binding is prevented by introducing point mutations in CDK4 (R24C) and CDK6 (R31C). The $CDK4^{R24C}$ mutation has been initially identified in hereditary melanoma and shows elevated CDK4 kinase activity (112). So far the $CDK6^{R31C}$ mutation has not been found in patients but is used to investigate CDK6-INK4 interactions. $CDK4^{R24C}/CDK6^{R31C}$ mice show a shortened survival caused by the onset of primary endocrine epithelial or hematopoietic malignancies. Mice injected with $CDK4^{R24C}/CDK6^{R31C}$ BCR-ABL1 transformed cell lines display accelerated tumor growth and reduced disease latency (113). This analysis highlights the crucial importance of INK4 binding to control CDK4/CDK6 activity in hematopoiesis. Therefore, it is attractive to conclude that CDK4/6 inhibitors are effective in patients that lack appropriate INK4-mediated control.

First evidence indicated that the *CDKN2* locus in human tumor cell lines derived from solid tumors is predominantly homozygously deleted and thereby $p16^{INK4a}$ becomes inactivated. This was later verified also for leukemia and lymphoma; only a low frequency of point mutations has so far been documented (114–118).

Studies in primary leukemia also identified alterations in $p15^{INK4b}$. The highest frequency of homozygous deletions of $p16^{INK4a}$ or $p15^{INK4b}$ occurs in ALL, while they are heterozygously deleted in chronic lymphocytic leukemia (CLL) (114, 119–121). T-ALL is most frequently associated with $p16^{INK4a}$ loss, while $p15^{INK4b}$ deletions are more often observed in pediatric ALL (70, 106, 119, 122–127). Initial studies focused their attention on the frequency of $p16^{INK4a}$ and $p15^{INK4b}$ mutations in adult and childhood ALL (70, 114, 120, 122, 128). Only at later stages the potential of these genes as prognostic factors was taken into account.

The overall incidence of $p16^{INK4a}$ deletion is higher than $p15^{INK4b}$. Patients with $p15^{INK4b}$ deletions harbor $p16^{INK4a}$ co-deletions, which is not consistently observed vice versa. Cases with homozygous $p16^{INK4a}$ deletion either maintain an unmutated $p15^{INK4b}$ gene or show a hemizygous $p15^{INK4b}$ deletion. These findings point at $p16^{INK4a}$ as the central target of deletions which play the central role for ALL leukemogenesis (70, 119, 120, 123).

The prognostic significance of $p16^{INK4a}$ and $p15^{INK4b}$ deletions remains a matter of debate with contradictory reports: some studies showed an adverse prognostic effect (122, 123, 127, 129–133), which was not confirmed by others (70, 134–136).

Analysis of mixed leukemia types, small patient cohorts or insensitive molecular techniques, like polymerase chain reaction (PCR), immunocytochemistry and fluorescence *in situ* hybridization (FISH) may have complicated the interpretation. The conclusion of some studies still leaves the potential implication of $p16^{INK4a}$ and $p15^{INK4b}$ deletions in patient prognosis elusive.

Point mutations in the *CDKN2A/CDKN2B* genes, encoding for $p16^{INK4a}$ and $p15^{INK4b}$ respectively, are sporadically found in human hematopoietic disorders. A comprehensive analysis of 264 T-ALL cases, searching for mutations in cell cycle genes, found *CDKN2A/CDKN2B* as the most mutated ones (137). Inactivation of $p15^{INK4b}$ and $p16^{INK4a}$ genes can also be based on hypermethylation of the 5' CpG islands in their promoter regions which induces transcriptional silencing (138). This mode of $p16^{INK4a}$ inactivation is commonly found in breast and colon cancer (139) but also in leukemia and lymphoma. Normal hematopoietic cells lack $p15^{INK4b}$ and $p16^{INK4a}$ promoter hypermethylation, which only occurs *de novo* upon malignant transformation (140). Interestingly, $p15^{INK4b}$ or $p16^{INK4a}$ seem unaffected at any stage of CML (140), whereas hypermethylation of $p15^{INK4b}$ and $p16^{INK4a}$ is a common event in multiple myeloma (MM) (141). Selective $p15^{INK4b}$ promoter hypermethylation, without $p16^{INK4a}$ alterations, is observed in acute myeloid leukemia (AML), myelodysplastic syndrome and ALL (140, 142–146), whereas Burkitt's lymphoma and Hodgkin's lymphoma present p16INK4a hypermethylation (140, 141, 147–150).

Overall, the current available data show that inactivation of *p15*^{INK4b} and *p16*^{INK4a} in human hematopoietic malignancies is caused by genetic deletion or promoter hypermethylation. Linking these alterations in a well-evaluated cohort of patients would be extremely precious to finally define their role for disease progression and their prognostic relevance. The frequency of their alterations in leukemia and lymphoma is indicative of a central role and renders them promising candidates for novel therapeutic approaches.

5.1.2 p18^{INK4c}

Being the functionally most relevant INK in HSC regulation under stress conditions, it is not surprising that the absence of p18^{INK4c} provokes hematopoietic abnormalities and extramedullary hematopoiesis (111). Mice lacking *p18*^{INK4c}experience the consequences of the absence of its tumor suppressor function and its role in controlling lymphocyte homeostasis (111, 151). *p18*$^{INK4c-/-}$ mice spontaneously develop neoplasia including angiosarcoma, testicular tumors, pituitary tumors and lymphoma.

p18^{INK4c} mutations in human hematopoietic malignancies are surprisingly rare in acute leukemias, as they have not been identified in AML and deletions have been reported in just some cases of adult ALL (70, 152, 153). *p18*^{INK4c} maps on the chromosomal region 1p32. In line with data showing no involvement of p18^{INK4c} in childhood AML (70), no alterations of the 1p region in childhood ALL have been found so far (154). Similarly, no evidence for *p18*^{INK4c} promoter hypermethylation in acute leukemia has been reported (155).

In MM, *p18*^{INK4c} is frequently deleted, whereas no point mutations have been detected (156, 157).

In normal B-cells, p18^{INK4c} controls the cell cycle and is involved in the terminal differentiation of B-cells into plasma cells through the inhibition of CDK6 (158, 159). Despite that role, *p18*^{INK4c} expression is preserved in most lymphoid malignancies (68, 118). The hemizygous loss of p18^{INK4c} has been reported in mantle cell lymphoma, but not in Hodgkin's lymphoma, where *p18*^{INK4c} is frequently repressed due to promoter hypermethylation (160–162).

The oncofusion protein PML-RARα which drives acute promyelocytic leukemia (APL) directly suppresses *p18*^{INK4c} expression which is downregulated in APL blasts compared to normal promyelocytes (163).

ChIP-seq experiments of MLL and AF9 in THP-1 cells reveal the *CDKN2C* locus, encoding for p18^{INK4c}, as a binding region. This indicates that *p18*^{INK4c} expression is subject to MLL-AF9 mediated regulation (164).

A detailed map of p18^{INK4c} regulation in different leukemic subtypes is still missing and would help clarifying the role of p18^{INK4c} in hematopoietic malignancies and leukemic stem cells (LSCs). The data currently available are indicative for sporadic alterations of *p18*^{INK4c} in hematologic malignancies.

5.1.3 p19^{INK4d}

The analysis of *p19*^{INK4d} knock-out mice failed to detect any tumor suppressing effects of p19^{INK4d}. Mice lacking p19^{INK4d} do not spontaneously develop tumors and no abnormalities of the hematopoietic system are evident (56). In line, alterations of *p19*^{INK4d} are not general hallmarks of hematopoietic neoplasms (76, 165) albeit the data available are scarce. The absence of a mouse phenotype in terms of enhanced cell proliferation and tumor development upon *p19*^{INK4d} loss suggests a functional compensation exerted by the other INK4 or Cip/Kip proteins.

5.2 Cip/Kip proteins in leukemia and lymphoma

5.2.1 p21$^{Cip1/Waf}$

p21$^{Cip1/Waf}$ is a key mediator of p53-dependent tumor suppressor functions (22) and acts as a negative regulator of cell cycle progression. p21$^{Cip1/Waf}$ and its role in cellular proliferation have been described in a vast body of literature. Its negative function on cell cycle progression indicates that p21$^{Cip1/Waf}$ may exert tumor suppressive roles and participates in leukemia development even under wild type p53 conditions.

p21$^{Cip1/Waf}$ deficient mice are viable and fertile (166, 167). In those mice, harboring wild type p53, spontaneous tumor development occurs late in life at an average age of 16 months. The variety of malignancies includes tumors of hematopoietic, vascular and epithelial origin. For instance, 14% of all tumors are B-cell lymphoma (168).

The tumor spectrum developed by *p21*$^{Cip1/Waf}$ deficient mice is remarkably similar to the one observed in p53 deficient mice, which is not surprising keeping in mind the p21$^{Cip1/Waf}$ activation by p53. However, p53 deficient mice are characterized by longer latency. However, *p21*$^{Cip1/Waf}$ deficient mice do not develop T-cell lymphoma, one of the most frequent tumors arising in p53 deficient mice.

The clinical relevance and potential as a prognostic marker of aberrant *p21*$^{Cip1/Waf}$ expression has been assessed in various types of human cancers.

Loss of p21$^{Cip1/Waf}$ protein levels correlates with a more advanced tumor stage and worse prognosis in pancreatic cancer (169), while its overexpression has been shown to be associated with poor prognosis in non-small cell lung cancer (170) and in esophageal squamous cell carcinoma patients (171).

Interestingly, other studies report low *p21*$^{Cip1/Waf}$ expression being associated with reduced survival in patients affected by esophageal carcinoma (172, 173).

The relationship between *p21*$^{Cip1/Waf}$ expression and gastric cancer remains controversial as well. Some authors reported a positive correlation between *p21*$^{Cip1/Waf}$ expression and favorable prognosis (174, 175), whereas others observed that *p21*$^{Cip1/Waf}$ expression is associated with poor survival (176).

Analysis of deletions and mutations of *p21*$^{Cip1/Waf}$ has been carried out in few human hematological malignancies and could be mapped in few subtypes. *p21*$^{Cip1/Waf}$ alterations are rare in typical mantle cell lymphoma (MCL), but loss of *p21*$^{Cip1/Waf}$ expression is present in aggressive MCLs harboring wild-type *p53* gene (177).

In a large cohort of AML patient blasts, high *p21*$^{Cip1/Waf}$ expression was found in AML1-ETO positive leukemia (178) with unknown significance. Given its role in maintaining the HSC-pool during normal hematopoiesis (37), one may speculate that it plays a role for LSCs by supporting their self-renewal capacity.

p21$^{Cip1/Waf}$ mutations appear to be not involved in childhood T-ALL pathogenesis, despite extensive studies no mutations were detected (179).

p21$^{Cip1/Waf}$ methylation status in leukemia still remains a debated topic. *p21*$^{Cip1/Waf}$ hypermethylation was observed in bone marrow cells derived from ALL patients, where it is indicative of a poor prognosis (180). Other studies failed to find any evidence for *p21*$^{Cip1/Waf}$ methylation in ALL and AML (155, 181, 182).

For instance, *p21*$^{Cip1/Waf}$ expression appears independent of its promoter methylation status in AML cell lines but correlates with demethylation of p73, a homologue of p53 and a known upstream transcriptional activator of *p21*$^{Cip1/Waf}$ (183). Treatment of AML cell lines with the methylation inhibitor 5-Aza-2′-deoxycytidine (5-Aza-CdR) results in the induced *p21*$^{Cip1/Waf}$ expression by p73 demethylation, provoking a cell cycle arrest in the G1 phase (184, 185). Decreased *p21*$^{Cip1/Waf}$ expression, without any signs of methylation, has been linked to higher disease aggressiveness in myelodysplastic syndrome (MDS). In line with the data from AML patients, reduced *p21*$^{Cip1/Waf}$ expression was commonly correlated to p73 methylation (186).

More studies are required to precisely understand how the *p21*$^{Cip1/Waf}$ methylation status interferes with disease progression and if p73 methylation can be used as a marker for the *p21*$^{Cip1/Waf}$ status.

In addition to growth arrest, p21$^{Cip1/Waf}$ is involved in apoptosis, DNA repair and senescence. For instance, one of the most extensively studied functions of p21$^{Cip1/Waf}$ is the protection of cells against apoptosis.

An example is given by the usage of histone deacetylase inhibitors (HDACI) to induce apoptosis (187–189). *p21*$^{Cip1/Waf}$ expression is upregulated by an increased histone acetylation of H3K4 at the *p21*$^{Cip1/Waf}$ promoter region, which is mediated by the HDACI SAHA (suberoylanilide hydroxamic acid) (190). *p21*$^{Cip1/Waf}$ overexpression confers resistance to SAHA-induced apoptosis which was shown in human AML cells. SAHA treatment promotes apoptotic cell death in leukemic cells by inducing pro-apoptotic genes such as TRAIL (TNF-related apoptosis-inducing ligand) and its downstream effector caspase-8. One mechanism through which p21$^{Cip1/Waf}$ exerts anti-apoptotic effects in AML cell lines is the inhibition of caspase-8 cleavage to suppress TRAIL-mediated apoptosis (191).

A second anti-apoptotic function of p21$^{Cip1/Waf}$ was also reported for AML blasts. There, high cytoplasmatic p21$^{Cip1/Waf}$ protein levels provide protection against cytotoxic agents. Blasts with cytoplasmatic p21$^{Cip1/Waf}$ levels show reduced etoposide (VP-16) mediated apoptosis (192). Similarly, the enforced expression of p21$^{Cip1/Waf}$ in CML blast cells confers resistance to Imatinib induced apoptosis (193). These studies suggest that p21$^{Cip1/Waf}$ expression should be investigated to act as a marker for therapeutic outcome.

p21$^{Cip1/Waf}$ expression is essential for the initiation and maintenance of leukemogenesis induced by PML/RAR-transformed HSCs. Under this condition p21$^{Cip1/Waf}$ is required to maintain the self-renewal capacity of LSCs and to limit DNA-damage. p21$^{Cip1/Waf}$ protects from functional exhaustion (194). In line p21$^{Cip1/Waf}$ is crucial for the maintenance of self-renewal and chemoresistance of LSCs in a murine model of T-ALL (195).

In MLL-AF10-induced AML *p21*$^{Cip1/Waf}$ suppression is achieved by the oncomir miR-17-91, that is associated with enhanced LSC self-renewal and decreased leukemia latency (196). Functional studies for the role of p21$^{Cip1/Waf}$ have been mainly carried out in cell lines from different leukemia subtypes. The literature on primary patient samples is scarce. It appears that the involvement of p21$^{Cip1/Waf}$ is highly context dependent and relies on the differentiation status of the cells and on the driver oncogenes.

The fact that *p21*$^{Cip1/Waf}$ is important to maintain stem cell self-renewal might provide a basis for novel attempts to target p21$^{Cip1/Waf}$ to induce exhaustion.

5.2.2 p27^{Kip1}

p27^{Kip1} regulates cell proliferation by inhibiting CDK complexes and arresting cell proliferation in response to anti-mitogenic signals (Figure 1) (8, 197–199).

Analysis of *p27*^{Kip1} knock-out mice highlighted the importance of p27^{Kip1} as cell cycle regulator: *p27*^{Kip1} deficient mice have an overall augmented cell proliferation which is reflected in increased body size and hyperplastic organs. Tumor formation becomes manifested spontaneously; pituitary and parathyroid tumors evolve and the mice show an increased susceptibility to tumorigenesis upon γ-irradiation or treatment by the chemical carcinogen N-ethyl-N-nitrosourea (ENU) (79, 80, 200). These studies defined p27^{Kip1} as tumor suppressor.

Mutations in the *p27*^{Kip1} gene and its homozygous inactivation are generally rare in human cancers. In people *CDKN1B*, encoding for p27^{Kip1}, has been identified as the second most common altered gene by frame-shift mutations in heterozygosity in hairy cell leukemia (HCL), a form of B-cell CLL. In most patients the *CDKN1B* mutation is clonal, thereby suggesting an early role in the pathogenesis of HCL (201, 202).

The subcellular location of $p27^{Kip1}$ and its concentration determine the impact on malignant transformation. On the one hand, $p27^{Kip1}$ acts as a tumor suppressor by inhibiting CDK-cyclin complexes and cell cycle progression when present in the nucleus. On the other hand, a localization shift of $p27^{Kip1}$ from the nucleus to the cytoplasm, may promote tumor formation by regulating cytoskeletal structure and cell migration (89).

Augmented levels of $p27^{Kip1}$ and its cytoplasmic localization have been correlated with poor prognosis and increased metastasis in diverse solid tumors including breast (94), cervix (97) and esophagus (95) carcinomas, as well as in some lymphoma and leukemia (91–93).

Despite a rare mutation rate, *p27^{Kip1}* deregulation is one of the key events promoting leukemogenesis. Several mechanisms altering *p27^{Kip1}* expression and localization have been described. miRNAs play a prominent role and abundance of $p27^{Kip1}$ subjected to miRNA-mediated regulation: oncogenic expression of miRNA targeting *p27^{Kip1}* translation can cause *p27^{Kip1}* loss (203). In CML patients, increased miR-152-3p promotes aggressive behavior of CML cells by targeting $p27^{Kip1}$ (204). Similarly, miR-148a correlates with low *p27^{Kip1}* expression and increased proliferation in MM cells (205).

In lymphoma, low $p27^{Kip1}$ levels correlate with a poor prognosis (206). Vice versa, high $p27^{Kip1}$ levels are associated with enhanced disease-free survival in AML, indicative for disease progression (207).

In contrast, AML patients with low $p27^{Kip1}$ due to deletion of the chromosomal region 12p13, have a better overall survival. Although together with *CDKN1B*, nine other genes are located in the 12p13 chromosomal region, the reported improved clinical outcome can be ascribed to reduced *CDKN1B* expression levels which might lead to higher cell proliferation which makes leukemic cells more susceptible to cytotoxic agents (208).

Besides the genomic alterations, also the phosphorylation sites play an important role for $p27^{Kip1}$ levels. $p27^{Kip1}$ is a substrate of FLT3 and FLT3-ITD in AML patient samples, where they phosphorylate $p27^{Kip1}$ at the residue Y88 which is required for subsequent $p27^{Kip1}$ phosphorylation at T187 by the CDK2-cyclin complex marking $p27^{Kip1}$ for SCF^{Skp2}-mediated degradation. FLT3 inhibition reduces pY88-$p27^{Kip1}$ and increases $p27^{Kip1}$ levels leading to cell cycle arrest (209).

High $p27^{Kip1}$ levels are associated with a poor outcome in B-cell chronic lymphocytic leukemia (B-CLL). In B-CLL disease progression does not result from uncontrolled cell proliferation but is the result of defective apoptosis and enhanced cell survival. High *p27^{Kip1}* expression is discussed to contribute to the protection against apoptotic stimuli like $p21^{Cip1/Waf}$ (93).

The presence of high $p27^{Kip1}$ levels in CLL was confirmed by others who also found an inverse correlation with c-Myc protein levels. C-Myc deregulation is a frequent event in leukemia and lymphoma (210, 211). Low Myc levels are associated with low expression of its target gene *Skp2*, a component of the SCF^{Skp2} ubiquitin ligase complex that degrades $p27^{Kip1}$. The reduced Skp2-mediated degradation leads to the $p27^{Kip1}$ accumulation which confers resistance to apoptosis (210).

In untransformed $CD34^+$ progenitor cells, β_1-integrin engagement increases $p27^{Kip1}$ nuclear levels, which in turn decrease CDK2 activity thus restraining G1/S-phase progression. BCR-ABL expression in CML $CD34^+$ cells induces elevated cytoplasmatic $p27^{Kip1}$ levels. In this context, such high $p27^{Kip1}$ levels do not restrain CML cell proliferation due to its cytoplasmatic relocation, thereby contributing to the loss of integrin-mediated proliferation inhibition observed in normal $CD34^+$ cells (212).

More recent studies demonstrate that BCR-ABL1 promotes leukemia by subverting nuclear $p27^{Kip1}$ tumor-suppressor function *via* two independent mechanisms. In a kinase-dependent manner, BCR-ABL1 induces SCF^{Skp2} expression through the PI3K pathway (213), promoting the degradation of nuclear $p27^{Kip1}$, thus compromising its tumor-suppressor activity. In a kinase-independent fashion it increases cytoplasmatic $p27^{Kip1}$ abundance, preventing apoptosis and thereby promoting leukemic cell survival (214, 215).

The overexpression of a stable $p27^{Kip1}$ harboring two point mutations which prevent its phosphorylation on sites responsible for its SCF^{Skp2}-mediated nuclear degradation (T187A) and for its PI3K-directed cytoplasmatic sequestration (T157A) causes a G1/S arrest, markedly inhibiting proliferation of BCR-ABL+ cells (216).

The complexity of the regulation mechanism regulation location and degradation require further investigations to define disease entities where $p27^{Kip1}$ may serve as clinical marker.

5.2.3 $p57^{Kip2}$

Based on its ability to inhibit G1-S phase cyclin-CDK complexes, $p57^{Kip2}$ is considered a tumor suppressor. As mentioned above for $p21^{Cip1/Waf}$ and $p27^{Kip1}$, $p57^{Kip2}$ is involved in many cellular processes including apoptosis, and cellular migration.

The fact that $p57^{Kip2}$ has a crucial role during embryogenesis and is required for normal embryonic development makes it unique under der CKI family. *p57^{Kip2}* knock-out mice show severe developmental defects and display increased embryonic and perinatal lethality (217, 218) which complicated further studies on tumorigenesis in mice and most studies rely on human patient samples.

Reduced *p57^{Kip2}* expression is associated with high tumor aggressiveness and poor prognosis in several types of tumors, such as gastric, colorectal, pancreatic, breast and lung carcinoma as well as leukemia (103, 104, 219–221). p57Kip2 expression is decreased in MDS, in particular in patients with a poor karyotype. Low expression results from an impaired response to the SDF-1/CXCR4 signal which induces *p57^{Kip2}* expression (222). *p57^{Kip2}* knock-out mice show hyperproliferation and

differentiation delay in several tissues (218), which are features associated with the pathogenesis of MDS (223).

Another described mechanism how $p57^{Kip2}$ expression is altered is promoter methylation. Hypermethylation of the *CDKN1C* gene, encoding for $p57^{Kip2}$, occurs in diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, ALL (224, 225) and nodal DLBCL (226). In the low-risk group of DLBCL, *CDKN1C* methylation is associated with a more favorable overall survival. The authors proposed aberrant *CDKN1C* promoter methylation as a biological marker in patients with DLBCL (226). Another study in DLBCL patients suggested that the analysis of *CDKN1C* methylation status may serve as a biomarker for the detection of minimal residual disease, underlining the importance of $p57^{Kip2}$ for determining leukemia relapse risk (227).

Analysis of the $p57^{Kip2}$ methylation status in adult and childhood ALL found a rate of 50% *CDKN1C* hypermethylation in adult ALL but only 7% hypermethylation in childhood leukemia (226). Interestingly, in 53% of the childhood ALL samples $p57^{Kip2}$ was absent without methylation and overall $p57^{Kip2}$ levels were 8-fold lower compared to normal lymphocytes. The low expression points at additional ways to regulate $p57^{Kip2}$ in this particular disease class (228). *In line,* $p57^{Kip2}$ *methylation and protein expression in adult ALL patients does not show any correlation* as 10 out of 15 patients with *CDKN1C* hypermethylation expressed $p57^{Kip2}$ (229).

Overall, methylation status of $p57^{Kip2}$ does not seem to be a reliable marker for $p57^{Kip2}$ levels. Conditional knockout mice would be a useful tool to study the role of $p57^{Kip2}$ in hematopoietic diseases in more detail.

6 Pharmacologic CDK inhibition in hematologic malignancies

CDK kinase inhibitors are under extensive investigation in numerous preclinical and clinical studies in a variety of solid tumors and they are currently tested in hematological neoplasms (230, 231).

Pan-CDK inhibitors represented the very first generation of CDK inhibitors with the function to restrain cell proliferation *via* the inhibition of the CDK enzymatic activity. Flavopiridol was the first CDK inhibitor used in clinical trials and tested for the treatment of ALL, AML and CLL (232–234). Due to their low selectivity causing severe cytotoxic effects in healthy cells and a wide range of side effects, pan-CDK inhibitors have been discontinued in clinical trials (113, 235).

Considering the key role of CDK6 in malignant hematopoiesis it represents an effective therapeutic target (236–238). This is underlined by the high frequency of $p15^{INK4b}$ and $p16^{INK4a}$ inactivation in leukemia and lymphoma. The development of more specific CDK inhibitors, including CDK4/6-kinase inhibitors, represented an exciting turn over in the field (239).

Palbociclib is a CDK4/6 kinase inhibitor that acts by blocking enzymatic functions by mimicking INK4 binding. Palbociclib has been FDA approved to treat breast cancer patients and clinical trials exploring its effects in hematological malignancies are ongoing. Richter et al. present in their recent work (231) an extensive and detailed collection of preclinical and clinical studies conducted with several CDK4/6 inhibitors in hematological diseases.

Palbociclib resistance is a common phenomenon in breast cancer patients (240, 241). In breast cancer and AML high levels of $p16^{INK4a}$ and $p18^{INK4c}$ are associated with resistance to Palbociclib and to a CDK6 protein degrader that is based on the structure of Palbociclib. Despite this correlation, low $p16^{INK4a}$ levels are not predictive for Palbociclib sensitivity (242). All INK4 proteins are in principle capable to prevent Palbociclib binding to CDK6 and thereby capable to induce resistance. Whether this fact is also true for other CDK inhibitors needs to be investigated. The cell-type specific expression of INK4 proteins needs also to be taken into consideration when studying CDK-inhibitors resistance.

The challenge in the development of novel inhibitors is in the design of molecules able to reduce the side effects and to overcome drug resistance. An innovative approach of CDK inhibition would consider the possibility to mimic the functions of INK4 proteins for a selective inactivation of CDKs. However, intensive research is needed to fill the need of X-ray crystal structures of most of the CDKs and CDKs/INK4/Cip/Kip complexes and to make this creative approach possible.

7 Discussion

INK4 and Cip/Kip proteins were initially identified as CDK inhibitors and negative regulators of cell cycle progression. Only recently, the involvement in other cellular processes including apoptosis and cell migration was uncovered. Thereby CKIs bridge cell cycle regulation to other cellular functions. Under certain circumstances CKIs may even promote cancer progression.

Tumor cells frequently display mutations in CKIs which underscores the significance of these proteins for tumorigenesis. We here summarize the dominant alterations of CKIs in hematopoietic malignancies and discuss their consequences for disease development, maintenance, and diagnosis.

Within the INK4 family, $p15^{INK4b}$ and $p16^{INK4a}$ are most frequently inactivated in leukemia and lymphoma either by deletion or hypermethylation of 5' CpG islands in their promoter regions (114–116, 118, 140–150). The prognostic importance of these alterations in distinct disease entities remains unclear. Considering the unique functions of each

INK4 proteins, especially their role under stress conditions, one could speculate that distinct expression patterns lead to different disease subtypes and dictates therapeutic outcomes.

CDK4/6 specific inhibitors represent a promising valuable choice for the treatment of hematological malignancies. However, resistance to CDK inhibitor therapy has been frequently observed. INK4 proteins are capable of inducing resistance by binding to CDK6. Studies are needed to evaluate whether this holds true for other CDK inhibitors.

As proliferation and cell cycle control are essential features of a cell, the components of the cell cycle machinery are present in multiple variants, which can substitute for each other. INK4 proteins share common tasks and, in a similar manner, CDKs may substitute for each other. This complexity makes it exceedingly difficult to generalize any consequence upon loss or mutations of a single player. Effects will also be context and cell type dependent.

This enormous plasticity of the cell cycle machinery to adapt ensures cell proliferation and presents a major challenge when it comes to predict therapeutic outcomes of drugs interfering with CDKs or INKs. The removal or inhibition of a single player may be rapidly compensated by a rearrangement of CDK complexes.

Another layer of complexity is induced by the emerging CDK6 kinase-independent functions that regulate transcriptional processes relevant for leukemia. The involvement of CDK6 in LSCs biology makes it an attractive target for leukemia therapy (238, 243). It is unclear how CKIs binding to CDK6 interferes with the transcriptional role of CDK6. It is also unknown whether INK4 or Cip/Kip binding to CDK6 alters the composition of CDK6 containing transcriptional complexes and/or chromatin location. We need to understand how CDK-CKIs complexes interfere with cell cycle-independent functions to reliable predict treatment outcomes. Moreover, effects of kinase inhibitor treatment on the kinase-independent functions of CDK6 are still enigmatic. The frequent upregulation of CDK6 (237, 235) in hematopoietic tumors (243, 244) and the fact that alterations of INK4 proteins are commonly found in hematopoietic tumors demands for the understanding of any CDK6-INK4 correlation in leukemia/lymphoma to exploit CDK4/6 inhibitors in hematopoietic malignancies.

Despite the importance of $p18^{INK4d}$ for HSC self-renewal under homeostatic and stress conditions (40, 52,53), $p18^{INK4d}$ mutations are not a hallmark of hematopoietic malignancies. $p18^{INK4d}$ deregulation is rarely observed in hematopoietic neoplasms. Alterations on the transcriptional/translational level cannot be entirely excluded. As such the oncogene MLL-AF9 regulates $p18^{INK4d}$. In line, the comparison of AML subtypes identified distinct INK4 expression patterns for different AML entities. The global analysis of the protein levels of individual CIKs in respect to their hematopoietic disease type is required to design tailored treatment strategies.

We are only starting to understand and appreciate functions of the Cip/Kip proteins in regulating apoptosis and cell migration. The involvement of Cip/Kip in tumorigenesis is an attractive emerging field of research and will open novel innovative therapeutic avenues.

$p21^{Cip1/Waf}$ has a dual context-dependent role in leukemogenesis and acts as tumor suppressor and promoter. In cell lines, the anti-apoptotic effect of cytoplasmatic $p21^{Cip1/Waf}$ confers a survival advantage and mediates chemoresistance. Inhibition of $p21^{Cip1/Waf}$ under these conditions bears the potential to sensitize leukemic cells to chemotherapy. Similarly, cytoplasmatic $p27^{Kip1}$ prevents apoptosis and may be exploited as potential therapeutic target. Most studies rely on cell lines and this only partially reflects the *in vivo* situation. The reality-check in patients is still missing to judge the clinical relevance of these observations. Therapeutic strategies that simultaneously target oncogenic Cip/Kip functions while preserving tumor suppressive functions would represent an innovative optimal approach.

Author contributions

All authors made substantial, direct, and intellectual contributions to the work. KK was the principal investigator and takes primary responsibility for the paper. AS, VS and KK wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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