Journal of Oncology

Changes in Cell Signaling Pathways as a Goal of Targeted Therapy of Solid Tumors

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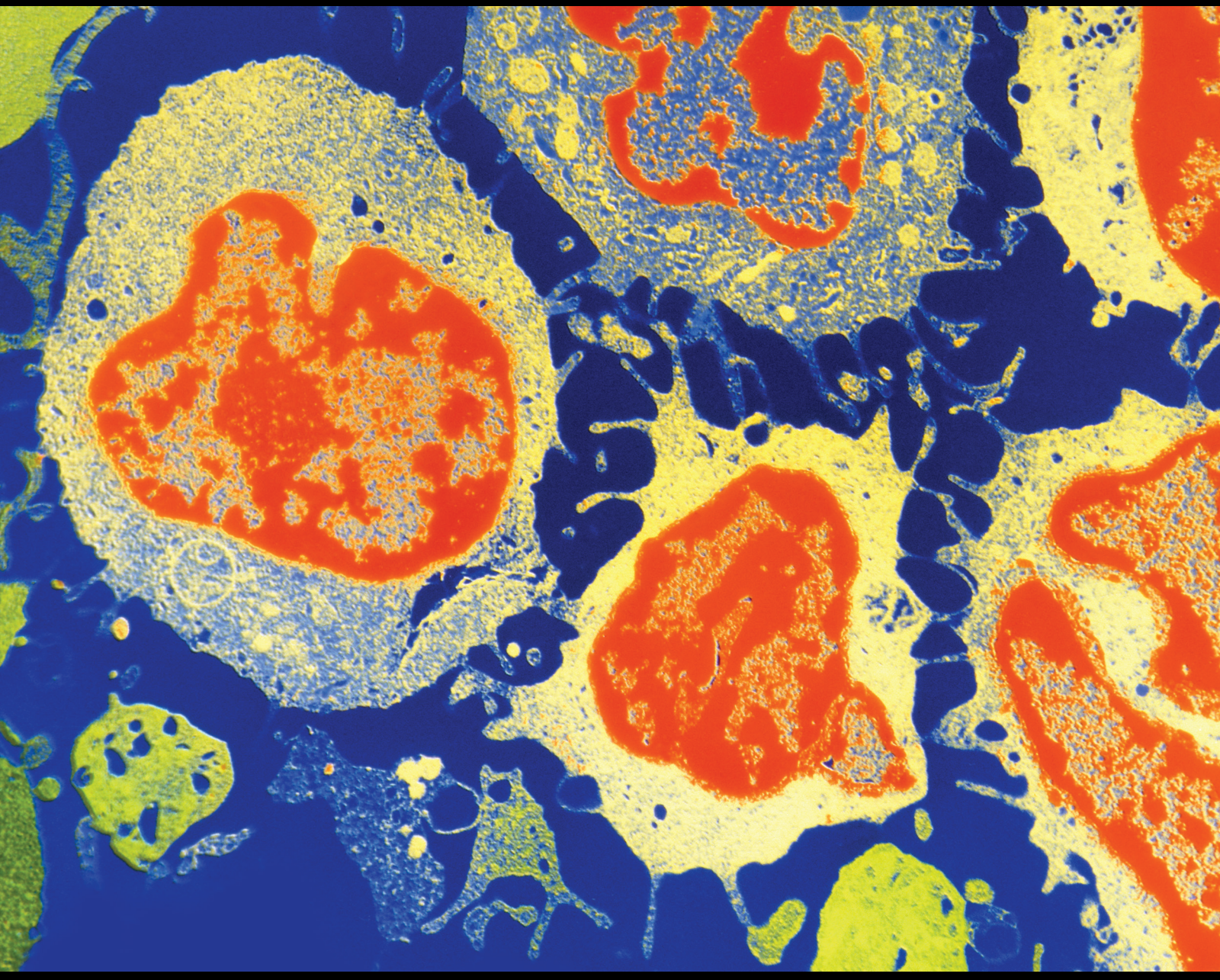

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Research Article

Genetic Profiles Playing Opposite Roles of Pathogenesis in Schizophrenia and Glioma

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Background. Patients diagnosed with schizophrenia were found having lower risks to develop cancers, including glioma. Based on this epidemiology, we hypothesized that there were gene profiles playing opposite roles in pathogenesis of schizophrenia and glioma. *Methods*. Based on GEO datasets and TCGA, key genes of schizophrenia genes on the opposite development of glioma were screened by different expressed genes (DEGs) screening, weighted gene coexpression network analysis (WGCNA), disease-specific survival (DSS), and glioma grading and verified by gene set enrichment analysis (GSEA). *Results*. First, 612 DEGs were screened from schizophrenia and control brain samples. Second, 134 key genes more specific to schizophrenia were left by WGCNA, with 93 key genes having annotations in TCGA. Third, DSS of glioma helped to find 42 key gene expressions of schizophrenia oppositely associated with survival of glioma. Finally, 24 key genes showed opposite expression trends in schizophrenia and different glioma grading, i.e., the upregulated key genes in schizophrenia expressed increasingly in higher grade glioma, and vice versa. CAMK2D and MPC2 were taken as the examples and evaluated by GSEA, which indeed showed opposite trends in the same pathways of schizophrenia and glioma. *Conclusion*. This workflow of selecting novel targeted genes which may have opposite roles in pathogenesis of two diseases was firstly and innovatively generated by our team. Some filtered key genes were indeed found by their potential effects in several mechanism studies, indicating our process could be effective to generate novel targeted genes. These 24 key genes may provide potential directions for future biochemical and pharmacotherapeutic research studies.

1. Introduction

The incidence of cancers in patients with schizophrenia was proposed lower than that of general population, firstly raised by the Board of Control of the Commissioners in Lunacy for England and Wales in 1909 [1]. A latest meta-analysis recruited schizophrenia patients from 16 cohort studies found decreased overall cancer incidence (RR = 0.90, 95% confidence interval (CI) 0.81–0.99), especially in lung cancer, colorectal cancer, liver cancer, stomach cancer, and prostate

cancer [2]. Interestingly, the overall incidence of cancer in patients with schizophrenia did not parallel their cancer risk factor exposures [3]. Noteworthy, many potential confounding factors, including sex, ethnicity, genetic background, environmental exposure, and antipsychotic medications, influenced that the cancer prevalence among schizophrenia patients did not decrease in all types of cancer [2]. Therefore, some factors specifically related to schizophrenia may influence the tumorigenesis.

Considering that schizophrenia was well known for its heritability and familial transmission, the genetic components of schizophrenia may weigh heavily in the development of cancers. Some studies revealed significantly decreased risks of cancers in persons with schizophrenia and their relatives, suggesting that the familiar/genetic factors contributing to schizophrenia may potentially inhibit tumorigenesis and lead to the better survival [4–6]. In cerebral cancers, Grinshpoon et al. [7] reported that the standardized incidence ratios (SIRs) of the cancers in brain sites were significantly lower than 1.0 among men with schizophrenia (SIR = 0.56, 95% CI 0.32–0.81), suggesting a decreased risk of cerebral carcinomas in this group of people [7].

Some epidemiological studies showed that persons with schizophrenia may less likely to suffer from glioma [7, 8]. Gao et al. reviewed several genes involved in pathogenesis of schizophrenia play opposite roles in the development of glioma, such as neural progenitor proliferation, neurite outgrowth, neuronal migration, synapse formation, neurogenesis, and synaptic transmission and consolidation [9]. Not only the epidemiological information, there were some antipsychotic agents, such as pimozide, trifluoperazine, and brexpiprazole sensitizing glioblastoma or glioma stem cells, partially indicating that schizophrenia and glioma may have crosstalk on pathological mechanisms [3, 10, 11]. Above information provides hints, and we hypothesize that key genes of schizophrenia were crosstalk and negatively associated with the development of glioma. However, limited studies directly unveiled the association between schizophrenia genes and the survival of glioma. Therefore, identification of key genes of crosstalk between schizophrenia and glioma will be helpful to guide more insightful investigations and excavate novel targets of biochemical and pharmacotherapy research of schizophrenia and glioma in the future.

In summary, our study using a special gene expression profiles, based on GEO datasets, TCGA, and CGGA, first time, directly found 24 key genes of schizophrenia genes on the opposite development of glioma through different expressed genes (DEGs) screening, weighted gene coexpression network analysis (WGCNA), disease-specific survival (DSS), and glioma grading, and they were verified by Gene Set Enrichment Analysis (GSEA). These identified key genes through this workflow help to determine novel therapeutic targets for the treatments of schizophrenia and glioma.

2. Materials and Methods

2.1. Analysis Overview. In this study, the dataset about schizophrenia (SCZ) was downloaded and reanalyzed, which was GSE35794 (platform GPL6244) from the National Center of Biotechnology Information Gene Expression Omnibus, the most acknowledged gene expression resource for scientific community submitted data. All samples in this dataset were from cadaver with proper consent. The data were divided into two groups (the SCZ group and control group). DEGs between SCZs and control tissues were screened using the R software. Once DEGs were identified, functional and pathway enrichment analyses were used to analyze connections of DEGs and to determine the interaction of DEGs on the molecular level. Meanwhile, WGCNA was used to find the key genes positively and negatively related to schizophrenia. Then, DSS was used to find the key genes' opposite influence of survival in glioma and GETx and TCGA were used to evaluate the expression levels of these key genes in different glioma grading. Additionally, two genes were exhibited as examples and analyzed by GSEA to check whether the common pathways of schizophrenia and glioma in the two genes showed opposite trends or not. Lastly, six key genes were reevaluated in another brain tumor database, CGGA (Chinese Glioma Genome Atlas), through gene expression in different glioma grading and survival curves in patients with high- or low-level gene expression. The processing flow is shown in Figure 1.

2.2. Microarray Data Preprocessing and DEGs Screening. The raw data of GSE35794 were filtered out by probes with a corresponding gene symbol, and the average value of the gene symbols was calculated with multiple probes. Between the two groups, the Linear Models for Microarray Data Analysis (limma) package was used to screen the DEGs [12]. Threshold values were set as $p < 0.05$.

2.3. Functional Analysis of the DEGs. Nowadays, the most commonly trusted gene function knowledge bases are gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). In this study, we used a clusterProfiler package to analyze function profiles (gene ontology, GO; Kyoto Encyclopedia of Genes and Genomes, KEGG) of genes and gene clusters to identify major biological functions of genes [13]. All DEGs went through KEGG pathway analyses and GO analyses including the biological process (BP) using clusterProfiler.

2.4. Gene Network Construction and Module Detection. WGCNA was used to identify modules of coexpressed genes within gene expression networks [14]. To construct the network, the absolute values of Pearson correlation coefficients were calculated for all possible gene pairs. Values were entered into a matrix, and the data were transformed so that the matrix followed an approximate scale-free topology. A dynamic tree cut algorithm was used to detect network modules. WGCNA R package was used to evaluate the correlation of schizophrenia and module membership by the 'p. weighted' function [15].

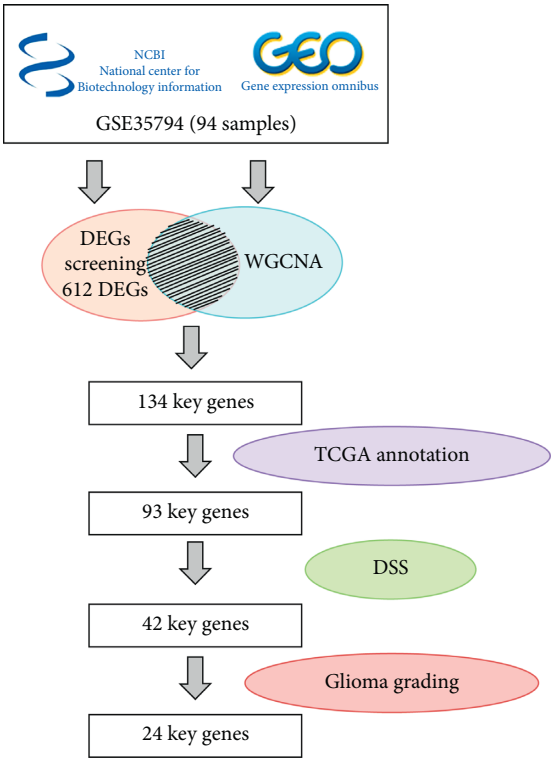


Figure 1: The workflow of screening the key genes playing opposite roles in schizophrenia and glioma.

2.5. Key Genes of Schizophrenia Evaluated by DSS and Glioma Grading. After the DEGs were evaluated by WGCNA, the left genes were tested by DSS from TCGA. The upregulated key genes were intersected with hazard ratio (HR) < 1 of glioma (generated from DSS in TCGA, p value < 0.05), and downregulated key genes were intersected with HR > 1 of glioma (generated from DSS in TCGA, p value < 0.05) to find out key genes that may play opposite roles in schizophrenia and glioma.

Following that, the left key genes were evaluated by TCGA and the Genotype-Tissue Expression (GTEx) databases to observe the gene expression in different glioma grading. Expression data of glioma and normal controls were obtained from TCGA and the Genotype-Tissue Expression (GTEx) databases.

2.6. Gene Set Enrichment Analysis (GSEA). GSEA helps to determine whether distinct sets of genes have significant differences using computational methods. We performed the GSEA analysis using the software clusterProfiler package of R language. Differences were considered statistically significant at $|NES| > 1$, nominal p value < 0.05, and FDR q value < 0.25. Then, the genes were used with the clusterProfiler package for analysis of the GO biological process. The cutoff value for the significant enrichment was set at $p < 0.05$.

2.7. Statistical Analysis. Statistical analyses and graphics were undertaken using R version 3.5.1. Student's t-test was used for the univariate analyses where appropriate. Survival rates of the expression level (high vs. low) were estimated by the Kaplan–Meier method with Rothman CIs. Survival curves were compared with the logrank test. The HR and 95% CI associated with the expressions of h-prune were estimated through a univariable Cox regression. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Data Preprocessing and DEGs Screening. Removing the bipolar and depression samples, the dataset of GSE35974 contained 94 samples of the human cerebellum from schizophrenia and unaffected control in total 144 samples. The data of GSE35974 contained the clinical characteristics of age and gender, but not antipsychotic drug treatment. From the 94 samples, 612 DEGs were screened out with a threshold of $p < 0.05$. The limma package was employed to filter, and 332 upregulated genes and 280 downregulated genes were recognized afterward. A volcano plot and heatmap were depicted with the full picture of DEGs in all cases (Figures 2(a)–2(b)).

3.2. Functional Analysis of DEGs. By examining functions of DEGs, we had a better view about disease progression of schizophrenia. The GO analysis and KEGG pathway were employed to sort out DEGs. In GO biological processes, the most overrepresented are gene silencing by miRNA, G1/S transition of mitotic cell cycle, regulation of histone modification, regulation of calcium ion transport into cytosol, positive regulation of mitochondrion organization, miRNA mediated inhibition of translation, regulation of nucleocytoplasmic transport, negative regulation of protein localization to membrane, positive regulation of ATP biosynthetic process, and response to leucine (Figure 2(c)). In the KEGG pathway analysis, DEGs were notably enriched in MicroRNAs in cancer, phospholipase D signaling pathway, neurotrophin signaling pathway, mTOR signaling pathway, insulin signaling pathway, synaptic vesicle cycle, insulin resistance, cell cycle, phosphatidylinositol signaling system, and Fc gamma Rmediated phagocytosis (Figure 2(d)).

3.3. Schizophrenia Genes Screened by WGCNA. Gene expression network analyses are an analyzing approach for describing the interactions among groups of transcripts so that the systematic alterations in expression could be observed. The modules identified by WGCNA were illustrated in a cluster dendrogram of modules identified by WGCNA, eigengene adjacency heatmap of module expression associations, module-trait relationship, and interesting genes in network heatmap (Figures 3(a)–3(d)), indicating that the clinical features were specific to schizophrenia. The module-trait relationship >0.3 was set as the modules positively related to schizophrenia, which were module midnight blue, red, and grey, and module-trait relationship < -0.3 was set as the modules negatively related to schizophrenia, which were module grey 60 and brown. Therefore, WGCNA was applied to evaluate gene expressions from SCZ and control

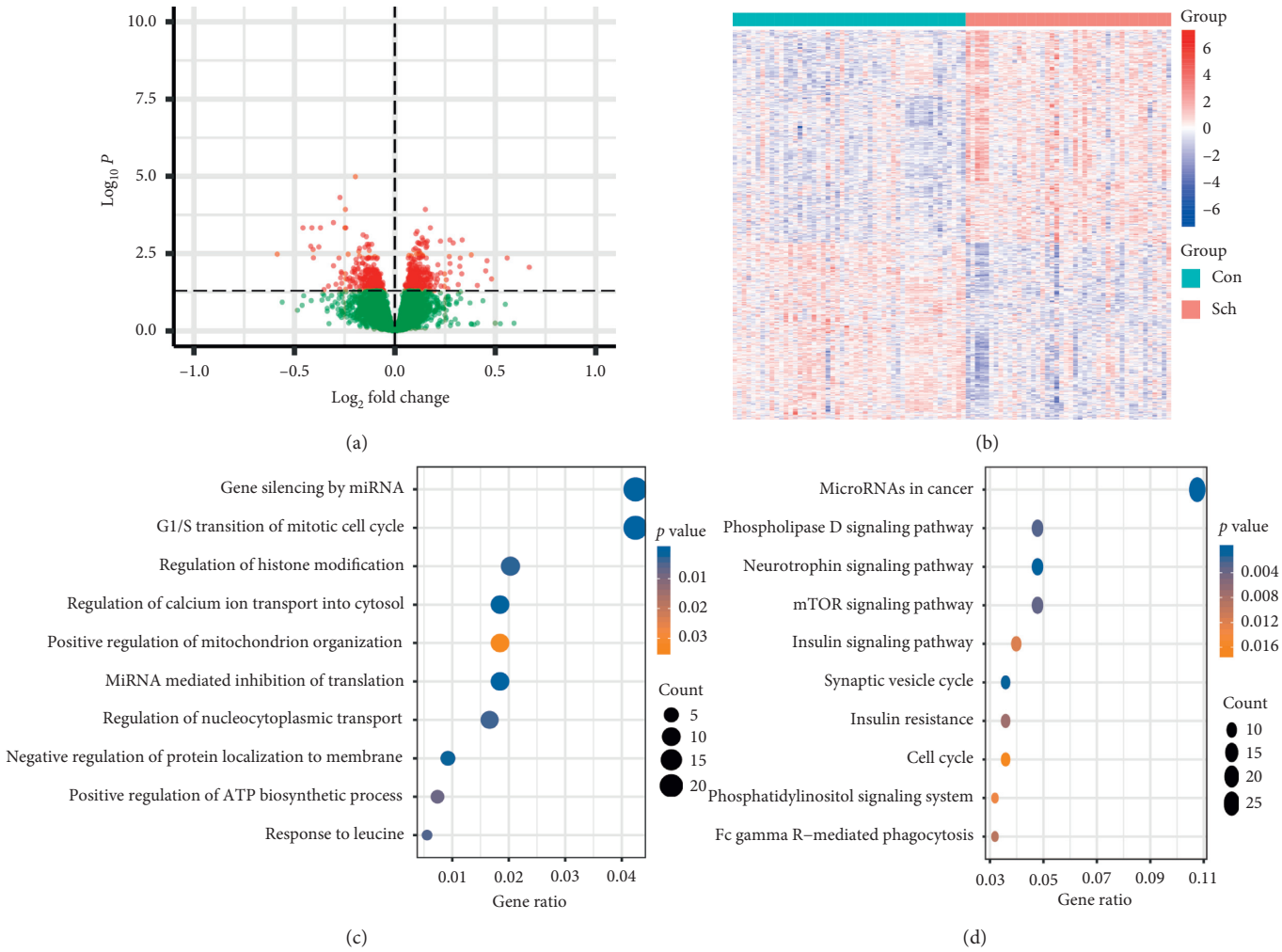


Figure 2: Functional Analysis of DEGs of schizophrenia. (a) Volcano plot for the DEGs. A total of 612 DEGs were screened out with a threshold of $p < 0.05$. (b) Heatmap showing the expression profiles of DEGs, with a gradual change in color from red to blue indicating high to low. (c) GO enrichment in molecular function with the 10 terms. (d) KEGG pathway enrichment analysis of common DEGs with 10 terms.

samples in GSE 35974. The intersections of module midnight red with upregulated DEGs and the intersections of grey 60 with downregulated genes were zero. Finally, there were 26 upregulated key genes and 107 downregulated key genes identified by WGCNA, which found the genes close to clinical data of schizophrenia.

3.4. DEGs Screened by DSS in Glioma. Since schizophrenia patients had lower cancer incidence, the opposite gene expressions in glioma were expected to identify among 134 key genes filtered by WGCNA. DSS of glioma, a survival rate specific to glioma, was recognized as the evaluation method. Among 134 key genes, 93 key genes with annotations in TCGA were chosen for DSS. There were 6 upregulated key genes intersected with high risk in glioma and 36 downregulated key genes intersected with low risk in glioma through DSS, shown in Table 1. 6 most significant DEGs were chosen as examples to exhibit the difference between schizophrenia and control (Figures 4(a)–4(c) and 4(g)–4(i), $^{**}p < 0.01$, $^{***}p < 0.001$), and their survival curves of glioma (Figures 4(d)–4(f) and 4(j)–4(l)). Other DEGs are shown in Table 1 for your reference in your advance research.

3.5. Key Genes Evaluated by Glioma Grading. Although 42 key genes were selected by WGCNA and DSS, the range of potential targeted genes for future biochemical and pharmacological research studies would be better to be compressed. Therefore, we considered that gliomas could be divided into low-grade gliomas (grade I and II) and high-grade gliomas (grade III and IV) according to the World Health Organization classification criteria. When the grade was higher (malignancy degree increased), the gene expression increased, meaning this gene may play a role in pathogenesis of glioma. Checking the expression levels of these genes in different glioma grading may help to observe the relationship of 42 key genes and the severity of glioma. If the expression level of a gene increases in higher-grade glioma, this gene may be closer to pathogenesis of glioma,

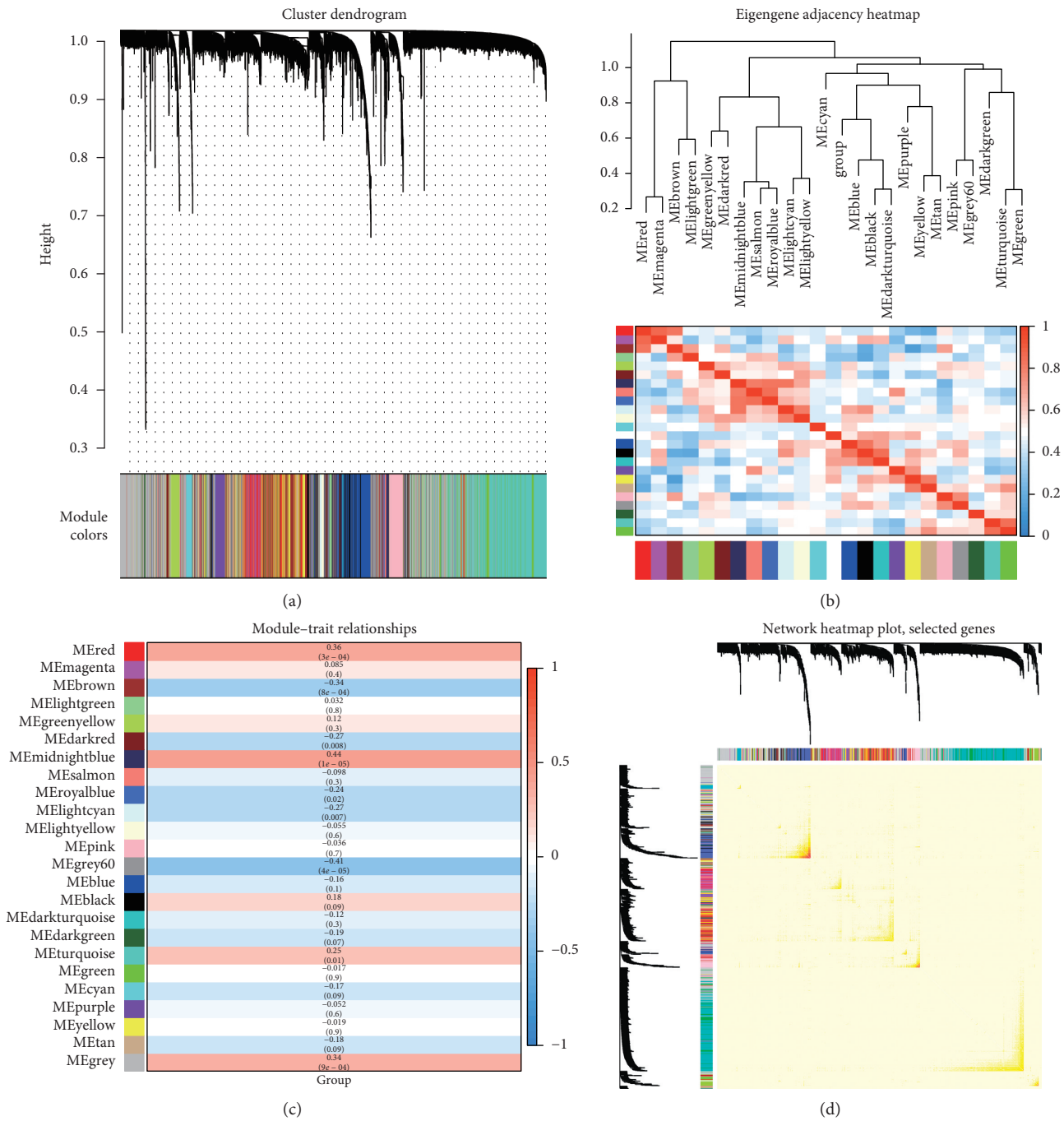


Figure 3: Modules features chosen by WGCNA. (a) Cluster dendrogram of modules identified by WGCNA. (b) Eigengene adjacency heatmap of module expression associations. (c) Module-trait relationships. (d) Network heatmap plot among selected genes.

and vice versa. Therefore, the upregulated key genes in schizophrenia were expected to find increased expression levels in higher-grade glioma, and downregulated key genes in schizophrenia decreased in lower-grade glioma. After observing the expression trends, 24 genes, i.e., ACOT9, ADA2, AP2M1, APMAP, APOO, ARPC2, CAMK2D, DHDDS, EIF3K, ERGIC3, EXTL2, FUNDC2, LZIC, MPC2, MYL12B, PAM, PRMT2, SLC35B4, TMEM167A, TMEM19, TSPAN13, VPS35, CNKSR2, and RTN4RL1, were accorded with the above expectation. 6 key genes (CAMK2D, EIF3K, MPC2, MYL12B, PAM, and SLC35B4) of schizophrenia are displayed as the expression trends in glioma grading in Figures 5(a)–5(f) ($^{**}p < 0.01$, $^{***}p < 0.001$). CAMK2D and MPC2 were selected as the examples and evaluated by GSEA. In the common pathways of schizophrenia and glioma, these two genes showed opposite trends in schizophrenia and glioma, shown in Figures 5(j)–5(k).

CGGA, another brain tumor database, stored Chinese glioma datasets over 2,000 samples with mRNA sequencing, mRNA microarray, and matched clinical data to benefit the

Table 1: 42 genes closely related to schizophrenia and glioma (TCGA) but may play opposite roles in the two diseases.

Upregulated key genes of schizophrenia intersected with high risk in glioma	CNKSR2, NPFFR1, RTN4RL1, WAPL, ZNF281, and ZNF519
Downregulated key genes of schizophrenia intersected with low risk in glioma	ACOT9, ADA2, AP2M1, APMAP, APOO, ARPC2, C19orf12, CAMK2D, CAP2, CFL1, CNR1, DHDDS, DYNLT3, EIF3K, ERGIC3, EXTL2, FDPSP2, FUNDC2, GPAT3, LAMTOR5P1, LZIC, MPC2, MRAP2, MYL12B, NRN1, PAM, PGK1, PRMT2, RHBG, SLC35B4, SNX10, TMEM159, TMEM167A, TMEM19, TSPAN13, and VPS35

correlation and survival analysis. CAMK2D, EIF3K, MPC2, MYL12B, PAM, and SLC35B4, selected from the schizophrenia dataset and TCGA, were reevaluated in CGGA through gene expression in glioma grading and survival curves between patients having high level of gene expression and those having low level of gene expression. Luckily, the analyzed datum was quite in line with the above datum. These six key genes expressed increasingly in a higher glioma grade, shown in Figures 6(a)–6(f) ($^{**}p < 0.01$, $^{***}p < 0.001$). Additionally, the survival rate was higher in patients with low gene expression level, shown in Figures 6(j)–6(k).

4. Discussion

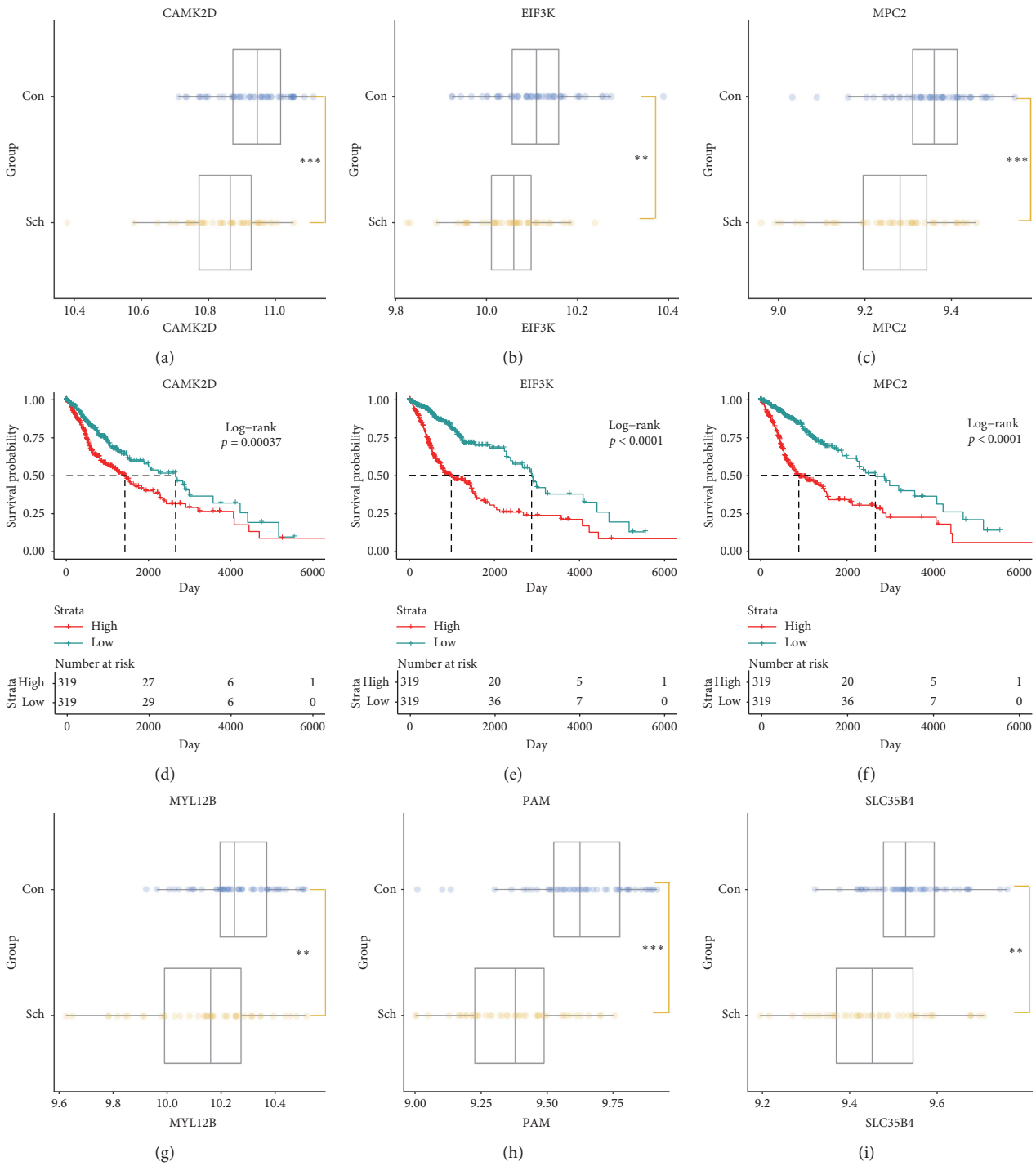
Schizophrenia patients were found to have reduced overall risk of cancers compared to the general population, including the cancers of lung, melanoma, brain, breast, corpus uteri, and prostate. Additionally, there were several antipsychotic agents presenting their influence on glioma cells. Although the epidemiology and pharmacological evidences provided the association of schizophrenia and incidence of cancers, the gene profiles associated with underlying mechanisms between schizophrenia and glioma were still unclear. Gene profiling, based on our workflow, may provide the most related genes that play opposite roles in the two cerebral diseases.

In our study, the workflow first used DEGs screening, WGCNA, DSS, and expression in glioma grading to find the key genes that were significantly and differently expressed in schizophrenia patients, closely related to clinical data of schizophrenia, opposite influence of survival of glioma, and opposite trends of gene expressions in glioma, respectively. Finally, 24 key genes of schizophrenia were screened out, showing opposite influences in the survival of glioma and opposite gene expression trends in glioma grading, i.e., key genes upregulated in schizophrenia and low risk of glioma and key genes down-regulated in schizophrenia but also having high risk glioma. This workflow is an innovation of gene profiling to nicely find the intersection containing the key genes playing opposite roles in the two brain diseases.

Based on our findings, several proteins expressed by above genes were found in peers' mechanism studies both in schizophrenia and glioma. Interestingly, these genes indeed showed totally two direction effects, i.e., the risky genes in schizophrenia showed in glioma patients with good survival, while genes may prevent the development of schizophrenia showed in glioma patients with worse survival. Due to the limited studies, there were CAMK2D, EIF3K, MPC2, MYL12B, PAM, and SLC35B4 from 24 genes with references discussing their different roles in the two cerebral diseases.

CAMK2D encodes one of the subfamilies of calcium/calmodulin-dependent kinase II (CaMK II), which regulate Ca^{2+} homeostasis. In mammalian cells, CaMK II is composed of four different chains: α, β, γ, and δ. The encoded protein is contributed to this kinase δ chain [16]. Calcium/calmodulin-dependent kinase II alpha knockouts mice presented schizophrenia features that showed remarkable elevated levels of D2 receptors in their high-affinity state [17]. This trend was consistent with our results that CAMK2D expression was significantly lower in schizophrenia patients. Meanwhile, the activity of calcium/calmodulin-dependent protein kinase II was upregulated in resistant glioma cells and its cDNA transfection in sensitive glioma cells lead to glioma cells resistance, indicating that CaMK II may be involved in malignant glioma cell resistance [18]. This association was in line with our finding that CAMK2D expression was associated with the decreased DSS in glioma patients. Although there were some studies revealed the cell resistant by CaMK II may be through the Fas pathway, our findings provided a new vision of CAMK2D that may regulate common pathogenesis of schizophrenia and glioma so that the potential treatments of schizophrenia could be found in the glioma pathway, and vice versa.

MPC2, encoding one subunit of the mitochondrial pyruvate carrier (MPC) complex, a transporter protein in the mitochondrial inner membrane to control pyruvate transportation into the mitochondria, therefore plays a crucial role in the pathways of pyruvate metabolism and citric acid (TCA) cycle and glucose/energy metabolism [19]. The MPC complex contains MPC1 and MPC2, two obligate protein subunits. The loss in any subunit results in the destabilization of the MPC complex and thus dysfunction of the MPC complex [20]. Recent GWAS and meta-analysis in East Asian population showed MPC2 variant rs10489202 may be a risk locus for schizophrenia [21–23], indicating that expression of MPC2 may play a role in pathogenesis of schizophrenia. Additionally, abnormal MPC function was found in several cancers and contributed proliferation of cancers [20]. A glioma study found that MPC1 distinguished improved survival but MPC2 worsened survival in 1p19q-intact tumors ($p < 0.01$) [24]. These studies partially supported our finding that MPC2 expression was striking lower in schizophrenia patients and associated with deteriorated survival in glioma. Therefore, MPC2 could be a novel targeting gene to investigate new mechanisms between the two diseases.



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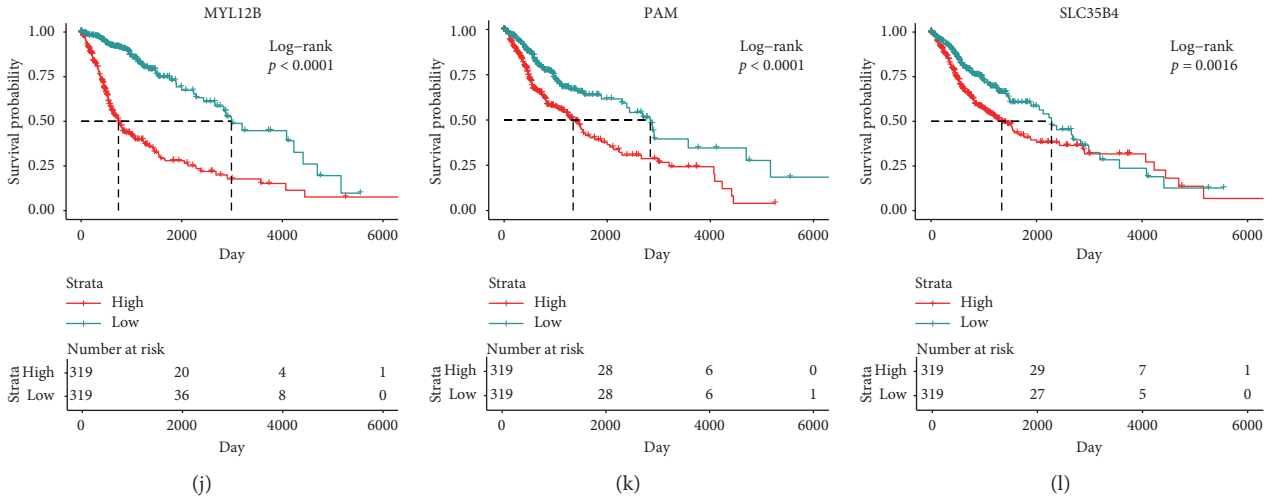
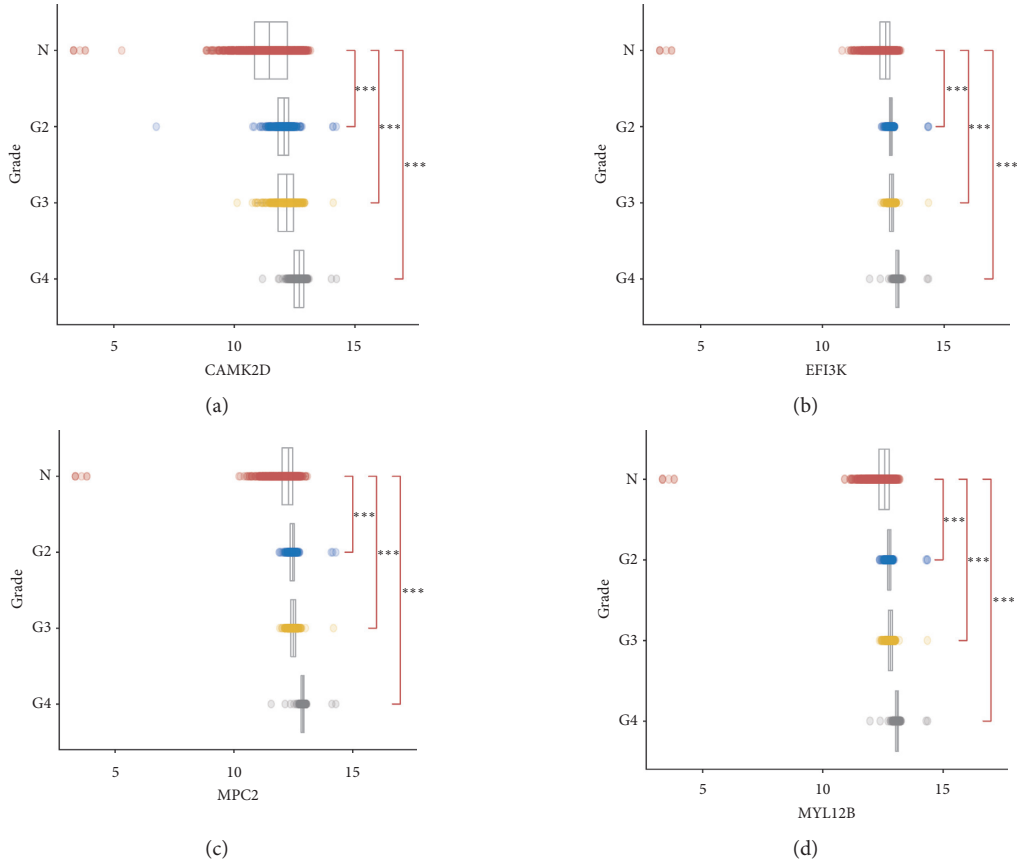


Figure 4: 6 key genes as examples exhibiting the gene expression differences in schizophrenia and survival curves in glioma (TCGA). (a b, c g, h i) The differences of gene expression of CAMK2D (a), EIF3K (b), MPC2 (c), MYL12B (g), PAM (h), and SLC35B4 (i) in schizophrenia and control. (d e, f, j, k l) The survival curves of CAMK2D (d), EIF3K (e), MPC2 (f), MYL12B (j), PAM (k), and SLC35B4 (l) in glioma patients (TCGA). $^{**}p < 0.01$, $^{***}p < 0.001$.



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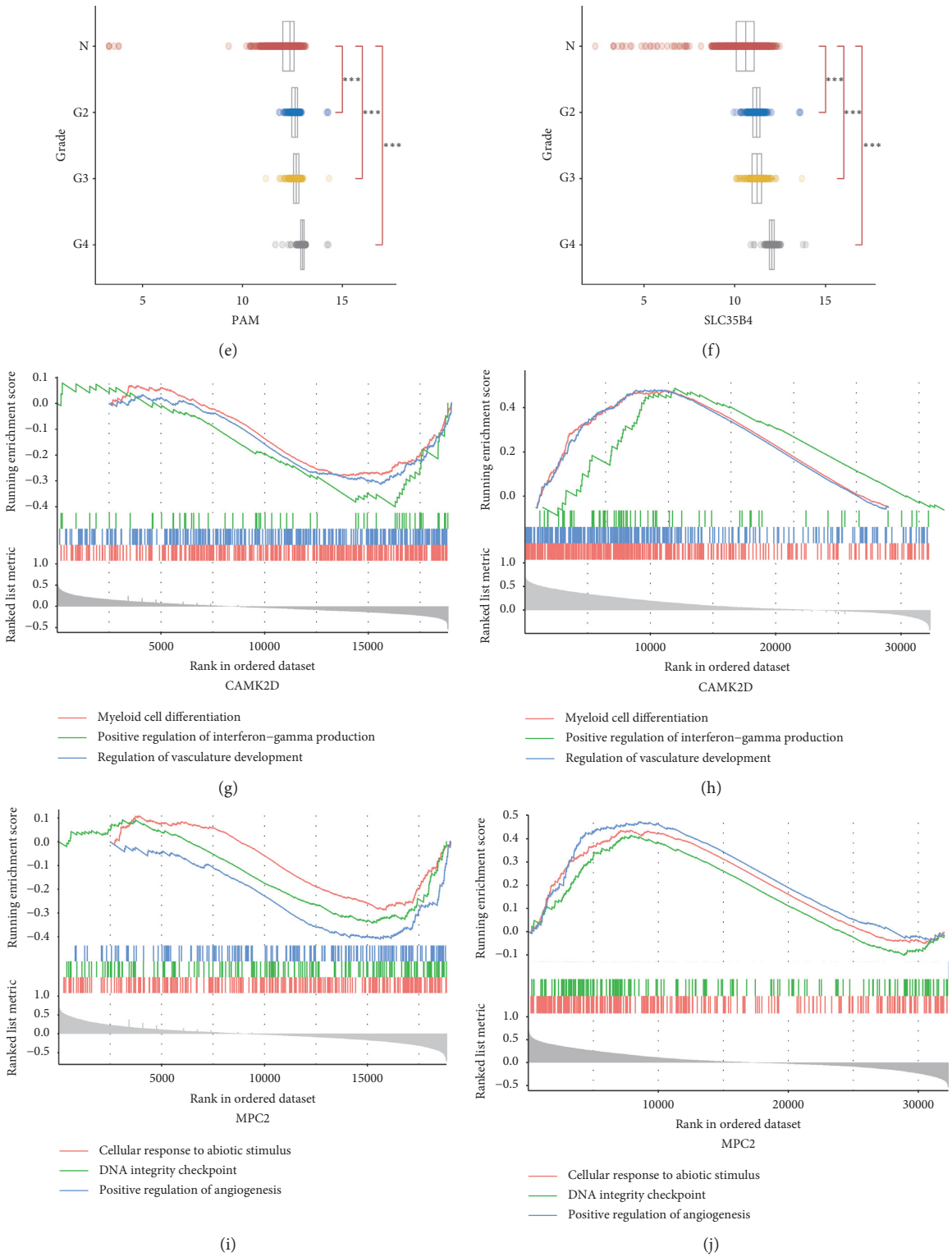
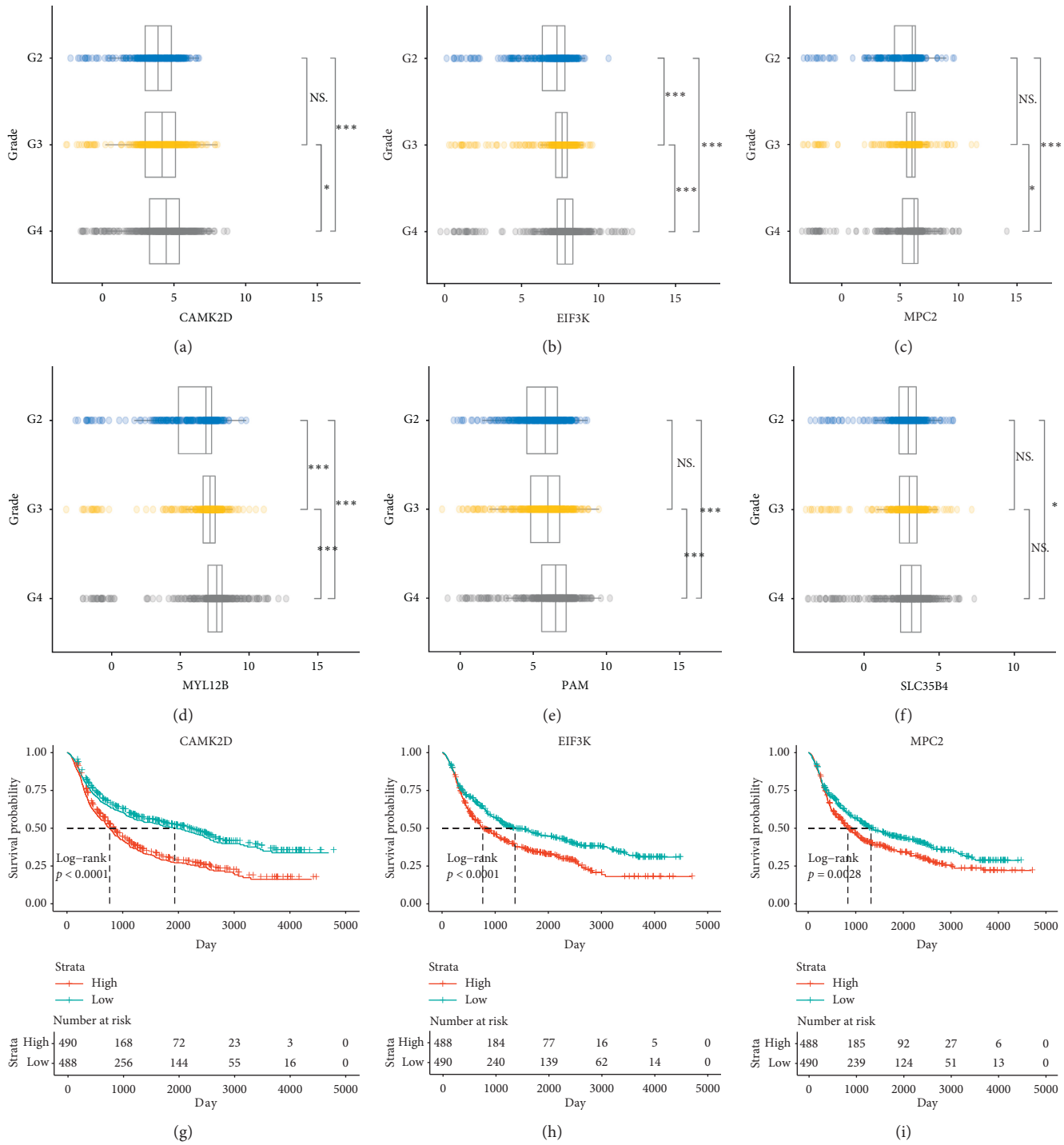


Figure 5: 6 key genes as examples exhibiting their expressions in different glioma grading (TCGA) and 2 genes as examples to show their pathways opposite in schizophrenia and glioma (a–f) The trends of gene expressions of CAMK2D, EIF3K, MPC2, MYL12B, PAM, and SLC35B4 in different glioma grading (TCGA). (g–h) The GSEA analysis of CAMK2D in schizophrenia (g) and glioma (h). (i–j) The GSEA analysis of MPC2 in schizophrenia (i) and glioma (j). $^{**}p < 0.01$, $^{***}p < 0.001$.

EIF3K encodes eukaryotic translation initiation factor-3 (eIF3) subunit k, assembling with other 12 subunits to form the largest eIF3 complex, which implicates in mRNA translation initiation, termination, ribosomal recycling, and the stimulation of stop codon readthrough [25]. Recent studies found that the changes of expression of a single eIF3



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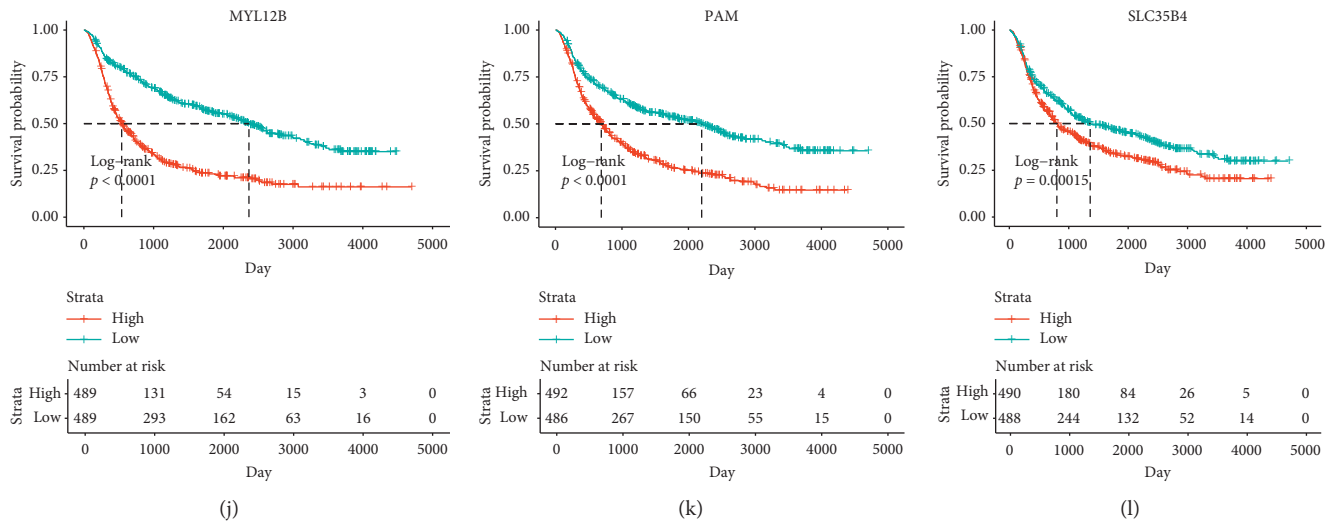

Figure 6: 6 key genes, CAMK2D, EIF3K, MPC2, MYL12B, PAM, and SLC35B4, were reevaluated by CGGA in glioma grading and survival curves. (a–f) The trends of gene expressions of CAMK2D, EIF3K, MPC2, MYL12B, PAM, and SLC35B4 in different glioma grading (CGGA). (g–i) The survival curves of CAMK2D (g), EIF3K (h), MPC2 (i), MYL12B (j), PAM (k), and SLC35B4 (l) in glioma patients (CGGA). $^{**}p < 0.01$, $^{***}p < 0.001$.

subunit influence other subunit expressions [26], suggesting that changes of expression of any single eIF3 subunit may promote human disorders, including neurodegenerative disease, cancer, and infection. eIF3 was interacted with the protein encoded by a candidate gene of schizophrenia, disrupted-in-schizophrenia 1 (DISC1) gene, which was disrupted by a balanced chromosomal translocation, *t* (1; 11) (*q*42.1; *q*14.3) [27]. The translocation reduced DISC1 protein expression [28]. Overexpression of DISC1 promoted the assembly of the eIF3 complex [27], generating a hypothesis that lacks DISC1 protein may be hard to stimulate the expressions of eIF3 subunits. These studies not only agreed well our finding that the expression of EIF3K was decreased in schizophrenia but also supported to hypothesize that the protein encoded by EIF3K may be involved in schizophrenia pathogenesis. Additionally, the subunits eIF3 a, b, c, e, and f have been found as the oncogene overexpressed in several cancers, including nonsmall-cell lung cancer, breast cancer, cervical carcinoma, esophagus squamous-cell carcinoma, gastric carcinoma, and osteosarcoma. [25]. Recently, systematically profiling found that the expression of eIF3b, eIF3i, eIF3k, and eIF3m was increased with the glioma grade and poorer overall survival [29]. More studies showed knockdown of EIF3B [30], decreasing EIF3C [31], and silencing EIF3D [32] and EIF3E [33] alleviated proliferation and migration of glioma cells. Our profiling results were in accordance with above studies that increased expression of EIF3K was associated with decreased survival of glioma patients and increased glioma grades. Based on the peer investigations of pathogenesis on both schizophrenia and glioma, we have the reason to advise EIF3K as the potential targets of the mechanism study of schizophrenia and glioma, resulting new therapies for both diseases.

MYL12B encodes a subunit of myosin regulatory light chain 2 (MYL2), which regulates the activity of nonmuscle myosin II [34]. Knockdown of MYL12A/12B leads to dramatic changes of cell morphology and dynamics in NIH 3T3 cells [34]. A GWAS study found that the susceptibility genes of schizophrenia were associated with mRNA levels of MYL2 ($p < 1.0E-4$) [35]. Another study conducted on postmortem brains from schizophrenia patients observed that MYL was phosphorylated in the anterior cingulate cortex [36]. Regarding to glioma, MYL was related to glioma cell migration [37], which can be blocked by inhibitors of myosin II [38]. Therefore, above studies of MYL and myosin II both on schizophrenia and glioma accorded with our findings that MYL12B was expressed lower in schizophrenia and was found decreasing DSS when glioma grade increasing. Although there were only several studies investigated on MYL12B, they could be the forerunner of exploring this potential intersecting gene, MYL12B, to excavate some novel minerals of the two diseases' mechanisms.

PAM encodes a preproprotein, peptidylglycine α-amidating monooxygenase (PAM), which is proteolyzed to generate the mature enzyme, including two distinct catalytic domains, a peptidylglycine α-hydroxylating monooxygenase (PHM) domain and a peptidylalphahydroxyglycine α-amidating lyase (PAL) domain [39]. These domains sequentially catalyze neuroendocrine peptides to active α-amidated products and regulate complex signaling between intestinal organs, peripheral neurons, and the central neuronal system [39]. PAM was recognized as one of the most promising candidate genes of schizophrenia [40]. Moreover, the expression of PAM was found increased in glioma cells [41]. There studies linked PAM to both schizophrenia and glioma, supporting our gene profiling that PAM may have a close relationship of both pathogenesis. Currently, there were limited mechanism studies of PAM on schizophrenia and glioma so that researchers may reveal deeper mechanisms that why an enzyme related to secreted peptides could conduct pathogenic or antipathogenic effects on the two diseases.

SLC35B4 encodes a subfamily of the solute carrier family of human nucleotide sugar transporters, which transport cytosolic nucleotide sugar to glycosyltransferases that reside in the lumen of the endoplasmic reticulum (ER) and/or Golgi apparatus [42]. Recently, SLC35B4 was evaluated as an empirically significant SNP of schizophrenia through a GWAS study [43]. In the cancer side, although there was no direct study of investigating the effects of SLC35B4 on glioma, several studies suppressed SLC35B4 expression to benefit the cancer therapies. SLC35B4 expression was markedly higher in gastric cancer tissues and involved in the progression of gastric cancer [44]. In advanced prostate cancer, a regulatory SNP, rs1646724, influenced SLC35B4 to promote the prostate cancer proliferation, migration, and invasion [45]. In glioma cells, SLC22A18 [46], SLC8A2 [47], SLC9A1 [48], and SLC34A2 [49] were examined as the risk genes of glioma. Our data also indicated that SLC35B4 may be involved in pathogenesis of both schizophrenia and glioma, which provide hints that researchers may shed light on this glycosyltransferase gene for drug development of both two diseases.

5. Conclusion

Through our process, 24 genes were sieved for future studies. Luckily, 6 genes were found by the mechanism studies both in schizophrenia and glioma. However, some biomedical investigations were not the direct indications. Additionally, the dataset of GSE35974 did not contain the clinical characteristics of antipsychotic drug treatment so that the influence of antipsychotic drug treatment could not be revealed. Therefore, further and deeper research could be conducted that why the two diseases shared gene profiles playing the opposite role in their pathogenesis. The negative overlap of risk genes between schizophrenia and glioma could hook more interests of investigators to discover novel pathways and potential pharmacotherapies based on our gene profiling.

Data Availability

The datasets generated during and/or analyzed during the current study are available in the GO, TCGA, and CGGA repositories.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

HEATR1 Deficiency Promotes Chemoresistance via Upregulating ZNF185 and Downregulating SMAD4 in Pancreatic Cancer

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Objective. To discover the correlated gene with HEATR1 in regulating chemoresistance of gemcitabine. *Methods*. Gene chip analysis was performed to find out differential genes between HEATR1-KD and control groups. The top 20 genes were subjected to high-content screening, and functional assay was implemented. Gene expression profiling was carried out to find the downstream target. Immunohistochemistry and survival analysis were performed. *Results*. ZNF185 fold change (4.5285) was the most significant between the HEATR1-KD and control groups. Knocking down ZNF185 could promote the chemosensitivity, apoptosis, and proliferative inhibition, with SMAD4 significantly upregulated. Patients with high HEATR1 and SMAD4 or low ZNF185 exhibited better survival. *Conclusion*. HEATR1, ZNF185, and SMAD4 could affect the chemosensitivity of gemcitabine and may be the indicators of gemcitabine selection in the chemotherapy of pancreatic cancer.

1. Introduction

Pancreatic cancer is the most malignant GI tract cancers with the worst prognosis around the world. It is predicted to reach the second leading cause of cancer-related death by 2030 in the United States [1]. Gemcitabine has been the cornerstone chemotherapeutic agent of pancreatic cancer in the past 20 years [2]. Recently, the novel chemotherapeutic regimen including FOLFIRINOX (fluorouracil, leucovorin, irinotecan, and oxaliplatin) [3] or gemcitabine combined with albumin-bound paclitaxel [4] seemed to improve the survival but only limited to the patients with good performance because of the side effect of intolerable toxicity of FOLFIRINOX or paclitaxel. Therefore, gemcitabine is still the first-line chemotherapeutic agent for pancreatic cancer, and understanding the mechanisms of the resistance will provide significant clinical strategy. Unfortunately, the mechanism of gemcitabine resistance has not been fully elucidated although the previous research was focused on the molecular and cellular changes including gemcitabine metabolism enzymes, inhibition of the apoptotic pathway, activation of the cancer stem cells (CSC), or epithelial-to-mesenchymal transition (EMT) [5].

HEAT repeat-containing protein 1 (HEATR1) contains HEAT repeats, initially found in some proteins including huntingtin, elongation factor 3, and the PR65/A subunit of phosphatase 2A [6]. The human HEATR1 gene is located on chromosome 1q43 and encodes a high molecular weight (236 KDa) protein with 2144 amino acids. Our team discovered the effect of HEATR1 on the chemosensitivity of gemcitabine and published the original research on *Cancer Research* in 2016, explaining the possible mechanism in that HEATR1 enhanced the chemosensitivity to gemcitabine by facilitating the interactions between AKT and PP2A and promoting Thr308 dephosphorylation [7]. In this study, our team aim is to discover novel functional genes correlated with HEATR1 in sensitizing pancreatic cancer cells to gemcitabine, which may help to search for a new therapeutic target and improve the efficacy of gemcitabine in the pancreatic cancer.

2. Materials and Methods

2.1. Ethical Statement. The study protocol was approved by the Independent Ethics Committee at Zhongshan Hospital, Fudan University. Written informed consent forms were signed by all the participating patients, and all the

experiments were in accordance with the Declaration of Helsinki revised in 2013 [8].

2.2. Cell Lines. Human pancreatic cancer cell lines PANC-1, SW 1990, MIA-PaCa2, Patu-8988, and Capan-1 were purchased from ATCC, and the identification of all the cell lines was confirmed by STR profiling at GeneChem Company (Shanghai, China). PANC-1 and MIA-PaCa2 were cultured in a medium containing high-glucose Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco) in a humidified 37°C and 5% CO_2 incubator, while Patu-8988, Capan-1, and Sw 1990 cell lines were cultured with the RPMI-1640 medium (Gibco) instead of DMEM.

2.3. Quantitative Real-Time PCR. Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) from human pancreatic cancer cell lines. Standard cDNA synthesis reactions were implemented using the M-MLV Reverse Transcriptase kit (Promega, USA) following the instructions [9]. For qRT-PCR analysis, reverse transcribed products were amplified using SYBR Premix Ex Taq (TaKaRa, Japan). PCR reactions were carried out using the ABI 7500 Real-Time PCR (Applied Biosystems, CA, USA) and repeated three times. Relative mRNA levels were normalized to GAPDH, and the relative expression of transcripts was analyzed by using the $2^{-\triangle\triangle Ct}$ methods. The following primers were used: human HEATR1 (Gene accession no. NM_018072): 5-TTCACTTGTCGCCTTACTTCC-3 (forward) and 5-CCAGAACCATCTGTGCTTTGA-3 (reverse); human ZNF185 (Gene accession no. NM_007150):5-GATCCGAGACTGTCCAAAGAT-3 (forward) and 5-AAT GGTGTCACGGTGAATGA-3 (reverse); human SMAD4 (Gene accession no. NM_005359):5-ACGAACGAGTTG-TATCACCTGG-3 (forward) and 5-TGCACGATTACTTGG TGGATG-3 (reverse); human GAPDH: 5-TGACTTCAA-CAGCGACACCCA-3 (forward) and 5-CACCCTGTTGCT GTAGCCAAA-3 (reverse).

2.4. Western Blot Analysis. Total proteins extracted with the cell lysate buffer and protease inhibitors were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were incubated with the primary antibody: HEATR1 (1 : 10000, ab241610, Abcam, CA, USA), FLAG (1 : 2000, F1804, Sigma, USA) , SMAD4 (1 : 1000, AF2097, R&D systems, MN, USA), and GAPDH (1 : 2000, sc-32233, Santa Cruz, Texas, USA) overnight after being blocked with 5% nonfat milk in TBS-T. Protein expression was detected with the Image System using Tanon 5200 (Tanon, Shanghai, China).

2.5. Stable Knockdown of HEATR1 and ZNF185. The GV115 puromycin lentiviral vectors were designed and constructed by GeneChem Co., Ltd. (Shanghai, China). The human HEATR1 siRNA target sequence was GCTGAA-CAAGTCCGAATAGAA. The GV115 puromycin lentiviral vectors were used as controls. Stably transfected clones for HEATR1 were validated by western immunoblot analysis. The human ZNF185 siRNA target sequence was TCCAAAGATTACCCTAGAA, and the target sequence was cloned into the GV115 lentiviral vector. ZNF185 knockdown efficiency was validated by qPCR in PANC-1 cells, and western blot validation was carried out in 293T cells expressing 3 × FLAG-ZNF185 fused protein. After knockdown efficiency validation, stable shHEATR1 cells were transfected with the shZNF185 lentivirus, and functional experiments were arranged.

2.6. Cell Proliferation Assay. For the cell proliferation assay, 5000 cells in every well dispensed in 100 μl aliquots were seeded in a 96-well plate, and the viable cells were counted after 72 hours after treated with gemcitabine (10 μM). Cells were incubated in 10% CCK-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA) diluted in a normal culture medium for an additional 2 h. For estimating the viable cells and IC50 values, the absorbance at a wavelength of 450 nm was measured in every well. Finally, the cells were fixed with 1% paraformaldehyde for 30 minutes and stained with 0.1% (w/v) crystal violet for 30 min [10]. The numbers of cell colonies were counted using the Image-Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD, USA).

2.7. Cell Apoptosis Assay. Apoptosis was measured by using the Annexin V-APC Kit (BD Bioscience) after 72 hours after treated with gemcitabine (10 μM). Briefly speaking, the cells were harvested with trypsin, washed twice with ice-cold PBS, and resuspended 1 × binding buffer. Then, 10 ul of annexin V-APC was added into 200 ul of cell suspensions. After incubation for 15 mins, the population study of the target cohort was performed by a FACS Aria II flow cytometer (BD Bioscience) [11].

2.8. Gene Chip Analysis. Total RNAs were extracted from cells and then subjected to gene chip analysis to find out differential expression genes (DEGs) (fold change >1.5 and P value < 0.05) between the two groups (HEATR1-KD vs NC). DEGs were further analyzed, and 30 genes were selected. Selection principles were as follows: (1) literature review was carried out to ensure that the selected DEGs were not reported with functions previously in pancreatic cancer; (2) transmembrane protein-encoding genes were ruled out due to their low knockdown efficiency; (3) fold change of selected DEGs was larger than 2.4. These 30 selected genes were tested for their expression levels in HEATR1-KD PANC-1 cells using quantitative PCR.

2.9. High-Content Screening. The high-content screening (HCS) was carried out by the method mentioned in the previous reports [12]. In brief, PANC-1 cells were transfected with HEATR1 knockdown lentivirus (HEATR1-KD cells). Then, different RNAi sequences targeting 20 genes

were transfected to HEATR1-KD cells using the lentiviral vector containing GFP tags to monitor cell viability. The GFP-positive cells were cultured and observed for 5 days. Live cells were counted and analyzed for 5 days using the HCS instrument software (CQ1, Yokogawa). Differentially expressed genes from HCS screening were uploaded to Ingenuity Pathway Analysis (IPA, Ingenuity System) for pathway analysis and molecular pathway identification.

2.10. Immunohistochemical Analysis. A tissue microarray was constructed following the standard tissue protocols as described previously [13]. Primary antibodies were HEATR1 (rabbit monoclonal; 1 : 500; Abcam, USA), ZNF185 (rabbit polyclonal; 1 : 1000: Invitrogen, USA), and SMAD4 (rabbit monoclonal; 1 : 100; Abcam, USA) followed by incubation with the secondary antibody. The positive staining was measured by a computerized image system including a Leica-CCD camera connected to a Leica-DM-IRE2 microscope. Pictures of representative fields were captured by the Leica QWin Plus v3 software. The specimens with negative or weak intensity (±) of HEART1, ZNF185, and SMAD4 were graded as low expression, while those with moderate or strong (++/+++) were graded as high expression.

2.11. Patients' Specimens and Follow-Up. From January 12, 2012, to March 3, 2017, the same pancreatic surgical group in our hospital performed radical resection for pancreatic cancer on 80 consecutive patients. Overall survival (OS) was defined as the interval between the date of surgery and death or the last follow-up day. The last follow-up day was 1 November 2019. None of the patients received any preoperative treatment, and all of the patients received a postoperative chemotherapeutic regimen of six cycles of standard gemcitabine [14]. Clinicopathologic features were staged according to the 2002 International Union Against Cancer's tumor-node-metastasis (TNM) classification system [15].

2.12. Statistical Analysis. SPSS 20.0 (Chicago, IL, USA) was used for statistical analysis. Qualitative variables were compared using the Pearson χ^2 test or the Fisher exact test. Quantitative data were recorded as mean ± SD and compared using the *t*-test or one-way ANOVA. Significance was determined at $P < 0.05$. Kaplan–Meier analysis and log-rank test were used to compare the survival. The Cox regression model was used to perform multivariate survival analysis.

3. Results

3.1. HEATR1 Promotes the Chemosensitivity to Gemcitabine in Pancreatic Cell Lines. After we examined the HEATR1 mRNA levels in 5 pancreatic cell lines, we firstly knocked down HEATR1 expression in the pancreatic cancer cell line PANC-1 using the RNA interference technique through the lentivirus vector (shHEATR1) and the scrambled sequence as the negative control (shCtrl) (Figure 1(a)). We found that the chemoresistance pancreatic cancer to gemcitabine was much more significant after HEATR1 knockdown (Figure 1(b)), and the apoptotic percentage decreased (Figure 1(c)) when proliferation increased dramatically (Figure 1(d)) on day 5 after HEATR1 was knockdown.

3.2. Gene Chip Analysis of Differentially Expressed Genes and HCS Screening after HEATR1 Knockdown. The top 20 out of 30 selected genes with high expression levels were subjected to high-content screening (HCS) in HEATR1-KD cells to testify whether they could affect cell proliferation activity when knocking down their expressions, with or without gemcitabine treatment, respectively. After the selection from the gene chips and the bioinformatical analysis, we discovered ZNF185, whose fold change (4.5285) was the most significant in these 20 genes between the HEATR1 knocking down and control groups (Figure 2(a)). Then, we tested the expression levels of these 30 selected genes in HEATR1-KD PANC-1 cells using quantitative PCR (Figure 2(b)). By high-content screening (HCS), we confirmed that knocking down ZNF185 in the pancreatic cancer cells could promote the chemosensitivity of gemcitabine (Figure 2(c)).

3.3. ZNF185 Enhances the Chemoresistance to Gemcitabine in HEATR1 Knockdown Pancreatic Cancer Cells. We confirmed the chemosensitive function of ZNF185 by observing the elevation of apoptotic percentage (Figure 2(d)) and proliferative inhibition (Figure 2(e)) of pancreatic cancer cells after knocking down both HEATR1 and ZNF185 and treated with gemcitabine. Moreover, we found that after knocking down ZNF185, the pancreatic cancer cells in the *S* phase and the G2/M phase increased, while the cells in the G1 phase decreased (Figure 2(f)), both of which indicated that the cellular proliferation was inhibited in the *S* phase.

3.4. Expression of SMAD4 Increases in the ZNF185 Knockdown Pancreatic Cancer Cells. To determine the possible downstream genetic target of ZNF185, we performed gene expression profiling of PANC-1 cells transduced with either control or shZNF185 lentivirus. Genes were upregulated and downregulated; red denotes the upregulated genes, and green denotes the downregulated genes (Supplementary Materials (available here)). Enriched canonical pathways were analyzed using IPA (Ingenuity Pathway Analysis). We discovered that the SMAD4 significantly increased. The expression of qRT-PCR (Figures 3(a) and 3(b)) and western blots (Figure 3(c)) for ZNF185 and SMAD4 were consistent with the gene expression profiling data.

3.5. Expression of HEATR1, ZNF185, and SMAD4 in the Pancreatic Cancer Tissues and Correlation with the Survival. We analyzed the expression levels in pairs of pancreatic cancer and normal pancreatic tissues. Representative IHC of HEATR1, ZNF185, and SMAD4 is shown in Figure 4. HEATR1 showed mainly low expression of 80% (64/80) in cancer and high expression of 65% (52/80) in normal pancreas (Figures 4(a) and 4(b)). We also observed that

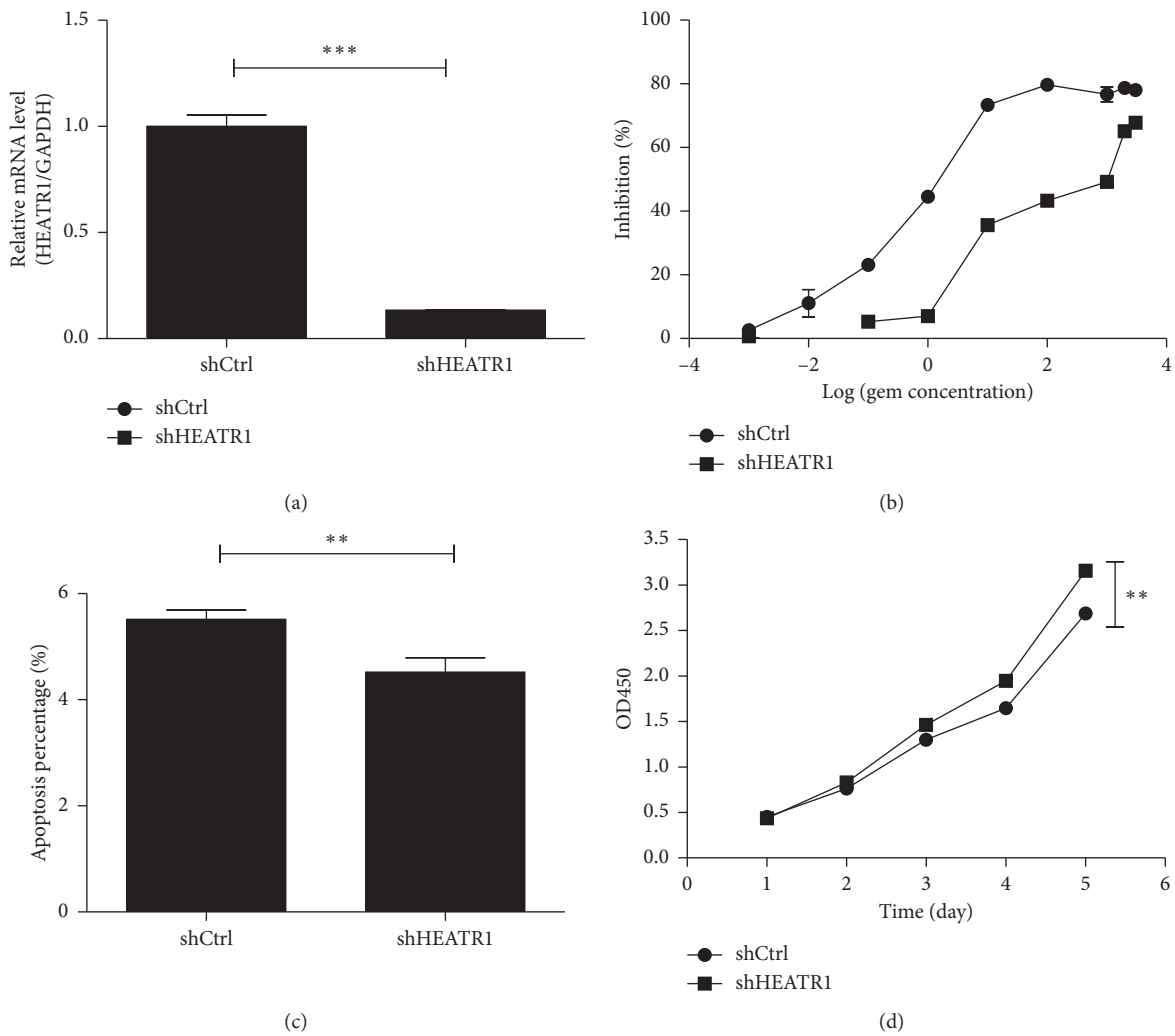


Figure 1: HEATR1 promotes the chemosensitivity to gemcitabine in pancreatic cell lines. (a) HEATR1 knockdown in PANC-1. (b) Increased chemoresistance of PANC-1 to gemcitabine after HEATR1 knockdown. (c) Apoptotic percentage decreased after HEATR1 knockdown when treated with gemcitabine. (d) Proliferation increased on day 5 dramatically after HEATR1 knockdown when treated with gemcitabine.

ZNF185 was mainly low (56.3%, 45/80) in the normal pancreas and high (51.2%, 41/80) in cancer (Figures 4(c) and 4(d)). SMAD4 was low (50%, 40/80) in the normal pancreas and mainly low (73.8%, 59/80) in cancer (Figures 4(e) and 4(f)). The χ^2 test showed significant differences in the expression of HEATR1 ($\chi^2 = 37.143$, $P < 0.001$), ZNF185 ($\chi^2 = 20.077$, $P < 0.001$), and SMAD4 ($\chi^2 = 23.309$, $P < 0.001$) between the cancer and the normal pancreas.

When analyzing the relationships between HEATR1, ZNF185, and SMAD4 expression grading and the clinicopathological factors (Table 1), we found that a higher HEATR1 expression grading showed a significant correlation with normal CA19-9 ($\chi^2 = 9.879$, $P = 0.002$) and higher differentiation ($\chi^2 = 9.135$, $P = 0.002$), while higher ZNF185 expression was correlated with higher CA19-9 ($\chi^2 = 4.121$, $P = 0.042$) and poorer differentiation ($\chi^2 = 31.223$, $P = 0.001$), and higher SMAD4 was correlated with normal CA19-9 ($\chi^2 = 7.944$, $P = 0.005$) and higher differentiation ($\chi^2 = 9.399$, $P = 0.002$).

The median survival time of the cohort was 12 months. After the patients were treated with gemcitabine chemotherapy, patients with higher HEATR1 ($P < 0.001$) and SMAD4 ($P < 0.001$) staining or lower ZNF185 ($P < 0.001$) staining exhibited better overall survival (Figure 5). From the multivariate survival analysis, we observed that high TNM staging, poorer differentiation, higher staining of HEATR1, or lower staining of ZNF185 in pancreatic cancer were the independent prognostic factors of pancreatic cancer patients (Table 2).

4. Discussion

Gemcitabine has long been the first-line chemotherapeutic agent for pancreatic cancer during the past 20 years, but the clinical response was always unsatisfactory mainly caused by the chemoresistance within the several weeks after starting the treatment [16]. The possible mechanism of the chemoresistance in the previous study included inadequate

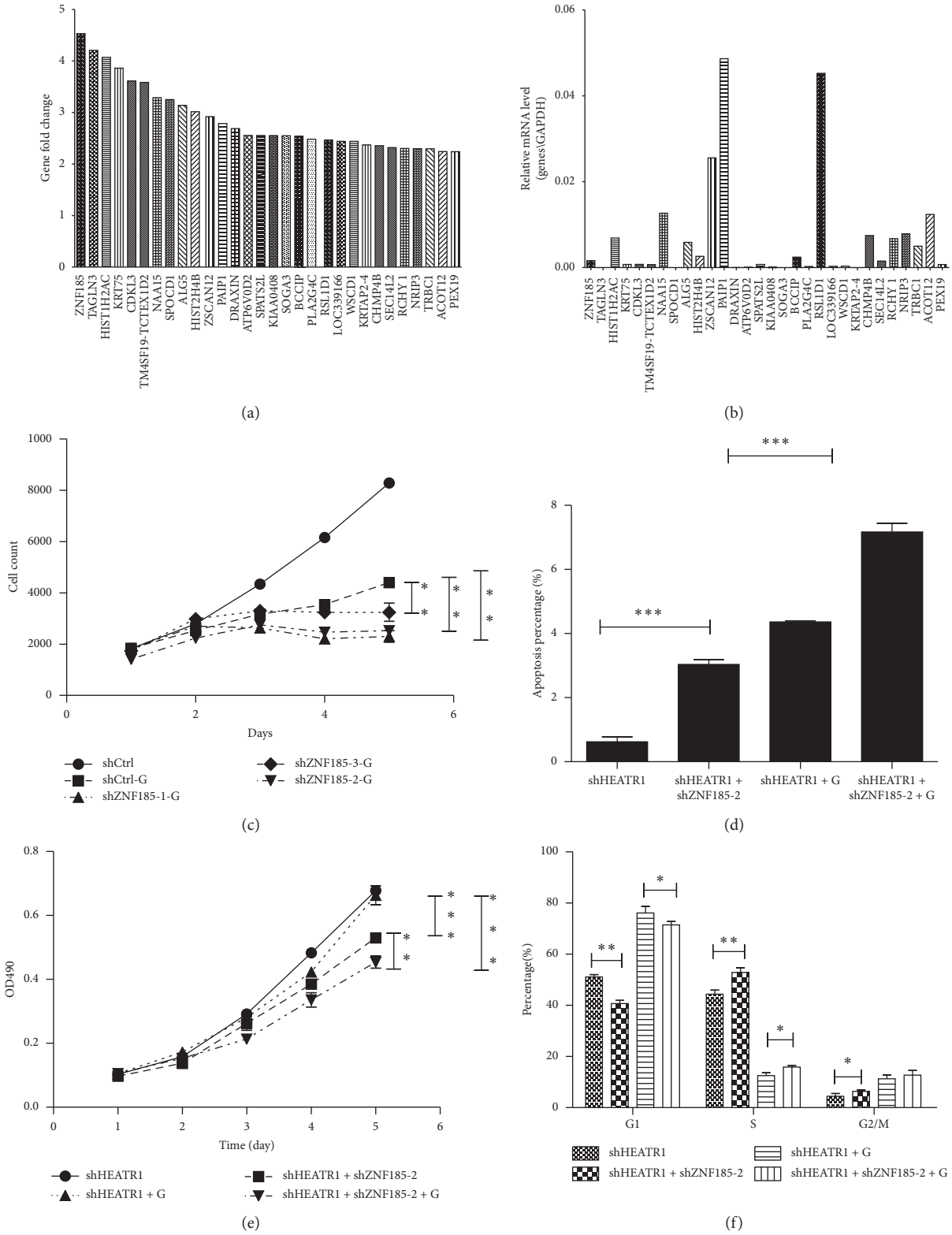


Figure 2: Gene chip analysis of differentially expressed genes and HCS screening after HEATR1 knockdown. (a) Differential expression fold change (4.5285) of ZNF185 was the most significant in the selected 30 genes between the HEATR1-KD and control groups in gene chips. (b) Quantitative PCR of the selected 30 genes. (c) In HCS screening, knocking down ZNF185 in the pancreatic cancer cells could promote the chemosensitivity of gemcitabine; confirmation of chemosensitive function of ZNF185 by observing the increased apoptotic percentage (d) and proliferative inhibition (e) when HEATR1 and ZNF185 were both knocked down. (f) After knocking down ZNF185, the pancreatic cancer cells in the S phase and the G2/M phase increased, while the cells in the G1 phase decreased.

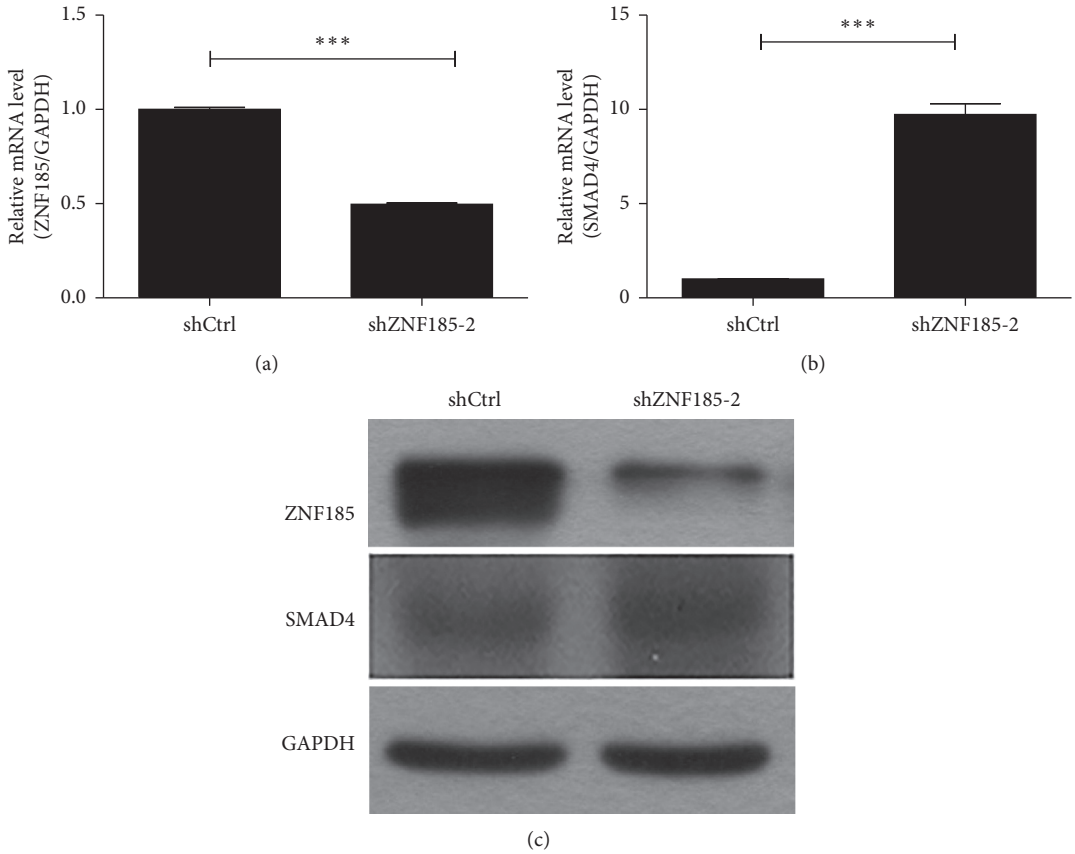


Figure 3: Expression of SMAD4 increased in the ZNF185 knockdown pancreatic cancer cells. (a) qRT-PCR of ZNF185 and (b) SMAD4 when ZNF185 was knocked down. (c) Western blots of ZNF185 and SMAD4 when ZNF185 was knocked down.

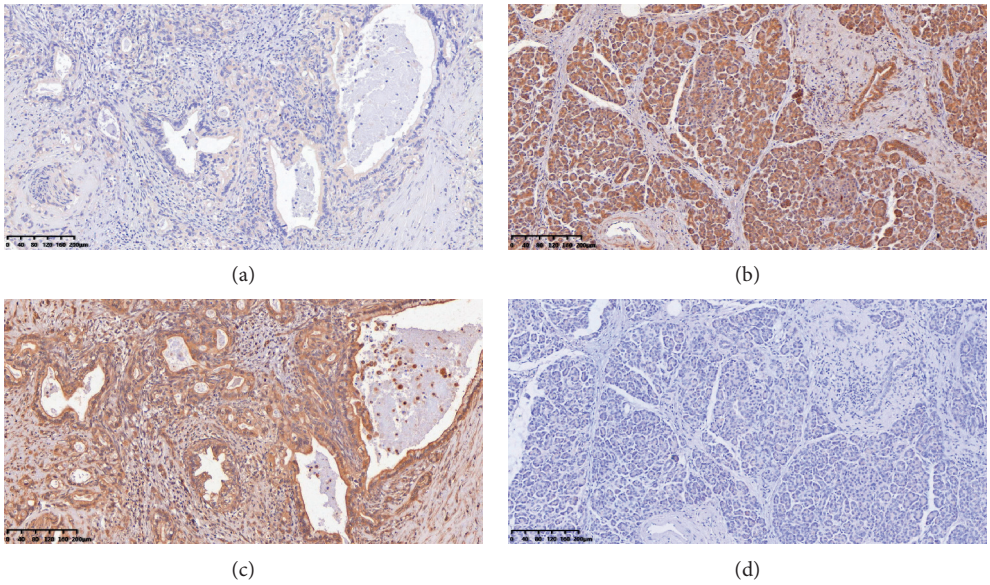
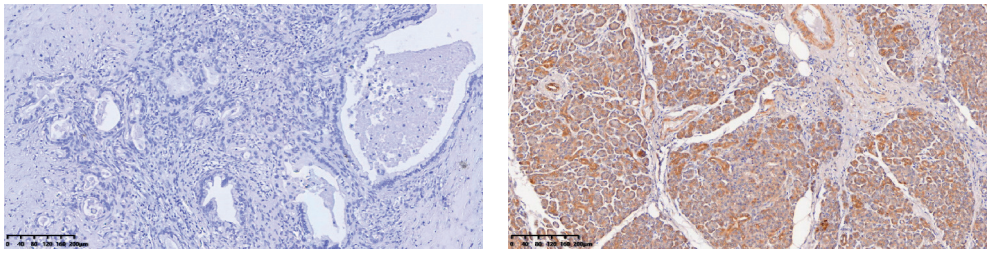


Figure 4: Continued.



(e) (f)

Figure 4: Expression status in pancreatic ductal adenocarcinoma. Representative images of expression of HEATR1 in cancer (a) and normal (b) tissues. Representative images of expression of ZNF185 in cancer (c) and normal (d) tissues. Representative images of expression of SMAD4 in cancer (e) and normal (f) tissues.

Table 1: Relationship between HEATR1, ZNF185, and SMAD4 expression and clinicopathological features of pancreatic cancer.

Group	HEATR1 expression		*P* value	ZNF185 expression		*P* value	SMAD4 expression		*P* value
	Low	High		Low	High		Low	High	
Gender									
Female	28	11	0.074	19	22	0.659	34	7	0.056
Male	36	5		20	19		25	14	
Age (year)									
<60	24	8	0.361	15	17	0.784	23	9	0.756
≥60	40	8		24	24		36	12	
CA19-9 (U/ml)									
<37	9	8	**0.002**	12	5	**0.042**	8	9	**0.005**
≥37	55	8		27	36		51	12	
Tumor diameter (cm)									
<3	17	4	0.899	10	11	0.904	17	4	0.382
≥3	47	12		29	30		42	17	
TNM staging									
IA~IIA	35	13	0.052	26	22	0.235	34	14	0.468
IIB~IV	29	3		13	19		25	7	
Lymphatic positive									
No	43	13	0.272	28	28	0.733	42	14	0.698
Yes	21	3		11	13		17	7	
Tumor location									
Head	45	7	0.046	23	29	0.270	42	10	0.052
Body and tail	19	9		16	12		17	11	
Differentiation									
High and medium	25	13	**0.003**	31	7	**0.001**	22	16	**0.002**
Poor	39	3		8	34		37	5	
Vessel invasion									
No	54	14	0.754	35	33	0.247	49	19	0.413
Yes	10	2		4	8		10	2	
Vessel metastasis									
No	40	13	0.156	26	27	0.939	36	17	0.097
Yes	24	3		13	14		23	4	
Perineural invasion									
No	45	12	0.711	26	31	0.377	43	14	0.589
Yes	19	4		13	10		16	7	

The bold values indicate *P* values less than 0.05.

transmembrane transport of the agent due to hypovascularity [17], antiapoptotic pathways [18], and epithelial-mesenchymal transition [19]. In our previous study, we discovered that downregulation of HEATR1 in pancreatic cancer causes resistance to gemcitabine. We hypothesized that HEATR1 promotes gemcitabine efficacy through inhibiting AKT phosphorylation [7]. But, whether is there any functional downstream gene of HEATR1 that synergistically regulates the chemosensitivity of gemcitabine is still an interesting further objective.

Here, we found that knocking down ZNF185 could synergistically promote the chemosensitivity of gemcitabine

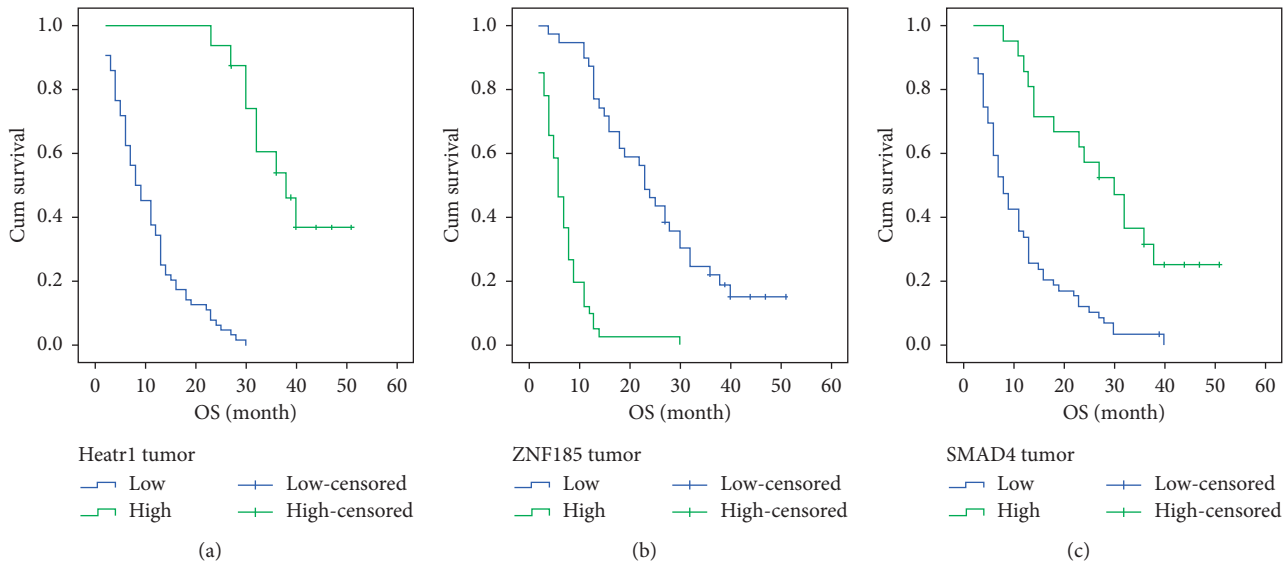


Figure 5: Expression of HEATR1, ZNF185, and SMAD4 in the pancreatic cancer tissues and correlation with the survival. (a) Univariate analysis of HEATR1 expression and OS. (b) Univariate analysis of ZNF185 expression and OS. (c) Univariate analysis of SMAD4 expression and OS.

in pancreatic cancer cells. ZNF185 belonging to the ZNF family, located on the DXS52 region of the long arm of chromosome Xq28, is one kind of the actin-cytoskeleton-associated Lin-l 1, Isl-1, and Mec-3 (LIM) domain-containing protein [20]. The knowledge about the function of ZNF185 is still lacking, while some previous studies have shown its role in cell proliferation, cell differentiation, and cell apoptosis in the prostate, lung, and head and neck squamous cell carcinoma [21]. Our observation is the first to identify a relationship between ZNF185 regulation and gemcitabine chemosensitivity of pancreatic cancer. Moreover, we also preliminarily discovered that knocking down ZNF185 served as increasing the chemosensitivity via SMAD4 in pancreatic cancer which was confirmed by *in vitro* and *in vivo* analysis. The tumor suppressor gene SMAD4 is first referred to as pancreatic cancer deletion gene4 (DPC4) because the deficiency in its expression was first discovered in pancreatic cancer [22]. A previous study showed that genetic alterations or homozygous deletion of SMAD4 can influence the normal signalling of the TGF-β pathway and uncontrolled cell growth and pancreatic tumorigenesis [23], and low expression of SMAD4 is correlated with poor survival in pancreatic cancer, but there are very few studies on SMAD4 with chemosensitivity of gemcitabine in pancreatic cancer [24]. Besides, the relevance of our study was confirmed in the samples of pancreatic cancer patients, and we demonstrated that HEATR1, ZNF185, and SMAD4 expression are significantly associated with the prognosis with pancreatic cancer in the patients treated with gemcitabine chemotherapy. Therefore, it is possible that patients with high IHC staining of HEATR1 or low staining of ZNF185, which indicate the chemosensitive markers, are suitable for gemcitabine treatment and may have better prognosis.

Our study also had some limitations. First, all of the functional assays were implemented in the HEATR1-KD cells, while we failed to perform the overexpression of HEATR1 because of the large molecular weight of HEATR1. Second, in the past 5 years, there were increasing number of the different chemotherapeutic regimens for pancreatic cancer, including gemcitabine monotherapy or combination with other agents (e.g., oxaliplatin and albumin-bound paclitaxel), or even more toxic regimen of FOLFIRINOX. Although these regimens showed encouraging prognostic benefit in the USA or European patients [25], most of the Chinese patients still cannot endure the toxicity of FOLFIRINOX or Abraxane because of the side effects such as diarrhea [26]. Besides, according to the NCCN guidelines in 2019, no definite standard has been established in the adjuvant treatment of pancreatic cancer so far. Chemotherapy alone with gemcitabine (category 1), 5-FU/leucovorin (category 1), gemcitabine/capecitabine (category 1), or continuous infusion 5-FU is listed in the guidelines as options for adjuvant treatment. Therefore, gemcitabine is still the cornerstone chemotherapy agent of pancreatic cancer in the East Asian, especially the Chinese pancreatic patients. Whether the HEATR1-ZNF185-SMAD4 pathway exerts the same chemosensitive mechanism in these agents will need further research.

Taken together, it is the first time to reveal that HEATR1, ZNF185, and SMAD4 are correlated in the chemosensitivity of gemcitabine in pancreatic cancer. These results require further studies with a larger patient cohort to confirm and chemoresistance mechanism for pancreatic cancer. A better understanding of the causes of gemcitabine chemoresistance is critical to the development of novel comprehensive treatment strategies for pancreatic cancer.

Table 2: The clinicopathological stigma and multivariate survival analysis.

Variables	Patients ($n = 80$)	OS		
		Univariate P value	Multivariate P value	Hazard ratio (95% CI)
Gender				
Female	39	0.532		
Male	41			
Age (years)				
<60	32	0.577		
≥60	48			
CA19-9 (U/ml)				
<37	17	**0.005**	0.202	
≥37	63			
Tumor diameter (cm)				
<3	21	0.847		
≥3	59			
TNM staging				
IA~IIA	48	**<0.001**	**0.027**	2.597 (1.113–6.057)
IIB~IV	32			
Lymphatic positive				
No	56	**0.014**	0.439	
Yes	24			
Tumor location				
Head	52	0.301		
Body and tail	28			
Differentiation				
High and medium	38	**<0.001**	**<0.001**	4.257 (2.105–8.609)
Poor	42			
Vessel invasion				
No	68	0.158		
Yes	12			
Vessel metastasis				
No	53	0.266		
Yes	27			
Perineural invasion				
No	57	0.523		
Yes	23			
HEATR1 tumor expression				
Low	64	**<0.001**	**<0.001**	0.099 (0.027–0.360)
High	16			
ZNF185 tumor				
Expression	39	**<0.001**	**0.004**	2.765 (1.385–5.519)
Low	41			
High				
SMAD4 tumor				
Expression	59			
Low	21	**<0.001**	0.355	
High				

The bold values indicate P values less than 0.05.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yuan Fang, Xu Han, and Jianang Li contributed equally to this article.

Acknowledgments

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Supplementary Materials

Gene expression profiling of PANC-1 cells transduced with either control or shZNF-185 lentivirus. Genes were upregulated, and genes were downregulated; red denotes the upregulated genes, and green denotes the downregulated genes. H3608-1, H3608-2, and H3608-3 indicate 3 repeated gene profiling after transduced with shCtrl lentivirus.

H3609-1, H3609-2, and H3609-3 indicate 3 repeated gene profiling after transduced with ShZNF185. (*Supplementary Materials*)

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Review Article

EGFR Polymorphism and Survival of NSCLC Patients Treated with TKIs: A Systematic Review and Meta-Analysis

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Tyrosine kinase inhibitor- (TKI-) based therapy revolutionized the overall survival and the quality of life in non-small-cell lung cancer (NSCLC) patients that have epidermal growth factor receptor (*EGFR*) mutations. However, *EGFR* is a highly polymorphic and mutation-prone gene, with over 1200 single nucleotide polymorphisms (SNPs). Since the role of *EFGR* polymorphism on the treatment outcome is still a matter of debate, this research analyzed the available literature data, according to the PRISMA guidelines for meta-analyses. Research includes PubMed, Scopus, ISI Web of Science, and 14 of genome-wide association studies (GWAS) electronic databases in order to provide quantitative assessment of the association between ten investigated *EGFR* SNPs and the survival of NSCLC patients. The pooled HR and their 95% CI for OS and PFS for different *EGFR* polymorphisms using a random or fixed effect model based on the calculated heterogeneity between the studies was applied. The longest and the shortest median OSs were reported for the homozygous wild genotype and a variant allele carriers for rs712829 (-216G>T), respectively. Quantitative synthesis in our study shows that out of ten investigated *EGFR* SNPs (rs11543848, rs11568315, rs11977388, rs2075102, rs2227983, rs2293347, rs4947492, rs712829, rs712830, and rs7809028), only four, namely, rs712829 (-216G>T), rs11568315 (CA repeat), rs2293347 (D994D), and rs4947492, have been reported to affect the outcome of TKI-based NSCLC treatment. Of these, only -216G>T and variable CA repeat polymorphisms have been confirmed by meta-analysis of available data to significantly affect OS and PFS in gefitinib- or erlotinib-treated NSCLC patients.

1. Introduction

For the past several decades, lung cancer remains one of the major causes of mortality worldwide [1–3]. According to the World Health Organization, it is the most commonly diagnosed cancer and the leading cause of cancer death, with over 2 million of new cases and more than 1.7 million deaths in 2018 [4, 5]. Of those, over 85% is due to non-small-cell lung cancer (NSCLC), which exhibits better prognosis than its complement, i.e., small cell lung cancer [1], yet displays low long-term survival and reduced quality of life [6, 7]. Although cigarette smoking represents the primary risk factor for NSCLC development [8], numerous investigations confirmed that genetics plays one of the leading roles in the process [9–11]. Gene variations that have been identified as conferring higher risk of NSCLC could be either germline or somatic, with some of the most common lung cancer-related driver mutations linked to epidermal growth factor receptor gene (*EGFR*) [12].

EGFR is a transmembrane tyrosine kinase receptor that, upon activation, becomes a transducer of signals for cell proliferation [13]. *EGFR* overexpression, often due to genetic alterations, has been firmly and consistently associated with carcinogenesis [13–15], and *EGFR* itself recognized as a potential target of an important therapeutic approach to cancer. Namely, it has been observed that drugs that inhibit tyrosine kinases, enzymes important for tumor cell proliferation, growth, and metastasis, display target-specific antitumor activity against different types of malignancies, including lung, breast, colorectal, and prostate cancer [16]. Since the discovery of gefitinib, the first tyrosine kinase inhibitor (TKI) aimed *EGFR* [17], several similar drugs have been approved for the treatment of NSCLC, including erlotinib [18, 19]. Compared with chemotherapy as a former treatment of choice, TKI-based therapy revolutionized the overall survival and the quality of life of NSCLC patients, especially if they are carriers of the *EGFR* driver mutations [20–23]. Still, for the majority of patients, the prognosis of NSCLC remains unfavorable, mainly as a consequence of either intrinsic or acquired resistance to TKI. While acquired resistance develops during the treatment, mostly due to occurrence of secondary *EGFR* mutations, intrinsic resistance usually implies the presence of inherited variations, including *EGFR* single-nucleotide polymorphisms (SNPs) [24–27].

EGFR is highly polymorphic and mutation-prone gene, with over 1200 SNPs [28] and over 2700 mutations [29] described so far. *EGFR* mutations have been extensively studied in relation to NSCLC, and some of them, including alterations in the tyrosine kinase domain, were clearly associated with better response to TKI-based therapy [30]. Yet, the role of *EFGR* polymorphism on the treatment outcome is still a matter of debate, as published research studies offer inconsistent results [31, 32], and available meta-analyses lack the comprehensiveness in terms of included SNPs [25, 33]. Therefore, the aim of our study was to review and analyze the available literature on TKI-based therapy, in order to provide quantitative assessment of the association between *EGFR* polymorphism and the survival of NSCLC patients.

2. Methods

2.1. Literature Search and Study Selection. To identify the studies on the association between *EGFR* polymorphisms and the survival in NSCLC patients treated with TKI therapy, a systematic search of the available literature according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines for meta-analyses and systematic reviews was performed [34]. Three electronic databases, namely, PubMed [35], Scopus [36], and ISI Web of Science [37], were thoroughly explored, with a search query consisting of a specific combination of subject headings and text words. For searching the PubMed database, the following combination of terms was used: ((“receptor, epidermal growth factor” [MeSH Terms] OR *EGFR* [All Fields]) AND (gene[tiab] OR “polymorphism, genetic”[MeSH Terms]) AND (“carcinoma, non-small-cell lung” [MeSH Terms] OR NSCLC [All Fields]) AND ((((“drug therapy” [Subheading] OR treatment [All Fields] OR “erlotinib hydrochloride” [MeSH Terms] OR TKI OR “TK inhibitors” OR “tyrosine kinase inhibitors” OR “Tyrosine-kinase inhibitor”) AND response [All Fields]) OR Prognosis [MeSH]) AND (humans [MeSH])). Other two databases, i.e., Scopus and ISI Web of Science, were searched using the appropriately modified initial PubMed search query (details are available upon request). In addition, detailed search of several publically available databases of genome-wide association studies (GWAS) was carried out, including the GWAS Central [38], the Genetic Associations and Mechanisms in Oncology (GAME-ON) [39], the Human Genome Epidemiology (HuGE) Navigator [40], the National Human Genome Research Institute (NHGRI GWAS Catalog) [41], the database of Genotypes and Phenotypes (dbGaP) [42], the GWASdb [43], the Italian Genome-Wide Database (IGDB) [44], and the GRASP: Genome-Wide Repository of Associations between SNPs and Phenotypes [45]. Finally, we separately searched the bibliographies of eligible studies to look for additional studies. We considered studies published until February 09, 2018 and written in English, Italian, or Russian.

Studies were considered eligible if they assessed the association between the *EGFR* polymorphism in NSCLC patients treated with EGFR-TKI, and survival, expressed as the progression-free survival (PFS), time to progression (TTP), or the overall survival (OS). PFS was defined as the time from the first day of EGFR-TKI treatment until tumor progression or death from any cause while censoring the patients that were lost to follow-up [46]. Time from initiating the therapy until the disease progression as the event of interest was considered as the TTP [47]. Finally, OS was defined as the period from the first day of EGFR-TKI therapy to the date of death or final follow-up, whichever arrived first [47]. Hazard ratios (HRs) with corresponding 95% CIs were used to evaluate the quantitative aggregation of the survival for different genotypes of *EGFR*.

After all potentially eligible studies were collected, cross-linking of the studies from different electronic databases was performed in order to remove duplicates. Two reviewers (J.O. and V.V.) independently screened the titles and abstracts of the relevant articles, and any disagreement was resolved through discussion. Full texts of the potentially eligible studies were subsequently retrieved and assessed for final inclusion, according to the reported criteria. Namely, we only included studies conducted on patients with histopathologically confirmed NSCLC, who received EGFR-TKIs based therapy and where the measures of outcome were reported according to the *EGFR* genotype. On the other hand, reviews, meta-analyses, editorials, case reports, and studies conducted only on cell lines were excluded. When there were multiple publications on the same or overlapping study population, we only included the most comprehensive one.

2.2. Data Extraction and Quality Assessment. Two investigators (J.O. and V.V.) independently extracted data from each article into a database using a structured sheet. The

following items were considered: (a) general: first author, year, country, study design, study period, and number of patients; (b) study subjects: median age, gender, ethnicity, percentage of smokers, clinical stage, and median follow-up period (months); (c) therapy: preparation therapy, main therapy line used; (d) *EGFR* genotype: genotyping platform used, variant location, the dbSNP-ID, and number of patients per genotype; (e) outcome: OS, TTP, and PFS with 95% CI.

The same investigators evaluated the methodological quality of included studies using the widely accepted Newcastle–Ottawa Quality Assessment Scale (NOS) for cohort studies [48] and the Jadad Scale for the randomized control trials (RCTs) [49]. The NOS for cohort studies evaluates three perspectives of the methodological quality: the selection of the study groups (four points); the comparability of the groups (two points); and the ascertainment of exposure or outcome of interest for cohort studies (three points) and assigns a total of maximum 9 points. The Jadad scale for reporting randomized controlled trials evaluates the risk of bias in three domains: randomization, double blinding, and description of withdrawals and dropouts with a final score from 0 to 5. Any disagreements between the reviewers were resolved through discussion or in consultation with other authors.

2.3. Statistical Analysis. Meta-analysis was conducted when at least two studies on the same genetic variant were available. We calculated the pooled HR and their 95% CI for OS and PFS for different *EGFR* polymorphisms using a random or fixed effect model based on the calculated heterogeneity between the studies [50]. The $\chi 2$-based Q statistics and the I^2 statistics [51] were used to evaluate the between study heterogeneity, with $I^2 = 0\%$ indicating no observed heterogeneity, 25% regarded as low, 50% as moderate, and 75% as high [52]. When Q test or the I^2 test indicated significant heterogeneity between the studies ($p < 0.10$, $I^2 > 50\%$), the random-effect model was used, otherwise, the fixed-effect model was applied. Additionally, Galbraith's plot was constructed to explore the weight each study had on the overall estimate and the contribution to the Q statistics for heterogeneity [53].

We also performed a one-way sensitivity analysis to check stability of the results. To assess the publication bias (where appropriate), we conducted Egger's asymmetry test (level of significance $p < 0.05$) [54]. All statistical analyses were performed using the STATA software package v.15 (STATA Corporation, College 162 Station, TX, USA), and statistical significance was set at $p < 0.05$.

3. Results

3.1. Search Results and Study Characteristics. Of 5467 records obtained through the screening of PubMed, ISI WOS, Scopus, and 14 GWAS databases, 3699 remained after removing the duplicates. After reading the titles and abstracts, 42 full text articles were assessed for the inclusion. We further excluded 33 papers for not fulfilling the inclusion criteria, leaving 9 studies as eligible. After inspection of references of the included studies, we additionally identified two studies, arriving to the 11 studies to be finally included in the review. Ultimately, 5 studies were incorporated in the quantitative synthesis for OS, and 4 were considered for PFS (Figure 1).

Our search results consisted of 10 cohort studies [25, 26, 31, 32, 55–60] and 1 randomized controlled trial (RCT) [61], conducted in high-income Western countries and in Asia. Overall quality of the included study was good, with two studies [56, 60] scoring maximum point on the NOS. Highest scores were demonstrated for most of the evaluated domains, while the domain of follow up adequacy was with the lowest score (Supplementary Table 1). Sample size varied from 62 to 760 patients while median age from 55.2 to 67.0 years. Majority of patients were in clinical stages III and IV. Reported medium follow-up time ranged from 11.4 months up to 62.7 months, and TKIs used in the studies were gefitinib and erlotinib. Detailed description of the included studies is presented in Table 1.

All included studies investigated the OS and reported their findings for 10 different *EGFR* SNPs, namely, rs11543848, rs11568315, rs11977388, rs2075102, rs2227983, rs2293347, rs4947492, rs712829, rs712830, and rs7809028. The longest and the shortest median OS were reported for the homozygous wild genotype and a variant allele carriers for rs712829 (-216G>T), respectively [25]. Increased HR was observed in patients lacking wild-type (CA)16 allele (rs11568315) as compared with carriers of at least one (CA) 16 allele [58]. On the other hand, lower HR (0.29; 95% CI: 0.10–0.83) indicating better prognosis was reported in homozygous carriers of less common g.106268G allele (rs4947492) [31], in carriers of at least one variant -216T (rs712829) allele (HR = 0.67; 95% CI: 0.48–0.94) [61], as well as in carriers of lower number of CA repeats (HR = 0.43; 95% CI: 0.23–0.78) [59], as compared with their corresponding genotypes. Interestingly, carriers of one or both variant alleles, as compared with homozygous wild genotype for 181946C>T (rs2293347), displayed increased HR according to one [31] and decreased HR according to other [32] investigated study. The details of OS, HR, and RR for each investigated SNP across the included studies are presented in Table 2.

Of all included studies, six [25, 26, 32, 56, 59, 61] reported PFS in relation to five different SNPs, with only one of them reporting the median PFS time [25]. On the other hand, TTP was reported in only two studies [55, 57] and were stratified according to four different SNPs (Table 3). Based on the PFS reports, better prognosis was associated with rs11568315, rs2293347, and rs712829 polymorphisms, i.e., with the presence of lower number of CA repeats [55] and variant 181946T [32] and -216T [56] alleles. In addition, lower number of CA repeats (rs11568315) was also associated with better TTP [55].

3.2. Quantitative Synthesis. Five studies [31, 32, 56, 59, 61] reported enough information about OS to be included in our meta-analysis, and the forest plot with pooled HR and their

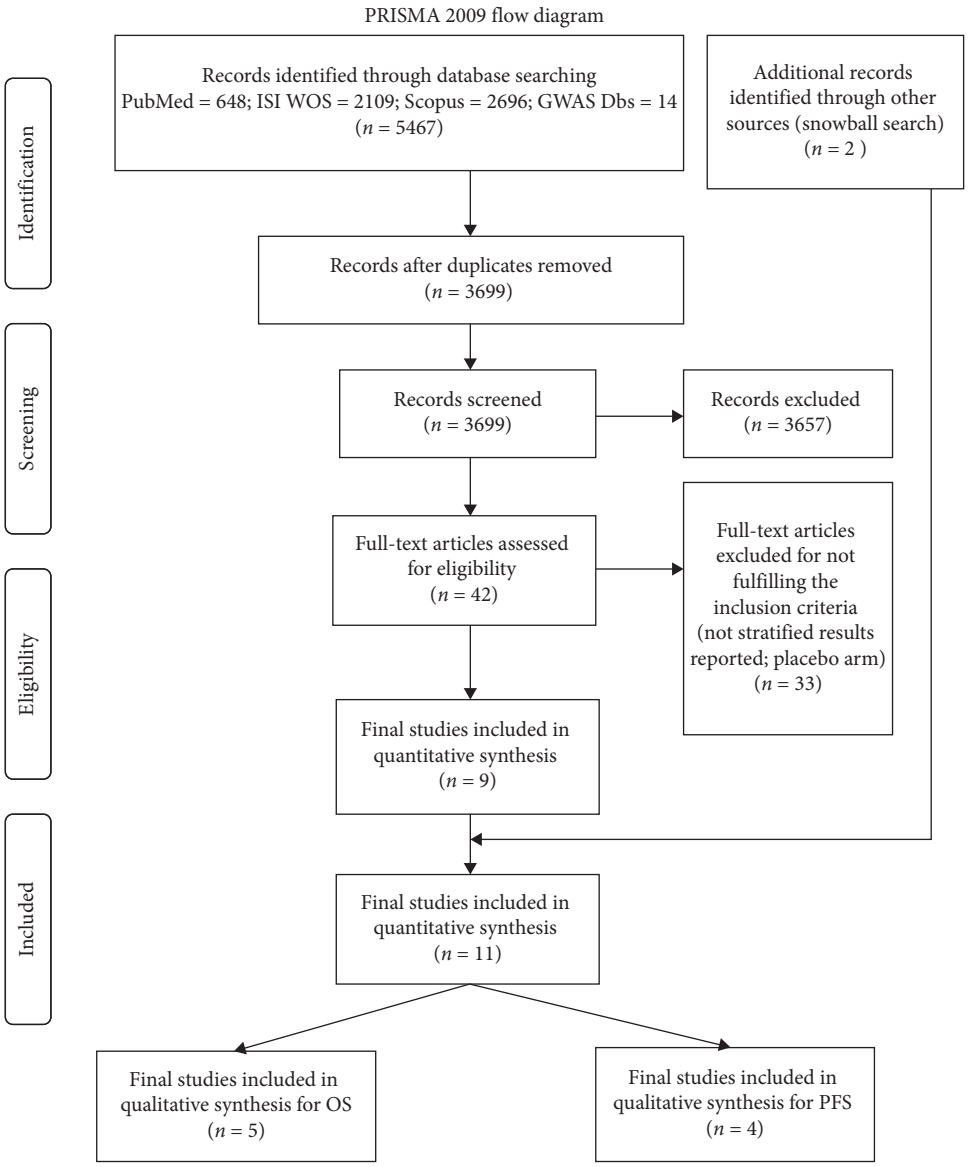

Figure 1: Flowchart depicting the literature search and study selection process.

95% CI of OS available for four SNPs, namely, rs11568315, rs712830, rs712829, and rs712830, is presented in Figure 2. Due to significant heterogeneity between the studies, the random effect model was applied. Egger test and Begg's correlation method demonstrated no evidence of publication bias. Our analysis revealed rs712829 (HR = 0.80, 95% CI: 0.67–0.96, $p = 0.01$; heterogeneity $I^2 = 0\%$, $p = 0.37$) and rs11568315 (HR = 0.56, 95% CI: 0.32–0.99, $p = 0.046$; heterogeneity $I^2 = 51.9\%$, $p = 0.15$) polymorphisms, more precisely the presence of at least one -216T variant allele and the presence of ≤16CA repeats, respectively, as the only positive prognostic factors for the OS, with no observed heterogeneity (Figure 2). The Egger test demonstrated no statistical evidence of publication bias for rs712829 and rs712830 ($p = 0.48$ and $p = 0.6$, respectively). As Galbraith's plot, performed to explore the potential sources of heterogeneity, identified the study of Winther-Larsen et al. [32], this study was omitted in one-way sensitivity analysis of rs712829. Yet, the overall HR remained significant and was only slightly changed to 0.69 (95% CI: 0.52–0.91, $p < 0.008$; heterogeneity $I^2 = 0\%$, $p = 0.78$). Other investigated SNPs demonstrated increased pooled HR but without statistical significance.

Four studies [32, 56, 59, 61] were included in the pooled analysis of PFS in patients stratified according to genotyping data available for three *EGFR* SNPs, i.e., rs11568315, rs712829, and rs712830. As there was no significant heterogeneity between the studies, the fixed effect model was applied. No significant publication bias was demonstrated by Egger tests (rs712829 and rs712830, $p = 0.19$ and $p = 0.08$, respectively) even though these tests for exploring the publication bias are underpowered with only few studies included. Again, the only significant factors, indicating better prognosis in NSCLC treated with TKIs, were the presence of at least one -216T variant allele (HR = 0.81, 95% CI: 0.68–0.96, $p = 0.02$; heterogeneity $I^2 = 0\%$, $p = 0.37$) and

Table 1: General characteristics of the included studies.

Author, year	Country	Study period	No. of patients	Median age, years (range)	Gender (males, %)	Ethnicity (%)	Smokers (%)	Clinical stage (%)	Median follow-up in months	TKI (dose)	Additional therapy
Han et al., 2007 [55]	Korea	Jan 2002–Dec 2004	86	61 (30–87)	57.0	NR	55.8	IIIB (5.8); IV (94.2)	16.9	Gefitinib (250 mg/d)	NR
Ichihara et al., 2007 [26]	Japan	Nov 2000–May 2006	98	66 (NR)	63.0	NR	62.0	NR[b]	11.4[c]	Gefitinib (250 mg/d)	88.0% of patients previously treated with chemotherapy
Liu et al., 2008 [56]	USA and Canada	Dec 2000–Feb 2003	92	61 (36–87)	41.0	Caucasian (95); Asian (3); African American (2)	79.0	IIIB (7.0); IV (93.0)	28.5 (PFS); 29.9 (OS)	Gefitinib (NR)	85.0% of patients were previously, and 95% concurrently treated with chemotherapy
Giovannetti et al., 2010 [57]	Italy	NR	96	64 (NR)	57.3	NR	68.8	IIIB (9.4); IV (90.6)	NR	Gefitinib (250 mg/d)	84.5% of patients were previously treated with chemotherapy[d]
Tiseo et al., 2010 [58]	Italy	NR	91	67 (40–85)	61.5	Caucasian (100)	78.0	III (11.0); IV (89.0)	NR	Gefitinib (250 mg/d)	All patients were previously treated with chemotherapy
Nie et al., 2011 [60]	China	Jun 2002–Sep 2006–Jul 2010[a]	115[e]	57 (NR)	56.5	NR	NR	IV (83.5)	54.0	Gefitinib (250 mg/d) or erlotinib (150 mg/d)	All patients were previously treated with chemotherapy
Jung et al., 2012 [25]	Korea	Jan 2007–Dec 2010	71[f]	59 (34–85)	62.0	Asian (100)	57.7	NR	12.7	Gefitinib (250 mg/d) or erlotinib (150 mg/d)	All patients were previously treated with chemotherapy
Zhang et al., 2013 [31]	China	Jan 2008–Dec 2010	128	55 (32–80)	48.4	NR	32.0	IIIB (25.0); IV (75.0)	16.6	Gefitinib (250 mg/d)	All patients were previously treated with one or two other therapy options
Winther-Larsen et al., 2014 [59]	Denmark	Jan 2007–Oct 2011	62[g]	65 (33–88)	40.0	Caucasian (100)	16.0	IV (100)	52.2	Erlotinib (150 mg/d, dose reduced in case of side effects grade 2 or higher)	NR
Winther-Larsen et al., 2015 [32]	Denmark	Jan 2007–Apr 2014	331	64 (34–89)	46.0	Caucasian (100)	26.0	IV (100)	62.7	Erlotinib (150 mg/d, dose reduced in case of side effects grade 2 or higher)	84.0% and 10% of patients were previously treated with platinum-based and pemetrexed and/or docetacel-based chemotherapy, respectively
Kim et al., 2017 [61]	Italy and Canada	NR	760	62 (27–81)	66.3	East Asian (3.2); other (96.8)	79.3	IIIB (10.9); IV (89.1)	36.0	Erlotinib (150 mg/d)	50% of patients were treated with erlotinib, followed by cisplatin and gemcitabine at progression; other 50% were treated with cisplatin and gemcitabine, followed by erlotinib at progression

NR: not reported. [a]Last follow-up. [b]Based on the Eastern Cooperative Oncology Group performance status, there were 61.0% with grade 0/1 and 39.0% with grade 2/3/4. [c]For survivors. [d]Additional 127 chemotherapy-treated/gefitinib-nontreated NSCLC patients were used as a comparison. [e]70 on gefitinib and 45 on erlotinib. [f]37 on gefitinib and 34 on erlotinib. [g]All were *EGFR* mutation positive.

Table 2: *EGFR* genotype and overall survival of NSCLC patients treated with TKIs.

dbSNP-ID	Variant type, location, and/or consequence	Author, year (ref)	Genotyping platform used	Genotype	No. of patients (%)	Median OS (95% CI) in months	HR (95% CI)	RR (95% CI)
rs11534848	Missense variant, 1562G>A, R521K	Zhang et al., 2013 [31]	MassARRAY system	AA	48 (35.5)	10.0 (4.2–15.8)	Reference	NA
				AG	66 (48.9)	16.8 (7.0–26.6)	1.53 (0.94–2.51)	NA
				GG	21 (15.6)	29.4 (4.9–53.9)	1.84 (0.86–3.95)	NA
rs11568315	Intron variant, g.55020560_55020561AC [n]	Giovannetti et al., 2010 [57]	TaqMan assay	Both alleles ≤ 16CA	30 (31.9)	7.9 (3.5–12.2)	NA	NA
				At least 1 allele > 16CA	64 (68.1)	11.6 (6.5–16.7)	NA	NA
		Nie et al., 2011 [60]	PCR-RFLP and sequencing	At least 1 allele ≤ 16CA	66 (57.4)	15.9 (9.4–22.4)	NA	Reference
				Both alleles > 16CA	49 (42.6)	10.7 (4.7–16.8)	NA	0.81 (0.55–1.19)
		Tiseo et al., 2010 [58]	Fluorescent PCR and capillary electrophoresis	At least 1 allele = 16CA	56 (74.0)	12 (9.0–15.0)	Reference	NA
				Both alleles ≠ 16CA	20 (26.0)	4 (1.0–8.0)	**1.95 (1.12–3.7)**	NA
		Ichihara et al., 2007 [26]	PCR and sequencing	Short allele ≥ 19CA or the sum of alleles ≥ 39CA	63 (64.3)	NA	Reference	NA
				Short allele < 19CA or the sum of alleles < 39CA	35 (35.7)	NA	0.96 (0.50–1.86)	NA
		Kim et al., 2017 [61]	TaqMan assay and sequencing	Both alleles ≤ 16CA	74 (28.0)	NA	Reference	NA
				At least 1 allele > 16CA	188 (72.0)	NA	0.89 (0.64–1.24)	NA
		Liu et al., 2008 [56]	PCR-RFLP	At least 1 allele > 16CA	59 (64.0)	NA	Reference	NA
				Both alleles ≤ 16CA	33 (36.0)	NA	0.72 (0.45–1.16)	NA
		Winther Larsen et al., 2014 [59]	PCR-RFLP and capillary electrophoresis	Any allele ≤ 16CA	44 (71.0)	19.6 (11.9–27.3)	**0.43 (0.23–0.78)**	NA
				Both alleles > 16CA	18 (29.0)	8.4 (5.0–11.9)	Reference	NA
rs11977388	Intron variant, g.150522T>C	Zhang et al., 2013 [31]	MassARRAY system	TT	50 (39.4)	13.2 (6.1–20.3)	Reference	NA
				TC	64 (50.4)	16.8 (7.2–26.4)	1.26 (0.80–2.00)	NA
				CC	13 (10.2)	16.5 (0.0–40.4)	1.34 (0.56–3.18)	NA
rs2075102	Intron variant, g.171581C>A	Zhang et al., 2013 [31]	MassARRAY system	CC	90 (70.3)	11.8 (5.9–17.7)	Reference	NA
				CA	33 (25.8)	16.8 (12.8–20.8)	0.91 (0.55–1.49)	NA
				AA	5 (3.9)	29.4 (NA)	1.15 (0.34–3.90)	NA
rs2227983	Missense variant, 1562G>A, R497K	Giovannetti et al., 2010 [57]	TaqMan assay	GG or GA	81 (88.0)	7.4 (6.5–8.4)	NA	NA
				AA	11 (12.0)	8.0 (0.0–17.3)	NA	NA
		Nie et al., 2011 [60]	PCR-RFLP and sequencing	AA	43 (37.4)	17.4 (3.4–31.5)	NA	Reference
				AG	47 (40.9)	14.3 (7.0–21.6)	NA	0.81 (0.49–1.34)
				GG	25 (21.7)	12.3 (1.1–23.4)	NA	0.71 (0.43–1.17)
		Liu et al., 2008 [56]	PCR-RFLP	AA	43 (47.0)	NA	Reference	NA
				GG or GA	49 (53.0)	NA	1.24 (0.74–1.95)	NA

Table 2: Continued.

dbSNP-ID	Variant type, location, and/or consequence	Author, year (ref)	Genotyping platform used	Genotype	No. of patients (%)	Median OS (95% CI) in months	HR (95% CI)	RR (95% CI)
rs2293347	Synonymous variant, 181946C>T, D994D	Zhang et al., 2013 [31]	MassARRAY system	GG	59 (46.1)	21.0 (14.0–27.9)	Reference	NA
				GA	59 (46.1)	15.0 (8.3–21.8)	**1.75 (1.08–2.86)**	NA
				AA	10 (7.8)	2.0 (0.0–5.4)	**2.44 (1.06–5.56)**	NA
		Winther-Larsen et al., 2015 [32]	AS-PCR or PCR followed by sequencing	CC	252 (80.0)	NA	Reference	NA
				CT or TT	64 (20.0)	NA	**0.73 (0.54–0.97)**	NA
rs4947492	Intron variant, g.106268G>A	Zhang et al., 2013 [31]	MassARRAY system	AA	55 (43.0)	14.9 (6.9–22.8)	Reference	NA
				AG	60 (46.9)	11.8 (3.6–20.0)	0.86 (0.53–1.39)	NA
				GG	13 (10.1)	24.6 (NA)	**0.29 (0.10–0.83)**	NA
rs712829	5′ UTR variant, g.5031G>T, -216G>T	Giovannetti et al., 2010 [57]	TaqMan assay	GG	34 (36.2)	8.0 (3.0–13.0)	NA	NA
				GT or TT	60 (63.8)	11.6 (5.7–17.5)	NA	NA
		Jung et al., 2012 [25]	PCR-RFLP or PCR followed by sequencing	GG	63 (88.7)	29.5 (17.4–41.7)	NA	NA
				GT	8 (11.3)	1.4 (3.7–39.5)	NA	NA
		Kim et al., 2017 [61]	Taqman PCR and sequencing	GG	78 (32.0)	NA	Reference	NA
				GT or TT	162 (68.0)	NA	**0.67 (0.48–0.94)**	NA
		Winther-Larsen et al., 2015 [32]	AS-PCR or PCR followed by sequencing	GG	134 (42.0)	NA	Reference	NA
				GT or TT	182 (58.0)	NA	0.89 (0.70–1.13)	NA
		Liu et al., 2008 [56]	PCR-RFLP	GG	34 (37.0)	NA	Reference	NA
				GT or TT	58 (63.0)	NA	0.73 (0.45–1.19)	NA
rs712830	5′ UTR variant, g.5056A>C, -191C/A	Giovannetti et al., 2010 [57]	TaqMan assay	CC	78 (83.0)	7.9 (7.0–8.7)	NA	NA
				CA or AA	16 (17.0)	6.0 (2.8–9.2)	NA	NA
		Kim et al., 2017 [61]	Taqman PCR and sequencing	CC	195 (81.0)	NA	Reference	NA
				CA or AA	45 (19.0)	NA	1.19 (0.80–1.78)	NA
		Winther-Larsen et al., 2015 [32]	AS-PCR or PCR followed by sequencing	CC	236 (75.0)	NA	Reference	NA
				CA or AA	80 (25.0)	NA	0.95 (0.73–1.25)	NA
		Liu et al., 2008 [56]	PCR-RFLP	CC	81 (88.0)	NA	Reference	NA
				CA or AA	11 (12.0)	NA	1.09 (0.52–2.29)	NA
rs7809028	Regulatory region variant, g.198953G>A	Zhang et al., 2013 [31]	MassARRAY system	GG	60 (49.2)	20.9 (7.4–34.4)	Reference	NA
				GA	53 (43.4)	16.5 (10.2–20.4)	0.74 (0.45–1.21)	NA
				AA	9 (7.4)	2.0 (1.0–3.1)	0.52 (0.22–1.21)	NA

OS: overall survival; HR: hazard ratio; RR: relative risk; NA: not available.

the presence of ≤16CA repeats (HR = 0.48, 95% CI: 0.33–0.7, $p < 0.01$; heterogeneity $I^2 = 0\%$, $p = 0.45$) (Figure 3). The Galbraith plot identified the study of Winther-Larsen et al. [32], thus we omitted this study in the one-way sensitivity analysis for rs712829. The results confirmed statistical significance of rs712829-related HR, which was only slightly

Table 3: *EGFR* genotype and survival (PFS and TTP) of NSCLC patients treated with TKIs.

dbSNP-ID	Variant type, location, and/or consequence	Author, year (ref)	Genotyping platform used	Genotype	No. of patients (%)	Progression-free survival (PFS) (95% CI)	Time-to-progression (TTP) (95% CI)
rs11568315	Intron variant, g.55020560_55020561AC [n]	Giovannetti et al., 2010 [57]	TaqMan assay	Both alleles ≤ 16CA	30 (31.9)	NA	3.2 (0.7–5.7)[b]
				At least 1 allele > 16CA	64 (68.1)	NA	3.1 (2.4–3.8)[b]
		Han et al., 2007 [55]	PCR and fragment length analysis	Both alleles ≥ 38CA	46 (53.5)	NA	Reference
				Both alleles ≤ 37CA	40 (46.5)	NA	**0.54 (0.34–0.88)**[a]
		Ichihara et al., 2007 [26]	PCR and sequencing	Short allele ≥ 19CA or the sum of alleles ≥ 39CA	63 (64.3)	Reference	NA
				Short allele < 19CA or the sum of alleles < 39CA	35 (35.7)	1.08 (0.63–1.86)[a]	NA
		Kim et al., 2017 [61]	TaqMan assay and sequencing	Both alleles ≤ 16CA	74 (28.0)	Reference	NA
				At least 1 allele > 16CA	188 (72.0)	0.94 (0.71–1.25)[a]	NA
		Liu et al., 2008 [56]	PCR-RFLP	At least 1 allele > 16CA	59 (64.0)	Reference	NA
				Both alleles ≤ 16CA	33 (36.0)	**0.54 (0.33–0.88)**[a]	NA
		Winther Larsen et al., 2014 [59]	PCR-RFLP and capillary electrophoresis	Any allele ≤ 16CA	44 (71.0)	**0.39 (0.22–0.70)**[a]	NA
				Both alleles > 16CA	18 (29.0)	Reference	NA
rs2227983	Missense variant, 1562G>A, R497K	Giovannetti et al., 2010 [57]	TaqMan assay	GG or GA	81 (88.0)	NA	3.3 (2.4–5.0)[b]
				AA	11 (12.0)	NA	3.1 (1.5–4.7)[b]
		Liu et al., 2008 [56]	PCR-RFLP	AA	43 (47.0)	Reference	NA
				GG or GA	49 (53.0)	1.54 (0.98–2.42)[a]	NA
rs2293347	Synonimous variant, 181946C>T, D994D	Winther-Larsen et al., 2015 [32]	AS-PCR or PCR followed by sequencing	CC	252 (80.0)	Reference	NA
				CT or TT	64 (20.0)	**0.74 (0.55–0.99)**[a]	NA
rs712829	5′ UTR variant, g.5031G>T, -216G>T	Giovannetti et al., 2010 [57]	TaqMan assay	GG	34 (36.2)	NA	3.2 (2.6–3.8)[b]
				GT or TT	60 (63.8)	NA	3.2 (0.8–5.7)[b]
		Jung et al., 2012 [25]	PCR-RFLP or PCR followed by sequencing	GG	63 (88.7)	5.1 (2.7–7.5)[b]	NA
				GT	8 (11.3)	16.6 (5.8–27.5)[b]	NA
		Kim et al., 2017 [61]	Taqman PCR and sequencing	GG	78 (32.0)	Reference	NA
				GT or TT	162 (68.0)	0.78 (0.59–1.03)[a]	NA
		Winther-Larsen et al., 2015 [32]	AS-PCR or PCR followed by sequencing	GG	134 (42.0)	Reference	NA
				GT or TT	182 (58.0)	0.90 (0.70–1.14)[a]	NA
		Liu et al., 2008 [56]	PCR-RFLP	GG	34 (37.0)	Reference	NA
				GT or TT	58 (63.0)	**0.62 (0.38–0.99)**[a]	NA

Table 3: Continued.

dbSNP-ID	Variant type, location, and/or consequence	Author, year (ref)	Genotyping platform used	Genotype	No. of patients (%)	Progression-free survival (PFS) (95% CI)	Time-to-progression (TTP) (95% CI)
rs712830	5′ UTR variant, g.5056A>C, -191C/A	Giovannetti et al., 2010 [57]	TaqMan assay	CC	78 (83.0)	NA	3.2 (2.5–3.9)[b]
				CA or AA	16 (17.0)	NA	3.2 (3.0–3.4)[b]
		Kim et al., 2017 [61]	Taqman PCR and sequencing	CC	195 (81.0)	Reference	NA
				CA or AA	45 (19.0)	1.13 (0.8–1.58)[a]	NA
		Winther-Larsen et al., 2015 [32]	AS-PCR or PCR followed by sequencing	CC	236 (75.0)	Reference	NA
				CA or AA	80 (25.0)	1.15 (0.88–1.51)[a]	NA
		Liu et al., 2008 [56]	PCR-RFLP	CC	81 (88.0)	Reference	NA
				CA or AA	11 (12.0)	0.86 (0.40–1.85)[a]	NA

[a]Hazard ratio (HR); [b]Median (months); NA–not available; in bold, significant result at the level <0.05.

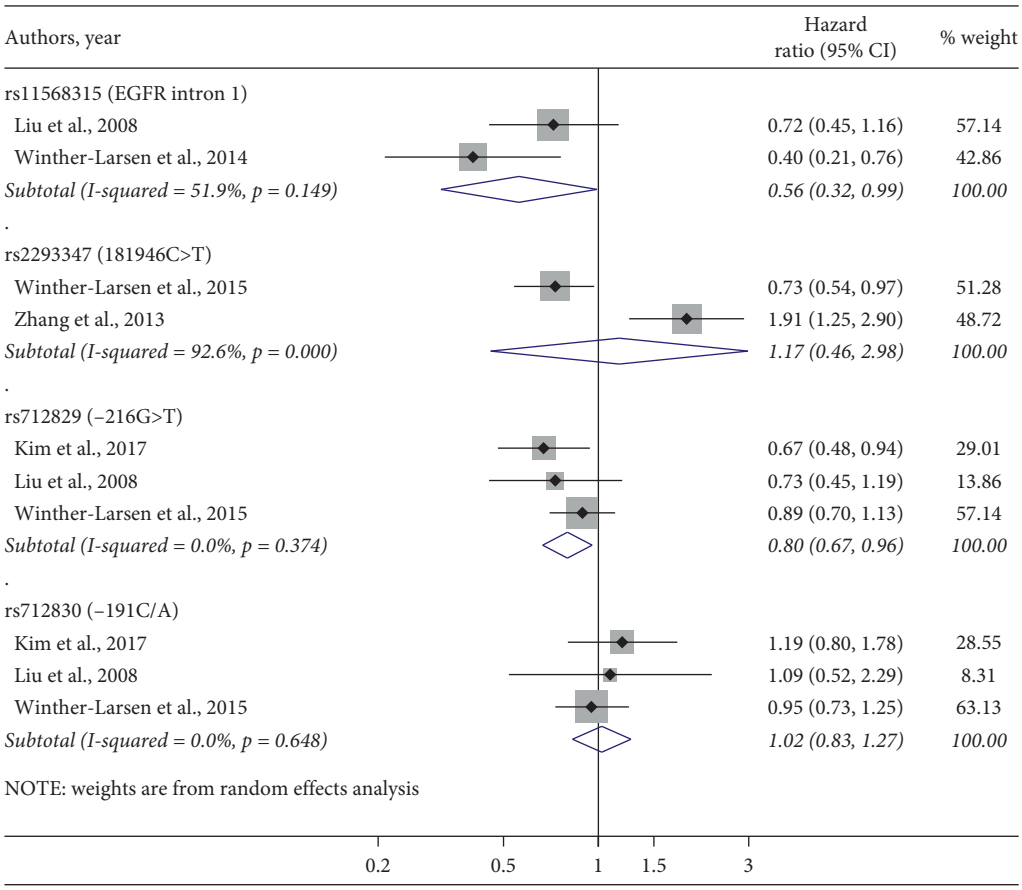

Figure 2: Forest plot reporting pooled HR and their 95% CI of four SNPs for the OS. The square size indicates the weight of each study and of pooled data.

changed to 0.74 (95% CI: 0.58–0.94, $p = 0.01$; heterogeneity $I^2 = 0\%$, $p = 0.42$). The other investigated SNPs, namely, -191C>A (rs712830), displayed increased, albeit insignificant, pooled HR.

4. Discussion

The discovery of activating mutations in the *EGFR* gene from fifteen years ago represented a major breakthrough in the

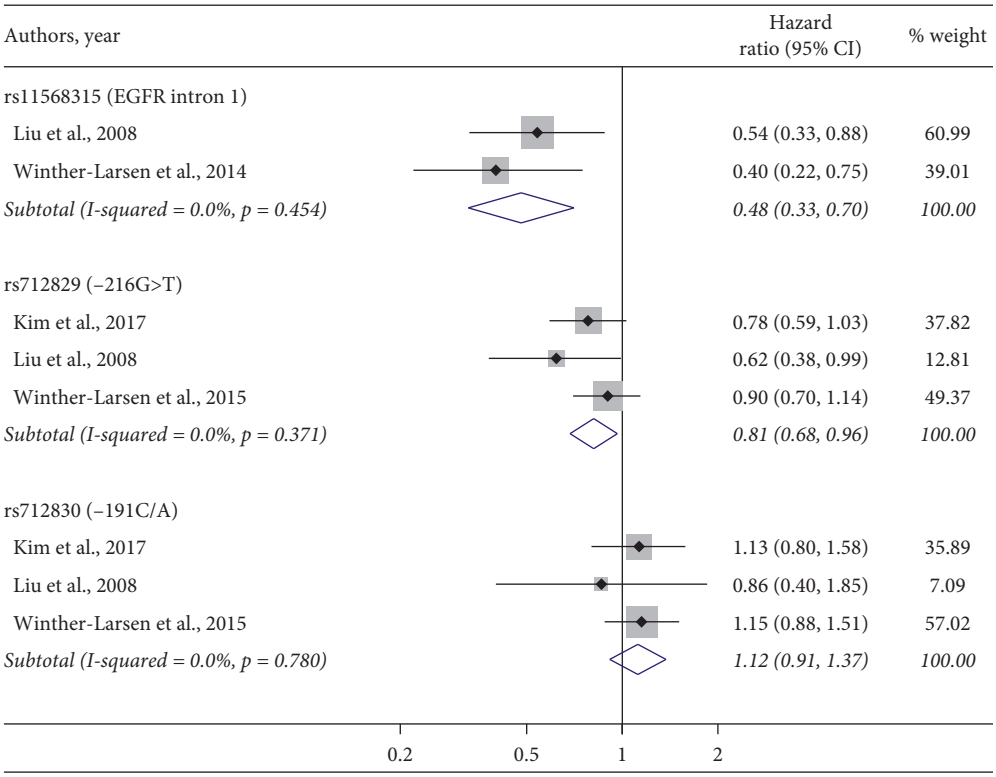

Figure 3: Forest plot reporting pooled HR and their 95% CI of three SNPs for the PFS. The square size indicates the weight of each study and of pooled data.

treatment of NSCLC [22, 23], as clinical responsiveness to TKIs, promising new treatment alternatives [17], turned out to be highly dependent on the presence of so-called "sensitizing" *EGFR* mutations [62]. Consequently, both of the first-generation TKIs, gefitinib and erlotinib, have been approved for the treatment of patients with metastatic NSCLC, but only if their tumors harbor *EGFR* exon 19 deletions or exon 21 (L858R) substitution mutations [63]. Nevertheless, neither *EGFR* mutation testing nor full TKI response is easy to achieve, as former postulates availability of samples of biopsied/resected tumor tissue or pleural effusion and appropriate methodology, expertise and equipment [64], while later is undermined by intrinsic or acquired resistance to TKI that exists or develops in majority of patients [24–26].

In overcoming these issues, numerous studies have been performed to disclose other important factors involved in response to TKI, aiming for those which could be more easily detected and also already present at the beginning of the treatment, hence useful as potential prediction markers for TKI-based therapy outcome. Significant research load has been focused on *EGFR* as the therapy target, revealing that certain germline variants of the *EGFR* gene could confer altered prognosis in their NSCLC-diagnosed carriers treated with TKI [31, 32, 55, 56, 58, 61]. However, the studies were either underpowered [25, 55, 56] or yielded conflicting results [26, 32, 58, 61], leaving the possibility of *EGFR* SNP-associated role in clinical responsiveness to TKIs insufficiently explored. To assess, consolidate, and integrate the available knowledge on this subject, we performed systematic review and meta-analysis of published reports on association between *EGFR* polymorphism and the survival of NSCLC patients. Of 10 *EFGR* SNPs evaluated in our study, four were reported to affect the response to TKI, namely, rs712829 (-216G>T) [56, 61], rs11568315 (CA repeat) [55, 56, 58, 59], rs2293347 (D994D) [31, 32], and rs4947492 [31]. However, pooled analysis of the available data revealed that only *EGFR* -216G>T and variable CA repeat polymorphisms significantly affect the prognosis of TKI-treated NSCLC patients, with longer OS and PFS associated with the presence of variant -216T allele and ≤16CA repeats.

The 5′-flanking region of the *EGFR* gene acts as a promoter by binding Sp1 transcription factor [65]. *EGFR*-216G>T SNP is located in one of the Sp1 binding sites, thus affecting initiation of the *EGFR* transcription [66]. Namely, it has been discovered that the replacement of G by T at this position increases the promoter activity and gene expression by 30% and 40%, respectively [67]. Furthermore, this effect proved to be unaffiliated to the presence of other polymorphisms in this region, as well as to the cell type or *EGFR* expression level [67]. The observation that the response on TKI only partly depends on the presence of *EGFR* activating mutations [68] opened the question of the yet-unexplained difference in therapy outcome, for which -216G>T polymorphism seemed like a reasonable answer. Therefore, most of the studies investigating the association between *EGFR* polymorphism and NSCLC TKI-based treatment included -216G>T. Many of them revealed that it significantly improves treatment outcome [25, 56, 69] and increases the risk of treatment-related toxicity [56, 57, 70]. However, some

failed to observe such associations [32, 61, 69, 71], thus the overall conclusion regarding the importance of -216G>T has not been reached so far. In the present meta-analysis, whose advantage over previous publications lies in higher validity, reliability of the results [72], *EGFR* -216G>T was significantly associated with both OS and PFS in TKI-treated NSCLC patients. Our results therefore suggest the possibility that this *EGFR* polymorphism can be used as an easy-to-obtain and ever-present additional predictive factor in these patients, which could simplify the decision-making process during prescribing and improve the outcome of the therapy. It should be noted, however, that all the reports included in our study were based on either gefitinib or erlotinib treatment, thus our conclusions might not be necessarily relevant to the therapies based on newer TKIs, whose mechanism of action is slightly different [19, 73].

The first intron of *EGFR* has an important regulatory function, which relies on the presence of an enhancer element that stimulates promoter activity [74]. *EGFR* SNP rs11568315 is located close to enhancer in *EGFR* intron 1 and represents a variable simple sequence repeat (SSR) consisting of 14 up to 21 CA dinucleotides [75]. It has been observed that the transcription activity of *EGFR* declines with the increasing number of CA repeats, most probably due to alteration in DNA secondary structure, but also that this effect can be outweighed by other regulatory mechanisms [76]. To determine the possible role of this polymorphism in response to TKIs, numerous studies investigated NSCLC, but also other types of cancer whose therapy is *EGFR*-targeted. Some of the published reports conform in conclusion that the number of CA repeats affects the outcome of TKI-based therapy, with lower number of CA repeats corresponding to higher response rate [55, 60, 77, 78], longer time-to-progression [55, 56, 58, 59, 70], longer survival [58, 59, 77], and increased toxicity [79]. However, others did not detect any significant association [25, 26, 57, 61], deeming this *EGFR* polymorphism to be clinically unimportant. Out of eight CA repeat-related studies involved in our systematic review, in three the influence on survival has been reported, with carriers of 16 CA repeats (representing the shorter and the most frequent allele [80]) having longer OS [58, 59], and carriers of alleles shorter than or equal to 16 CA having longer PFS [56, 59], as compared with other NSCLC patients on gefitinib therapy. The present meta-analysis confirmed the observed effect of variable CA repeats on OS. The possible reasons of conflicting results in the literature might be the lack of consensus in regard to cutoff values defining shorter versus longer CA repeats [26, 81], the presence of linkage disequilibrium with other functional SNPs that remained undetected or unexplored [56, 66, 78], or interethnic differences in the allelic distribution [80]. Yet, our results indicate that the length of CA repeat in *EGFR* intron 1 could be used as another predictive marker for the outcome of TKI-based therapy in NSCLC patients.

The last two *EGFR* SNPs reported to affect the outcome of TKI-based treatment of NSCLC, namely, rs2293347 (D994D) and rs4947492, are currently the least explored. Both are localized within regulatory regions, as former resides in exon 25, i.e., within C-terminal domain [82], and later in the first intron of *EGFR* [83]. So far, the role of rs2293347 in the treatment of NSCLC patients has been investigated in three different studies [31, 32, 78], and all of them reported significant association of this polymorphism and the response to TKIs. Yet, while Ma et al. [78] and Zhang et al. [31] associated the presence of variant allele with shorter OS, shorter PFS, and lower response rate, Winther-Larsen et al. [32] reported the opposite, with variant (albeit major) allele carriers on gefitinib therapy exhibiting higher disease control rate and longer OS and PFS. This *EGFR* SNP is synonymous; hence, it does not lead to a change of the amino acid sequence. Nevertheless, it has been confirmed that even synonymous variations could alter protein amount, structure or function, by affecting mRNA stability, translational kinetics, and splicing [84]. Having in mind the localization of rs2293347, i.e., its proximity to TK domain [82], as well as the contradictory reports regarding its role in TKI efficacy and safety [31, 32, 78], this *EGFR* SNP could be considered a good candidate for future clinical trials. On the other hand, only one study of NSCLC treatment with TKIs evaluated the role of rs4947492 [31], reporting significant association of the variant allele with shorter OS [31]. This variation is believed to alter *EGFR* expression [31], yet linkage disequilibrium with other SNPs could also explain or affect its role in TKI-related treatment [83]. Anyhow, the observed effect would need further confirmation.

The present study harbors several limitations, including the lack or incompleteness of data regarding additional treatments used in included studies, which could affect the overall outcome of the therapy. Also, linkage disequilibrium that has been described among different *EGFR* SNPs, or between SNPs and *EGFR* activating mutations, was not always taken into account. In addition, only publically available reports were included in our study, thus the possibility of a publication bias cannot be completely excluded. Other types of bias that might affect included studies, e.g., selection bias and information bias, might be present too. Also, it would have been valuable to stratify our findings according to sociodemographic characteristics and/or environmental effect modifiers, but this was not feasible since the original datasets were not available to us. Finally, the number of available studies for most of the investigated SNPs was insufficient for any sound conclusion to be drawn. Nevertheless, our study has several advantages. We used comprehensive and rigorous methodology to obtain all available eligible studies. Quality of the included studies was rather high, confirmed by the appropriate quality measurement tools. Statistical power of our analyses was considerably increased in respect to any single study, because of a bigger number of cases that were pooled for different SNPs.

In conclusion, our study shows that out of ten investigated *EGFR* SNPs (rs11543848, rs11568315, rs11977388, rs2075102, rs2227983, rs2293347, rs4947492, rs712829, rs712830, and rs7809028), only four, namely, rs712829 (-216G>T), rs11568315 (CA repeat), rs2293347 (D994D) and rs4947492, have been reported to affect the outcome of TKI-based NSCLC treatment. Of these, only -216G>T and variable CA repeat polymorphisms have been confirmed by meta-analysis of available data to significantly affect OS and

PFS in gefitinib- or erlotinib-treated NSCLC patients. To ascertain whether these SNPs affect the response to other TKIs, as well as whether other *EGFR* SNPs have a role in NSCLC treatment, additional studies are warranted.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Vladimir Jurisic and Vladimir Vukovic contributed equally to this work.

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Supplementary Materials

Supplementary Table 1: methodological quality of the included studies: based on A, the Newcastle-Ottawa Quality Assessment Scale for cohort studies, and on B, Jadad scale for RCT. Supplementary Figure 2: funnel plot for publication bias evaluation: overall survival for rs712829 (-216G>T). Supplementary Figure 3: funnel plot for publication bias evaluation: overall survival for rs712830 (-191C/A). Supplementary Figure 4: Galbraith plot for assessing heterogeneity: overall survival for rs712829 (-216G>T). Supplementary Figure 5: Galbraith plot for assessing heterogeneity: overall survival for rs712830 (-191C/A). Supplementary Figure 6: funnel plot for publication bias evaluation: progression-free survival for rs712829 (-216G>T). Supplementary Figure 7: funnel plot for publication bias evaluation: progression-free survival for rs712830 (-191C/A). Supplementary Figure 8: Galbraith plot for assessing heterogeneity: PFS for rs712829 (-216G>T). Supplementary Figure 8: Galbraith plot for assessing heterogeneity: PFS for rs712830 (-191C/A). (*Supplementary Materials*)

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Review Article

Signal Transduction Pathways in Breast Cancer: The Important Role of PI3K/Akt/mTOR

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Breast cancer is the cancer with the highest prevalence in women and is the number-one cause of cancer mortality worldwide. Cell transduction is a fundamental process in the development and progression of cancer. Modifications in various cell signalling pathways promote tumour cell proliferation, progression, and survival. The PI3K/Akt/mTOR pathway is an example of that, and it is involved in growth, proliferation, survival, motility, metabolism, and immune response regulation. Activation of this pathway is one of the main causes of cancer cell resistance to antitumour therapies. This makes PI3K/Akt/mTOR signalling a crucial object of study for understanding the development and progression of this disease. Thus, this pathway may have a role as a potential therapeutic target, as well as prognostic and diagnostic value, in patients with breast cancer. Despite the existence of selective PI3K/Akt/mTOR pathway inhibitors and current clinical trials, the cellular mechanisms are not yet known. The present review aims to understand the current state of this important disease and the paths that must be forged.

1. Introduction: Current State of the Disease

Breast cancer is the most prevalent cancer type in women as well as the leading cause of cancer mortality in this population worldwide, with a peak incidence between 45 and 65 years of age [1]. Although it is not common, breast cancer can also occur in men, with a frequency of 1 in 100 diagnosed cases, representing less than 1% of all cancers in men [2].

Among the most important risk factors associated with breast cancer are ageing, family history, nulliparity, hormonal factors, such as early menarche or late menopause, and other factors related to lifestyle, such as alcohol consumption, obesity, and physical inactivity [3, 4].

Breast cancer can be hereditary or sporadic. The most frequent mutations associated with hereditary cancer include those that affect DNA damage repair (DDR) genes, the most important of which are mutations in the BRCA1, BRCA2, and TP53 genes [5]. Sporadic cancer represents approximately 85% of all cases of breast cancer and is associated with some of the risk factors mentioned above; however, it has also been associated with exposure to carcinogens, such as air pollutants [6], electromagnetic radiation [7], and DDR gene expression dysregulation [8].

According to their presentation, ductal carcinoma *in situ* is the most diagnosed breast cancer type, followed by lobular carcinoma *in situ* [9]. Breast cancer, in turn, is divided into different subtypes based on the presence or absence of the

estrogen receptor (ER), progesterone receptor (PR), and HER2 receptor. Thus, we can distinguish between a luminal subtype, being ER/PR+, an Her2+ subtype, which has this receptor overexpressed, and a triple negative or basal-like subtype (TNBC). Following this classification, the luminal subtypes can be divided into luminal A, characterized by ER/PR+, HER2–, and low Ki67 expression, and luminal B, characterized by ER/PR+, HER2+, and high Ki67 expression. Subtype Her2+ is ER/PR negative, and the triple negative indicates a lack of all these receptors [10–12].

Cell signal transduction is a fundamental process in the development and progression of cancer. Hanahan and Weinberg [13] noted that tumour cells exhibit a set of characteristics or hallmarks, including uncontrolled proliferation, genomic instability, and apoptosis evasion. To this end, modifications to various cell signalling pathways promote tumour cell proliferation, progression, and survival [14]. These alterations are due to mutations in oncogenes that overexpress certain proteins, mutated proteins that present uncontrolled activity, or inactivation of tumour suppressor genes that favour these processes [15].

Many alterations in breast cancer cells that affect cell signalling pathways have been described. In fact, variations have been described in the responses mediated by calcium-sensitive receptors [16, 17] or hypoxia-inducible factor [18] or even in the apoptotic cell mechanisms themselves [19]. However, the alterations most studied and most directly involved in the progression and development of breast cancer pathways are those mediated by the ER and human epidermal growth factor type-2 receptors (HER2/Neu or c-ErbB2) [19]. The activity of HER2 receptors in turn promotes the signalling of other pathways such as the mitogen-activated protein kinases (MAPKs) or cell components like glycogen synthase kinase-3 (GSK-3) and PI3K/Akt/mTOR pathways, both represented in Figure 1, denoting the importance of signal integration and transduction processes in the progression and development of breast cancer [20–23].

2. Importance of the PI3K/AKT/mTOR Pathway in Cancer

PI3K/Akt/mTOR is a cell signalling pathway involved in growth, proliferation, survival, motility, metabolism, and immune response regulation [24, 25]. This pathway has also been associated with a great variety of diseases and syndromes, such as tuberous sclerosis, Parkinson's disease, and vascular diseases [26–28].

Studies on PI3K/Akt/mTOR have also focused on cancer research. Alterations to this pathway have been found in practically all human tumours, including breast cancer, where up to 60% of the tumours present different variations that hyperactivate this pathway [29].

The dysregulation of this pathway has been related to a wide variety of cancer hallmarks, including uncontrolled proliferation, genomic instability, and metabolic reprogramming in tumour cells [13, 30]. In addition, PI3K/Akt/mTOR pathway activation is one of the main causes of cancer cell resistance to antitumour therapies [31]. This makes the PI3K/Akt/mTOR pathway a crucial object of study for understanding the development and progression of this disease, the role of this pathway as a potential therapeutic target, and the prognostic and diagnostic value of this pathway in patients with breast cancer [32, 33].

3. PI3Ks in Tumours and Existing Therapies

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that integrate signals from growth factors, cytokines, and other extracellular stimuli and are related to the cell's response to these kinases [34]. Three different classes of PI3Ks are known, which in turn are divided into different subclasses according to their affinity for certain substrates, their sequence homology, and the functions that each of these classes have in cell signal transduction; class I PI3Ks are the most studied and the most clearly related to oncogenic processes [35]. Class I PI3Ks are divided into the following classes: PI3K IA, activated by receptor tyrosine kinases (RTK) receptors, G-protein-coupled receptors (GPCRs), and oncogenes such as Ras; and PI3K IB, regulated exclusively by GPCRs [36]. Among these, class IA is the most directly implicated in cancer. PI3K class IA consists of a regulatory subunit, which can be p85α, p55α, p50α, p85β, or p55γ, and a catalytic subunit, which can be p110α, p110β, or p110δ [37].

When a cellular receptor is activated, it dimerizes and autophosphorylates in various regions, phosphorylating, in turn, different adapter proteins and being recognized by the p85 subunit, which binds to these phosphorylated residues and generates a conformational change that releases p110, the subunit that exerts catalytic activity. Consequently, a phosphate group is added to phosphatidylinositol 4,5-bisphosphate (PIP2), transforming it into phosphatidylinositol (3,4,5)-trisphosphate (PIP3) [37]. Phosphatase and tensin homolog (PTEN) phosphatase, which can remove a phosphate from PIP3 to convert it into PIP2, is the most important negative regulator of this pathway and one of the tumour suppressors with the greatest effect on different types of cancer [38]. PIP3 levels can also be regulated by another tumour suppressor known as inositol polyphosphate-4-phosphatase type II B (INPP4B), which has the same effect as PTEN. INPP4B loss has been considered a marker of aggressiveness in TNBC tumours [39, 40].

Activating mutations in PIK3CA, the gene that encodes the p110α catalytic subunit, have been identified as potent oncogenic mechanisms implicated in the hyperactivation of this pathway (Figure 2); these mutations are especially noteworthy in breast cancer, where up to 27% of patients have mutations in this gene [41]. Mutations in PI3KCA are more common in luminal A subtype cancers, where they are detected in 45% of tumours, followed by HER2+ mutations with a frequency of 39%; luminal B represents 30% of cancers, and TNBC alterations appear in 9% of cases [24]. These mutations affect mainly the helical domain of the p110α subunit, reducing its repression by regulatory subunits or facilitating its interaction with IRS1 [42, 43]. PI3K activation is a critical step in oncogenesis and plays a role in treatment resistance in ER+/HER2+ breast cancers. There

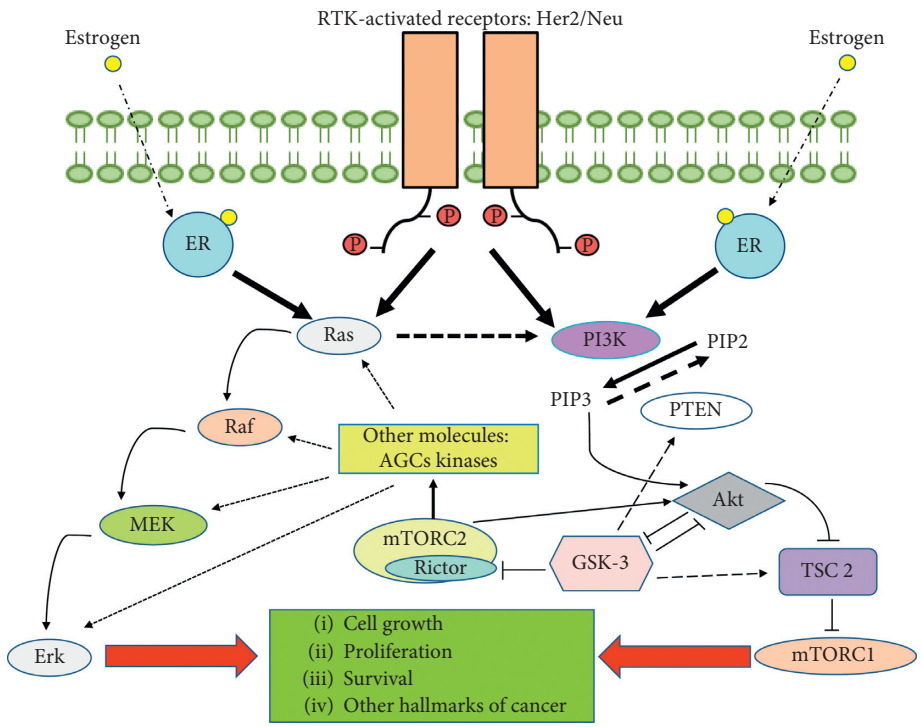

Figure 1: Overview of cell signalling mediated by the tyrosine kinase receptors (RTKs) Her2/Neu and estrogen receptors (ERs), two key components of breast cancer development. Their activation initiates the PI3K/Akt/mTOR and MAPK pathways, finally promoting cell growth, proliferation, survival, and other hallmarks of cancer. Although this is a review of PI3K/Akt/mTOR signalling, it is important to understand that the different pathways are connected by different points. In this figure, we have presented two examples: Ras, promoting PI3K activation, and how some AGC kinases (such as SGK-3) activated by mTORC2 also interact with the MAPK pathway. Additionally, GSK-3 plays an important role as well in the regulation of these pathways, represented in the figure. GSK-3 is an example of how complex those interactions are, by the inhibition and activation of different molecules implicated in PI3K and MAPK pathways.

are currently PI3K inhibitors, which are being implemented in clinical trials [44, 45].

Mutations in PTEN also result in important alterations in cell signalling in patients with breast cancer. The relationship between the loss of PTEN gene function and unfavourable predictive factors has been observed in various types of cancer, such as gastric, prostate, and colorectal cancer [46–48]. Lu et al. [49] described how the activity exerted by this phosphatase slows cell growth and induces apoptosis and anoikis in breast cancer cells. The results of a meta-analysis of 27 studies and 10,231 cases performed by Li et al. [50] showed that there seems to be an association between PTEN expression loss and tumour aggressiveness in breast cancer patients, especially those with ER, PR, and TNBC tumours, thus demonstrating the involvement of PTEN in the initiation and malignancy of breast cancer tumour cells.

There are multiple PI3K inhibitors that have been developed or are in the study phase. These are classified according to their specificity for each isoform and can be divided into (1) first-generation inhibitors, also known as pan-inhibitors of PI3K, which target all the different PI3K class I isoforms (α, β, δ, or γ), (2) second-generation inhibitors, which are specific to any of these isoforms, or (3) dual PI3K/mTOR inhibitors. However, the efficacy shown by these types of inhibitors as therapeutic agents is far from that expected due to the coexistence of various mutations present in tumour cells, compensatory feedback cycles, or the toxicity associated with these treatments [24]. That is why research has focused on these inhibitors in combination with other drugs, although much work remains.

Of the first-generation inhibitors, breast cancer studies have focused on the use of pictilisib and buparlisib. The role of pictilisib as a pan-inhibitor (GDC-0941) in the inhibition of the metastatic phenotype in thyroid carcinomas has been demonstrated due to its action on PI3K and HIF-1α [51]. Zou et al. [52] observed the synergistic role that this drug had with the MEK inhibitor UO126 in inhibiting NSCLC cell growth. A study conducted by Schmid et al. [53] showed how the combination of pictilisib with anastrozole obtained a better response in inhibiting breast cancer cell proliferation. However, in a study conducted by Krop et al. [54], the combined use of pictilisib with fulvestrant was not associated with an improvement in the treatment of patients with advanced-stage breast cancer resistant to endocrine therapy, regardless of the presence or absence of PI3KCA mutations, measured in terms of progression-free survival (PFS).

Buparlisib (BKM120), another pan-inhibitor of PI3K, has demonstrated synergistic growth inhibitory effects in combination with agents targeting the HER2 receptor in preclinical studies [45]. Several early clinical trials have shown positive results for the use of buparlisib in breast cancer patients [55–57]. Another phase II clinical trial developed by Loibl et al. [58] studied the combination of

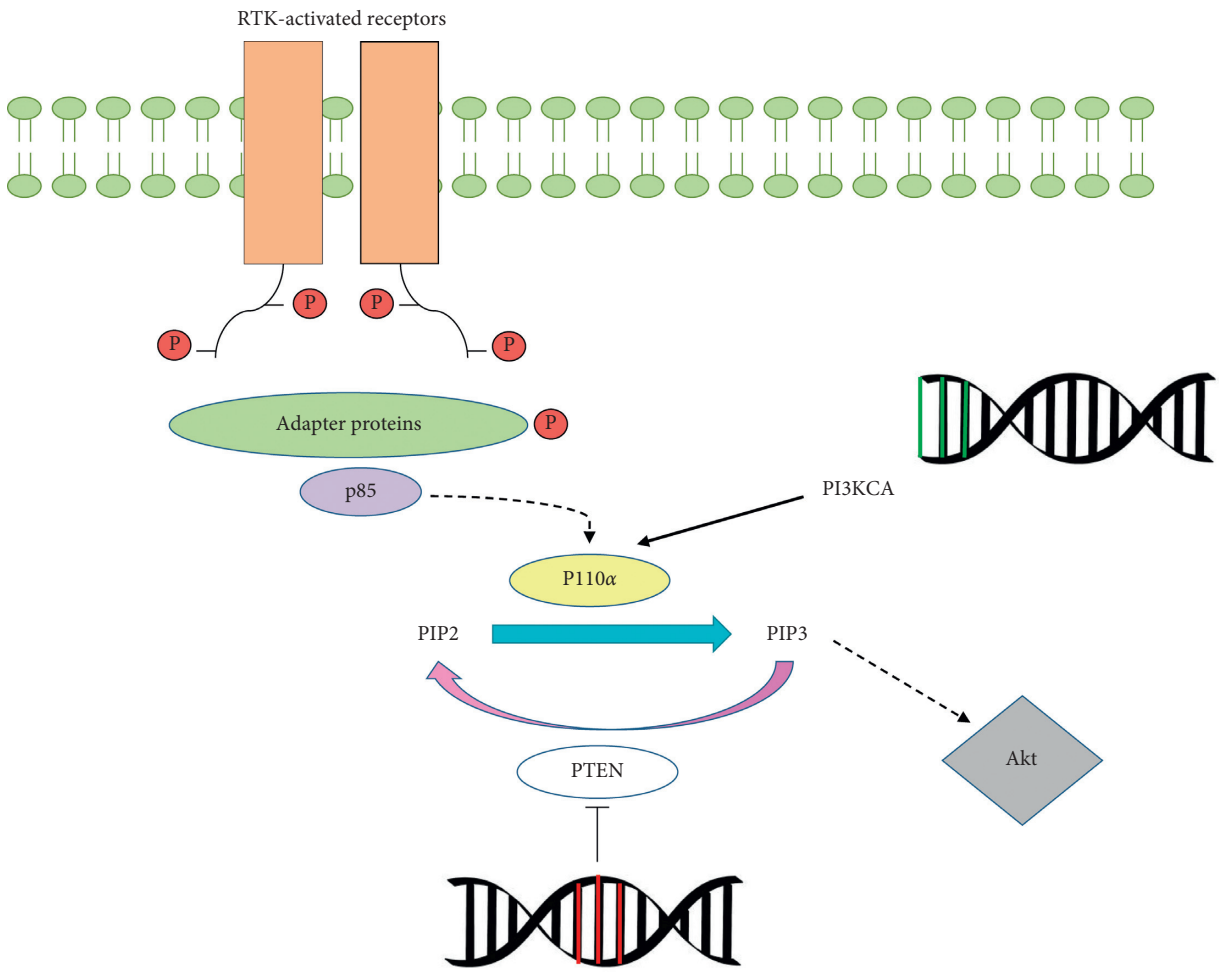

Figure 2: Mechanism of action of PI3K and PTEN. RTK receptors are activated, phosphorylating themselves and other series of adapter proteins, such as IRS-1. In this case, the regulatory subunit p85 binds to these residues and releases the catalytic subunit p110α, which adds a phosphate to PIP2 and transforms it into PIP3, which will subsequently activate Akt. PTEN prevents this activation by dephosphorylating PIP3. The loss of function of this gene, represented in red, or activating PI3KCA mutations, shown in green, can overactivate this route, favouring the development of cancer.

buparlisib with trastuzumab and paclitaxel in women with primary HER2+ tumours. The results showed little reliability in its use; however, high rates of objective response and a reduction in the Ki67 cell proliferation marker in the ER+HER2+ subgroup denote the importance of future investigations of therapies targeting this pathway. Furthermore, two important clinical trials need to be mentioned here: BELLE-2 and BELLE-3. Both were double-blind, randomized, placebo-controlled phase 3 trials in which the use of buparlisib was analysed in hormone receptor-positive Her2-negative postmenopausal women. BELLE-2 aimed to evaluate the combination of buparlisib plus fulvestrant versus placebo and fulvestrant. The results showed that the use of this PI3K inhibitor combined with endocrine therapy was associated with a significant improvement compared to fulvestrant alone. However, this combination was also found to have considerable toxicity, limiting its efficacy [59]. In BELLE-3, 432 postmenopausal women who had previously received endocrine therapy and mTOR inhibitors were divided again into two groups: a group that received fulvestrant and buparlisib and a control group that received only fulvestrant. Patients with fulvestrant plus buparlisib had a longer PFS than those who received fulvestrant alone, but again, the toxicity of this combination restricts its use [60]. On the whole, these works highlight the importance of performing further studies with PI3K-α-specific inhibitors to provide the greatest benefits.

Currently, research is also aimed at the use of second-generation PI3K inhibitors, such as alpelisib (BYL719) and taselisib (GDC0032), which target the PI3K-α isoform, as a higher-safety profile is expected compared to previous inhibitors [61]. Numerous previous studies seem to indicate that mutations in PI3KCA in ER + tumours decrease the response rate of breast cancer tumours to antiestrogenic therapy [62, 63]. A series of preclinical cell models developed by Fritsch et al. [64] demonstrated how cells with mutations in PIK3CA had greater sensitivity to alpelisib, while cell lines without this mutation had selective sensitivity. A phase III clinical trial showed how patients with advanced tumours and mutations in PIK3CA benefited from the use of alpelisib in combination with fulvestrant; compared with fulvestrant with placebo, this combination increased the PFS and

response rates [65]. In fact, alpelisib has recently become the first PI3K inhibitor approved by the FDA for the treatment of breast cancer, so the study of this type of inhibitor is a growing point of interest in actual research. Other preclinical studies also reported the efficacy of taselisib in cellular models with mutations in PIK3CA in breast cancer [66]. SANDPIPER is an ongoing randomized, double-blind phase III clinical trial with 631 postmenopausal women with ER + HER2- PI3KCA mutations who showed progression or recurrence during or after aromatase inhibitor (AI) therapy. This trial aims to investigate the efficacy and safety of taselisib plus fulvestrant in comparison to a fulvestrant alone control group (NCT02340221). SOLAR-1 is another ongoing phase III clinical study designed to evaluate the use of alpelisib plus fulvestrant in ER/PR + Her2– advanced cancer patients after treatment with AI (NCT02437318). Currently, GDC-0077, another second-generation PI3K-α inhibitor, is being tested in a phase I clinical trial in patients with PIK3CA mutant solid tumours that are locally advanced or metastatic, including breast cancer, and GDC-0077 is being tested in combination with targeted therapies focused on the ER/PR+Her2– subtype (NCT03006172).

Regarding third-generation inhibitors or dual PI3K/mTOR inhibitors, the role of some of these inhibitors, such as BEZ235, has been studied in combination with other inhibitors, such as everolimus (RAD-001), and these inhibitors have been shown to exert a synergistic action by decreasing the growth of some TNBC cell lines, such as MDA-MB-231 cells or the ER + MCF-7 line [67]. The combination of BEZ-235 with autophagy inhibitors has also been studied, and these combinations have resulted in inhibited proliferation and increased apoptosis in breast cancer cells [68]. However, clinical studies of BEZ-235 have not yielded promising outcomes. A phase I/IB study of BEZ-235 in patients with Her2+ advanced breast cancer showed that the safety profile of this inhibitor was not adequate [69]. Nevertheless, clinical studies of BEZ235 are ongoing in patients with metastatic breast cancer [70]. Some previous studies indicate how AIs can be combined with PI3K/mTOR inhibitors for treating patients with resistance to endocrine therapy who present with ER+HER-metastatic cells. In addition, it has been studied how the PF-04691502 inhibitor is currently used for combined treatment with tamoxifen for treating breast cancer stem cells (BCSCs); in this manner, the resistance they presented when tamoxifen was applied in the absence of another drug was avoided, and the importance of this pathway in therapy resistance and in the formation of mammals was shown [71]. Finally, another dual PI3K/mTOR inhibitor, GDC-0980, has been shown to be particularly effective in BRCA-competent TNBC when combined with a PARP inhibitor and carboplatin because it inhibits the DDR system [72]. Studies on the efficacy of DDR inhibitors in breast cancer therapy are discussed in previous reviews [73].

4. AKT in Tumours and Existing Therapies

RAC-alpha serine/threonine-protein kinase (Akt), also known as protein kinase B (PKB), is a serine-threonine kinase that has three different isoforms encoded by three different genes: Akt1, Akt2, and Akt3. However, it is Akt1 that has been most associated with cancer [74]. Akt1 binds to PIP3 via pleckstrin homology (PH) domains [75]. This interaction localizes Akt to the cell membrane, resulting in the subsequent phosphorylation of the Thr308 and Ser473 residues by 3-phosphoinositide-dependent protein kinase-1 and -2 (PDK1 and PDK2). While PDK1 is activated by PIP3, PDK2 forms part of the mTORC2 complex [76].

The effects of Akt activation include the activation of MDM2, a p53 inhibitor, and the inhibition of other proteins involved in stimulating cell survival in stress situations [77]. The activation of Akt has also been associated with the inhibition of p21 and p27, which are involved directly in cell cycle control [78], and GSK-3β, as shown in Figure 3. GSK-3β inactivation is directly related to cell metabolism reprogramming and, more specifically, to the uptake and use of glucose, which favours the Warburg effect [79]. However, the role of GSK-3β in cancer remains controversial. Some studies have reported that GSK-3β may function as a tumour promoter. GSK-3β knockdown cells were associated with decreased levels of Bcl-2 and VEGF, thus suggesting the role of GSK-3β in inhibiting apoptosis and promoting angiogenesis [80]. On the other hand, previous findings have indicated GSK-3β as a tumour suppressor in breast cancer because it increases the sensitivity of chemotherapy and inhibits PI3K/Akt and Wnt signalling, thus playing a key role in the cell cycle and survival [81, 82]. The interaction of GSK-3 with PI3K/Akt pathway is represented in Figure 1. The activation of Akt is also related to the activation of mTORC1. In addition, Akt is involved in the activation and inactivation of various transcription factors; for example, it binds 14-3-3 proteins and prevents the translocation of FOXO to the nucleus [83]. Akt is also important in the regulation of NF-$\kappa\beta$-dependent gene transcription and CREB-1 overactivation, thus promoting the expression of antiapoptotic genes such as Bcl-2 and mcl-1 [84, 85].

The hyperactivation of Akt and consequently mTOR may facilitate the resistance that some patients with breast cancer have to endocrine therapies; in these patients, an inverse correlation was established between Akt activation and a partial treatment response [86, 87].

In breast cancer, the most frequent mutation is E17K-Akt1, representing 2% of all breast cancers. This mutation increases the affinity of the PH domain for lipids, resulting in the constitutive localization of Akt in the cell membrane [88]. Currently, there are phase I and II clinical trials with allosteric and catalytic Akt inhibitors. Hyman et al. [89] proposed the use of the E17K-Akt1 mutation as a predictive marker of the response to the catalytic inhibitor AZD5363, with promising results. However, studies with the allosteric inhibitor MK-2206 combined with hormone therapy reported benefit ratios of only 42% [57].

In addition, recent studies indicate that there are other proteins also activated by PI3K that contribute to the development of cancer. Lien et al. [30] described that Akt is not always hyperactivated in the presence of PI3KCA or PTEN mutations; the authors also showed the role that other PI3K-dependent proteins may play in the resistance to pathway inhibitors and how these proteins can substitute for Akt-

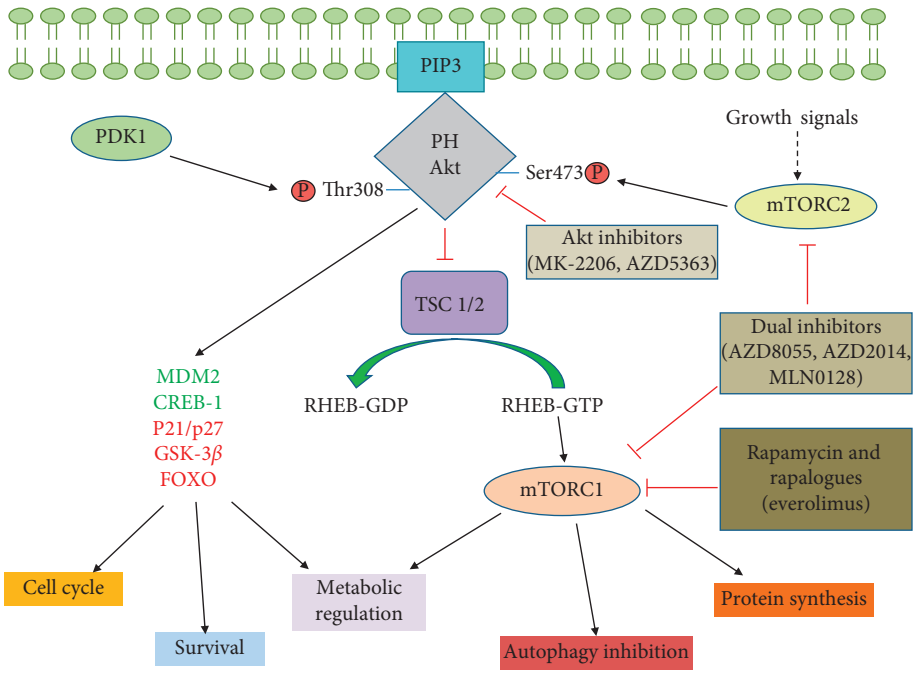

Figure 3: Activation of Akt after joining to PIP3 by its PH domain. PDK1 and PDK2, present in mTORC2, phosphorylate Akt, which will activate and inhibit a series of genes, transcription factors, and proteins, represented in red, such as the TSC complex, which will eventually activate mTORC1, resulting in a series of cellular responses. We have remarked in green the components which are stimulated or overactive in these cells. This figure also shows the main therapies developed to focus on this pathway.

mediated signalling, indicating that further knowledge regarding these pathways is important for developing promising future therapies (Figure 3).

5. mTOR in Tumours and Existing Therapies

The mammalian target of rapamycin (mTOR) is a protein kinase comprising two distinct complexes, mTORC1 and mTORC2. Both complexes are associated with a set of proteins that can be common or specific to each complex [90]. mTORC1 is a complex formed by the mTOR, Raptor, Deptor, Pras40, and mLST8 proteins [91]. This complex is activated by Akt via inhibition of the tuberous sclerosis complex (TSC) [92]. In turn, TSC inhibits mTORC1 due to its ability to inactivate RHEB, a GTPase that activates this complex. mTORC1 responds to amino acids, stress, oxygen levels, energy needs, and growth signals [24]. The activation of mTORC1 seems to be involved in multiple cellular processes, including protein synthesis control, through phosphorylation of the ribosomal proteins S6K1 and 4E-BP1, the regulation of metabolism, and the inhibition of autophagy [93].

mTORC2 is a complex that shares certain components with mTORC1, such as mTOR kinase, mLST8, and Deptor. In addition, it contains the Rictor protein and the subunits Sin1 and Protor 1/2 [91]. This complex is insensitive to rapamycin, unlike the mTORC1 complex [94]. mTORC2 is activated by growth signals and controls cell proliferation and survival processes, as well as the cytoskeleton, mainly by phosphorylating other proteins such as Akt, which, in turn, is the main activator of mTORC1 [95]. In fact, mTORC2 phosphorylates AGC kinase family members, which are Akt, SGK, and PKC (Figure 1), all of which have oncogenic effects [96, 97]. The role of SGKs is also interesting in cancer because they interact at various levels with MAPK signalling and are related to the tumourigenesis process in PI3K mutants through Akt-independent but SGK-3-dependent malignant transformation (Figure 1) [98]. mTORC2 also interacts with the retinoblastoma (Rb) tumour suppressor. Previous studies have demonstrated that Rb may inhibit Akt activation by inhibiting the mTORC2/PDK1 phosphorylation of Ser-473 Akt [99]. Furthermore, mTORC2 has been associated with the control of the expression of glycolytic genes by epigenetic regulation of H3K56Ac levels in glioma cells [100], suggesting the different and important roles that this complex may have in cancer progression and regulation.

Everolimus is one of the main drugs that targets mTOR, and numerous studies have demonstrated its effectiveness in breast cancer therapy [101]. Du et al. [102] reported the efficacy of everolimus in the inhibition of antiapoptotic proteins such as Bcl-2, as well as in breast cancer cell cycle and growth arrest and in disease progression. Other studies have shown the efficacy of everolimus in the treatment of advanced ER + PR + breast cancer [103]. Everolimus is an allosteric inhibitor of mTORC1 but not mTORC2 and can increase Akt phosphorylation by not binding the latter inhibited complex. Dual inhibitors of mTOR, affecting both complexes, have been studied. Leung et al. [104] demonstrated *in vitro* the synergy that this drug presented with other pathway inhibitors, such as dual mTOR inhibitors, representing new strides for greater efficacy of the developed therapies targeting this pathway.

Among the dual inhibitors are AZD8055, AZD2014, and MLN0128, which are in clinical development but have already demonstrated efficacy in numerous studies [105, 106]. These inhibitors show better results compared to mTORC1 inhibitors in blocking the PI3K/Akt/mTOR pathway as measured by 4-EBP1, SK6, and p-Akt inhibition [107]. The effectiveness of these inhibitors has been demonstrated even in everolimus resistance acquired by mutations in the rapamycin binding domain of mTOR (Figure 3) [108]. Here, it is important to discuss the role of mTOR inhibitors and their relationship with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs), another key component of the DDR system. Low levels of DNA-PKcs protein expression were related to higher tumour grade, dedifferentiation and mitotic index, and poor survival [73, 109]. CC-115, a dual inhibitor of mTOR and DNA-PKcs, has been shown to inhibit cell growth *in vitro* by blocking DDR pathways, thus inducing apoptosis in many cancer lines, including breast cancer cells [110]. Currently, a phase I clinical trial with CC-115 is ongoing. Other studies also report the role of the DNA-PK inhibitor NU7441 in sensitizing breast cancer cells to ionizing radiation and doxorubicin [111].

Recently, an mTORC2-selective inhibitor based on nanotechnology for HER2 amplification combined with the HER2 inhibitor lapatinib was validated in TNBC, showing its promising efficacy for both subtypes [112].

6. IRS4 in Tumours and the Relationship with PI3K/Akt/mTOR in Breast Cancer

Insulin receptor substrates (IRSs) are a set of adaptive cytoplasmic proteins that were originally identified by their role in insulin signalling [113]. The IRS family comprises six members. IRS1 and IRS2 are the best studied variants due to their broad expression in different tissues, and they strongly resemble each other in their structures [114]. IRS3 has been found in rodents but not in humans [115]. IRS5/DOK4 and IRS6/DOK5 are two distant members of the family with greater resemblance to each other than to the other IRSs [116]. The present study will review IRS4, which has been implicated in some types of cancer, such as breast cancer, squamous carcinoma of the lung, sarcomas, and acute lymphoblastic leukaemia [117, 118].

IRS phosphorylation occurs at tyrosine residues, generally through RTK-type receptors, thus inducing a signalling cascade that, in turn, promotes the activation of other pathways such as PI3K/Akt or MAPK (Figure 1) [114, 119]. However, Ikink et al. [120] demonstrated that IRS4, unlike other family members, did not require growth factors, leading to the proliferation of mammary epithelial cells due to elevated basal activation of the PI3K/Akt/mTOR pathway. This activation is achieved through the interaction of IRS4 with the p85 subunit [121]. In addition, an association was found between adenovirus infection or certain retroviruses and increased levels of IRS4 [121, 122].

Other studies have shown that there is a positive correlation between IRS4 levels and other proteins involved in breast cancer, such as Breast Tumor Kinase (Brk), a kinase that is overexpressed in 80% of breast tumour cells, indicating the importance of this protein in the development of breast cancer [123, 124].

It has also been observed that IRS4 induces resistance to trastuzumab and lapatinib, two treatments directed against HER2+ breast cancer cells, because it is involved directly in the hyperactivation of the PI3K/Akt/mTOR pathway, which is ultimately responsible for this resistance, as shown in previous studies; thus, IRS4 is a promising therapeutic target for these types of tumours that are resistant to treatment [125, 126].

7. Future Directions

The study of cellular events that occur in oncogenic processes is essential to understand the treatment actions we can take. Furthermore, it is important to understand that carcinogenesis involves a wide range of changes in tumour cells that enable their malignant transformation. From this perspective, the PI3K/Akt/mTOR cell signalling pathway is one of the key points of study that could result in improvements in the existing survival curves after good anatomopathological studies and analyses of the efficacy and efficiency of the inhibition. Despite the existence of selective PI3K/Akt/mTOR inhibitors and current clinical trials, the underlying cellular mechanisms are not yet known. Studying the existing scientific literature helps to understand the current state and the possible paths to be taken. To maximize the efficacy of the inhibitors and decrease their toxicity, PI3K/Akt/mTOR targeting is commonly combined with additional treatments, such as endocrine therapy or DDR inhibitors. Current challenges may be to minimize the side effects of these therapies as well as to increase their specificity, thus bringing more benefits to the patients who need to receive this type of treatment.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Miguel A. Ortega and Oscar Fraile-Martínez contributed equally to this work. Natalio García-Honduvilla and Santiago Coca share senior authorship of this work.

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Research Article

Th17 Cells and IL-17 As Novel Immune Targets in Ovarian Cancer Therapy

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Ovarian cancer (OC) is usually diagnosed at an advanced stage and is related with poor prognosis. Despite numerous studies, the pathogenesis of OC is still unknown. Recent studies indicate the role of the immune system in the development and spread of OC. The identification of factors and mechanisms involved in that process and their modulation is crucial for creating effective antitumor therapy. We investigated the potential role of Th17 cells in OC patients ($n = 71$) by analyzing the frequencies of Th17 cells in three different environments, i.e., peripheral blood (PB), peritoneal fluid (PF), and tissue (Th17 infiltrating cells), and the concentration of IL-17A in plasma and PF of patients in terms of their clinical and prognostic significance. Th17 cells were analyzed by flow cytometry as a percentage of $CD4^+$ lymphocytes that expressed intracellular expression of IL-17A. The level of IL-17A in plasma and PF were determined by ELISA. Our results showed accumulation of Th17 cells among tumor-infiltrating $CD4^+$ lymphocytes ($p < 0.001$ in relation to PB). Moreover, the percentage of Th17 cells in both PB and PF of OC patients was significantly lower than that in benign tumors group ($n = 35$). There were no significant differences in the percentage of Th17 cells in PB, PF, and tissue in relation to clinicopathological characteristics of OC patients and survival. The lower percentage of Th17 cells in the PB and PF of OC patients may promote evasion of host immune response by cancer cells. The concentration of IL-17A in plasma of OC patients was higher ($p < 0.0001$) than that in both benign tumors and control group ($n = 10$). The PF IL-17A level in OC patients was higher ($p < 0.0001$) than that in women with benign ovarian tumors, indicating its synthesis in OC microenvironment. Higher IL-17A level in PF is correlated with longer (median: 36.5 vs. 27 months) survival of OC patients.

1. Introduction

Ovarian cancer (OC) is usually diagnosed at an advanced stage of development, which translates into adverse effects of treatment. Despite numerous studies, the pathogenesis of OC is still unknown. The mechanisms of tumor escape from immune surveillance, tissue infiltration, and metastasis are not yet explained. Under normal conditions, the immune system produces cells with anticancer potential. However, the response they induce is suppressed in the tumor microenvironment (TME). Recent studies indicate the involvement of the immune system in the development and spread of ovarian cancer [1–5]. In patients with OC, tumor infiltration by tumor-associated macrophages (TAMs), regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) is observed, which stimulate immunosuppression and angiogenesis through various mechanisms, thereby contributing to disease progression and metastasis [6]. Abnormal vasculature is a hallmark of most solid tumors. It facilitates escape from immune surveillance and

impairs perfusion and the entry of drugs from the circulation, limiting their anticancer effect [1, 5].

Th17 cells are a subpopulation of T helper cells ($CD4^+$) that show high expression of interleukin 17 (IL-17A). They were described for the first time in 2003 by Aggarwal et al. [7]. Th17 cells develop from naive T cells in the presence of IL-6 and TGF-β. Their differentiation is controlled by transcription factors such as retinoic acid-related orphan receptors (RORγt, RORα) and signal transducer and activator of transcription (STAT3) [8–10], whereas the presence of IL-1, IL-6, and IL-23 conditions their migration [11]. In addition to IL-17A, Th17 lymphocytes secrete IL-17F, IL-6, IL-21, IL-22, CCL20, and TNF-α [12–15]. Studies to date indicate a pleiotropic activity of IL-17 [16, 17]. On the one hand, IL-17 exhibits anticancer activity; on the other hand, it can promote progression of cancer [18, 19].

It has been proved that Th17 lymphocytes intensify migration of neutrophils to inflammatory foci [20] and are involved in the pathogenesis of autoimmune and allergic diseases. An increased percentage of these cells has been found in patients with lupus erythematosus, psoriasis, multiple sclerosis, rheumatoid arthritis (RA), and bronchial asthma [14]. Chang et al. showed a higher percentage of Th17 cells and cytokines associated with their differentiation (IL-6, TGF-β) in the peritoneal fluid (PF) of patients with endometriosis. Interestingly, the percentage of IL-10-producing Th17 cells was higher in advanced stages of the disease [21]. It should be emphasized that the role of Th17 cells in the pathogenesis of malignant tumors is not yet fully understood. However, their presence has been demonstrated in many types of cancer, especially in colorectal cancer, breast cancer, lymphoma, prostate cancer, gastric cancer, melanoma, and hepatocellular carcinoma [22]. Few studies have reported an increased percentage of Th17 lymphocytes in patients with ovarian cancer [18, 23]. A study by Kato et al. suggested that interleukin 17 secreted by Th17 cells may stimulate tumor progression through proangiogenic effects [24]. Further researchers confirmed this suggestion, showing an increased percentage of $CD4^+$/IL-17^+ lymphocytes among cells infiltrating ovarian cancer [25]. Kryczek et al. demonstrated the presence of Th17 cells in peripheral blood (PB), in PF, and among tumor-infiltrating cells in patients with advanced ovarian cancer [18]. The chemokines CCL20, CCL17, CCL22, RANTES, macrophage migration inhibitory factor (MIF), MCP1, and CCL4, secreted by cancer cells, fibroblasts, and dendritic and myeloid cells, were responsible for their recruitment to the tumor microenvironment. Th17 lymphocytes, which are detected in malignant tumors, are characterized by increased expression of CXCR4 and CCR6 receptors, respectively, for the chemokine (C-X-C motif) ligand-12; CXCL12 (SDF-1α) and chemokine (C-C motif) ligand 20 (CCL20) [26]. The SDF-1α chemokine participates closely with VEGF in the induction of angiogenesis. VEGF has been shown to induce CXCR4 expression for CXCL12 on endothelial cells, thereby increasing their sensitivity to the SDF-1α signal, and, synergistically with CXCL12, to induce migration of endothelial cells to TME. SDF-1α intensifies VEGF-induced endothelial cell proliferation and–synergistically with VEGF–protects them against apoptosis [27]. Furthermore, CXCL12 may enhance tumor vascularization also by regulating cellular composition in its microenvironment. SDF-1α has been shown to increase the migration of plasmacytoid dendritic cells (pDCs) into the tumor microenvironment [27, 28]. Curiel et al. demonstrated that pDCs present in the PF of patients with ovarian cancer produced high levels of proangiogenic cytokines (IL-8, TNF-α) and stimulated the development of new tumor blood vessels [29].

Other researchers have shown that Th17 lymphocytes may exhibit features of stem-like cells, whose activity may be partially controlled by signalling pathways induced by hypoxia-inducible factors, especially by HIF-1 [30].

The anticancer activity of Th17 lymphocytes is associated with their effect on other cells of the immune system [18]. Th17 cells have been shown to increase migration of cytotoxic lymphocytes (CTL), NK cells, macrophages, and neutrophils. In addition, they stimulate maturation of DCs, resulting in increased expression of surface molecules of the major histocompatibility complex (MHC). Recruited cells interact through different mechanisms with cancer cells, leading to their death. Activation of cytotoxic lymphocytes by DCs presenting tumor antigens induces an anticancer response and may lead to inhibition of tumor growth, but data are ambiguous [31].

2. Aim of the Study

In this study, we investigated the potential role of Th17 cells in ovarian cancer patients by analyzing the frequencies of Th17 cells in three different environments, i.e., peripheral blood, peritoneal fluid and tissue (Th17 infiltrating cells) and the concentration of IL-17A in plasma and PF of patients in terms of their clinical and prognostic significance.

3. Materials and Methods

3.1. Study Population. A total of 71 patients with proven OC diagnosis were qualified to the research. The examined clinical materials were the drawing peripheral blood, peritoneal fluid, and tumor tissue. They were collected from patients that were operated from 2012 to 2017 at The First Department of Oncological Gynecology and Gynecology in Independent Public Teaching Hospital No. 1 in Lublin (Poland). The patients did not receive antitumor treatment before and drugs that might have influence on immune system. The exclusion criteria were also infections, allergy, and autoimmunological diseases. Patient characteristics at the time of OC diagnosis are presented in Table 1.

Moreover, the researches were conducted in the reference group of patients ($n = 35$) at the age 22–78 (median 28 years) with benign cyst of ovary (serous cyst of ovary) and in the control group of healthy women ($n = 10$) at the age 21–51 (median 29 years). The PB of control group were obtained thanks to Regional Centre of Blood Donation and Blood Treatment in Lublin. Every patient signed the written acquiescence to participation in the study. The conducted

Table 1: The clinical characteristics of ovarian cancer patients.

The chosen clinical data	Ovarian cancer ($n = 71$)
Age (median), years (range)	57 (22–79)
FIGO stage, n (%)	
Early (I-II)	25 (35.21%)
I	14 (19.72%)
II	11 (15.49%)
Advanced (III-IV)	46 (64.79%)
III	43 (60.56%)
IV	3 (4.23%)
Degree of histological differentiation, n (%)	
Intermediate grade (G2)	35 (49.30%)
Low grade (G3)	36 (50.70%)
The OC classification according Kurman and Shih, n (%)	
Type I (serous G2, mucinous, endometrioid)	42 (59.15%)
Type II (serous G3)	26 (40.85%)
Ca125 range, median (U/ml)	435.30 (5.71–23935.36)

research received approval of Bioethics Committee at Medical University of Lublin (KE-0254/280/2015).

3.2. Cell Isolation. Peripheral blood samples were collected before the surgical procedure into heparinised tubes (Sarstedt, Germany) and immediately processed. Mononuclear cells (MNCs) were separated by density gradient centrifugation with Gradisol L (AquaMedica, Poland) for 20 minutes at 700 ×g at room temperature. Interphase cells were removed, washed twice, and resuspended in phosphate-buffered saline (PBS, PAA Laboratories GmbH, Austria).

Peritoneal fluid and tissue were collected aseptically during the operation. MNCs from PB/PF were isolated by density gradient centrifugation on Gradisol L (AquaMedica, Poland) for 20 minutes at 700 ×g at room temperature. For isolation of tumor-infiltrating MNCs, freshly resected tissue was minced, placed into a gentleMACS C tube, and processed using Tumor Dissociation Kit (MiltenyiBiotec). The resulting cell suspension was filtered through 70 mm meshfilted (BD Biosciences) and subjected to the density centrifugation as described above. Mononuclear cells were isolated within 2 h of draw and used for *in vitro* cell culture and flow cytometry analysis.

3.3. Intracellular IL-17A Staining. In vitro mononuclear cell cultures were used to analysis of Th17 cells subpopulation. Taking into account viability and MNCs concentration, intracellular IL-17A analysis was performed on fresh PB ($n = 59$), PF ($n = 35$) and tumor ($n = 17$) samples from ovarian cancer patients and on fresh PB and PF samples from the reference group ($n = 32$).

Mononuclear cells (2×10^6 cells/ml) were cultured in RPMI 1640 supplemented with 2 mmol/l L-glutamine, 5% human albumin (ZLB Bioplasma, Bern, Switzerland), 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were stimulated with 25 ng/ml of Phorbol 12-Myristate 13-Acetate (PMA) and 1 mg/ml of ionomycin (Sigma, Germany) in the presence of BD GolgiStop (BD Pharmingen, USA) for 4 hours at 37°C in a 5% CO_2 atmosphere. Cultured cells were washed twice in PBS, divided into tubes, and then stained with monoclonal antibodies (MoAb) against the cell-surface markers anty-CD3 FITC, anty-CD4 PerCP-Cy5.5 (BD Bioscience, USA). Following membrane staining, cells were fixed and permeabilized using IntraPrep Kit (Immunotech, France) according to the manufacturer's instructions. Cells were then intracellularly stained with anty-IL-17A PE (e-Bioscience, USA) or a PE mouse IgG1, κ isotype control (e-Bioscience, USA). Finally, cells were washed and the percentage of Th17 cells was analyzed by flow cytometry (FACSCanto I Becton Dickinson, USA). Th17 cells were analyzed as percentage of $CD4^+$ that expressed intracellular expression of IL-17A.

For each analysis, 100,000 events were acquired and analyzed using FacsDiva software. Isotype-matched MoAb were used to verify the staining specificity and as a guide for setting the markers to delineate positive and negative populations. Dot plots, illustrating the analysis method for the identification of $CD3^+$ $CD4^+$IL-17^+ T cells, are shown in Figure 1.

3.4. ELISA. Plasma/PF samples were rendered cell-free by centrifugation (1000 ×g/15 min) and the supernatants were stored at −80°C until the time of analysis. The concentrations of IL-17A in plasma and PF were determined by ELISA (enzyme linked immunosorbent assay) according to the manufacturer's instruction. Human IL-17A High sensitivity ELISA kit (Bender MedSystems, USA) and sensitivity of 0.01 pg/ml were used. Measurement was estimated through ELISA El × 800 reader (BIO-TEK Instruments, USA).

3.5. Statistical Analysis. The statistical analysis of results was conducted using Statistica 12.0 PL.

The Wilcoxon paired test was used to compare the results in PB, PF, and tissue. The Mann-Whitney U test was applied to the results of statistical comparison between the studied groups (Control/OC/Reference group). Relationships between two parameters were investigated using Spearman's rank correlation test. The probabilities of overall survival (OS) were estimated using the Kaplan–Meier method and differences in survival curves were calculated using the logrank test; p value less than 0.05 was considered statistically significant. The data are presented as medians, minimum, and maximum.

The dot plots show representative data from OC patients, illustrating the analysis method for identification of $CD3^+$/$CD4^+$/IL-$17A^+$ (Th17) cells. An acquisition gate was established based on FSC and SSC that included mononuclear cells. A population P1 was drawn around the lymphocytes. Next, the P1 gated events were analyzed for CD3 FITC and CD4 PerCP-Cy5.5 staining and positive cells ($CD3^+$/$CD4^+$) were gated (region Q2). The final dot plots CD4 PerCP-Cy5.5 versus mouse IgG1 PE and CD4 PerCP-Cy5.5 versus IL-17A PE were established by combined gating of events using population P1 and region Q2. The

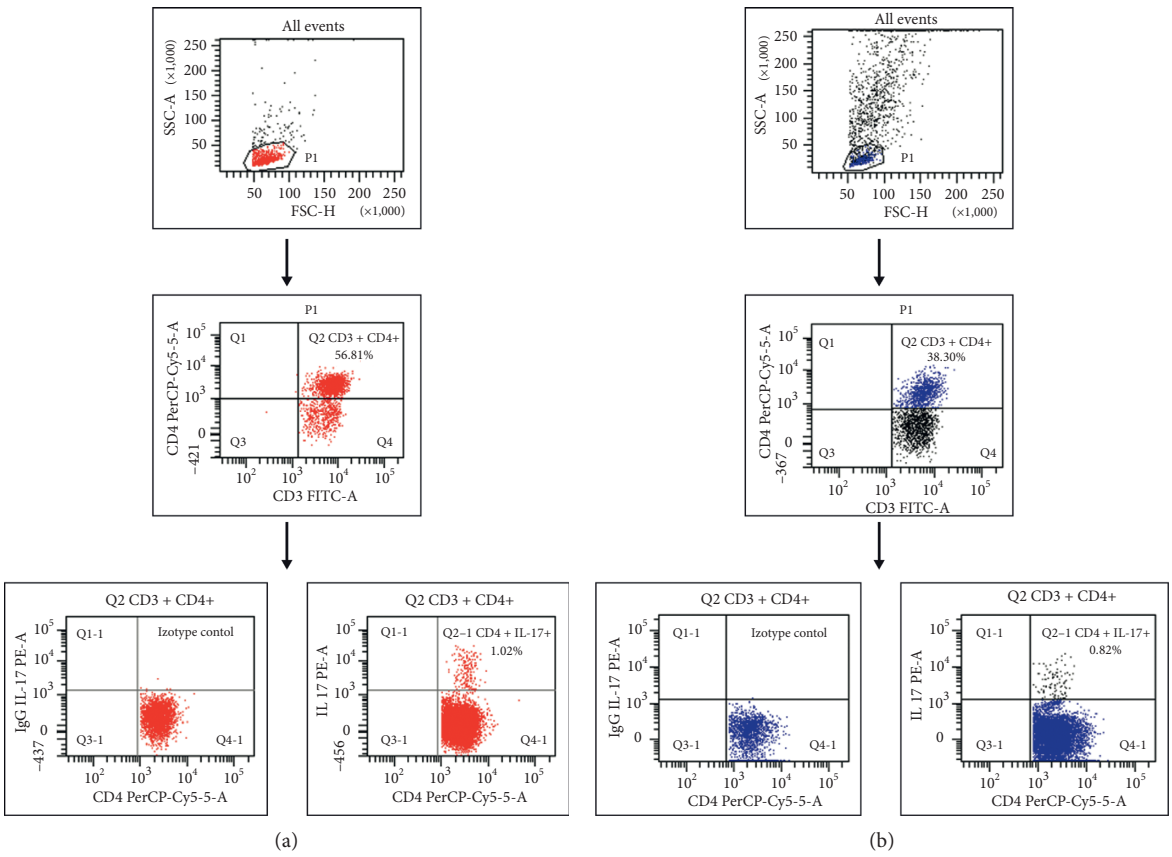

Figure 1: Flow cytometric analysis of Th17 cells in the peripheral blood (a) and tumor (b) of serous ovarian cancer patient.

number in the upper right quadrant in the region Q1-2 represents the percentage of $CD3^+/CD4^+/IL\text{-}17A^+$ (Th17) cells.

4. Results

Accumulation of Th17 cells among ovarian cancer infiltrating cells in OC patients.

The highest percentage of $CD4^+$ lymphocytes expressing IL-17 was detected among $CD4^+$ T cells infiltrating ovarian cancer and it was significantly higher ($p = 0.001$) compared to PB. The percentage of $CD4^+IL\text{-}17^+$ cells was higher in the tumor than that in the peritoneal fluid; however, the difference did not reach statistical significance ($p = 0.08$). There was no statistically significant difference ($p > 0.05$) in the percentage of $CD4^+IL\text{-}17^+$cells detected in peripheral blood and PF. The obtained results are presented in (Figure 2).

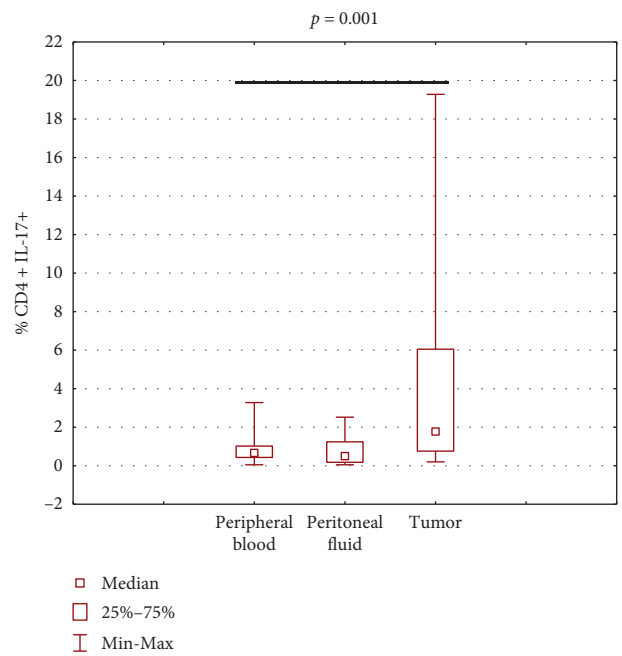

Figure 2: The percentage of Th17 cells in peripheral blood, in peritoneal fluid, and among ovarian cancer infiltrating cells.

4.1. Clinical Significance of Th17 Cells in the Peripheral Blood, Peritoneal Fluid, and Tumor Tissue of OC Patients. To investigate the clinical potential of $CD4^+IL\text{-}17^+$ cells, we determined its association with the patients' clinicopathological characteristics. There was no statistically significant difference ($p > 0.05$) in the percentage of $CD4^+IL\text{-}17^+$ cells in PB, PF, and tumor depending on the FIGO stage, histological grade, and type of OC according to Kurman and Shih classification. There was also no statistically significant difference ($p > 0.05$) in the percentage of $CD4^+IL\text{-}17^+$ cells depending on the menopausal status of patients.

Relationship between the percentage of Th17 cells in PB, PF, and tumor and concentration of IL-17A and clinicopathological characteristics of OC patients are shown.

There was no significant correlation ($p > 0.05$) between the percentage of Th17 cells in the PB and the plasma IL-17A level and between the percentage of Th17 cells and concentration of IL-17A in the PF of ovarian cancer patients.

There was no significant correlation ($p > 0.05$) between the percentage of Th17 cells and FIGO stage, histological grade, and type of OC according to Kurman and Shih classification, menopausal status, and plasma Ca125 level.

The percentage of Th17 cells in peripheral blood and peritoneal fluid of patients with ovarian cancer and in the group with benign ovarian tumor is shown.

In both peripheral blood and peritoneal fluid, the percentage of $CD4^+$ lymphocytes expressing IL-17 was significantly lower ($p < 0.0001$) in the ovarian cancer group than in the benign ovarian tumor group (Figure 3).

4.2. The Percentage of Th17 Cells in Peripheral Blood and Peritoneal Fluid of Patients with Ovarian Cancer or in the Group with Benign Ovarian Tumor. The percentage of Th17 cells in OC patients was lower in PF than that in PB; however, the difference did not reach statistical significance. The percentage of Th17 cells in patients with benign tumors was significantly higher ($p = 0.009$) in PF than that in PB (Figure 4).

4.3. The Analysis of Relationship between Th17 Cells Percentage and Survival of Ovarian Cancer Patients. In order to analyse relationship between Th17 cells and survival of ovarian cancer patients, the patients were classified depending on the examined factor values that were qualified as higher or lower than the median reached for the group of patients. The patients' survival was measured from the time of histopathological diagnosis to death or the end of observation time in living patients. In any of the examined microenvironments (PB, PF, or tumor), we have not demonstrated statistically significant relationships ($p > 0.05$) between the percentage of Th17 cells and five-year survival of ovarian cancer patients.

4.4. Accumulation of IL-17A in Plasma of Patients with Ovarian Cancer in Relation to the Group with Benign Ovarian Tumors and the Control Group. The highest concentration of IL-17A was detected in the plasma of ovarian cancer patients and it was significantly higher ($p < 0.0001$) compared to both the group of benign ovarian tumors and the control. The obtained results are presented in Table 2 and in (Figure 5).

There was no statistically significant difference ($p > 0.05$) in both plasma and PF IL-17A concentration depending on FIGO stage, histological grade, and type of OC according to Kurman and Shih classification. There was also no statistically significant difference ($p > 0.05$) in plasma IL-17A concentration depending on the menopausal status of the patients. What is interesting, IL-17A concentration in the PF of premenopausal patients was significantly higher ($p = 0.04$) than that in the postmenopausal group (Figure 6).

4.5. Relationship between the Concentration of IL-17A in Plasma and PF and Clinicopathological Characteristics of OC Patients. There was no significant correlation ($p > 0.05$) between the plasma level of IL-17A and FIGO stage, histological grade, and type of OC according to Kurman and Shih classification, menopausal status, and Ca125 level.

There was also no significant correlation ($p > 0.05$) between the PF level of IL-17A and FIGO stage, histological grade, and type of OC according to Kurman and Shih classification, and Ca125 level. What is interesting, in the PF, there was significant correlation (R Spearman −0.221, t $(N-2)$ −2.084, $p = 0.04$) between the level of IL-17A and menopausal status of patients.

4.6. Comparison of Plasma and Peritoneal Fluid IL-17A Levels in Patients with Ovarian Cancer or Benign Ovarian Tumors. The plasma IL-17A concentration was higher ($p = 0.05$) compared to that detected in the PF of patients with ovarian cancer (Figure 7). The plasma IL-17A concentration in patients with benign ovarian tumors was higher than that in PF; however, the difference did not reach the level of statistical significance ($p > 0.05$).

4.7. Assessment of IL-17A Concentration in the Peritoneal Fluid of Patients with Ovarian Cancer and in the Group with Benign Ovarian Tumors. The IL-17A concentration in the PF of ovarian cancer patients was significantly higher ($p < 0.0001$) compared to the group with benign ovarian tumors (Figure 8).

4.8. The Analysis of Relationship between Concentration of IL-17A in Plasma and PF and Survival of OC Patients. There was no statistically significant correlation between plasma IL-17A concentration and five-year survival of OC patients ($p > 0.05$). What is interesting, the survival of OC patients did not depend on IL-17A level in the peritoneal fluid and was significantly longer (the median: 36.5 months vs 27 months) in patients with higher IL-17A level than that in patients with lower IL-17A level in PF (Figure 9). The relationship between IL-17 concentration, clinical parameters, and five-year survival of OC patients is shown in Figure 10.

5. Discussion

In our study we evaluated the percentage and distribution of Th17 cells in ovarian cancer patients in three different environments, i.e., peripheral blood, peritoneal fluid, and tissue (Th17 infiltrating ovarian cancer), as well as the concentration of proinflammatory cytokine IL-17A in plasma and PF in terms of their clinical and prognostic significance.

It should be emphasized that the role of Th17 cells in ovarian cancer immunobiology is not clear. As a result of

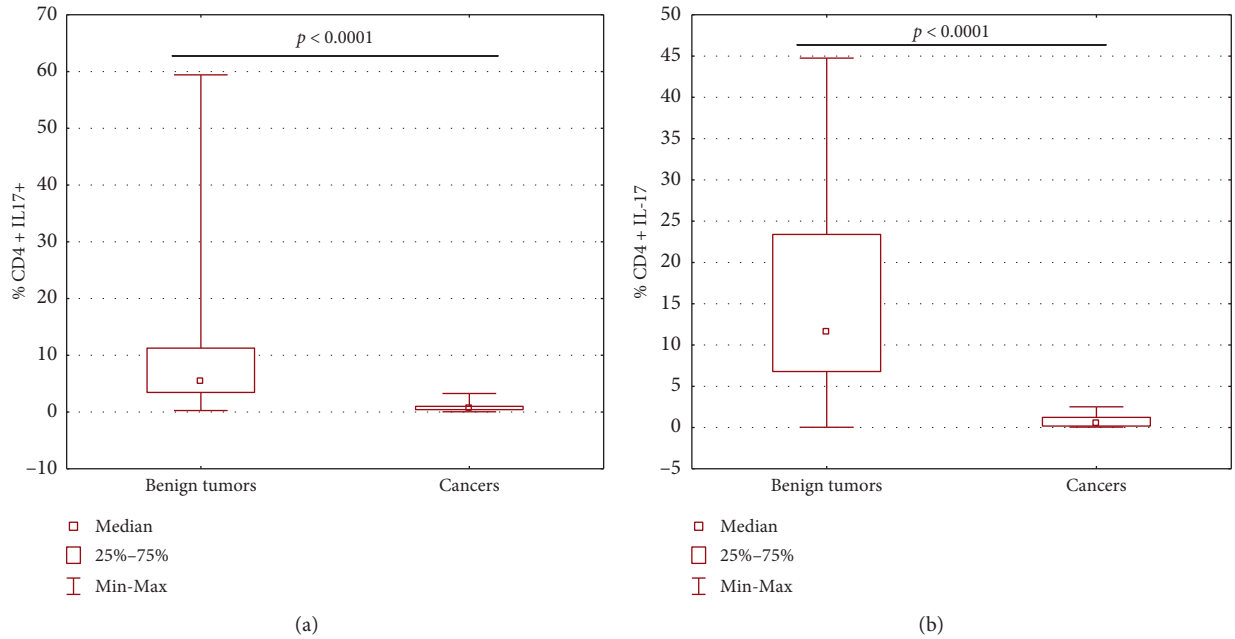

Figure 3: The percentage of Th17 cells in peripheral blood (a) and peritoneal fluid (b) of patients with ovarian cancer and in the group with benign ovarian tumor.

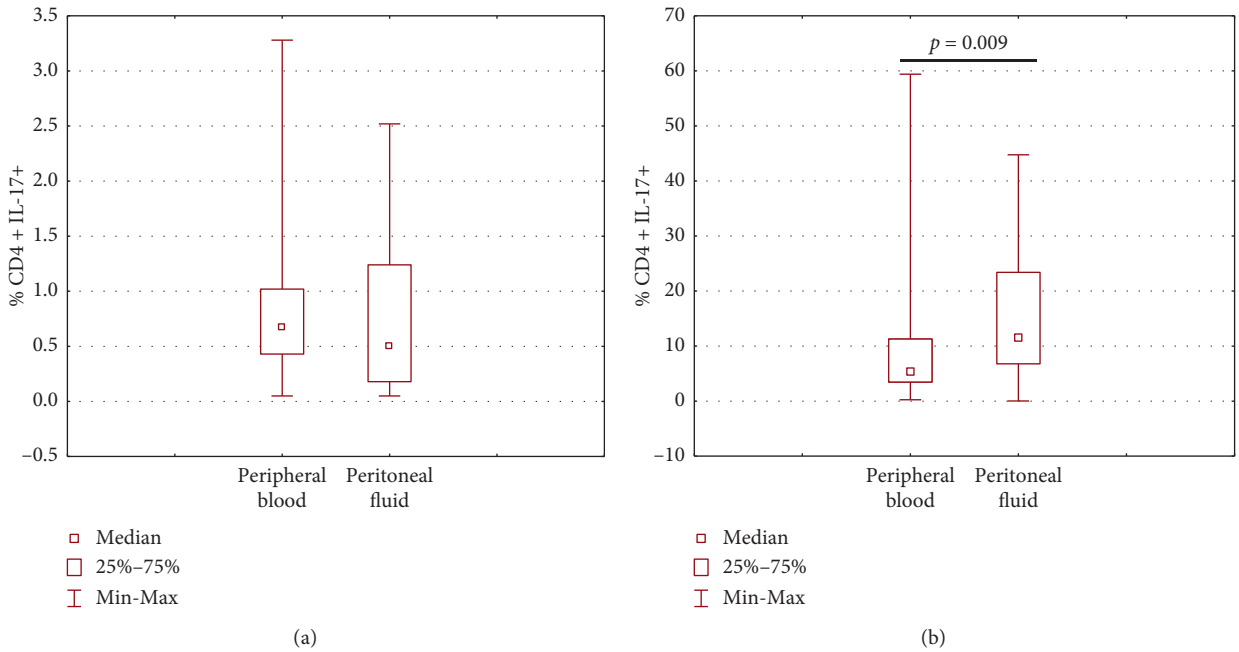

Figure 4: Comparison of Th17 cells percentage in PB and PF of ovarian cancer (a) and in the group with benign ovarian tumor (b).

contact with cancer cells, they can produce both pro- and anticancer factors. According to few reports, the population of Th17 has proangiogenic properties [24, 32]. However, the mechanisms of this activity in ovarian tumors are still not fully understood. In addition, it should be noted that these issues are not widely addressed in the available literature.

Our own research showed differences in the percentage and distribution of Th17 cells in peripheral blood, peritoneal fluid, and the tumor. The highest percentage of $CD4^+$ lymphocytes with intracellular expression of IL-17A was observed among $CD4^+$ cells infiltrating OC and it was significantly higher than that in peripheral blood. Similarly, Kryczek et al. demonstrated an accumulation of Th17 cells among cells infiltrating ovarian cancer, compared to PB and lymph nodes [18]. Other researchers also showed a higher concentration of Th17 subpopulations in tissue in patients

Table 2: Plasma IL-17A concentration in studied group of patients.

Group of patients	Plasma IL-17A concentration (pg/ml)		
	Median	Minimum	Maximum
Ovarian cancer ($n = 71$)	3.80*	0.042	30.00
Benign tumors ($n = 35$)	0.229	0.076	1.696
Control group ($n = 10$)	0.533	0.076	1.295

* $p < 0.0001$ compared to both the group of benign ovarian tumors and the control. Clinical significance of IL-17A in the plasma and peritoneal fluid of OC patients.

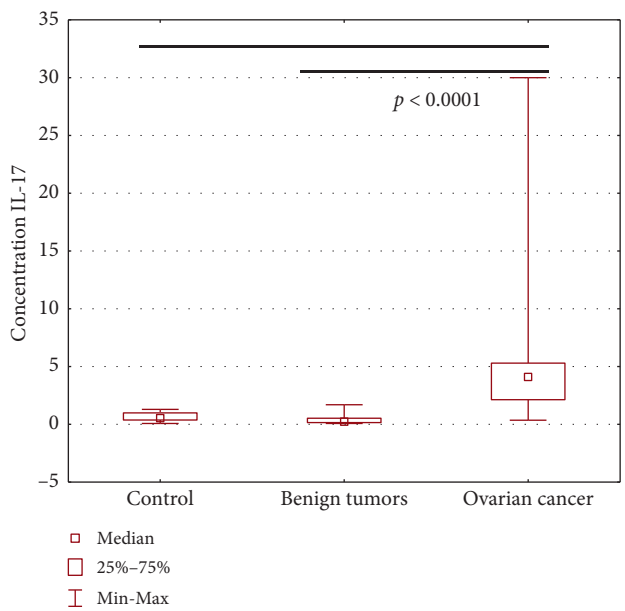

Figure 5: Plasma IL-17A concentration in patients with ovarian cancer, in the group with benign tumors and in the control group.

with OC, although the difference they found did not reach statistical significance [33]. The accumulation of Th17 cells in tumors observed in our research suggests the existence of factors increasing their migration and/or differentiation in the OC microenvironment. Kryczek et al. proved that Th17 cells infiltrating the tumor showed high expression of molecules such as CXCR4, CCR6, and CD161. They stated that these molecules may increase the migration of Th17 cells into the tumor microenvironment. Some *in vitro* studies also demonstrated the effect of other immune cell populations on inducing Th17 cell differentiation. Tumor-associated macrophages, which induced Th17 cells differentiation to the greatest extent, were particularly important in this respect. Further studies have shown that cytokines such as IL-1β and IL-23 are an important mediator of Th17 induction by TAMs in patients with ovarian cancer [18].

In our research, we showed a significantly lower percentage of Th17 cells in both PB and PF in ovarian cancer patients, compared to the group with benign ovarian tumor. The causes of the observed phenomenon are not fully understood. However, it suggests the existence of factors inhibiting the differentiation of Th17 cells in the examined female population. Other researchers have shown that Th17 cell differentiation in ovarian cancer patients can be inhibited by Tregs cell activity. It was observed in *in vitro* studies that Treg cells inhibited not only Th17 cell differentiation but also IL-17 synthesis by these cells. Kryczek et al. demonstrated that Treg cells isolated from OC express the CD39 molecule, an ectonucleotidase that converts ATP to adenosine, thereby participating in suppressing Th1 cell differentiation through the adenosinergic pathway [18]. Interestingly, Ye et al. noticed that Tregs and Th17 cells can transform each other and exhibit different functions in the tumor microenvironment. As a result of conversion into IFN-γ^+FoxP3$^+$ T lymphocytes, Th17 cells acquire strong immunosuppressive properties [34]. It is possible that this conversion is one of the mechanisms by which the cancer escapes from immune surveillance. IL-2 has been shown to regulate the balance between Tregs and Th17 cells in a tumor, reducing the percentage of Th17 cells in the TME and increasing the number of Tregs [35]. Other researchers have demonstrated that local administration of IL-2 increases the percentage of Th17 producing IFN-γ and TNF-α among tumor-infiltrating lymphocytes and induces the conversion of Tregs to Th17 [36].

Literature data suggest that Th17 cells induce synthesis of chemokines (CXCL9, CXCL10), which increase migration of effector cells that induce a Th1-type response in the OC microenvironment. It has been shown that tumor-infiltrating Th17 lymphocytes display low expression of activation markers and effector functions, such as HLA-DR, CD25, granzyme B, and perforin, and they do not mediate direct cytotoxic activity targeted against cancer cells. However, they recruit other immune system effector cells. According to this concept, IL-17 and IFN-γ secreted by Th17 synergistically induced synthesis of CXCL9 and CXCL10 chemokines, which in turn increased migration of effector T lymphocytes into the OC microenvironment. It was observed that the level of these chemokines correlated with the percentage of CD8$^+$ and NK cells infiltrating ovarian cancer [18]. In addition, the Th17 subpopulation stimulates cancer cells to secrete CCL20, a chemokine that enhances migration of dendritic cells to the TME [37]. In this respect, Th17 cells may participate in the induction of anticancer response in patients with OC. The reduced percentage of Th17 cells in patients with ovarian cancer observed in our own studies may be one of the strategies by which cancer escapes from the immune system.

Another evidence of the protective, anticancer role of Th17 cells is the results obtained by Hirahara et al., who observed inhibition of OC growth in a hamster after implantation of IL-17-gene-transfected tumor cells [38]. Other reports show that immunotherapy based on inhibition of the activity of the enzyme indoleamine 2,3-dioxygenase (IDO) increases the anticancer activity of Th17 cells [39].

Contradictory results were obtained by Zhu et al., who compared the concentration of Th17 cells infiltrating ovarian cancer and benign tumors by confocal microscopy. Their results indicate a significantly higher percentage of Th17 cells in malignant ovarian tumors, compared to benign lesions, which suggests the involvement of Th17 in cancer progression [40].

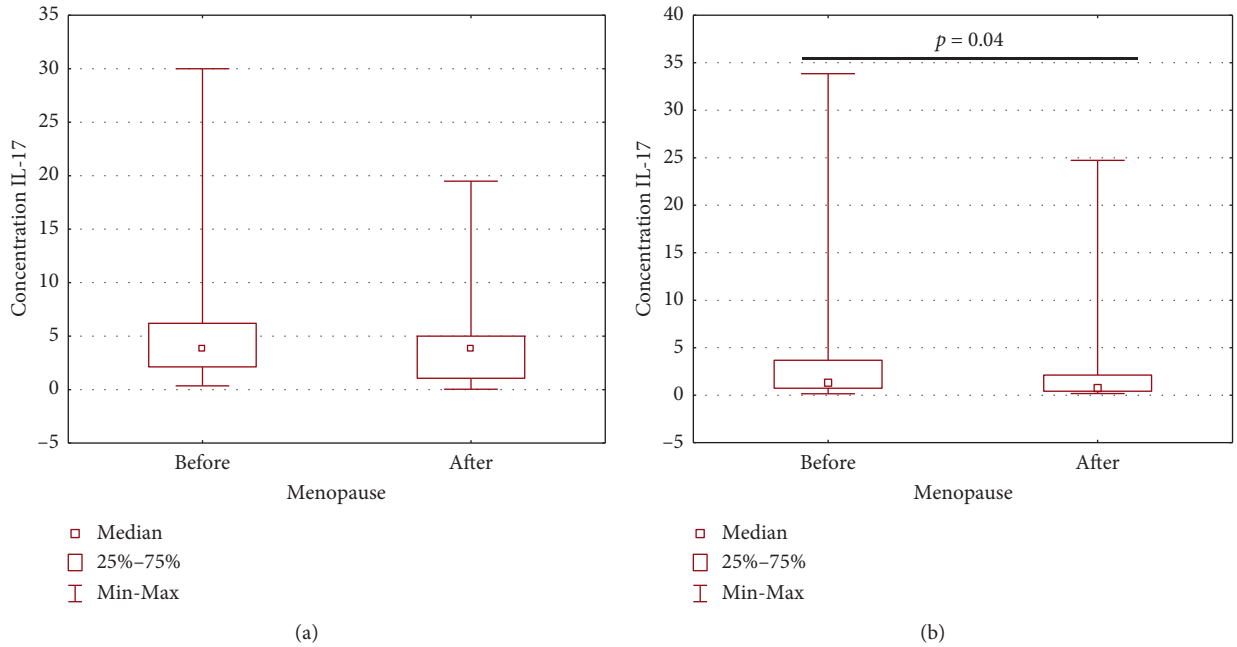

Figure 6: Plasma (a) and PF (b) IL-17A concentration in patients with ovarian cancer before and after menopause.

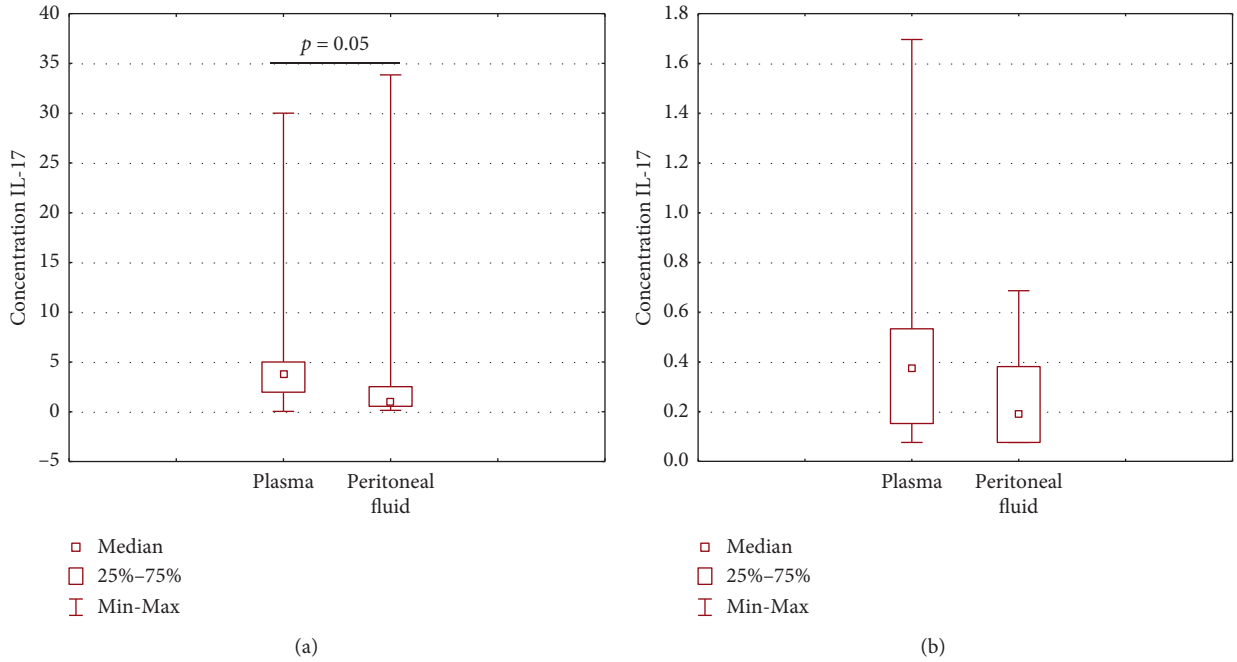

Figure 7: Comparison of plasma and PF IL-17A concentration in patients with ovarian cancer (a) or in the group with benign ovarian tumors (b).

Numerous studies emphasize the role of other subpopulations of tumor-infiltrating lymphocytes (TILs) in the induction of anticancer responses [41, 42]. They have been proved to be a prognostic factor in many types of cancer, including melanoma, colorectal cancer, and ovarian cancer [43–47]. Zhang et al. conducted a study on 186 patients with OC and showed that as many as 55% of women with $CD3^+$ TILs achieve a 5-year survival rate of 38%, while only 4.5% of women without TILs achieve a similar level [48]. The importance of TILs in cancer is also confirmed by a meta-analysis by Hwang et al. of 10 clinical studies involving 1815 patients with OC. As a result, it was established that the presence of both $CD3^+$ and $CD8^+$ TILs is associated with increased long-term survival. It has also been shown that a

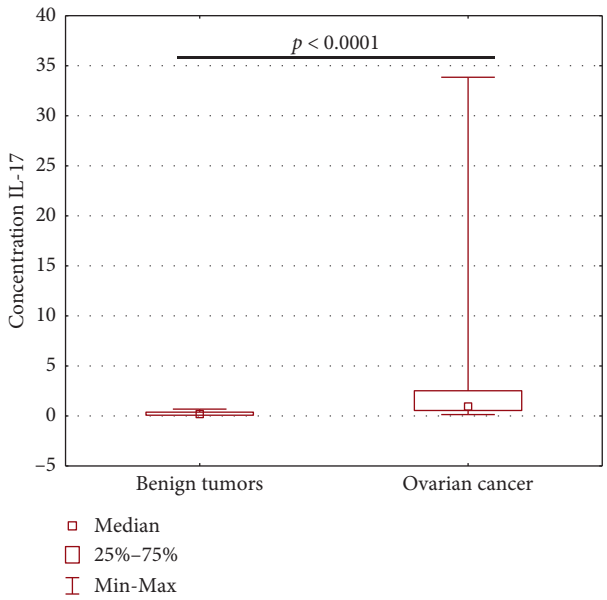

Figure 8: IL-17A concentration in the PF of patients with ovarian cancer and in the group with benign ovarian tumors.

high ratio of $CD8^+$ TILs to $FoxP3^+$ Treg cells in patients with OC is a positive prognostic factor [49]. Furthermore, a high percentage of $CD8^+$ TILs has been shown to be a prognostic factor for longer survival in patients with low-differentiated ovarian cancer [50].

There are few studies in the available literature assessing the relationship between the percentage of Th17 cells and the survival of ovarian cancer patients. In our research, we found no statistically significant relationship between the percentage of Th17 cells and the survival of patients with OC in any of the examined environments. Similarly, Winkler et al. showed no significant relationship between the percentage of $CD4^+IL\text{-}17^+$ cells either infiltrating the tumor or those detected in peripheral blood and the survival time of the patients [33]. It should be emphasized that the results of the assessment of the described relationship in cancer patients are divergent. 18 of the 27 studies analysed by Punta et al. showed that Th17 cells infiltrating tumors were a negative prognostic factor. Interestingly, five studies showed a positive correlation between Th17 cells and survival, including in cervical cancer and recurrent ovarian cancer. In the remaining four analysed reports, no significant correlations were found between the assessed cells and survival, including in epithelial ovarian cancer [19].

It was interesting to observe in our study that there was a significantly higher concentration of IL-17A in both the plasma and PF of patients with ovarian cancer, compared to the group with benign ovarian tumors. In contrast, Wang et al. did not observe any statistically significant differences in the concentration of IL-17 in the plasma of ovarian cancer patients, compared to the group with benign ovarian tumors and the group of healthy women [51]. The results we obtained are consistent with the results of Malekzadeh et al. who showed a higher concentration of IL-17A in the serum of patients with papillary adenocarcinoma, compared to the control group. They also observed a higher concentration of the assessed cytokine in the serum of patients with OC with a low degree of histological differentiation, compared to cancer with a high and medium degree of differentiation. According to them, this observation may suggest the existence of various sources of IL-17 (Th17 or cancer cells) involved in the production of this cytokine at particular stages of tumor progression [52]. Tang et al. showed a higher level of IL-17 expression in the tissue of ovarian malignant tumors, compared to benign tumor tissue and controls. The level of IL-17 expression assessed in the study correlated with the stage of the disease according to FIGO and the degree of histological differentiation of the tumor [53].

The higher concentration of IL-17A in the peritoneal fluid of OC patients noted in our study may contribute to local dissemination of cancer by affecting the formation of peritoneal implants. In addition, IL-17 may induce local IL-6 synthesis in the peritoneal environment, increase macrophage infiltration into the TME, and induce inflammatory processes and angiogenesis. Kato et al. showed a proangiogenic role of IL-17 in their study on an ovarian cancer mouse model. They found a significantly higher average number of blood vessels within ovarian tumors that were characterized by increased IL-17 mRNA expression [24]. Numasaki et al. noted that IL-17 directly affects endothelial cells, induces the production of proangiogenic cytokines and lymphokines, and enhances their activity. They confirmed the results on a SCID mouse model with implanted human non-small cell lung cancer (NSCLC). Using the RT-PCR technique, they showed that high expression of IL-17 in the tissue of primary NSCLS is associated with increased vascularization of the tumor, and thus with worse prognosis and shorter survival [14]. Other researchers report that IL-17 stimulates synthesis of proangiogenic chemokines, i.e., CXCL1, CXCL5, and CXCL8. IL-17 has been shown to promote tumor progression in colorectal cancer, lung cancer, breast cancer, stomach cancer, hepatocellular carcinoma, and pancreatic cancer [54–58]. It has been found that IL-17 mediates inflammation and may induce the production of other inflammatory factors, including TNF-α, IL-6, and IL-1β [59]. In turn, secreted IL-6 activates STAT3, which increases the level of expression of genes inducing tumor progression and metastasis [36]. Tartour et al. observed that transfection of human cervical cancer cells with IL-17 potentiated tumor growth after transplanting them into mice. Interestingly, mice lacking IL-17 exhibited slowed growth of melanoma (B16) and bladder cancer (MB49), which suggests the role of IL-17 in promoting tumor development [60]. IL-17 has been shown to promote VEGF secretion, enhancing tumor angiogenesis, cancer cell invasion, and metastasis [61]. Recent research indicates increased IL-17 expression in cervical cancer. According to the researchers, IL-17 may promote angiogenesis, proliferation, and invasion of cells in this tumor through the NF-κB signalling pathway [15].

Our own research showed significantly higher IL-17A concentration in the plasma of ovarian cancer patients, compared to that in peritoneal fluid. Similar results were obtained by Giuntoli et al., who showed a significant lower

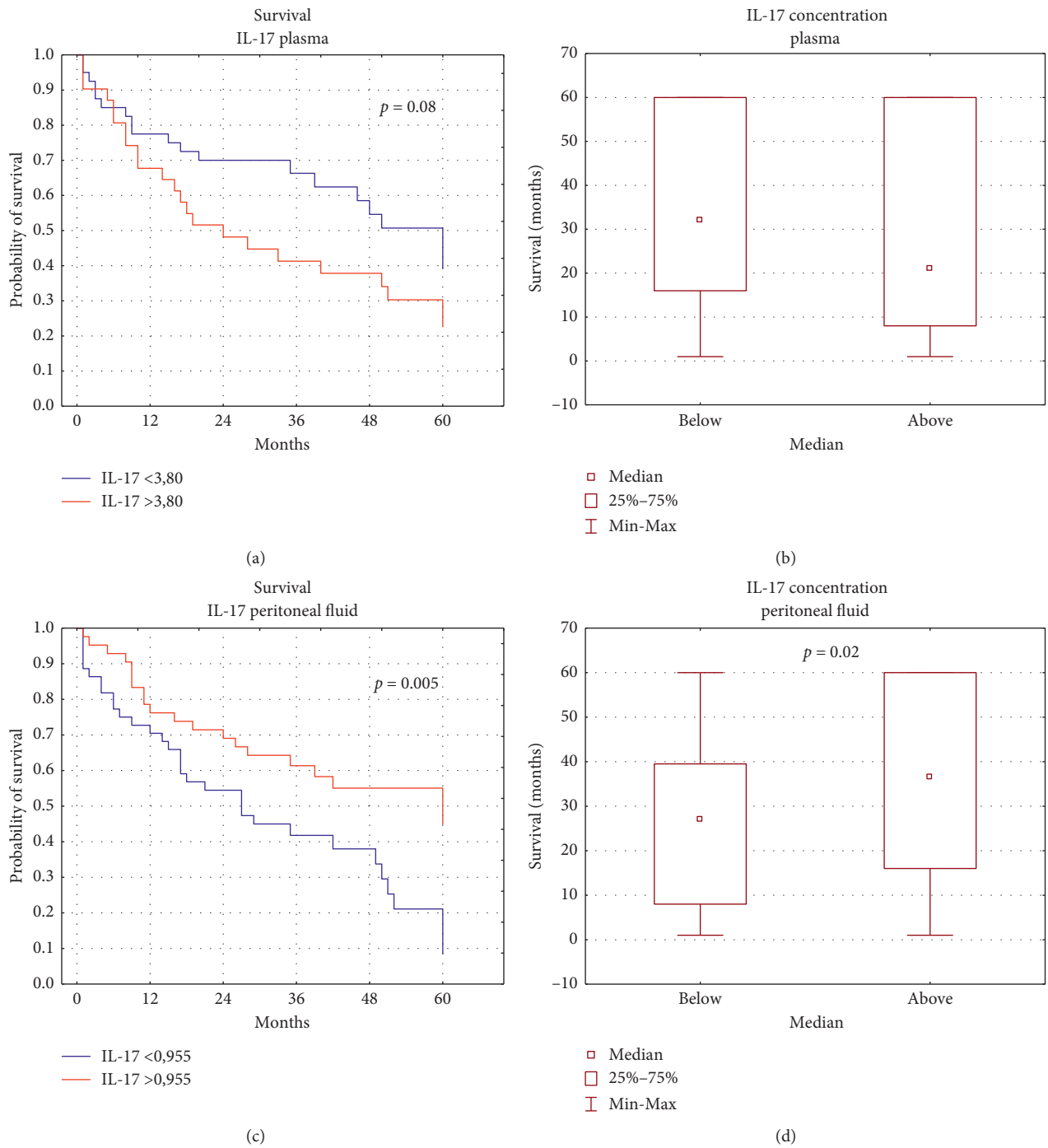

Figure 9: Relationship between the level of IL-17 in the plasma (a, b) and PF (c, d) and survival of OC patients.

concentration of IL-17 in PF, compared to the plasma of patients with advanced ovarian cancer [62]. According to the literature, the source of IL-17 is Th17 cells, as well as monocytes, neutrophils, NK cells, NKT-17, $\gamma\delta$-17, $CD8^+$ lymphocytes, and epithelial cells [63]. In our own research, we showed a significantly higher percentage of Th17 cells in the peripheral blood of patients with OC than that in PF. This observation indicates more intensified activity of the assessed cell population in the synthesis of IL-17 in the PB of OC patients.

In this context, interesting results were obtained by Kryczek et al., who showed a relationship between the concentration of IL-17 in peritoneal fluid and the survival time of OC patients. In patients whose PF contained high levels of IL-17, the average survival time was 78 months. In turn, patients with low levels of IL-17 in the PF lived shorter (27 months). It should be emphasized that this is the only report demonstrating a positive prognostic value of IL-17 in PF in ovarian cancer patients [18]. The results we obtained confirm the reports by Kryczek et al. We showed that the

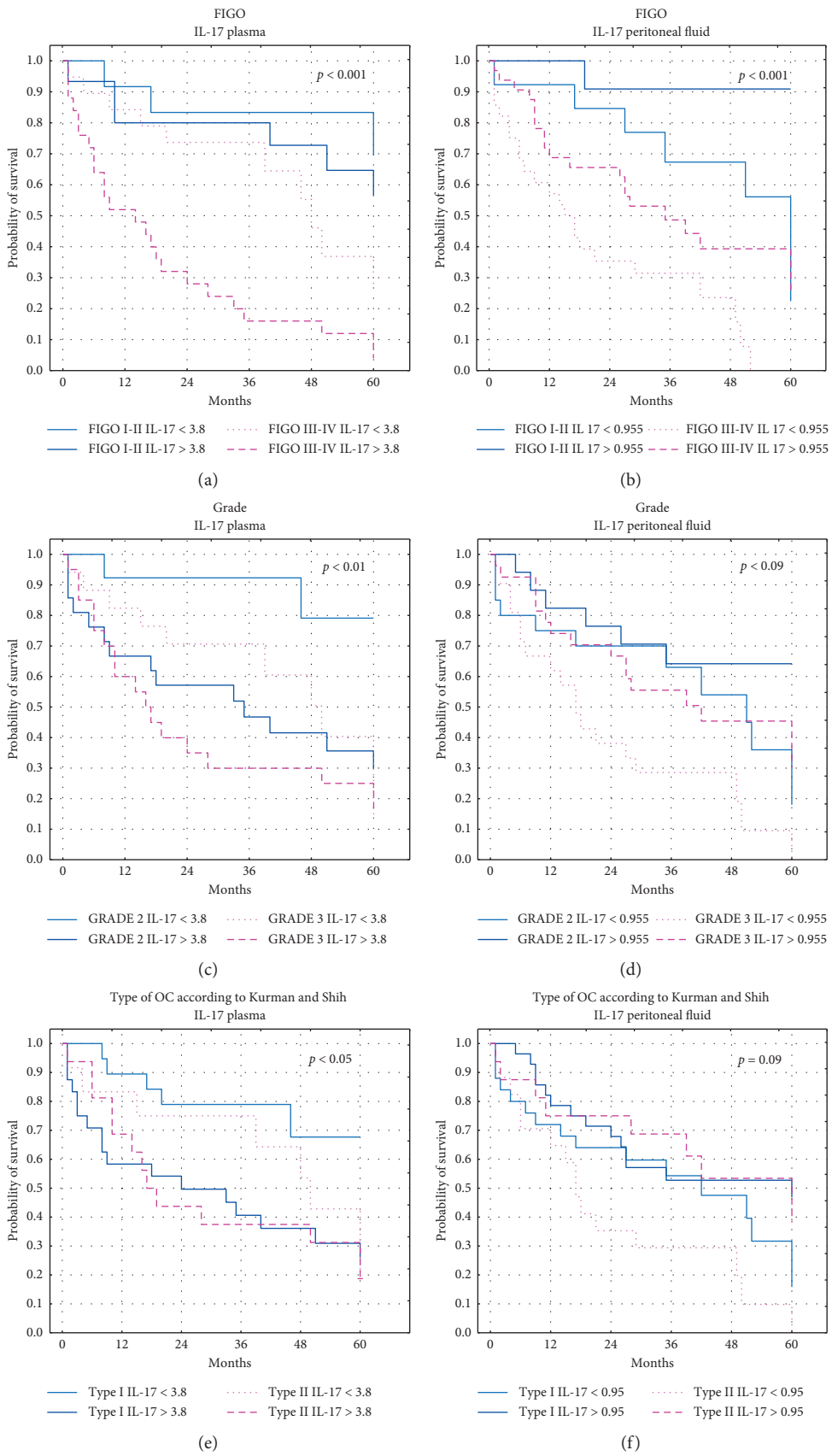

Figure 10: The relationship between IL-17 concentration, clinical parameters, and five-year survival of OC patients.

survival of patients with higher levels of IL-17A in peritoneal fluid was significantly longer than that in patients with lower concentration of IL-17A in PF (median: 36.5 months vs. 27 months). The results of the assessment of the relationship between IL-17 concentration in serum and the survival time of cancer patients are inconclusive. According to some studies, high levels of IL-17 correlated with poor prognosis, as demonstrated in patients with NSCLC, colon cancer, hepatocellular carcinoma, leukaemia, and gastric cancer. Other researchers did not observe any significant relationships or showed a positive relationship between IL-17 concentration and survival, as in the case of acute myeloid leukaemia [19]. Similar to our own study, Winkler et al. showed no significant correlations between IL-17A concentration in serum and survival time of ovarian cancer patients [33]. Using immunohistochemistry, Lan et al. showed a higher level of IL-17 expression in OC tissue, compared to controls. In addition, they showed a correlation between a high level of IL-17 expression and longer progression free survival (PFS) in patients with advanced ovarian cancer. However, no significant differences were found in the overall survival time of OC patients [64]. It is suggested that the reason for the observed discrepancies may be the microenvironment of individual tumors, especially the type of cell population responsible for IL-17A synthesis. Th17 cells may dominate in one environment, and neutrophils or mast cells in another [19].

Kryczek et al. showed a significantly higher concentration of IL-17A in the peritoneal fluid of patients with stage III OC according to FIGO, compared to the group with stage IV clinical cancer [18]. We did not observe such differences in our own research. The concentration of IL-17 we found in both plasma and PF did not differ significantly depending on the clinical stage of the disease according to FIGO, the degree of histological differentiation, and the type of cancer according to Kurman and Shih.

However, what is interesting is the significant differences we found in IL-17A concentration in the PF of premenopausal patients with OC, compared to postmenopausal patients. The concentration of IL-17A in the PF of premenopausal OC patients was significantly higher than that in postmenopausal patients. It is known that ovarian cancer mostly affects patients over 60 years of age. Malutan et al. showed a significantly higher concentration of selected proinflammatory cytokines (IL-1β, IL-8, and TNF-α) in women after natural and surgical menopause without cancer, compared to the control group [65]. They also evaluated the concentration of IL-17 in women depending on menopausal status. Contrary to our observations, no statistically significant differences in IL-17 levels in pre- and postmenopausal women were found in this study. Currently, research is conducted on female sex hormones and their effect on IL-17 concentration in systemic diseases. Perhaps it will help us understand the role of this cytokine in the pathophysiology of cancer. Interleukin 17 induces inflammation and contributes to joint damage in rheumatoid arthritis. The female sex hormone 17β-estradiol (E2) inhibits experimentally induced arthritis. As the main source of IL-17 is $\gamma\delta$T cells, the purpose of this study was to investigate whether E2 affects IL-17$^+$ $\gamma\delta$ cells during the development of arthritis in various experimental models. The researchers showed that treatment with E2 reduces the concentration of IL-17$^+\gamma\delta$T cells in joints, but increases the concentration of IL-17$^+\gamma\delta$T cells in draining lymph nodes, suggesting that E2 may prevent migration of IL-17$^+\gamma\delta$T cells from lymph nodes into joints [66].

In our studies, we did not find a statistically significant difference in the percentage of CD4$^+$IL-17$^+$ cells depending on the menopausal status of the patients. The first studies have been published on the effect of changes in sex hormone levels on the percentage of Th17 in autoimmune diseases. Andersson et al. observed the effect of estradiol on the percentage of Th17 in rheumatoid arthritis. Mice transfected with 17β-estradiol (E2) showed a reduction in the severity of arthritis and a lower percentage of Th17 in the joints, compared to the control group. Interestingly, mice treated with E2 showed a higher percentage of Th17 in the lymph nodes in the early stages of the disease, depending on ERα. E2 was found to increase the expression of C-C chemokine receptor 6 (CCR6) and the corresponding C-C20 ligand (CCL20) on Th17 cells in lymph nodes. These data suggest that this may be related to the CCR6-CCL20 pathway, which is important for Th17 cell migration [66]. Recent studies have shown a significantly higher concentration of IL-17 in the serum of women with a pregnancy complicated by fetal growth restriction (FGR) and preeclampsia, compared to healthy pregnant women. In conjunction with a reduced TGF-β level detected in pregnancy complicated by FGR associated with preeclampsia (PE), it may induce an inflammatory response and, consequently, lead to placental insufficiency [67]. Perhaps the conducted research not only will allow us to understand the role of hormonal balance in the development of autoimmune diseases, but will also open up new opportunities for research on the impact of female sex hormones on the immune system in ovarian cancer [66].

The available literature does not include any studies assessing the occurrence of Th17 cells and IL-17 cells in patients with types I and II ovarian cancer according to Kurman and Shih. According to the latest data, the effects of chemotherapy in patients with type II OC are worse than those in patients with type I ovarian cancer. It is estimated that about 90% of patients with type I OC survive five years, while about 90% of patients with type II cancer die within five years of diagnosis [68]. Although the molecular basis of the OC types according to Kurman and Shih seems to be well characterized [68, 69], little is known about how the immune system works in particular types of cancer. The conducted studies did not show any statistically significant differences in the percentage of Th17 cells and IL-17 levels in the examined environments depending on the type of cancer according to Kurman and Shih. Given the significant relationship between OC type and survival described in the literature, research on a larger number of patients with a particular type of cancer according to Kurman and Shih seems advisable.

6. Conclusions

To sum up, the results of our own research and the data quoted from the literature indicate a proinflammatory nature of the ovarian cancer microenvironment (high level of IL-17A in PF and a high percentage of Th17 infiltrating OC). These results suggest that Th17 cells/IL-17A may play a beneficial role in OC immunity. What is interesting, higher IL-17A level in the PF correlated with markedly more favorable clinical outcomes of OC patients.

On the other hand, the percentage of Th17 cells was lower in the PB and PF of ovarian cancer patients in comparison to the group with benign ovarian tumor. It may promote evasion of host immune response by cancer cells. The direct mechanism of Th17 action in OC has not been yet studied and remains unexplained. It seems justified to continue the preliminary research to thoroughly understand the mechanisms that play a key role in the process of infiltration of neighboring OC tissues not only in the context of their significance in the biology of OC, but also in the novel target for cellular immunotherapy.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' Contributions

I. Wertel conceived and designed the experiments. E. Zakrzewska, M. Bilska, M. Gogacz, and D. Suszczyk were involved in acquisition of data (acquired and managed patients, provided facilities, performed the experiments, etc.). A. Chudzik, A. Pawłowska, and I. Wertel were involved in analysis and interpretation of data (e.g., statistical analysis, biostatistics, and computational analysis). M. Bilska, A. Pawłowska, I. Wertel, and A. Chudzik were responsible for writing, reviewing, and/or revision of the manuscript. E. Zakrzewska and D. Suszczyk were responsible for administrative, technical, or material support (i.e., reagents/materials/analysis tools, constructing databases). M. Gogacz and I. Wertel revised the article critically.

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Review Article

AXL as a Target in Breast Cancer Therapy

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AXL is a receptor tyrosine kinase (RTK) that has been implicated in diverse tumor-promoting processes such as proliferation, migration, invasion, survival, and apoptosis. AXL therefore plays a role in cancer progression, and AXL has been implicated in a wide variety of malignancies from solid tumors to hematopoietic cancers where it is often associated with poor prognosis. In cancer, AXL has been shown to promote epithelial to mesenchymal transition (EMT), metastasis formation, drug resistance, and a role for AXL in modulation of the tumor microenvironment and immune response has been identified. In light of these activities multiple AXL inhibitors have been developed, and several of these have entered clinical trials in the U.S. In breast cancer, high levels of AXL expression have been observed. The role of AXL in cancer with a focus on therapeutic implications for breast cancer is discussed.

1. Introduction

AXL is an RTK that is part of the TAM (TYRO3, AXL, and MER) family of RTKs. AXL was originally identified in 1988 during a screen for genes involved in the progression of chronic myelogenous leukemia (CML) to blast crisis [1]. Approximately three years after AXL's initial discovery, two groups independently cloned and identified AXL as an RTK with transforming potential [2, 3]. While AXL was found to be necessary for transformation, it alone was not sufficient [3]. Initially, the intracellular role of AXL remained a mystery, as evidenced by one of these groups giving AXL the name "UFO" in reference to its unknown function [2]. Since then, AXL has been shown to be involved in a variety of cellular processes, including cell growth, proliferation, survival, apoptosis, and adhesion. Given these functions of AXL, it is not surprising that AXL plays a role in cancer progression, and indeed AXL has been implicated in a wide variety of malignancies from solid to liquid tumors. In breast cancer AXL expression has been observed in all of the main transcriptional subtypes, and AXL expression in primary breast tumors is strongly predictive of reduced patient survival and poor outcome [4, 5].

2. AXL Signaling Axis

Structurally, AXL, like the other TAM family members, consists of two immunoglobulin- (Ig-) like domains and two fibronectin III domains which comprise the extracellular portion of the receptor (Figure 1) [6]. It is through the fibronectin domains that AXL is thought to exert its effects on adhesion, which relates to such cellular processes as EMT, whereby polarized epithelial cells undergo a shift to a more mesenchymal-like state. The intracellular portion of AXL consists of a receptor tyrosine kinase domain containing a KWIAIES motif that is conserved among the TAM family members, though in TYRO3 the isoleucines are substituted with leucine residues [3, 7].

Canonical AXL activation involves binding of the ligand growth arrest-specific 6 (GAS6) to the Ig-like domains on AXL, resulting in receptor dimerization. GAS6 is able to bind the other TAM family receptors, but it shows a much higher affinity for AXL [8]. Up until recently protein S was thought to exclusively be a ligand for TYRO3 and MER, but recently it has been shown to be capable of binding to and activating AXL in glioblastoma cells [9]. Activation of AXL is not complete until a further interaction with the phospholipid phosphatidyl

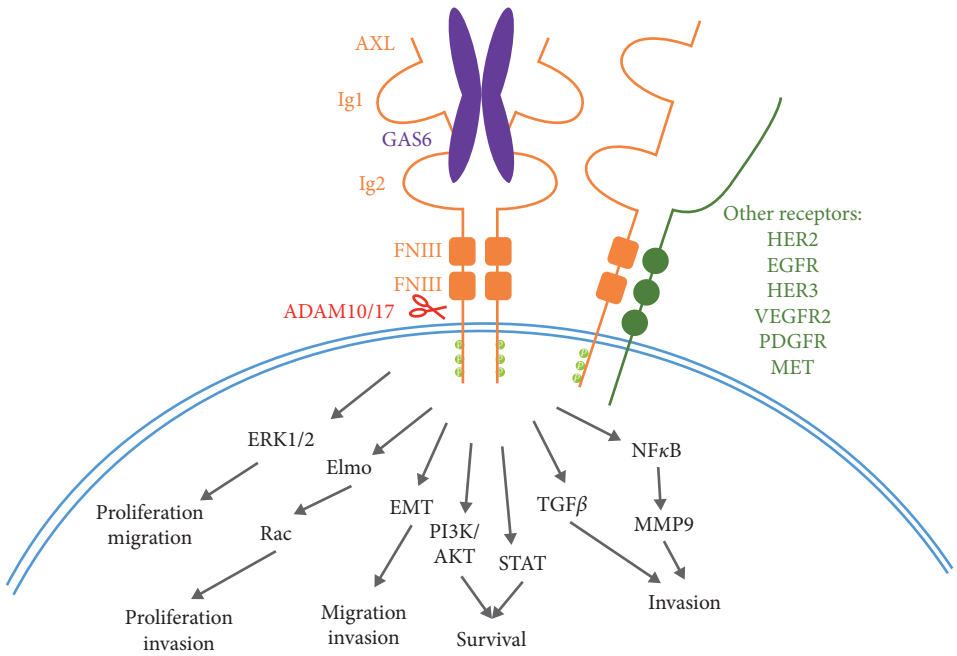

Figure 1: In breast cancer, AXL (orange) can be activated through either binding of GAS6 (purple) or through interaction with other receptors (green) to activate a variety of downstream signaling pathways (gray). Cleavage by ADAM10/17 (red) can result in release of the extracellular domain which retains ligand-binding abilities.

serine (PS) occurs, mediated by the gamma-carboxyglutamic acid (Gla) domain on GAS6 following its posttranslational modification [10]. PS is a phospholipid that is normally restricted to the intracellular portion of the phospholipid bilayer but is externalized in apoptotic cells or cells that are otherwise stressed, such as in virally infected cells. The tumor microenvironment also contains a high level of externalized PS due to the increased apoptotic index of tumors, metabolically stressed tumor cells, vasculature within the tumor, and tumor-derived exosomes [11].

Activation of AXL results in autophosphorylation on tyrosine residues in the cytoplasmic domain of the receptor and subsequent phosphorylation and activation of adaptor signaling proteins resulting in a signaling cascade and phosphorylation of downstream targets. The phosphorylation sites on AXL and activation of downstream pathways are highly context-dependent. Multiple tyrosine phosphorylation sites have been identified in the intracellular domain of AXL: Y698, Y702, Y703, Y779, Y821, and Y866, and three of these have been shown to be phosphorylated in breast cancer or breast cancer cell lines: Y698, Y702, and Y703 [12–15]. These three tyrosines represent sites of autophosphorylation and thus activation of AXL, with the remaining residues thought to be involved in signaling and docking of adaptor proteins.

3. Alternative Methods of Activation

Ligand-independent activation of AXL has also been observed. In MCF-7 cells for instance, activation of AXL independently of GAS6 binding stimulated nuclear factor-kappa B (NF-κB) mediated activation of MMP-9, although the exact mechanism by which AXL was activated in this case was not determined [16]. Similarly, in breast cancer stem cells GAS6 did not have an effect on AXL activation status, and while AXL was expressed at high levels in these cells GAS6 transcripts were only found at low levels, indicating that AXL activation in breast cancer stem cells is likely GAS6-independent [17].

AXL can be activated by interaction with other receptors or membrane-bound molecules, including by itself. Ectopic overexpression of AXL can result in receptor homodimerization and activation in cell lines such as NIH 3T3 cells; however, it is not known if this occurs in an *in vivo* setting [18].

AXL has been shown to interact with EGFR (epidermal growth factor receptor) and other EGFR family members. In triple-negative breast cancer (TNBC) lines AXL can be activated by EGFR [19]. This activation by EGFR expands the downstream signaling pathways beyond those seen when EGFR is activated alone. AXL can also promote the translocation of EGFR to the nucleus [20]. Meyer et al. also observed an interaction between AXL and other EGFR family members, namely HER2 and HER3 in breast cancer cells, as well as an interaction between AXL and MET (hepatocyte growth factor receptor) and AXL and PDGFR (platelet-derived growth factor receptor). Interactions between AXL and these other RTKs lead to enhanced downstream signaling. An interaction between AXL and EGFR has also been observed in glioblastoma cell lines where EGF can activate AXL, and the interaction between EGFR and AXL can lead to MMP9 activation to promote invasion [21].

The effect of the interaction between AXL and HER2 in breast cancer has recently been clarified. AXL can interact with HER2, both when HER2 is ectopically overexpressed in

MCF10a cells and endogenously in the HER2-amplified SKBR3 cell line [4]. This interaction leads to increased levels of AXL localized to the cell membrane and HER2 trans-phosphorylation of AXL, although AXL is not able to phosphorylate HER2. This interaction enhanced invasiveness and other functions important for metastasis formation. Importantly, this interaction between AXL and HER2 was also observed in $HER2^+$ patient tumors [4].

Additionally, reverse-phase protein array analyses in breast tumors revealed evidence of cross-talk between AXL and MET, along with potential physical interaction between these two receptors [22]. In neuronal cells, AXL exhibits cross-talk with MET, whereby regulation of neuronal survival by AXL is reliant on MET, not GAS6 [23].

Other means of ligand-independent activation of AXL have also been reported in other cell types through proteins such as vascular endothelial growth factor receptor-2 (VEGFR-2) and C1-TEN, although these have not been analyzed in breast cancer cells [24, 25]. Activation of AXL through interaction with other RTKs suggests that combination therapies that target both AXL and its interacting partners may be necessary in the clinical setting.

4. Post-Transcriptional and Post-Translational Modifications

Three alternative splicing variants have been identified for AXL, two of which encode 4.7 kb long mRNAs which only differ by 27 basepairs which are internal in the mRNA. Therefore, both of these isoforms contain intact extracellular ligand-binding domains and intracellular kinase domains, and both can effect cellular transformation [3]. The third isoform is the shortest, owing to exclusion of five alternative exons in the $5'$ coding region of the mRNA. It is not clear if there are specific differences in the function of these isoforms in cancer.

Post-translational modification of AXL can also alter AXL's role in the cell. The phosphorylation of AXL which occurs following its activation targets AXL for ubiquitinated-mediated degradation [26, 27]. An alternative method of degradation is proteolytic cleavage by matrix metalloproteinases and A Disintegrin and Metalloproteinase Domain (ADAM) family members in the extracellular domain, which releases a soluble 85 kDa N-terminal fragment of AXL (sAXL) [28]. The extracellular domain contains intact ligand-binding capabilities, and therefore sAXL can inhibit ligand-mediated AXL signaling [29]. sAXL levels are elevated in some cancer types, including hepatocellular carcinoma, and sAXL is being investigated as a biomarker in certain cancers and other inflammatory diseases [30–32].

AXL is post-translationally modified by glycosylation in the golgi. The extracellular domain of AXL contains six N-linked glycosylation sites. Fully glycosylated AXL is 140 kDa, with partial glycosylation yielding a protein of approximately 120 kDa. Inhibition of N-acetylglucosamine (GlcNAc) transferase results in accumulation of a 100 kDa unglycosylated form of AXL [33]. It is thought that glycosylation is important for AXL's function, as inhibition of glycosylation results in a decrease in cell proliferation and invasion in hepatocellular carcinoma cell lines and in metastasis formation *in vivo* [33].

5. Downstream Signaling Targets and Effectors in Breast Cancer

Like that of most RTKs AXL can signal through a variety of downstream effectors leading to influences on a variety of intracellular processes that are highly context-dependent. In breast cancer, AXL can stimulate a variety of downstream pathways, including phosphatidylinositide 3-kinase/RAC-α serine/threonine protein kinase (PI3K/AKT), extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), and the NF-κB pathway, as well as signal transducer and activator of transcription signaling (STAT) pathways [34]. Activation of these pathways in breast cancer can stimulate a variety of tumor-promoting abilities including breast cancer cell motility, invasion, proliferation, survival, angiogenesis, and other processes (Figure 1).

5.1. GAS6 Independent Pathways. In $AXL^{-/-}/HER2^+$ mouse tumor models, a decrease in EGF, Rho-GTPase, and TGF-β (transforming growth factor β) signaling is observed compared to in AXL-competent cells, indicating that AXL promotes signaling through these pathways [4]. These downstream effectors affect cellular processes such as extracellular matrix organization, cell migration, cytoskeletal organization, and EMT [4]. Stimulation of AXL and these subsequent downstream pathways appears to be ligand-independent in $HER2^+$ breast cancer [4].

In MCF-7 cell lines as well as in inflammatory breast cancer, AXL can promote cell invasion by inducing MMP-9 through NF-κB independently of GAS6 binding [16, 35]. AXL activation via interaction with EGFR amplifies signaling through the AKT pathway and elaborates on downstream EGFR signaling to include downstream pathways such as GSK3 (glycogen synthase kinase 3), ERK, and P38 in TNBC cells [19]. These pathways affect cellular processes such as proliferation and migration.

Reverse-phase protein array analysis in breast tumors revealed evidence of cross-talk between AXL and the RTK MET, along with potential physical interaction between these two receptors [22]. Treatment of MDA-MB-231 cells with the MET ligand HGF resulted in phosphorylation of AXL, and knockdown of MET reduced AXL mRNA levels [22]. Although the two receptors could coimmunoprecipitate this is not entirely a ligand-independent interaction, as MET was observed to be required for signal transduction by GAS6 [22]. The downstream effectors of this interaction include AKT and ERK and are thought to be important for apoptosis and migratory ability in MDA-MB-231 cells.

5.2. Ligand-Mediated Downstream Signaling Pathways. Several different downstream pathways can be activated through ligand-dependent activation of AXL. A reverse-phase protein analysis of human breast tumors indicated that GAS6 can promote phosphorylation of several

downstream targets including AKT, CREB, GSK3α/β, ERK1, c-Jun, MEK1, S6, Stat3, as well as NFκB [22]. Similarly, stimulation of MDA-MB-231 cells with GAS6 resulted in activation of various downstream signaling cascades including PI3K-AKT, MAP kinase, NF-κB, and JAK-STAT pathways [36].

Immunoprecipitation of AXL from lapatinib-resistant HER2$^+$ cells contained a subunit of PI3K, indicating that AXL and PI3K may have a functional interaction [37]. Additionally, knockdown of AXL in MDA-MB-231 cells leads to a decrease in proangiogenic factors released into the media, including VEGF, thrombospondin-1, endothelin-1, and uPA [38]. Therefore, AXL could also mediate effects on the recruitment of blood vessels to the solid breast tumor.

Another means by which AXL can promote invasion is through GAS6-mediated phosphorylation of the Elmo scaffold proteins by AXL to ultimately activate Rac GTPase, which also promotes breast cancer cell proliferation [39]. AXL activation through binding to macrophage-produced GAS6 can lead to downstream activation of AKT and STAT3, promoting survival in a p53 $^{-/-}$ model of early-stage mammary tumors [40].

6. Regulation of AXL Expression in Breast Cancer

Despite the elevated levels of AXL seen in breast malignancies, less than 2.5% of breast cancers exhibit any alteration in *AXL* (mutation, rearrangement, etc.), and amplifications specifically are also exceedingly rare (less than 2%) [41–43]. Therefore, it is likely that the contribution of AXL to transformation is likely due to overexpression of the wild-type receptor, which can be mediated through various mechanisms.

Several transcription factors have been found to directly regulate AXL expression in breast cancer. A ChIP-seq analysis in MDA-MB-231 cells identified AXL as a target of the Hippo transducers YAP/TAZ [44]. This confirmed earlier reports in other cancer types that AXL is regulated by YAP/TAZ [45, 46]. Vimentin and other EMT transcription factors can also regulate AXL, including Twist, Snail, and Slug [5, 47].

Other transcription factors have been implicated in the expression of AXL in other cancer types, but these have not been directly studied in breast cancer. The transcription factor HIFα can promote AXL in renal clear cell carcinoma, and AP1 can promote AXL expression in leukemia [48, 49]. The AP1 transcription factors c-JUN and c-FOS can mediate AXL overexpression in head and neck and esophageal cancers [50]. Exogenous expression of the Sp1 and Sp3 transcription factors can increase AXL transcripts in several cancer cell lines, although this has not been examined in breast cancer [51]. Additionally, MZF1 binds to the AXL promoter in cervical and colorectal cancer cell lines and enhances transcription of AXL [52].

In addition to regulation by transcription factors, several other players have been shown to affect AXL transcript levels in breast cancer cells. Knockdown of the MET RTK results in a reduction of AXL mRNA levels in MDA-MB-231 cells [22]. A systems modeling approach identified decreased AXL expression following MEK inhibition in TNBC cells [53]. AXL expression has been shown to correlate with that of the estrogen (ER) and progesterone (PR) receptors in breast cancer, indicating that estrogen and/or progesterone could regulate AXL expression [54, 55]. Indeed, blocking estrogen through either use of an estrogen antagonist or estrogen deprivation results in lower AXL mRNA levels in HER2$^+$ ER$^+$ breast cancer cells that have acquired resistance to lapatinib [37].

Epigenetic modifications such as promoter methylation and histone acetylation can also affect AXL transcript levels. Decreased methylation of the AXL promoter was observed in lapatinib-resistant HER2$^+$ breast cancer cells compared to sensitive cells, which correlated with higher levels of AXL expression in the resistant cells [37]. In colorectal and cervical cancer cell lines, methylation of certain transcription factor binding sites in the AXL promoter lead to decreased expression of AXL [51]. In lung cancer, mutant p53 bound to the AXL promoter and could reduce histone acetylation, leading to upregulation of AXL expression and enhanced cell growth and motility [56]. And finally, in acute myeloid leukemia (AML), methylation of the AXL promoter was inversely correlated with response to chemotherapy drugs [57].

MicroRNAs have also been implicated in regulation of AXL post-transcriptionally. miR-34a targets the *AXL* 3′ UTR in TNBC (MDA-MB-231 cell line). mIR-34a is found at a low level in MDA-MB-231 cells compared to non-transformed MCF10a cells or other lines representing other breast cancer subtypes [58, 59]. When miR-34a was overexpressed, AXL was downregulated [58]. Other microRNAs can regulate AXL in other cancer types, including miR-199a-3p in osteosarcoma and miR-139 in prostate cancer [60–62].

AXL can also be regulated at the protein level. The membrane protein TIG1 binds and stabilizes AXL in breast cancer, preventing its proteasomal degradation leading to enhanced proliferative, migratory, and invasive abilities via activation of NF-κB and MMP-9 [35]. As mentioned above, protease cleavage can lead to release of the cytoplasmic domain of the receptor, which retains ligand-binding capabilities. Therefore, regulation of cleavage could alter AXL activity levels.

7. The Role of AXL in the Tumor

Up until recently, AXL expression was thought to be limited to TNBC cell lines. However, analysis of patient tumors indicates that AXL is present across all of the main transcriptional subtypes, and there is no significant association between AXL expression and TNBC [4, 63]. Additionally, others have shown that AXL expression correlates with the presence of the estrogen receptor in breast carcinomas, indicating that despite high-level expression of AXL in TNBC, AXL is not restricted to TNBC tumors [54]. Indeed, a role for AXL in the progression of various breast cancer subtypes has been identified. These include in inflammatory breast cancer [35], TNBC [64], and ER-positive [54], as well as HER2-amplified [4] breast cancers. AXL has also been

implicated in more rare forms of breast tumors, including in phyllodes tumors of the breast, which are tumors that originate in stromal tissue [65], as well as in male breast cancer [66]. The role of AXL in the processes of EMT, metastasis, and the microenvironment/immune system in breast cancer is discussed below.

8. EMT

EMT describes the process whereby cells undergo a morphological transition from the epithelial polarized phenotype to the mesenchymal fibroblastoid phenotype. This process is defined by loss of cell-cell adhesion molecules, such as E-cadherin, downregulation of epithelial differentiation markers, and upregulation of mesenchymal molecular markers, such as vimentin. This process is crucial for normal embryonic development, and it also plays a role in tissue regeneration, organ fibrosis, inflammation, and tumor progression. In cancer, it is hypothesized that EMT cells gain migratory, invasive, and survival potential at the expense of proliferative ability. EMT has therefore been implicated in the process of metastasis. AXL expression strongly correlates with EMT markers across a spectrum of cancer types, including breast, lung, colorectal, bladder, endometrial, and ovarian cancers [67].

Originally, AXL was identified as a downstream target of EMT in breast cancer, whereby vimentin was shown to upregulate Slug and ultimately AXL [47]. Similarly, induction of EMT in the nontumorigenic epithelial MCF10a cell line through overexpression of the EMT transcription factors Snail, Slug, Twist, or Zeb2 resulted in elevated levels of AXL expression [5]. AXL is expressed at higher levels in mesenchymal mammary cell lines compared to parental epithelial lines, and if the mesenchymal cells are induced to become more epithelial like through a mesenchymal to epithelial transition, then these cells have less AXL expression [17]. Induction of EMT in MCF10a cells was also observed to promote autocrine GAS6 signaling [5]. These studies indicate that AXL is downstream of EMT.

However, more recently AXL has been identified as also being able to promote EMT, and therefore functions as part of a positive feedback loop to promote EMT as well as be upregulated in response to EMT transcription factors in normal and immortalized mammary cells [17]. Overexpression of AXL in human mammary epithelial cells (HMLE) resulted in a decrease in E-cadherin and an increase in the mesenchymal markers N-cadherin, vimentin, and Snail, consistent with an EMT [17]. Additionally, treatment of TNBC cell lines with recombinant GAS6 results in increased expression of EMT-associated genes, such as *SNAIL*, *SLUG*, and *VIM*, which effected increased migration and invasion [68].

siRNA knockdown of vimentin leads to a decrease in AXL expression in MCF10a cells, which are immortalized mammary cells but are not transformed [47]. A correlation was seen between the mesenchymal marker vimentin and AXL in both migratory cells at the wound edge in cell-based assays as well as in mammary tumors [47]. Interestingly, AXL and vimentin expression also correlated in normal mammary tissue samples from patients, not just in tumor tissue [47]. When vimentin is targeted by siRNAs in the mesenchymal MDA-MB-231 cell line, AXL protein levels decreased by 56%; however, no change in vimentin levels was observed when AXL was targeted [47].

AXL does not just play a role in mesenchymal cancers. Neu$^+$: *AXL*$^{-/-}$ tumors grown in mice had decreased expression of EMT genes compared to those with wild-type AXL expression and AXL expression in HER2$^+$ tumors correlated with EMT markers [4]. HER2$^+$ cancers retain epithelial features, but there is evidence that their metastasis requires EMT [69].

9. Metastasis

AXL has been shown to promote breast cancer cell motility, invasion, proliferation, survival, and anoikis resistance, often in conjunction with its role in EMT. Not surprisingly then AXL has been implicated in breast cancer metastasis. AXL levels have been observed to be increased in metastases compared to the primary tumor of the same patient [5].

Overexpression of AXL can induce increased invasiveness and motility of the normally weakly invasive MCF-7 cell line [70], and AXL knockdown in the mesenchymal/invasive MDA-MB-231 cell line results in decreased migratory and invasive ability [5, 47, 71].

AXL expression is also associated with increased tumorigenecity of breast cancer cells. In mouse breast cancer cells sorted into two groups based on the level of AXL expression, it was observed that cells expressing high levels of AXL were more tumorigenic than their low AXL-expressing counterparts [17]. In a mouse model of HER2$^+$ breast cancer, only slight differences were observed in tumor formation in AXL knockout mice compared to controls [4]. This is in contrast to tumor formation by MDA-MB-231 cells, a mesenchymal triple-negative cell line, where AXL was shown to be required for primary tumor formation, and knocking down AXL expression in established tumors reduces their size [5, 71]. In a mouse model of radioresistant breast cancer, MMTV-PyMT tumors selected for resistance to radiation therapy, AXL knockout reduced tumor growth compared to control tumors [72].

A direct role for AXL in promoting breast cancer metastasis has also been observed. In an *in vivo* comparison between metastasis formation of MDA-MB-231 cells with and without AXL knockdown, control cells metastasized to the lymph nodes, lungs, ovaries, and kidneys, while no macro- or micrometastases were observed from the AXL knockdown cells [5]. A similar experiment looked for metastasis specifically to the lung. While AXL knockdown in MDA-MB-231 xenografts was able to form tumors, there were no observable metastases to the lungs in AXL knockdown tumors [71].

Despite not showing a significant effect on primary tumor formation, in a model of HER2$^+$ breast cancer when metastasis formation was tracked, AXL knockout mice showed reduced lung metastases compared to controls [4]. GAS6 knockout did not affect metastasis formation in these same studies, indicating that the metastasis-promoting effect

of AXL occurs independently of GAS6 and is thought to involve interaction between AXL and HER2 [4]. AXL was shown to be important for all steps of the metastatic process in this model. By analyzing the amount of circulating tumor cells in orthotopic xenografts derived from $HER2^+$ control and AXL knockout cells, researchers observed a reduced number of circulating cells derived from xenografts in the AXL knockout cell lines compared to controls, indicating that AXL is important for initial intravasation of the primary tumor [4]. A similar decrease in extravasation was seen with AXL knockdown, and AXL was shown to be required for the maintenance of $HER2^+$ metastatic lesions as induction of shRNAs targeting AXL in the period after extravasation into the lungs had occurred resulted in smaller metastases [4].

The role of AXL in metastasis of other subtypes of breast cancer is less clear. When injected into the tail vein of mice, MDA-MB-231 cells with AXL knockdown extravasated into the lungs significantly less than for cells without AXL knockdown, indicating that AXL is important for extravasation [47]. However, AXL was shown to be dispensable for the maintenance of already established lung metastases from MDA-MB-231 xenografts, where induction of AXL knockdown after lung colonization had occurred resulted in the same amount of lung foci compared to controls without AXL knockdown [71]. In a model of TNBC breast cancer, xenografts of MDA-MB-231 cells showed reduced tumor growth with AXL knockdown [5]. AXL was also observed to be essential for metastasis of these tumors as well as metastasis from the highly metastatic mouse 4T1 tumor model [5]. This aligns with other evidence cited above that AXL is primarily responsible for metastasis formation and not primary tumor growth in epithelial-like cancers (ER^+, $HER2^+$), while it may play a role in primary tumor formation (and metastasis) if the primary tumor is mesenchymal-like, as is the case for most TNBC cancers.

AXL has therefore been implicated in intravasation, extravasation, and maintenance of metastases in $HER2^+$ breast cancer and in extravasation in other models of mammary tumors. In $HER2^+$ cancers at least, GAS6 appears to be dispensable for metastasis [4]. This does not mean GAS6 is uninvolved in metastasis as it can play an important role in reprogramming of the metastatic niche to promote metastasis outgrowth, discussed below.

10. Microenvironment and Immune System

A role for GAS6 has also been observed in modulating the tumor microenvironment. In a mouse $p53^{-/-}$ model of breast cancer, increased GAS6 levels were observed in preinvasive lesions that have high levels of infiltrating macrophages [40]. These immune system macrophages can produce GAS6, stimulating AXL and leading to activation of downstream pathways AKT and STAT3, resulting ultimately in decreased E-cadherin expression [40]. Increased GAS6 in preinvasive hyperplastic lesions compared to the normal mammary gland was observed, but decreased GAS6 levels were seen in invasive tumors compared to preinvasive lesions, even though macrophages continued to increase. In $GAS6^{-/-}$ knockout mice, early stage progression and time to tumor formation are decreased, but established tumor growth is unaffected [40]. In sum these data suggest that stromal GAS6 is involved in early changes that promote the switch from preinvasive to invasive cancer.

In a study of mouse 4T1 cells, which are highly metastatic breast cancer cells, leukocyte-derived GAS6 was shown to promote tumor growth *in vivo* [73]. In $GAS6^{-/-}$ mice, 4T1 tumors can grow, but they are smaller than those grown in $GAS6^{+/+}$ mice, likely owing to decreased proliferation, not increased apoptosis in these tumors. In $GAS6^{-/-}$ mice, metastasis to the lungs is also reduced but not eliminated [73].

A direct role for AXL in modulation of the immune system has also been observed. In the MMTV-PyMT mouse model of breast cancer, AXL was observed to be upregulated in mesenchymal extravasated cells and to be important for activating lung fibroblasts in the stroma. These activated fibroblasts in turn secrete factors which favor the epithelial state, resulting ultimately in a mesenchymal to epithelial transition (MET) that promotes metastasis growth [74]. Thus, AXL promotes reprogramming of the metastatic niche that favors both an initial EMT and a subsequent MET at the metastatic site.

High-level expression of AXL in mouse mammary tumors that were unresponsive to ionizing radiation in combination with immune checkpoint therapy has also been observed [72]. When AXL was deleted from these resistant tumors they became radiosensitive, which was mediated by immunological alteration, including enhanced antigen presentation and altered cytokine secretion [72]. AXL can suppress MHCI expression and thus antigen presentation and promote the release of cytokines that further contribute to decreasing the antitumor immune response.

In other cancer types, AXL can upregulate immune checkpoint proteins and alter chemokine signaling in lung adenocarcinoma, suggesting therapies that target immune checkpoint molecules like anti-PDL1 might be ineffective unless a combination therapy is used which also targets AXL [75]. Given the role of AXL in altering the immune system in breast cancer, a similar mechanism could be occurring in mammary tumorigenesis as well.

11. Clinical Implications

The above-discussed roles of AXL in the cell and tumor result in clinical implications for AXL in breast cancer. AXL and its ligand GAS6 could have prognostic and/or biomarker potential in the clinic. AXL is a strong negative predictor of patient survival in breast cancer, indicating its prognostic potential [5]. Similarly, AXL-associated tumor inflammation is correlated with poor prognosis in TNBC patients [76]. Additionally, levels of GAS6 could also be of prognostic significance in the clinical setting. Macrophage-derived GAS6 is a critical regulator of the transition from premalignant to invasive cancer and could be a biomarker of progression for patients with early-stage cancer [40].

sAXL, the cleaved extracellular domain of AXL, is being explored as a potential biomarker in certain cancers and other inflammatory conditions, such as in hepatocellular carcinoma, neurofibromatosis type 1, and in NSCLC

[30–32, 77]. sAXL was overexpressed in effusions from patients with breast carcinoma; however, this was not informative of chemoresponse or survival [78]. Therefore, it is currently unclear whether sAXL levels could be used as a biomarker in breast cancer.

12. Resistance to Therapy

AXL expression in the tumor has been implicated in resistance to a variety of therapies, both targeted and conventional, in several different cancer types including breast. Additionally, AXL expression in the microenvironment can contribute to drug resistance and epithelial plasticity [79].

12.1. Targeted Therapies. AXL has been implicated in resistance to EGFR-targeted therapy such as erlotinib and lapatinib in TNBC. This resistance is associated with EGFR-induced ligand-independent transactivation of AXL [19]. Activation of AXL has also been implicated in EGFR inhibitor resistance in HER2$^+$ breast cancer [37]. AXL expression is upregulated in HER2$^+$ cell lines with acquired resistance to lapatinib, a dual HER2 and EGFR inhibitor [37]. siRNA-mediated knockdown of AXL in these resistant cells restored sensitivity to lapatinib and to trastuzumab, a monoclonal antibody directed against HER2 [37].

AXL's ability to mediate resistance to EGFR-targeted therapies has been well studied in NSCLC, where mutation of EGFR is often a driver, and resistance to EGFR-targeted inhibitors is a major clinical hurdle. Treatment of NSCLC lines with an AXL inhibitor in combination with erlotinib restored sensitivity to erlotinib [80]. AXL is also able to mediate resistance to a newer EGFR-targeted inhibitor, osimertinib, in NSCLC [81–83]. This resistance is thought to be mediated by AXL interacting with EGFR and HER3 [82]. Finally, AXL has also been implicated in resistance to cetuximab treatment, a monoclonal EGFR-targeted antibody, in head and neck squamous cell carcinoma and in NSCLC [84].

In HER2-amplified gastric cancer cells, AXL, along with MET, can mediate resistance to afatinib, a pan-HER inhibitor [85]. It is not known if a similar mechanism is at play in HER2-amplifed breast cancer.

MEK inhibition in breast cancer is associated with decreased cleavage of the extracellular domain of AXL by ADAM10/17, resulting in increased signaling through AXL. This can confer resistance to MEK inhibitors and indicates a possible point of regulation of AXL activity in these cells [28].

Additionally, AXL has been implicated in resistance to a variety of other targeted therapies in other cancer types, including vemurafenib, sunitinib, alpelisib, crizotinib, and imatinib [86–90]. Resistance to such diverse therapies is likely linked to AXL's role in mediating EMT, which has long been associated with chemoresistance.

12.2. Conventional Therapies. AXL can also promote resistance to nontargeted agents. AXL-depleted murine breast cancer cells with breast cancer stem-cell-like properties were more sensitive to paclitaxel and etoposide compared to AXL-competent cells [17]. Similarly, inhibition of AXL signaling in MCF-7 cells with acquired resistance to conventional chemotherapies restored their sensitivity [91]. In TNBC and NSCLC cells that have undergone an EMT, inhibition of AXL can synergize with antimitotic agents such as docetaxel and paclitaxel but not with gemcitabine, doxorubicin, or cisplatin [92]. AXL inhibition in combination with other antimitotic agents, such as aurora kinase inhibitors and polo kinase inhibitors, which result in mitotic arrest, similarly resulted in synergistic inhibition of mesenchymal cancer cell proliferation [92]. As discussed above, AXL has been shown to promote EMT, and acquisition of mesenchymal features has been associated with resistance to a variety of targeted and conventional therapies [92].

In other cancer types, AXL has been implicated in resistance to doxorubicin and cytosine arabinoside in AML [57], to cisplatin resistance in esophageal cancer [93], to multiple chemotherapies in NSCLC [94], and to 5-FU treatment in colorectal cancer [95].

13. Inhibitors and Clinical Trials

Several inhibitors of AXL signaling have been investigated both in *in vitro* settings and in *in vivo* preclinical models and also in clinical trials. The mechanism of action of these therapeutics is diverse, including small molecular inhibitors, monoclonal antibodies, and CAR T-based therapies. Agents that have been used in clinical trials as well as some newer promising agents in preclinical development are discussed as follows.

14. Small Molecule Inhibitors

Several selective AXL or dual AXL/MER inhibitors have entered clinical trials; in addition, there are other multitarget AXL inhibitors that have been developed which target AXL in conjunction with other RTKs such as MET [96, 97].

14.1. AXL-Selective Inhibitors

14.1.1. BGB324/Bemcentinib/R428. BGB324 is a small molecule AXL inhibitor that has entered clinical trials (Table 1). In preclinical models, BGB324 reduces the invasion of MDA-MB-231 and murine 4T1 cells, both of which are highly migratory and invasive cell lines [98]. In orthotopic models with 4T1 cells and intracardiac injection of MDA-MB-231 cells, BGB324 reduced the amount of metastases observed [98]. BGB324 alone or in combination with nivolumab, an anti-PD-1 antibody, prolonged the survival of mice with mesenchymal glioblastoma tumors [9]. Not just mesenchymal tumors have shown effects with BGB324 treatment. Long-term systemic treatment with BGB324 decreased circulating tumor cells and lung metastases in a mouse model of HER2$^+$ breast cancer but had no effect on primary tumor growth [4].

Results of phase II trials with BGB324 have recently been reported. 26 patients with AML received BGB324 in combination with cytarabine or decitabine, and 20 were evaluated for response. Four out of nine patients receiving

Table 1: AXL-targeted therapies currently in clinical trials in the U.S.

Therapeutic	Clinical trial	Phase	Disease
Small molecule inhibitor			
AXL selective			
BGB324/R428/bemcentinib	NCT02424617	Phase 1/2	NSCLC
	NCT02488408	Phase 1/2	Acute myeloid leukemia, myelodysplastic syndrome
	NCT02872259 (active)	Phase 1/2	Melanoma
	NCT02922777 (active)	Phase 1	NSCLC
	NCT03184558 (completed)	Phase 2	*TNBC, inflammatory BC
	NCT03184571 (active)	Phase 2	NSCLC, lung adenocarcinoma, metastatic lung cancer
	NCT03649321 (active)	Phase 1/2	Pancreatic
	NCT03654833 (active)	Phase 2	Mesothelioma
	NCT03824080 (active)	Phase 2	Acute myeloid leukemia, myelodysplastic syndrome
	NCT03965494	Early phase 1	Glioblastoma
SLC-391/SLC 0211	NCT03990454	Phase 1	Solid tumors
	NCT02729298 (active)	Phase 1	Solid tumors
TP-0903	NCT03572634 (active)	Phase 1/2	Chronic lymphocytic leukemia, small lymphocytic lymphoma
Dual AXL/MER			
INCB081776	NCT03522142 (active)	Phase 1	Solid tumors
ONO-7475	NCT03176277 (active)	Phase 1	Acute leukemia
Antibody-based			
Antibody-drug conjugate			
BA3011/CAB-AXL-ADC	NCT03425279 (active)	Phase 1/2	Solid tumors
Enapotamab vedotin/HuMax-AXL-ADC/AXL-107-MMAE	NCT02988817 (active)	Phase 1/2	Solid tumors
Monoclonal antibody			
BGB149	NCT03795142 (active)	Early phase 1	Healthy volunteers
Anti-AXL Fc fusion protein			
AVB-S6-500	NCT03607955	Phase 1	Ovarian, fallopian, peritoneal
	NCT03639246 (active)	Phase 1/2	Ovarian
	NCT04004442	Phase 2	Urothelial carcinoma
	NCT04019288	Phase 1/2	Ovarian, fallopian, peritoneal
	NCT04042623	Phase 2	IgA nephropathy
CAR T-based therapy			
CCT301-38	NCT03393936 (active)	Phase 1/2	Renal cell carcinoma

BGB324 plus cytarabine achieved a complete response with an incomplete hematologic recovery, and two others achieved stable disease. Of the 11 patients receiving decitabine plus BGB324, four achieved a complete response with an incomplete hematologic recovery, and one progressed to stable disease [99]. Another clinical trial evaluated BGB324 in combination with an anti-PD1 immunotherapy in patients with advanced NSCLC. Out of 29 patients, seven partial responses were reported, and 40% of patients with AXL-positive biopsies achieved objective responses. The median progression-free survival for patients expressing AXL was 5.9 months, compared to 4.0 months for AXL negative patients [100]. Clinical trials for BGB324 in TNBC and other cancers are ongoing [101].

14.1.2. SLC-391/SLC-0211. SLC-391/SLC-0211 is a relatively selective small molecule inhibitor of AXL that inhibited the growth of murine colon cancer tumors, likely through stimulation of an anti-immune response [102]. SLC-391/SLC-0211 has also been shown to be effective against AML cells that express high levels of AXL/GAS6 [103]. A clinical trial for safety profiling of SLC-391/SLC-0211 in patients with advanced solid tumors has been established but is not yet recruiting.

14.1.3. TP-0903. TP-0903 showed effectiveness in preclinical models of refractory CLL, neuroblastoma, AML, and other solid cancers, including those that are refractory to other treatments [104–109]. TP-0903 is currently in clinical trials for both advanced stage solid tumors as well as for patients with CLL and small lymphocytic lymphoma.

14.2. Dual AXL/MER Inhibitors

14.2.1. INCB081776. This small molecule inhibitor targets both AXL and MER. In xenografts of NSCLC, INCB081776

was shown to inhibit tumor growth in immunocompetent but not in immunocompromised mice, indicating that the effects of this compound could be mediated through an anti-tumor immune response [110]. INCB081776 is currently in an early-phase clinical trial for patients with advanced solid tumors.

14.2.2. ONO-7475. This small molecular inhibitor targets both AXL and MER and is effective against preclinical models of AML [111]. It is currently in clinical trials for acute leukemia.

14.3. Others. Several other inhibitors have been developed that are not specific for AXL nor are dual AXL and MER inhibitors. BMS777-607 is a tyrosine kinase inhibitor that blocks the activity of all of the TAM family members. In a murine TNBC model, BMS-777607 in combination with an immunotherapy (anti-PD-1) decreased tumor growth and lung metastases [112]. Additionally, several new AXL inhibitors have been developed, and many have shown promise in preclinical testing. The AXL inhibitor SGI7079 reduced the growth of established NSCLC xenografts and was able to restore sensitivity to EGFR inhibitors in erlotinib-resistant cell lines [80]. Mollard et al. designed a series of AXL kinase inhibitors that inhibited the growth of pancreatic cancer cell lines [113]. Newly developed compound 8i is selective for AXL compared to most other kinases except for FLT3, and it inhibited invasion and migration of MDA-MB-231 cells induced with TGF-β, which increases expression of EMT markers [114]. RU-301 and RU-302 are newly developed small molecular inhibitors of the TAM family of receptors by preventing GAS6 binding [115].

14.4. Other Considerations. Despite clinical promise, potential problems could arise with any targeted therapy. A recent study showed a positive feedback loop was present in MDA-MB-231 cells whereby treatment with BMS777607 resulted in increased levels of AXL at the cell surface due to inhibition of ubiquitin-mediated lysosomal degradation [26]. This could decrease the effectiveness of this therapy. Signaling through MERTK has also been shown to mediate resistance to AXL-targeted therapies in TNBC preclinical models [116], and activation of HER3 has been implicated in resistance to AXL inhibitors in MDA-MB-231 cells [117]. Therefore, future studies should investigate AXL inhibitors in combination with other targeted therapies or chemotherapeutics in order to be effective in the clinic. Decreased AXL transcript levels in lapatinib-resistant $HER2^+$ cells have been observed in response to treatment with estrogen blockers, suggesting clinical potential of AXL inhibitors in combination with estrogen receptor blockers such as fulvestrant or tamoxifen, or aromatase inhibitors, such as letrozole, for instance [37].

15. Antibody-Based Therapies

Due to the high level of AXL expression on many tumor types, antibody-based therapies which specifically target AXL could be beneficial in the clinic.

15.1. Antibody-Drug Conjugates

15.1.1. BA3011/CAB-AXL-ADC. BA3011 is a conditionally active biologic (CAB) antibody-drug conjugate (ADC) that has shown efficacy in inhibiting the growth of lung, prostate, and pancreatic xenografts [118]. It has entered clinical trials for advanced solid cancers [119].

15.1.2. HuMax-AXL-ADC. HuMax-AXL-ADC is an ADC that due to the antibody portion specifically targets AXL-expressing cells and exposes them to the microtubule-disrupting agent monomethyl auristatin E (MMAE) [120]. In a lung tumor xenograft model complete regression was observed after a single dose, and HuMax-AXL-ADC was also effective against patient-derived xenografts [120]. In preclinical testing HuMax-AXL-ADC has shown efficacy against EGFR-inhibitor-resistant NSCLC [121], as well as melanoma, including multidrug-resistant melanoma [122, 123]. This agent has entered clinical trials to establish the safety profile of HuMax-AXL-ADC in patients with various solid tumors (not including breast) [124].

15.2. Monoclonal Antibodies. An early phase 1 clinical trial of BGB149, a function blocking antibody directed against AXL, is currently recruiting (NCT03795142). In preclinical development, treatment of murine breast cancer xenografts with a different monoclonal antibody that blocks GAS6 binding, YW327.6S2, that binds both human and murine AXL, decreased tumor growth, inhibited the activity of tumor-associated macrophages, and decreased metastasis formation from MDA-MB-231 xenografts [125]. The anti-AXL monoclonal antibody 20G7-D9 has also been shown to be effective in management of TNBC breast cancer xenografts and patient-derived xenografts, where it blocks signaling, prevents EMT, reduces tumor growth, decreases migration and invasion, and also decreases metastasis formation [68, 126]. Other mAbs are being investigated as well. MAb173 inhibited the invasiveness of Kaposi sarcoma cells and decreased tumor formation and increased apoptosis in Kaposi sarcoma xenografts [127]. DAXL-88 decreased AXL signaling and migration of ovarian and lung cancer cell lines [128].

15.3. Anti-AXL Fc Fusion Protein. AVB-S6-500 is a fusion protein composed of the extracellular ligand-binding domain of the AXL receptor fused to the immunoglobulin G1 Fc domain. As such, this fusion protein binds to GAS6 and prevents its binding to AXL. AVB-S6-500 recently gained "fast-track" status for the treatment of patients with recurrent ovarian cancer, indicating that review of the drug will be expedited by the Food and Drug Administration due to its potential ability to fulfill an unmet life-threatening clinical need. In a mouse model of ovarian cancer treatment with AVB-S6-500 in combination with chemotherapy resulted in smaller tumors than those treated with chemotherapy alone [129]. AVB-S6-500 can also synergize with carboplatin and paclitaxel to reduce ovarian cancer cell growth [130].

Other AXL decoy receptors have been developed, including MYD1-72, which has a high affinity for GAS6 and therefore blocks the interaction between AXL and GAS6. This drug blunts tumor growth and metastasis of mouse mammary tumor cells [131]. The ligand-independent methods of activating in AXL discussed above however indicate that such ligand-blocking therapies could be of limited value since AXL is able to be activated through dimerization with itself and with other receptors, independent of ligand binding. Alternative therapeutic strategies, such as using agents which decrease receptor abundance or block the AXL kinase domain, might therefore be more effective.

15.4. CAR T-Based Therapy. CAR T (chimeric antigen receptor-modulated T lymphocyte) therapy targeting AXL was effective in a TNBC preclinical model [132]. Currently, a clinical trial is underway of CCT301-38 for patients with AXL-expressing metastatic renal cell carcinoma upon biopsy.

16. Conclusions

Thus far, research into AXL in breast cancer has clearly demonstrated an oncogenic role for this receptor in various breast cancer subtypes, as it is upregulated in breast cancer tumors and can promote tumor formation, EMT, metastasis, and chemoresistance through stimulation of an abundance of intracellular signaling pathways. However, a few key questions about AXL remain to be elucidated. As of yet, no activating mutations have been identified in AXL; thus, mutational status of the receptor cannot serve as a biomarker for therapeutic response. Identification of patient subsets that are likely to benefit from therapeutic inhibition of AXL will be crucial if the above-mentioned AXL inhibitors are to be successful in the clinic. It remains to be seen what the biomarker will be for AXL, whether it will be AXL expression levels, activation status, and/or presence of GAS6 or sAXL. Even once a biomarker has been identified through cell culture work, it will be necessary to determine if this marker can be effectively measured in clinical samples, and accurate analytical tools and perhaps more specific phospho-AXL-directed antibodies may need to be developed. The role of AXL in breast cancer is highly context-dependent, and therefore, it is possible that different biomarkers may be necessary for different breast cancer subtypes. For example, AXL has been shown to interact with HER2 in $HER2^+$ breast cancer, but this receptor is not overexpressed in TNBC [4].

As discussed above, AXL has been shown to promote a variety of downstream signaling pathways to mediate its various roles in cancer progression, again, many of which are highly context-dependent. It will be important to further elucidate which downstream pathways are responsible for various roles of AXL in order to better predict potential partnering agents with AXL inhibitors. The role of AXL in chemoresistance further suggests that a treatment approach inhibiting AXL in combination with inhibitors of other molecular targets could be an effective therapeutic strategy for breast cancer treatment. The above-discussed role of AXL in the immune system could suggest that AXL inhibition in concert with one of the newer immune checkpoint blocking therapies could be effective.

Finally, while AXL has been the main TAM family member that has been investigated in breast cancer, future work should also consider the role of other TAM family members, especially given the demonstrated cross-talk between these receptors and the recent finding that protein S can stimulate AXL [9]. If other TAM family members are shown to be involved in breast cancer progression, it could indicate that more broad-spectrum TAM inhibitors as opposed to more highly selective AXL inhibitors may be more effective in breast cancer treatment.

In sum, future work should seek to further uncover AXL's role in the breast tumor so that combination therapies could be developed that co-target AXL along with other intracellular players. AXL inhibitors are beginning to show efficacy in the clinic, and a deeper understanding of AXL's role in breast cancer could lead to a better identification of subsets of patients with potential to benefit from AXL-targeted therapy.

Conflicts of Interest

The author declares no conflicts of interest.

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Research Article

Screening of Pleural Mesothelioma Cell Lines for Kinase Activity May Identify New Mechanisms of Therapy Resistance in Patients Receiving Platin-Based Chemotherapy

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Background. Malignant pleural mesothelioma (MPM) is a rare, predominantly asbestos-related and biologically highly aggressive tumor associated with a dismal prognosis. Multimodal therapy consisting of platinum-based chemotherapy is the treatment of choice. The reasons underlying the rather poor efficacy of platinum compounds remain largely unknown. Kinase activity might influence cellular response to these regimens. *Materials and Methods*. For this exploratory study, we screened MPM cell lines (NCI-H2452, NCI-H2052, and MSTO-211H) differing in response to cisplatin and benign control fibroblasts (MRC-5) for overall phosphorylation patterns as well as kinase activity with respect to cellular response to cisplatin-based therapeutics. We analysed the cell lines for cellular kinases in a high-throughput manner using the highly innovative technique PamGene. Cell state analysis including apoptosis, necrosis, and cell viability was performed by using enzyme activity and fluorescent-based assays. *Results*. Cisplatin alters cellular phosphorylation patterns affecting cell cycle, migration, adhesion, signal transduction, immune modulation, and apoptosis. In cisplatin-responsive cell lines, phosphorylation of AKT1 and GSK3B was decreased but could not be influenced in cisplatin-resistant NCI-H2452 cells. Cisplatin-responsive cell lines showed increased phosphorylation levels of JNK1/2/3 but decreased phosphorylation in cisplatin-resistant NCI-H2452 cells. *Conclusion*. Kinase phosphorylation and activity might play a crucial role in cellular response to cytostatic agents. Cisplatin influences phosphorylation patterns with distinct features in cisplatin-resistant cells. These alterations exert a significant impact on cell cycle, migration, adhesion, signal transduction, immune modulation, and apoptosis of the respective tumor cells. Based on our results, the induction of p38 or JNK1/3, or inhibition of AKT1 by, for example, BIA-6, might offer a positive synergistic effect by induction of an apoptotic response to cisplatin-based treatment, thus potentially enhancing the clinical outcome of MPM patients.

1. Introduction

Malignant pleural mesothelioma (MPM) is a rare, predominantly asbestos-related tumor and associated with a dismal prognosis [1, 2]. In the US, approximately 2500 new cases of mesothelioma are diagnosed each year and the incidence of mesothelioma is expected to decline steadily [1, 3, 4]. In contrast, the incidence of mesothelioma in Europe continues to rise [1, 2, 5–7].

Besides pemetrexed, platinum compounds are standard chemotherapeutic agents and still a hallmark of chemotherapy for MPM [8]. In clinical practice, pemetrexed is used in combination with cisplatin [9] or carboplatin [10–13]. Platinum-containing regimens have a greater activity than nonplatinum containing combinations [14]. Cisplatin treatment shows a response rate of merely 14% and a median survival of fewer than 7 months [15]. Carboplatin treatment results in similar response rates ranging from 6 to 16% [15]. The reasons for this rather poor efficacy of platinum compounds are incompletely understood until now.

Platinum cytotoxicity is based on forming bulky DNA adducts by chemically altering DNA bases by covalent binding of platinum [13], leading to both DNA interstrand and (1 and 2 or 1 and 3) intrastrand cross-linking [16–23]. Platinum compounds prevent normal cell replication and trigger apoptosis [18, 22, 24], unless adducts from genomic DNA are repaired [21].

Resistance to antitumor agents such as platin compounds has been correlated to a broad spectrum of mechanisms. It is known since the early 1990s that the activity of several proteins involved in the development of antitumor drug resistance is regulated by protein phosphorylation [25]. Especially, the role of protein kinase C and others has been previously described [26]. During the past decade, our understanding of the underlying effect of platin-induced apoptosis has increased greatly by the identification of some of the major components involved in apoptosis and the processes regulating their activation. Kinases that have been suggested to play a role in apoptosis encompass the mitogen-activated protein kinase (MAPK) family, specifically p42/44 ERK, p38 MAPK, c-Jun N-terminal kinase (JNK), cyclic AMP-dependent protein kinase (PKA), protein kinase B (PKB), or AKT and protein kinase C (PKC) [27]. Furthermore, phosphorylation levels of different DNA damage genes such as ATM or ATR are known to influence cellular response to replicative stress induced by platinum containing drugs [28]. In addition, it has been shown in ovarian cancer and sarcoma cells expressing constitutively active JAK2 that cisplatin significantly inhibited tyrosine phosphorylation and kinase activity of JAK2 in a dose- and time-dependent manner [29].

Against this background, we aimed to investigate the impact of overall phosphorylation patterns as well as kinase activity in cellular response to cisplatin-based therapeutics. Therefore, we analysed different MPM cell lines for cellular kinases in a high-throughput manner using the highly innovative technique PamGene.

2. Materials and Methods

2.1. Cell Culture. MPM cell lines MSTO-211H (biphasic subtype and moderately cisplatin sensitive) and NCI-H2052 (epithelioid subtype and cisplatin sensitive) as well as the cell line NCI-H2452 (sarcomatoid subtype and cisplatin-resistant) were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Thermo Fisher Scientific, Massachusetts, USA). The human lung-fibroblast cell line MRC-5 was used as a control cell line. MRC-5 cells were cultured in Minimal Essential Medium (Thermo Fisher Scientific). All culture media were supplemented with 10% fetal calf serum and 1% penicillin and streptomycin (Thermo Fisher Scientific).

2.2. Treatment of MPM Cell Lines with Cisplatin. The effect of cisplatin on kinase activity was analysed in each cell line. Therefore, 1.6×10^5 cells/well were seeded in a 24-well plate. After 12 h of incubation at 37°C and 5% CO_2, 10 μM of cisplatin (Selleckchem, Houston, USA) was added to cells. After 48 h of incubation with cisplatin, protein isolation was performed according to the protocol 1160 from the PamGene platform (PamGene International B. V., Wolvenhoek, Netherlands). Therefore, cells were lysed by using M-PER Mammalian Protein Extraction Reagent containing HALT phosphatase inhibitor cocktail and HALT protease inhibitor cocktail EDTA-free (Thermo Fisher Scientific). Lysed cells were harvested by using a cell scraper. Lysates were stored in 5–20 μl aliquots at −80°C.

The protein concentration was determined via fluorometric quantification (Qubit, Thermo Fisher Scientific) using the protein assay kit according to the manufacturers' instructions.

2.3. Protein Tyrosine Kinase Assay. The Protein Tyrosine Kinase Assay (PTK Assay, PamGene) was performed according to the manufacturers' instructions. The PamChip®-4 was prepared by a blocking step with 30 μl of 2% BSA (PamGene). Master mix was prepared by using the reagent kit for PTK PamChip arrays (PamGene). 5 μg of sample protein lysate was applied. As required for the mastermix, ATP (4 mM) was diluted 1 : 25.

2.4. Serine/Threonine Kinase Assay. The Serine/Threonine Kinase Assay (STK Assay, PamGene) was performed according to the manufacturers' instructions. The PamChip®-4 was prepared by a blocking step with 30 μl of 2% BSA (PamGene). Master mix was prepared by using the reagent kit for STK PamChip arrays (PamGene). 0.5 μg of sample protein lysate was applied. As required for the mastermix, ATP (4 mM) was diluted 1 : 25.

2.5. Kinase Activity Determination. Analysis of the results of PTK and STK assay was performed by using the BioNavigator software (PamGene).

Image analysis and log2 transformation of the results were performed by using the BioNavigator software

(PamGene). Based on substrate phosphorylation pattern, kinase activities of each specific kinase were estimated using the kinase upstream analysis algorithm (BioNavigator). Each corresponding kinase was classified by specificity of each kinase, and dependency power levels were calculated. To visualize kinase activity changes before and after cisplatin treatment, kinase trees were generated by using the KinHub platform (http://www.kinhub.org/kinmap/).

2.6. Statistical Analysis. Statistical and graphical analyses of specific phosphosite phosphorylation levels were performed with the *R* statistical programming environment (v3.2.3).

Before starting the explorative data analysis, the Shapiro–Wilk test was applied to test for normal distribution of the data. Based on the results, either parametric or nonparametric test was used. For dichotomous variables, either the Wilcoxon Mann–Whitney rank sum test (nonparametric) or two-sided student's *t*-test (parametric) was applied. For ordinal variables with more than two groups, either the Kruskal–Wallis test (nonparametric) or ANOVA (parametric) was used to detect group differences.

Double dichotomous contingency tables were analysed using Fisher's exact test. To test dependency of ranked parameters with more than two groups, Pearson's chi-squared test was used. Correlations between metric variables were tested by using Spearman's rank correlation test as well as Pearson's product moment correlation coefficient for linear modelling.

Due to the multiple statistical testing, all p values were adjusted by using the false discovery rate (FDR). The level of statistical significance was defined as $p \leq 0.05$ after adjustment.

3. Results

3.1. Cisplatin Treatment Reveals Differences in Phosphorylation Pattern. MRC-5 cells showed minor changes in phosphorylation of phosphosites when comparing cisplatin treatment (highlighted by black bars) and medium (highlighted by grey bars) (Figure 1, green bars). MSTO-211H cells (blue bars) presented with changes in phosphorylation during cisplatin therapy. MSTO-211H has a distinct cluster in its phosphorylation pattern compared to the other MPM cell lines. During therapy, a similar shift in the phosphorylation pattern could be observed compared to the other two MPM cell lines. Untreated, a similar phosphorylation pattern could be detected for MSTO-211H cells. However, certain phosphosites showed a significantly enhanced signal. NCI-H2052 (red bars) and NCI-H2452 (yellow bars) presented with an overlap in their phosphorylation pattern and showed a much stronger phosphorylation of multiple phosphosites without exposition to cisplatin. MRC-5 presented in general with minor phosphorylation regardless of the respective phosphosites and treatment.

In sum, 54 phosphosites showed significantly altered phosphorylation during cisplatin therapy. 52 showed a reduction in phosphorylation status due to treatment, whereas two (PPR1A and FOXO3) showed an induction in their phosphorylation. In Figure 2, ten phosphosites with the most significant changes in phosphorylation after treatment with cisplatin are shown (p values are shown in Suppl. Table 1). A significant global reduction of tyrosine phosphosite phosphorylation could be outlined. No biologically relevant significance for serine/threonine kinases was monitored.

The comparison of significantly altered phosphorylation levels between the different cell lines revealed 62 alterations. The majority of differences [29] could be observed between MSTO-211H and the MRC-5 control cell line (Suppl. Table 2). In line with the results visualized in the heatmap, all targets showed significantly reduced signals. Between NCI-H2452 and MRC-5, 15 differences were observed, again, all with lower phosphorylation levels. Also, between NCI-H2052 and MRC-5, ten significantly higher phosphorylated sites could be observed in the tumor cells. Between MSTO-211H and NCI-H2452, three phosphosites (CD3Z, EGFR, and GSK3ß) showed a higher phosphorylation and four phosphosites (EFS, ENOG, EPHA7, and PTN6) showed lower phosphorylation levels in NCI-H2452 cells. Between NCI-H2052 vs. MSTO-211H, as well as between NCI-H2052 vs. NCI-H2452, no significantly altered phosphorylation levels could be observed.

3.2. Influence of Different Phosphorylation Patterns in Response to Cisplatin. Overall, high phosphorylation of phosphosites (Suppl. Table 3) lead to resistance against cisplatin therapy. In general, 24 phosphosites seem to impact cellular response to cisplatin-based therapeutic regimens. Respective phosphosites are shown in Suppl. Table 3. Especially, high phosphorylation of ESR1, LAT, PTN12, and PTN6 showed the strongest apoptosis-preventing effect. The circos plot (Figure 3) depicts the frequency of high or low phosphorylated phosphosites of kinases and their responsiveness to cisplatin.

3.3. Biological Relevance and Effected Cellular Pathways. Analysis of the phosphorylation pattern with respect to MAPK signaling pathway (KEGG hsa04010), cell cycle (KEGG hsa04110), and pathways in cancer (KEGG hsa05200) was performed for each cell line. Induction of phosphorylation by cisplatin is indicated by green labels, and reduction of phosphorylation is indicated in red labels (Suppl. Figures 1–3). In the MAPK signaling pathway, cisplatin reduces phosphorylation of receptor tyrosine kinases (especially EGFR, EPHA2, and KDR), and RASA1, RAF1, and AKT1 in each cell line. In the cell cycle pathway, GSK3B, CDK2, and CDK1 are reduced by cisplatin in each cell line, and in cancer pathways, cisplatin reduces phosphorylation of GSK3B, AKT1, PDGFRa/b, PLCG1/2, and CDK2. In NCI-H2452 cells, the reduction of phosphorylation by cisplatin is weaker than in other cell lines (indicated by light red).

Proteins contributing to cell adhesion/migration and membrane properties showed a cluster with remarkable response to cisplatin treatment in NCI-H2052 and MSTO-211H. EPHA1 (Suppl. Figure 4B), EPHA2 (Suppl.

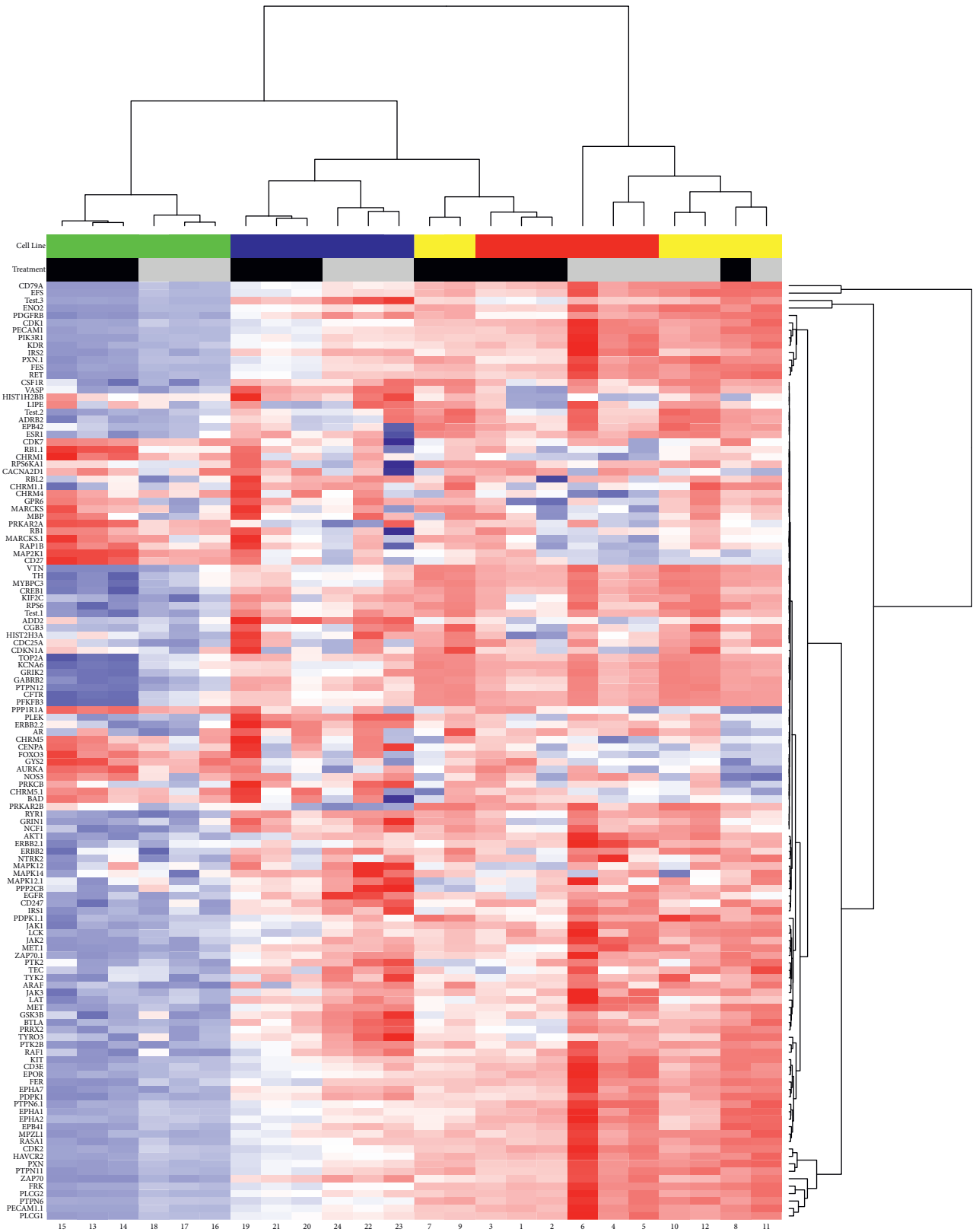

Figure 1: Heatmap of phosphorylation pattern between cells and treatment. MRC-5 is depicted by the green indicator on the *y*-axis and shows minor phosphorylation changes when comparing cisplatin treatment and medium. Blue indicators (*y*-axis) depict MSTO-211H and show that this cell line presented with changes in phosphorylation during cisplatin therapy. MSTO-211H cells have a distinct phosphorylation pattern compared to the other MPM cell lines. During therapy, a slight shift towards the phosphorylation pattern of the other two MPM cell lines can be seen. NCI-H2052 (shown in red) and NCI-H2452 (shown in yellow) present with an overlap in their phosphorylation pattern and show a much stronger phosphorylation of multiple phosphosites during therapy. Contrarily, MSTO-211H cells showed intermediate to slightly elevated phosphorylation. In contrast, MRC-5 cells present in general with minor phosphorylation regardless of the respective phosphosite and treatment.

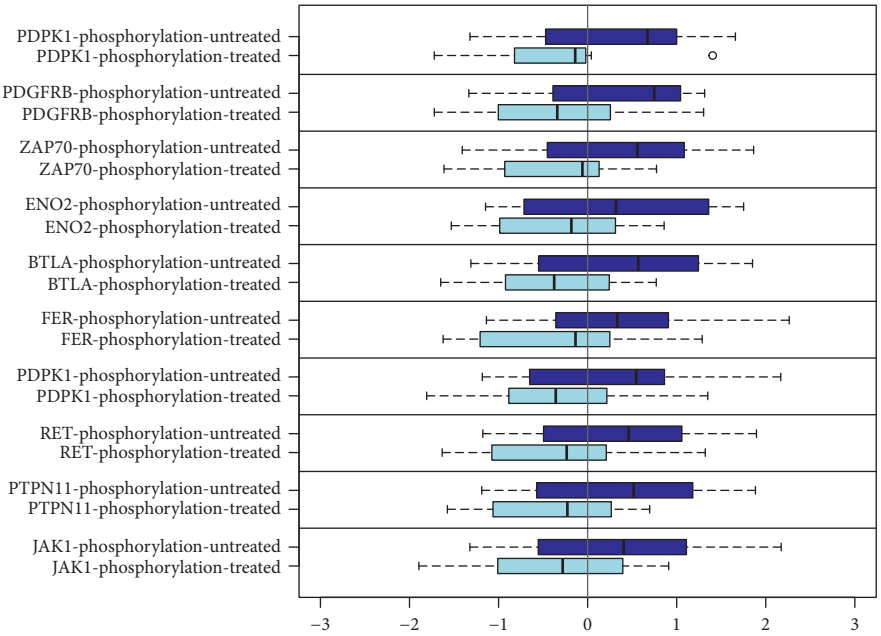

Figure 2: Top 10 influenced phosphosites during cisplatin therapy. On the *y*-axis, phosphosites of the respective protein are shown. Each box summarizes the results for all four cell lines, being measured in triplicates (dark blue = medium and light blue = cisplatin). To make the results comparable, the *x*-axis depicts a dimensionless *Z*-score. A significant, global reduction of tyrosine phosphosite phosphorylation could be observed. No significance for serine/threonine kinases was monitored. The figure focusses on the top 10 changes because further boxplots would be repetitive. In sum, 77 phosphosites showed significantly altered phosphorylation during cisplatin therapy. *p* values are depicted in Suppl. Table 1.

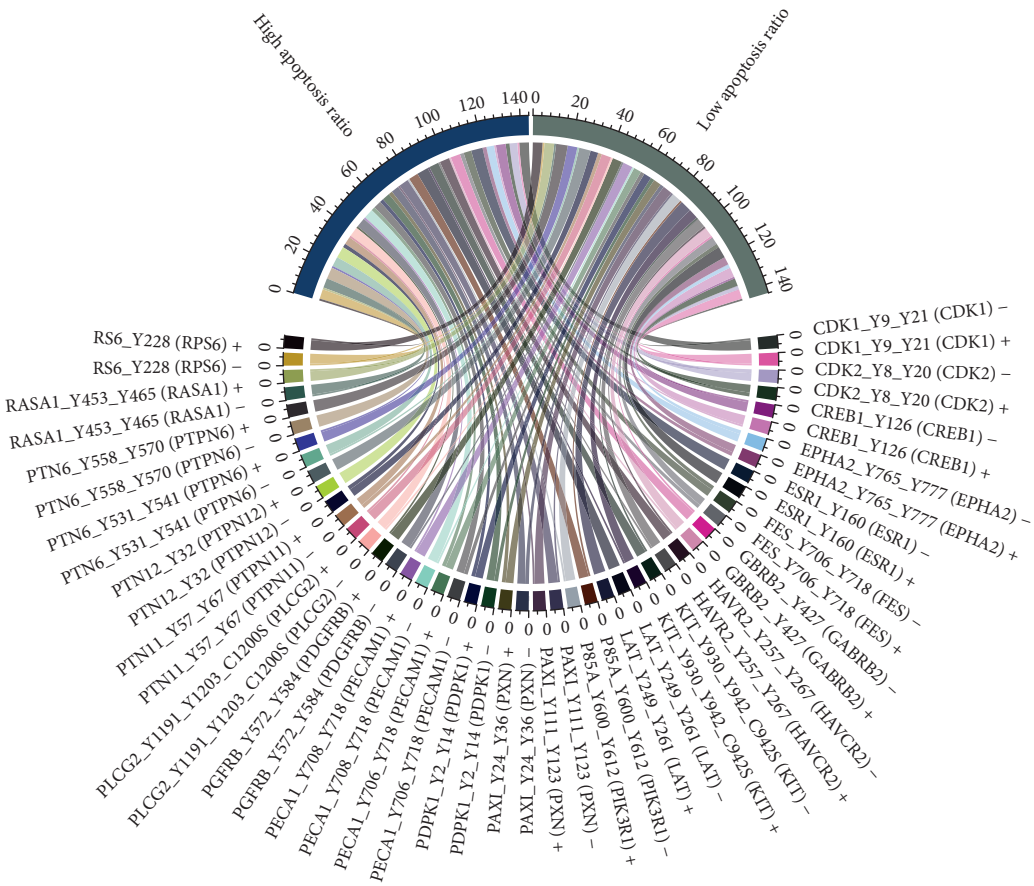

Figure 3: Circos plot of high and low phosphorylated phosphosites associated with high or low apoptosis ratio after cisplatin treatment. High and low phosphorylation of phosphosites were indicated with "+" or "−".

Figure 4C), EPHA7 (Suppl. Figure 4D), EFS (Suppl. Figure 6B), EPB41, PTK2B (Suppl. Figure 6D), FER, FES, KIT (Suppl. Figure 5A), PXN, PECAM1, PDGFRB, KDR (Suppl. Figure 6A), and ZAP70 showed comparable phosphorylation changes during cisplatin therapy (all $p < 0.01$ after Bonferroni correction; Table 1). Phosphorylation of the PTK phosphosites was low in fibroblasts in general. In NCI-H2452, cisplatin therapy led to a low reduction of phosphorylation with respect to the mentioned proteins. Instead, NCI-H2052 showed a much stronger reduction of phosphorylation of the mentioned phosphosites. MSTO-211H showed an intermediate to strong response during cisplatin therapy with respect to reduction of phosphorylation. Cisplatin therapy led to major changes in EFS phosphorylation, as phosphorylation decreased in all cell lines in a remarkable manner ($p < 0.0001$). In MRC-5 cells, the phosphosite was not anymore phosphorylated during cisplatin treatment. The result for EFS is shown in Suppl. Figure 6B.

Cisplatin leads to a reduction of AKT1 phosphorylation influencing one of the three AKT1 phosphosites (Suppl. Figure 6C). Phosphorylation was low in MRC-5 cells, regardless of the treatment. NCI-H2052 showed the strongest changes in AKT1 phosphorylation during therapy. Similar results were found for PTK2B (Suppl. Figure 6D), which is an upstream regulator of AKT1. Phosphorylation reduced during cisplatin therapy in NCI-H2052 and MSTO-211H, whereas the other cell lines showed minor response. PDGFRB and KDR phosphorylation showed comparable phosphorylation changes as mentioned above. Both contribute to the activation of AKT1.

Additional proteins that mediate inter- and intracellular signal transduction (ARAF, EPHA1, EPHA2, EPHA7, KIT, PTPN11, PIK3R1, PTPN6, and KDR) showed similar results as depicted in Suppl. Figures 4–6. The changes in phosphorylation are redundant to the results mentioned before.

BTLA, CD3E, CD247, CD79A, PTK2B, HAVCR2, PECAM1, and ZAP70 modulate immune response and showed differential phosphorylation during therapy. NCI-H2052 showed the strongest decrease in phosphorylation of these proteins during cisplatin therapy. In addition, phosphorylation was much higher before therapy compared to the other cell lines investigated. Similar high phosphorylation was also found in NCI-H2452 before therapy, but phosphorylation reduced less during therapy compared to NCI-H2052. Phosphorylation in MSTO-211H was much lower compared to the other two MPM cell lines. During therapy, only a minor reduction of phosphorylation was monitored for proteins modulating immune response. MRC-5 phosphorylation was low in general but decreased during therapy to a nonmeasurable extent.

Phosphorylation of proteins driving cell cycle control showed major changes during therapy (CDK1, CDK2, EPHA1, EPHA2, EPHA7, ENO2, PTK2B, FER, FES, FRK, KIT, PDGFRB, and KDR). The changes were similar to the above presented results. Again, NCI-H2052 showed the strongest changes, followed by NCI-H2452. Similar, but with general lower phosphorylation, MSTO-211H was comparable with the other two MPM cell lines. Again, MRC-5 presented with generally low phosphorylation of the reported proteins and reduced during therapy—in most cases, phosphorylation was absent due to cisplatin treatment.

3.4. Upstream Kinase Analysis. Kinase trees were created for each cell line (Suppl. Figures 9A–9D). The most affected kinases were in the family of tyrosine kinases (e.g., ALK, FES, and ZAP70) and CMGC kinases (e.g., CDK1, CDK2, and ERK1) in all four cell lines.

As depicted in the score plots and volcano plots (Suppl. Figures 7–8), NCI-H2052 cells showed a 2.5–3-fold decrease in kinase activity of FGFR1, FES, and ALK due to cisplatin treatment, with a high specificity score (2, dark red) for the respective phosphosites. In NCI-H2052, as well as in MRC-5 cells, kinase activity of ERK1/2 and CDK1 was 2.3-fold increased due to cisplatin treatment. In MRC-5 cells, kinase activity of HER2, FLT3, and EGFR showed a very strong decrease (6–9.5-fold) with a high specificity (2, dark red) for ten respective phosphosites. In MSTO-211H cells, kinase activity was decreased (3-4-fold) by 10 phosphosites with high specificity. Kinase activity was slightly decreased (0.4–0.7-fold) in NCI-H2452 for FAK1, Ron, SRC, CK1, and COT.

4. Discussion

Platinum compounds are standard chemotherapeutic agents and still a hallmark of chemotherapy for MPM in combination with pemetrexed [30]. Nevertheless, platin-containing regimens show unsatisfying response. Therefore, we investigated MPM cell lines, differing in their response to cisplatin (NCI-H2052: high apoptotic response, MSTO-211H: sparsely apoptotic response, and NCI-H2452: no response). We screened the cells for overall phosphorylation patterns as well as kinase activity with respect to cellular response to cisplatin-based therapeutics. We analysed the cell lines for cellular kinases in a high-throughput manner using the highly innovative technique PamGene.

In our study, we could demonstrate differences in the phosphorylation pattern in all cell lines due to cisplatin treatment. Overall, increase in phosphorylation after addition of cisplatin indicate an adaptive mechanism to escape from the effect of cisplatin. In particular, high phosphorylation of ESR1, LAT, PTN12, and PTN6 showed anti-apoptotic effects. PTN12 has dephosphorylation functions and therefore influences cellular signaling cascades [31]. It dephosphorylates cellular tyrosine kinases like ERBB2 and PTK2B. ERBB2 encodes HER2/neu that inhibits apoptosis by stimulation of proliferation via the RAS-MAP kinase pathway [32, 33]. In NCI-H2452 cells, phosphorylation levels of ERBB2 is not reduced, compared to other cell lines. Therefore, it could be suggested that this mechanism plays a role in this cell line, supporting its cisplatin resistance.

BTLA, CD3E, CD247, CD79A, PTK2B, HAVCR2, PECAM1, and ZAP70 modulate immune response and showed differential phosphorylation during therapy. The activation of BTLA leads to inhibition of $CD8^+$ cancer-specific T-cells [34]. BTLA showed decreased

Table 1: Significant phosphorylation changes after cisplatin treatment (p values and Bonferroni-adjusted p values).

Phosphosites	p value	Bonferroni-adj. p value
Tyrosine-protein phosphatase nonreceptor type 11 (EC 3.1.3.48) (protein-tyrosine phosphatase 2C) (PTP-2C) (PTP-1D) (SH-PTP3) (SH-PTP2) (SHP-2) (Shp2)._PTN11_57_67_Q06124	$1.58E-10$	$1.40E-08$
Gamma-enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydrolyase) (neural enolase) (neuron-specific enolase) (NSE) (enolase 2)_ENOG_37_49_P09104	$2.23E-10$	$1.99E-08$
Tyrosine-protein phosphatase nonreceptor type 6 (EC:3.1.3.48)_PTN6_531_541_P29350	$6.41E-09$	$5.71E-07$
Paxillin._PAXI_24_36_P49023	$8.74E-09$	$7.78E-07$
Tyrosine-protein kinase JAK2 (EC 2.7.10.2) (janus kinase 2) (JAK-2)_JAK2_563_577_O60674	$3.28E-08$	$2.92E-06$
Embryonal fyn-associated substrate (HEFS)_EFS_246_258_O43281	$3.38E-08$	$3.01E-06$
Hepatitis A virus cellular receptor 2, T-cell immunoglobulin and mucin domain-containing protein 3, T-cell membrane protein 3_HAVR2_257_267_Q8TDQ0	$4.21E-08$	$3.75E-06$
Proto-oncogene tyrosine-protein kinase fes/Fps (EC 2.7.10.2) (C-Fes)_FES_706_718_P07332	$5.51E-08$	$4.91E-06$
B-cell antigen receptor complex-associated protein alpha-chain precursor (Ig-alpha) (MB-1 membrane glycoprotein) (surface IgM-associated protein) (membrane-bound immunoglobulin-associated protein) (CD79a antigen)_CD79 A_181_193_P11912	$5.72E-08$	$5.09E-06$
Tyrosine-protein kinase FRK (EC 2.7.10.2) (FYN-related kinase) (nuclear tyrosine protein kinase RAK)_FRK_380_392_P42685	$6.73E-08$	$5.99E-06$
NA_ART_004_EAIYAAPFAKKKXC_NA	$7.47E-08$	$6.65E-06$
Tyrosine-protein kinase JAK1 (EC 2.7.10.2) (janus kinase 1) (JAK-1)_JAK1_1027_1039_P23458	$8.05E-08$	$7.17E-06$
Insulin receptor substrate 2_IRS2_626_638_Q9Y4H2	$9.51E-08$	$8.47E-06$
Hepatocyte growth factor receptor precursor (EC 2.7.10.1) (HGF receptor) (scatter factor receptor) (SF receptor) (HGF/SF receptor) (Met proto-oncogene tyrosine kinase) (c-Met)_MET_1228_1240_P08581	$1.22E-07$	$1.08E-05$
Paxillin._PAXI_111_123_P49023	$1.42E-07$	$1.26E-05$
Phosphatidylinositol 3-kinase regulatory subunit alpha (PI3-kinase p85 subunit alpha) (PtdIns-3-kinase p85-alpha) (PI3K)_P85 A_600_612_P27986	$1.81E-07$	$1.61E-05$
Proto-oncogene tyrosine-protein kinase receptor ret precursor (EC 2.7.10.1) (C-ret)_RET_1022_1034_P07949	$1.89E-07$	$1.68E-05$
1-Phosphatidylinositol-4,5-bisphosphate phosphodiesterase gamma-1 (EC 3.1.4.11) (phosphoinositide phospholipase C) (PLC-gamma-1) (phospholipase C-gamma-1) (PLC-II) (PLC-148)_PLCG1_764_776_P19174	$2.18E-07$	$1.94E-05$
Protein tyrosine kinase 2 beta (EC 2.7.10.2) (focal adhesion kinase 2) (FADK 2) (proline-rich tyrosine kinase 2) (cell adhesion kinase beta) (CAK beta) (calcium-dependent tyrosine kinase) (CADTK) (related adhesion focal tyrosine kinase) (RAFTK)_FAK2_572_584_Q14289	$2.55E-07$	$2.27E-05$
Linker for activation of T-cells family member 1 (36 kDa phospho-tyrosine adapter protein) (pp36) (p36-38)_LAT_249_261_O43561	$2.63E-07$	$2.34E-05$
Mast/stem cell growth factor receptor kit (EC:2.7.10.1), CD117_KIT_930_942_C942S_P10721	$2.91E-07$	$2.59E-05$
Tyrosine-protein kinase ZAP-70 (EC 2.7.10.2) (70 kDa zeta-associated protein) (syk-related tyrosine kinase)_ZAP70_313_325_P43403	$2.92E-07$	$2.60E-05$
Cyclin-dependent kinase 1 (EC:2.7.11.22, EC:2.7.11.23), cell division protein kinase 1, cell division control protein 2 homolog, p34 protein kinase (CDK1)_CDK1_9_21_P06493	$2.94E-07$	$2.61E-05$
Erythropoietin receptor precursor (EPO-R)_EPOR_361_373_P19235	$4.82E-07$	$4.29E-05$
1-Phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2 (EC:3.1.4.11) (phosphoinositide phospholipase C-gamma-2) (PLC-IV) (phospholipase C-gamma-2) (PLC-gamma-2)_PLCG2_1191_1203_C1200S_P16885	$5.38E-07$	$4.79E-05$
Platelet endothelial cell adhesion molecule precursor (PECAM-1) (EndoCAM) (GPIIA′) (CD31 antigen)_PECA1_708_718_P16284	$5.64E-07$	$5.02E-05$
Platelet endothelial cell adhesion molecule precursor (PECAM-1) (EndoCAM) (GPIIA′) (CD31 antigen)_PECA1_706_718_P16284	$5.64E-07$	$5.02E-05$
Cyclin-dependent kinase 2 (EC:2.7.11.22) cell division protein kinase 2 (EC 2.7.11.22) (p33 protein kinase)_CDK2_8_20_P24941	$6.07E-07$	$5.40E-05$

Table 1: Continued.

Phosphosites	p value	Bonferroni-adj. p value
RAC-alpha serine/threonine-protein kinase (EC:2.7.11.1) (PKB, RAC)_ AKT1_320_332_P31749	$8.65E-07$	$7.70E-05$
Ephrin type-A receptor 7 precursor (EC 2.7.10.1) (tyrosine-protein kinase receptor EHK-3) (EPH homology kinase 3) (receptor protein-tyrosine kinase HEK11)_ EPHA7_607_619_Q15375	$8.96E-07$	$7.98E-05$
Vascular endothelial growth factor receptor 2 precursor (EC 2.7.10.1) (VEGFR-2) (kinase insert domain receptor) (protein-tyrosine kinase receptor Flk-1) (CD309 antigen)_VGFR2_989_1001_P35968	$1.15E-06$	0.00010243
Myelin protein zero-like protein 1_MPZL1_236_246_O95297	$1.16E-06$	0.00010287
Tyrosine-protein phosphatase nonreceptor type 6 (EC:3.1.3.48)_ PTN6_558_570_P29350	$1.30E-06$	0.00011555
Proto-oncogene tyrosine-protein kinase FER (EC 2.7.10.2) (p94-FER) (c-FER) (tyrosine kinase 3)_FER_707_719_P16591	$1.47E-06$	0.00013057
Epidermal growth factor receptor precursor (EC 2.7.10.1) (receptor tyrosine-protein kinase ErbB-1)_EGFR_1165_1177_P00533	$1.68E-06$	0.00014992
Paired mesoderm homeobox protein 2 (PRX-2) (paired-related homeobox protein 2)_PRRX2_202_214_Q99811	$1.84E-06$	0.00016357
3-Phosphoinositide-dependent protein kinase 1 (EC 2.7.11.1) (hPDK1)_ PDPK1_2_14_O15530	$2.07E-06$	0.00018412
Receptor tyrosine-protein kinase erbB-2 precursor (EC 2.7.10.1) (p185erbB2) (C-erbB-2) (neu proto-oncogene) (tyrosine kinase-type cell surface receptor HER2) (MLN 19) (CD340 antigen)_ERBB2_870_882_P04626	$2.73E-06$	0.00024328
T-cell surface glycoprotein CD3 epsilon chain, T-cell surface antigen T3/Leu-4 epsilon chain, CD3e_CD3E_182_194_P07766	$2.84E-06$	0.00025288
3-Phosphoinositide-dependent protein kinase 1 (EC 2.7.11.1) (hPDK1)_ PDPK1_369_381_O15530	$3.02E-06$	0.00026873
Hepatocyte growth factor receptor precursor (EC 2.7.10.1) (HGF receptor) (scatter factor receptor) (SF receptor) (HGF/SF receptor) (Met proto-oncogene tyrosine kinase) (c-Met)_MET_1227_1239_P08581	$3.42E-06$	0.0003046
Ras GTPase-activating protein 1 (GTPase-activating protein) (GAP) (Rasp21 protein activator) (p120GAP) (RasGAP)_RASA1_453_465_P20936	$4.01E-06$	0.00035652
B- and T-lymphocyte attenuator, B- and T-lymphocyte-associated protein, CD272_BTLA_252_262_Q7Z6A9	$4.20E-06$	0.00037375
40S ribosomal protein S6 (phosphoprotein NP33)_RS6_228_240P62753	$1.57E-05$	0.00139465
Early E1A 32 kDa protein_E1A_ADE05_212_224P03255	$2.12E-05$	0.00188822
Tyrosine-protein kinase JAK3 (EC:2.7.10.2) (janus kinase 3) (JAK-3)_ JAK3_974_986_P52333	$2.20E-05$	0.00196214
cAMP-dependent protein kinase type II-beta regulatory subunit_KAP3_107_119P31323	$2.76E-05$	0.00245874
Insulin receptor substrate 1_IRS1_890_902_P35568	$3.07E-05$	0.00272913
Ephrin type-A receptor 2 precursor (EC 2.7.10.1) (tyrosine-protein kinase receptor ECK) (epithelial cell kinase)_EPHA2_765_777_P29317	$7.62E-05$	0.00677916
Proto-oncogene tyrosine-protein kinase LCK (EC 2.7.10.2) (p56-LCK) (lymphocyte cell-specific protein-tyrosine kinase) (LSK) (T-cell-specific protein-tyrosine kinase)_ LCK_387_399_P06239	0.00012233	0.01088757
T-cell surface glycoprotein CD3 zeta chain precursor (T-cell receptor T3 zeta chain) (CD247 antigen)_CD3Z_77_89_P20963	0.00013128	0.01168433
Protein 4.1 (Band 4.1) (P4.1) (EPB4.1) (4.1 R)_41_654_666_P11171	0.0001331	0.01184597
Pleckstrin (platelet p47 protein)_PLEK_106_118P08567	0.00013359	0.01188922
Tyrosine-protein kinase ZAP-70 (EC 2.7.10.2) (70 kDa zeta-associated protein) (syk-related tyrosine kinase)_ZAP70_485_497_P43403	0.00014176	0.01261638
Ephrin type-A receptor 1 precursor (EC 2.7.10.1) (tyrosine-protein kinase receptor EPH)_EPHA1_774_786_P21709	0.00019104	0.01700258
Glycogen synthase kinase-3 beta (EC:2.7.11.26), serine/threonine-protein kinase GSK3B (EC:2.7.11.1)_GSK3B_210_222_C218S_P49841	0.00030404	0.0270594
Receptor tyrosine-protein kinase erbB-2 precursor (EC 2.7.10.1) (p185erbB2) (C-erbB-2) (neu proto-oncogene) (tyrosine kinase-type cell surface receptor HER2) (MLN 19) (CD340 antigen)_ERBB2_679_691P04626	0.00041011	0.0364998
RAF proto-oncogene serine/threonine-protein kinase (EC 2.7.11.1) (Raf-1) (C-RAF) (cRaf)_RAF1_332_344_P04049	0.00051147	0.04552087

phosphorylation levels in all cell lines, but we could not detect any cellular effects of the differential phosphorylation patterns in cell state analysis. Nevertheless, we hypothesize this to be an important factor in MPM patients regarding cisplatin treatment. Resistance mechanisms possibly be challenged by kinase inhibitors, regulating immune response to cisplatin.

Gao et al. found that elevated expression and phosphorylation of AKT by GSK3B and PTEN was correlated with cell viability, migration, and apoptosis, and this might be explained by chemoresistance in breast cancer [35]. In NCI-H2052, we could reduce phosphorylation of AKT1 and GSK3B by cisplatin and therefore could induce apoptosis in this cell line. Cisplatin-treated and untreated NCI-H2452 cells showed no significant changes between phosphorylation of AKT1. Benzothienopyrimidine derivative (BIA-6), an AKT inhibitor, could effectively block the PI3K/AKT pathway in lung cancer cells in a dose-dependent manner and thus increase apoptosis [36]. Based on our data, a possible synergistic effect with platin-based treatment can be suggested. It could be possible that BIA-6 might also improve efficiency of cisplatin in NCI-H2452 cells.

In NCI-H2052, as well as in MRC-5 cells, kinase activity of p38 and ERK1/2 was increased due to cisplatin treatment. Hsieh et al. also assessed increased phosphorylation of ERK1/2 and p38 in nasopharyngeal carcinoma (NPC) and observed this effect by activation of caspases [37]. This confirms to our observation since NCI-H2052 and MRC-5 showed the highest apoptotic response to cisplatin. MSTO-211H, with lower apoptosis rates, showed only a slight increase of activity of p38 and ERK1/2, whereas non-responding NCI-H2452 cells showed decreased p38 kinase activity.

Zhao et al. observed associations between increased apoptosis by high expression of phosphorylated c-Jun N-terminal kinase (JNK) and subsequently elevated expression levels of p53 in ovarian cancer cells during treatment of platinum containing drugs [38]. This supports our observation in NCI-H2052 and MRC-5, showing elevated activity of JNK1/2/3 and decreased phosphorylation of JNK1/3 in cisplatin-resistant NCI-H2452 cells. Bar et al. found the activating transcription factor 3 (ATF3) as an important regulator of cisplatin cytotoxicity, being activated in platin-sensitive lung cancer cells due to cisplatin treatment [39]. In platin-sensitive cells, cisplatin induced activation of JNK and thus ATF3 induction. In their tested resistant cell lines, this JNK induction was missed. In their study, they tested the FDA-approved histone deacetylase inhibitor vorinostat demonstrating synergistic cytotoxicity in lung cancer cell lines Calu-6 and NCI-H23 cells together with cisplatin. As NCI-H2452 cells also show still activity of JNK, it would be interesting to test this histone deacetylase inhibitor also in this cell line.

5. Conclusions

Kinase phosphorylation and activity might play a crucial role in cellular response to cytostatic agents. Cisplatin treatment results in altered phosphorylation patterns in both the MPM cell lines and the lung fibroblast cell line. These alterations have consequences for cell cycle, migration, adhesion, signal transduction, immune modulation, and apoptosis of the cell. Cisplatin-resistant MPM cells showed a clearly distinct phosphorylation pattern compared to cells showing response to cisplatin, indicating a specific sensitivity-profile. Our results indicate that inhibition of AKT1 by, e.g., BIA-6, or, in another approach, induction of p38 or JNK1/3 of the MAPK pathway, might offer positive synergistic effects through induction of an apoptotic response to cisplatin-based treatment and thus potentially enhance patients' clinical outcome.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors state that they have no conflicts of interest to declare.

Authors' Contributions

Robert F. H. Walter and Fabian D. Mairinger contributed equally to the study.

Supplementary Materials

Suppl. Figure 1: MAPK signaling pathway of A: NCI-H2502, B: NCI-H2452, C: MSTO-211H, and D: MRC-5. Suppl. Figure 2: cell cycle pathway of A: NCI-H2502, B: NCI-H2452, C: MSTO-211H, and D: MRC5. Suppl. Figure 3: cancer pathways of A: NCI-H2502, B: NCI-H2452, C: MSTO-211H, and D: MRC-5. Suppl. Figure 4: phosphorylation level of A: ARAF, B: EPHA1, C: EPHA2, and D: EPHA7 in all cell lines. For each cell line, phosphorylation levels are depicted before (medium) and after cisplatin treatment (Cis). Suppl. Figure 5: phosphorylation level of A: KIT, B: PTPN11, C: PIK3R1, and D: PTPN6 in all cell lines. For each cell line, phosphorylation levels are depicted before (medium) and after cisplatin treatment (Cis). Suppl. Figure 6: phosphorylation level of A: KDR, B: EFS, C: AKT1, and D: PTK2B/FAK2 in all cell lines. For each cell line, phosphorylation levels are depicted before (medium) and after cisplatin treatment (Cis). Suppl. Figure 7: score plots and volcano plots of PTK upstream kinase analysis: A: score plot of PTK upstream kinase analysis for NCI-H2052 cells. B: volcano plot of PTK-upstream kinase analysis for NCI-H2052 cells. C: score plot of PTK-upstream kinase analysis for NCI-H2452 cells. D: volcano plot of PTK-upstream kinase analysis for NCI-H2452 cells. E: score plot of PTK-upstream kinase analysis for MSTO-211H cells. F: volcano plot of PTK-upstream kinase analysis for MSTO211H cells. G: score plot of PTK-upstream kinase analysis for MRC-5 cells. H: volcano plot of PTK upstream kinase analysis for MRC-5 cells. Suppl. Figure 8: score plots and volcano plots of STK upstream kinase analysis: A: score plot of STK upstream kinase analysis for NCI-H2052 cells. B: volcano

plot of STK upstream kinase analysis for NCI-H2052 cells. C: score plot of STK upstream kinase analysis for NCI-H2452 cells. D: volcano plot of STK upstream kinase analysis for NCI-H2452 cells. E: score plot of STK upstream kinase analysis for MSTO-211H cells. F: volcano plot of STK upstream kinase analysis for MSTO211H cells. G: score plot of STK upstream kinase analysis for MRC-5 cells. H: volcano plot of STK upstream kinase analysis for MRC-5 cells. Suppl. Figure 9: kinase-tree of A: NCI-H2052, B: MSTO-211H, C: NCI-H2452, and D: MRC-5. Suppl. Table 1: p values of the influenced phosphosites during cisplatin therapy. Suppl. Table 2: significantly altered phosphorylation levels between the different cell lines. Suppl. Table 3: association of phosphosite phosphorylation ("+" = high and "−" = low phosphorylation) to apoptosis ratio of cells after cisplatin treatment. (*Supplementary Materials*)

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Review Article

mTOR-Mediated Antioxidant Activation in Solid Tumor Radioresistance

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Radiotherapy is widely used for the treatment of cancer patients, but tumor radioresistance presents serious therapy challenges. Tumor radioresistance is closely related to high levels of mTOR signaling in tumor tissues. Therefore, targeting the mTOR pathway might be a strategy to promote solid tumor sensitivity to ionizing radiation. Radioresistance is associated with enhanced antioxidant mechanisms in cancer cells. Therefore, examination of the relationship between mTOR signaling and antioxidant mechanism-linked radioresistance is required for effective radiotherapy. In particular, the effect of mTOR signaling on antioxidant glutathione induction by the Keap1-NRF2-xCT pathway is described in this review. This review is expected to assist in the identification of therapeutic adjuvants to increase the efficacy of radiotherapy.

1. Introduction

Ionizing radiation is a therapeutic method that can induce cell death in tumors through direct or indirect damage, such as covalent bond breakage and reactive oxygen species (ROS) accumulation in cells [1–3]. Although radiotherapy efficacy is somewhat different depending on tumor type, excellent outcomes have been shown in many clinical reports [4]. Therefore, approximately 50% of patients with solid tumors undergo radiotherapy [5]. However, radioresistance has been observed in several types of tumors, including melanoma [6, 7]. In general, radiation upregulates radioresistant genes in tumor cells [8]. These upregulated genes are closely related to survival mechanisms, such as DNA repair, metabolic changes, tumor recurrence, and malignant transformation [9]. Thus, controlling the signals associated with radiation-induced resistant genes might be a major strategy in radiotherapy to reduce the irradiation burden in the host [10, 11]. For this reason, the discovery of adjuvants targeting radioresistant molecules is important for effective radiotherapy [12, 13].

mTOR is a major signaling protein that can affect cell survival through interaction with other cellular signal cascades. mTOR, which is a serine-threonine kinase, can form two distinct multiprotein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [14]. mTORC1 consists of five proteins, namely, mammalian target of rapamycin (mTOR), regulatory-associated protein of mTOR (Raptor), proline-rich AKT substrate 40 kDa (PRAS40), DEP-domain-containing mTOR-interacting protein (Deptor), and mammalian lethal with Sec13 protein 8 (mLST8) [15]. In general, mTORC1 activation is known to be regulated by energy status, amino acids, oxygen conditions, and various growth factors (Figure 1) [16]. Once mTORC1 is activated, lipid synthesis, protein synthesis, mitochondrial metabolism, and microtubule organization can be induced in cells [17]. However, autophagy is actually inhibited by mTORC1 as indicated in Figure 1. Sterol regulatory element-binding protein 1 (SREBP1) can stimulate lipid synthesis, but it is suppressed by lipin 1 in the nucleus [18]. However, activated mTORC1 promotes SREBP1-mediated lipid synthesis through inhibiting lipin 1

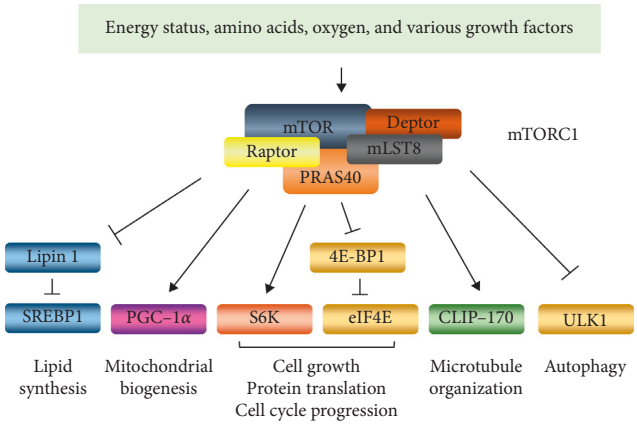

Figure 1: The functions of mTOR complex 1 (mTORC1). Intrinsic or extrinsic stimulators, such as energy levels, amino acids, oxygen levels, and various growth factors, induce mTORC1 activation. Once mTORC1 signaling is activated, lipid synthesis, mitochondrial biogenesis, and protein synthesis are promoted in the cells. mTORC1 also organizes microtubules and suppresses autophagy.

translocation into the nucleus by inducing its phosphorylation [19]. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) controls mitochondrial biogenesis and promotes mitochondrial metabolism [20]. In addition, PGC-1α regulates ROS generated during mitochondrial oxidative phosphorylation (OXPHOS) [21]. mTORC1 activates these functions of PGC-1α [22]. Furthermore, mTORC1 not only promotes microtubule organization through cytoplasmic linker protein-170 (CLIP-170) phosphorylation but also controls protein synthesis via the ribosomal protein S6 kinase beta-1 (S6K1) and the eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) [23, 24]. Moreover, mTORC1 inhibits Unc-51-like autophagy activating kinase 1- (ULK1-) mediated autophagy [25]. Because mTOR is connected to networks formed by various signaling pathways in the cell survival process, mTOR signaling is important in tumor studies.

The PI3K/AKT/mTOR pathway is associated with tumorigenesis, metastasis, and tumor therapeutic resistance [26]. In particular, the survival of tumor patients is affected by upregulated PI3K/AKT/mTOR cascade activity. mTOR phosphorylation was observed in 64.1% of tumor tissues obtained from gallbladder cancer (GBC) patients, and mTOR phosphorylation levels were associated with poor prognosis in these patients [27]. The poor survival of human hepatocellular carcinoma (HCC) patients was related to enhanced levels of phospho-AKT (491/528; 92.99%) and phospho-S6 (466/528; 88.26%) in their tumor tissues [28]. The induction of mTOR activation was also associated with poor prognosis in solid tumor patients, such as breast cancer, melanoma, gastric cancer, and urothelial carcinoma [29–32]. Although enhanced mTOR signaling activation has been reported to not be associated with the survival of some tumor patients, targeting mTOR signaling is still a reasonable approach for tumor radiotherapy [33–36]. Any protein in the PI3K/AKT/mTOR pathway can be targeted for the clinical inhibition of mTOR activation in tumor tissues [37, 38]. In particular, clinically targeting mTOR signaling during radiotherapy could increase tumor radiosensitivity [39]. Sirolimus analogs (rapalogs and mTOR inhibitors), such as everolimus, temsirolimus, and ridaforolimus, not only improved radiotherapy efficiency but also reduced tumor recurrence [40–43]. These previous studies implicate that blocking mTOR signaling in tumors might be an effective strategy in radiotherapy.

High levels of antioxidant molecules in tumors have been identified as factors that make antitumor therapy difficult in many previous studies [44, 45]. Therefore, targeting antioxidant mechanisms might be a good therapeutic strategy for efficient radiotherapy [46]. Recent studies have reported that antioxidant mechanisms can be regulated by mTOR signaling [47–49]. In this review, the effects of mTOR inhibition on the antioxidative Keap1-NRF2 pathway in solid tumors during radiotherapy and the underlying mechanisms are assessed.

2. mTOR Activates Antioxidant Defense Mechanisms in Radioresistant Solid Tumors

2.1. Mitochondrial ROS Generation and the KEAP1-NRF2 Pathway. NADH generated during glycolysis and the tricarboxylic acid (TCA) cycle transfers two electrons to complex I (NADH: ubiquinone oxidoreductase) in the mitochondrial matrix [50]. $FADH_2$ produced in the TCA cycle also transfers electrons to complex II (succinate dehydrogenase) [51]. Electrons from complexes I and III are subsequently transferred to ubiquinone, complex III (coenzyme Q cytochrome C reductase), and complex IV (cytochrome c oxidase) [52]. Complex IV transfers electrons to oxygen, and then, reduced oxygen forms water via a reaction with two hydrogen ions [53]. H^+ ions are pumped into mitochondrial intermembrane spaces through complexes I, III, and IV during electron transfer processes, and a hydrogen ion gradient is generated around the inner mitochondrial membrane [54]. ATPs are synthesized using the energy induced by H^+ ions entering into the intermembrane spaces through complex V (ATP synthase) [55]. Electrons leaked from complexes I and III partially reduce oxygen to form ROS, such as superoxide during OXPHOS [56]. Approximately 90% of intracellular ROS are estimated to be produced during OXPHOS in mitochondria [57]. Proper ROS levels stimulate cell proliferation, mediate signal cascades, and initiate immune responses, but excessive ROS levels lead to cell death [58]. Therefore, when ROS are induced, the cell activates antioxidant mechanisms for homeostasis [59]. Once superoxide is produced in mitochondria, manganese superoxide dismutase (MnSOD/SOD2) converts it to hydrogen peroxide [60]. Hydrogen peroxide, one of the ROS, must be converted to water. Glutathione (GSH) and thioredoxin (TRX) systems controlled by the Kelch-like ECH-associated protein 1-nuclear factor erythroid 2-related factor 2 (KEAP1-NRF2) pathway are required for this chemical change (Figure 2) [61]. Intracellular oxidative stress can directly and immediately promote the expression of antioxidant enzymes via the KEAP1-NRF2 pathway [62]. KEAP1 induces NRF2 degradation through its polyubiquitination on the NRF2-ECH

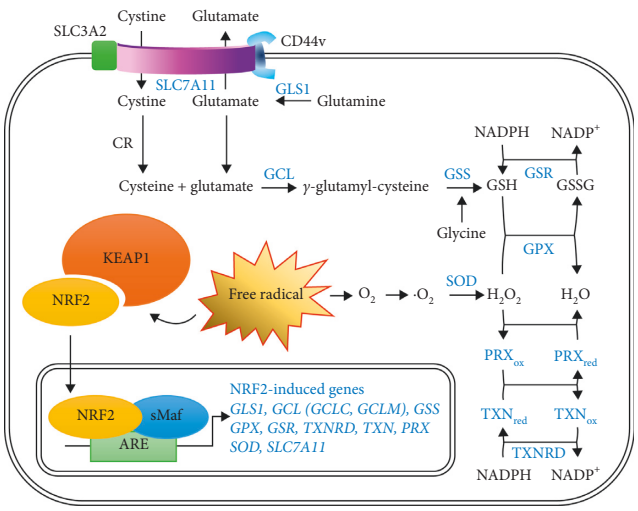

Figure 2: NRF2-mediated pathway of glutathione synthesis. The expression of antioxidant proteins (blue) can be induced by the Keap1-NRF2 pathway. Cystine introduced into the cytoplasm via xCT (SLC7A11) is converted to cysteine by cystine reductase (CR). Glutamine is converted to glutamate by glutaminase 1 (GLS1). Glutamate and cysteine bind and are transformed to γ-glutamyl-cysteine by glutamate-cysteine ligase (GCL). Glutathione synthetase (GSS) induces glutathione (GSH) synthesis via inducing a covalent bond between γ-glutamyl-cysteine and glycine. Glutathione peroxidase (GPX) oxidizes GSH to GSSG with H_2O_2 reduction to H_2O. GSSG can then be reduced by glutathione reductase (GSR). In addition to the GSH system, the thioredoxin (TRX) system (thioredoxin reductase (TXNRD), thioredoxin (TXN), and peroxiredoxin (PRX)) also comprises NRF2-mediated antioxidant proteins.

homology-like domain 2 (Neh2) domain of NRF2 in cells under normal conditions [63]. However, when intracellular ROS are induced, ROS oxidize cysteine residues in the intervening region (IVR) of KEAP1, and structurally modified KEAP1 is detached from NRF2 [64]. Free NRF2 translocated into the nucleus forms a heterodimer with small musculoaponeurotic fibrosarcoma (sMaf), and the transcriptional factors then bind to antioxidant response elements (AREs) to promote antioxidant gene expression [65]. SLC7A11 (xCT) forms a complex known as the xCT system, with CD98 (SLC3A2 or 4F2) and the CD44 variant isoform (CD44v) [66]. CD98 and CD44v contribute to the stability of the xCT system; in particular, CD44v helps to locate xCT on the cell surface [67]. The xCT system, a cystine-glutamate antiporter, mediates the influx of extracellular cystine and the efflux of glutamate transformed by glutaminase 1 (GLS1) from glutamine [68]. One cystine molecule transported into the cell is reduced to two cysteine molecules by cystine reductase (CR) [69]. Cysteine and glutamate are synthesized to γ-glutamyl-cysteine via heterodimeric glutamate-cysteine ligase (GCL) consisting of GCLC (catalytic subunit) and GCLM (modulating subunit), and the ligated product is then converted to glutathione via the glutathione synthetase-(GSS-) mediated addition of glycine [70]. Glutathione peroxidase (GPX) promotes H_2O_2 reduction to H_2O during the oxidation of GSH (reduced form) to glutathione disulfide (GSSG, oxidized form) [71]. Oxidized GSSG is returned to the reduced form of GSH by glutathione reductase (GSR) [72]. In addition to the GSH system, NRF2 induces gene expression of the TRX system [73]. Thioredoxin reductase (TXNRD) oxidizes NADPH to $NADP^+$ to reduce the oxidized form of thioredoxin (TXN) [74]. The reduced TXN induces H_2O_2 reduction to H_2O through the redox reaction of peroxiredoxin (PRX) [75]. Thus, the KEAP1-NRF2 pathway can be regarded as an essential mechanism for antioxidant defense.

2.2. mTOR-Dependent Antioxidant Mechanism in Solid Tumors. ROS can be controlled by NRF2-mediated antioxidant mechanisms in normal cells, consequently maintaining cellular homeostasis [76]. However, in various solid tumors, because antioxidant mechanisms are activated at higher levels than in normal cells, tumor cells can be more tolerant to excessive ROS levels than normal cells [77]. NRF2-dependent antioxidant enzymes such as SOD, glutathione peroxidase (GPX), glutathione reductase (GSR), peroxiredoxin (PRX), and thioredoxin reductase (TXNRD) are upregulated in tumor cells, and high expression of these proteins is associated with poor prognosis in tumor patients (Table 1) [78–83]. Mitochondrial SOD2 was shown to be highly expressed in ovarian cancer patients and contribute to antitumor therapy resistance [84]. Non-small-cell lung cancer (NSCLC) cells overexpressing GPX1 were resistant to cisplatin-induced ROS through PI3K/AKT pathway activation [85]. In pancreatic cancer cells, GPX4 was shown to be essential for the maintenance of stemness, and PRX1 was required for p38-mediated invasion [86]. A high level of PRX2 in colorectal cancer patients was associated with tumor progression and poor diagnosis [87]. Inhibition of GSR and TXNRD attenuated tumor growth in an NSCLC patient-derived xenograft model [88]. High γ-glutamylcysteine synthetase (γ-GCS) activity in human HCC cells was related to radioresistance [89]. In general, radiation promotes GSH synthesis [90]. Because rapamycin and everolimus can affect GSH synthesis, the use of mTOR inhibitors might increase the effectiveness of radiotherapy [91, 92]. In addition to antioxidant enzymes, the NRF2-dependent xCT system could promote the radioresistance of solid tumors. xCT was related to radioresistance in human breast cancer and mouse melanoma cell lines [93, 94]. CD98 contributed to radioresistance in head and neck squamous cell carcinoma [95]. CD44v induced radioresistance in human pancreatic cancer cells via increasing xCT stability [96]. These results suggest that antioxidant proteins controlled by the KEAP1-NRF2 pathway could be factors that make tumor therapy difficult. Therefore, targeting the KEAP1-NRF2 pathway might be a strategy to improve the efficacy of radiotherapy. In a recent study, xCT was reported to be modulated by mTOR signaling in human melanoma subjected to radiation [97]. The transcriptional activity of NRF2 can be regulated by mTOR inhibitors. Temsirolimus suppressed NRF2 translocation into the nucleus in RCC4 cells, a human renal cell carcinoma (RCC) cell line [98]. Everolimus reduced the phosphorylation of NRF2 in ARPE-19 adult retinal pigment epithelial cells [99]. In general, KEAP1 is important for the

Table 1: Glutathione synthesis-related proteins in the poor prognosis of tumor patients.

	Type of cancer	Level	Effects	Ref.
Glutathione peroxidase (GPX)	Breast cancer	High	Metastasis↑ Patient mortality↑	[80]
	Bladder cancer	High	Recurrence↑	[91]
	Oral squamous cell carcinoma	High	Metastasis↑ Poor survival↑	[92]
	Gastric carcinoma	High	Metastasis↑ Progression↑	[108]
	Hepatocellular carcinoma	High	Poor prognosis↑ Recurrence↑	[109]
Glutathione reductase (GR)	Glioblastoma	High	Drug resistance↑ Poor survival↑	[81]
Glutamate-cysteine ligase (GLC)	Hepatocellular carcinoma	High	Progression↑ Drug resistance↑ Poor survival↑	[110]
	Breast cancer	High	Drug resistance↑	[111]
	Melanoma	High	Malignancy↑	[112]
	Lung adenocarcinoma	High	Recurrence↑ Poor survival↑	[113]
Glutaminase 1 (GLS1)	Colorectal cancer	High	Metastasis↑ Poor prognosis↑ Poor survival↑	[114]
	Hepatocellular carcinoma	High	Malignancy↑ Poor prognosis↑	[115]
	Breast cancer	High	Cancer stem cells In HER2-type↑	[116]
SLC7A11 (xCT)	Colorectal cancer	High	Recurrence↑ Poor survival↑	[117]
	Non-small-cell lung carcinoma	High	Poor survival↑	[118]
	Glioma	High	Poor survival↑	[119]
	Hepatocellular carcinoma	High	Poor survival↑	[120]
	Breast cancer	High	Poor prognosis↑	[121]

transcriptional regulation of NRF2. Intracellular ROS promote NRF2 activity through the structural transformation of KEAP1 [64]. KEAP1 can also be dissociated from NRF2 by mTORC1. mTORC1 phosphorylates serine 351 in the Keap1-interacting region (KIR) of sequestosome 1 (SQSTM1/p62) [100]. Phosphorylated SQSTM1/p62 promotes KEAP1 degradation during selective autophagy [101]. Degradation of KEAP1 via autophagy activation does not necessarily occur in an mTOR-dependent manner [102, 103]. Moreover, mRNA expression of NRF2 is dependent on the transcriptional activity of eIF4F [104]. Activated mTORC1 signaling disturbs the inhibitory effect of 4E-BP1 on eIF4E through the phosphorylation of 4E-BP1 [105]. In other words, mTORC1 signaling activation could mediate NRF2 expression. Tuberous sclerosis complexes 1 and 2 (TSC1 and TSC2), also known as hamartin and tuberin, respectively, attenuate mTORC1 activity via inhibiting Ras homolog enriched in brain (Rheb) [106]. In a TSC1-null bladder cancer xenograft model, not only mTORC1 hyperactivation but also upregulated NRF2, GCLM, GCLC, and GSR expressions were observed [107]. Overall, previous studies suggest that the antioxidant mechanism could be mediated by NRF2 and that NRF2 expression and activation could be dependent on mTOR signaling (Figure 3).

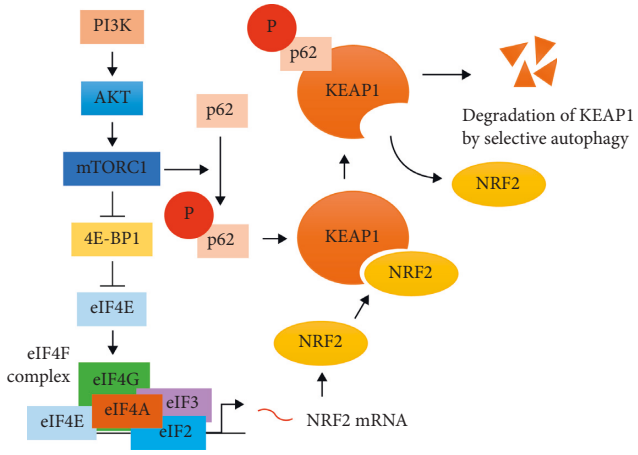

Figure 3: The effect of mTORC1 on NRF2 expression and activation. The activation of mTORC1 promotes the transcriptional activation of eIF4F. The eIF4F complex can induce NRF2 mRNA expression. NRF2 translocation into the nucleus is restricted because of its attachment to KEAP1. However, mTORC1 activation can induce the phosphorylation of p62 (at serine 351) and promote NRF2 translocation into the nucleus through p62-mediated KEAP1 degradation.

2.3. The Regulation of Antioxidant Defense via mTOR Inhibition in Solid Tumors Subjected to Radiation. Tumor tissues are formed by the uncontrolled proliferation of cancerous

cells [122]. As tumor tissues develop, it is difficult for some tumor cells to obtain nutrients and oxygen because blood vessels are unevenly distributed in tissues [123]. In particular, OXPHOS and ROS production in mitochondria depend on the supply of oxygen [124]. Therefore, the oxygen levels in the tumor microenvironment should be considered in the regulation of antioxidant defense through mTOR inhibition in solid tumors. Mitochondrial OXPHOS is stably induced in tumor cells under sufficient oxygen supply, but not under pseudo-hypoxic conditions [125]. Although higher ROS levels are induced in tumor cells than in normal cells, ROS levels could be properly controlled through the KEAP1-NRF2 pathway [126]. Because tumor cells are exposed to nutrients and various growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), mTOR signaling activation is promoted in the cells under normoxic conditions [127]. Moreover, radiation promotes not only ROS generation but also OXPHOS. In general, radiation causes mitochondrial damage and ROS production [128]. Irradiated tumor cells might use metabolism through glycolysis rather than OXPHOS because of mitochondrial damage [129]. However, irradiation with a single dose of 5 Gy promoted OXPHOS via mTOR-mediated enzymatic inhibition of hexokinase II in tumor cell lines (breast cancer MCF-7 cells, colon cancer HCT116 cells, and glioblastoma U87 cells) associated with the Warburg effect [130]. This implies that radiation might enhance OXPHOS in rapidly proliferating tumor cells. Thus, the inhibition of mTOR signaling might make tumor cells sensitive to radiation-induced ROS via the attenuation of NRF2-mediated antioxidant mechanisms under normoxic conditions. Unlike normal oxygen conditions, hypoxia can be caused in tumor cells located in tumor tissues with little blood vessel distribution [131]. The levels of OXPHOS and ROS generation could be reduced in tumor cells under persistent hypoxic conditions [132]. Hypoxia promotes the stability of hypoxia-inducible factor-1α (HIF-1α) [133]. HIF-1α induces regulated in development and DNA damage response 1 (REDD1) expression [134]. mTOR activity is completely inhibited in REDD1-overexpressing cells under normoxic conditions, and continuous hypoxia can weaken its activity in tumor cells [135, 136]. Therefore, it might be difficult to expect the regulatory effects of mTOR inhibition on tumor antioxidant defense under extreme hypoxic conditions. However, under these conditions, not only might all mitochondrial OXPHOS, ROS generation, and mTOR activity be reduced but also necrotic cell death could be induced in hypoxic tumor tissues [137]. Therefore, the inhibition of mTOR signaling is expected to make tumor cells sensitive to radiation-induced ROS via KEAP1-NRF2 pathway attenuation, except for in the continuously hypoxic regions in the tumor tissues (Figure 4).

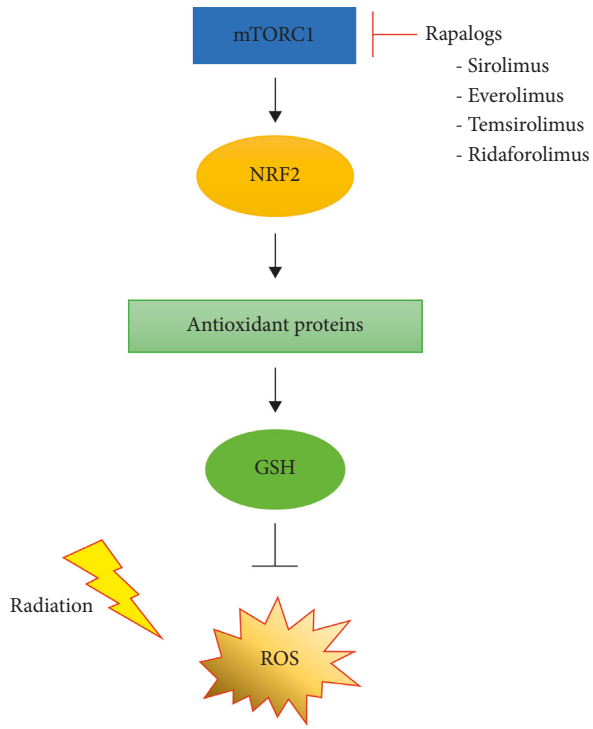

Figure 4: A strategic proposal for enhancing tumor sensitivity to radiation via inhibiting mTOR signaling. Activated mTORC1 signaling in tumors might effectively eliminate radiation-induced ROS through promoting antioxidant mechanisms. However, inhibiting mTOR signaling might attenuate the antioxidant KEAP1-NRF2 pathway. Therefore, blockade of mTOR signaling in tumors might be an alternative strategy to increase tumor susceptibility to radiation.

3. Cell Death Mechanisms Induced by mTOR Inhibition during Radiotherapy

3.1. The Promotion of Cell Death by mTOR Inhibition in Irradiated Tumors. Radiation induces reductions in tumor volumes and inhibits tumor metastasis [138, 139]. mTOR inhibitors, such as rapalogs, have been demonstrated to increase the radiosensitivity of tumors in previous studies. Rapamycin is an efficient drug to enhance radiosensitivity in various tumor types [39, 140]. Everolimus suppressed epithelial-mesenchymal transition (EMT) and angiogenesis [35, 40]. Everolimus also inhibited tumor stemness and recurrence [141, 142]. Therefore, the use of mTOR blockers might be a rational approach when treating tumor patients using radiotherapy. Ionizing radiation mainly causes apoptosis in tumor cells [143]. Various types of cell death including necrosis, apoptosis, pyroptosis, ferroptosis, and autophagy could be induced depending on the properties of adjuvants [144–147]. The inhibition of mTOR signaling induces autophagy [148]. Tumor cells can survive via autophagy under nutrient-depleted conditions, but autophagy can cause cell death in tumors depending on the surrounding environment [149]. Resistance to apoptosis and radiation has been observed in various tumor types [8]. Thus, targeting mTOR signaling can be effective in inducing autophagic cell death in apoptosis-resistant tumors during radiotherapy [150].

3.2. mTOR Inhibition and Ferroptosis in Radiotherapy. Induction of cell death in tumors is an important strategy to promote tumor radiosensitivity. The induction of ferroptosis has recently been reported to improve antitumor therapy in a tumor study [151]. Ferroptosis is a type of cell death with morphology

similar to necrosis, accompanied by excessive iron accumulation and lipid peroxidation [152]. However, unlike necrosis, ferroptosis is a form of regulated cell death mediated by the Fenton reaction [153]. A recent study showed that IFN-γ secreted by $CD8^+$ T cells can induce ferroptosis in tumor cells during immunotherapy with anti-CTLA-4 and anti-PD1 [154]. However, further investigations are needed to determine whether ferroptosis can be induced in tumors to improve radiosensitivity during tumor radiotherapy. The inhibition of mTOR activation can induce autophagy in tumor tissues. Autophagy promotes the degradation of ferritin, which contains Fe^{2+}, thereby increasing the Fe^{2+} level in the cytoplasm [155]. mTOR overexpression suppressed ferroptosis, whereas mTOR depletion enhanced ferroptosis in cardiomyocytes [156]. High levels of NRF2 and GSH inhibited ferroptosis, but mTOR inhibition reduced the inhibitory effects of both NRF2 and GSH on ferroptosis [157]. This indicates that ferroptosis might be associated with the autophagic process [158]. In a recent study, nanoparticles bound to erastin and rapamycin efficiently suppressed tumor growth [159]. Therefore, the inhibition of mTOR signaling might induce ferroptosis as well as autophagy in irradiated solid tumors.

4. Conclusion

Ionizing irradiation should not only reduce tumor volume but also suppress tumor recurrence in tumor radiotherapy. However, because radioresistance is observed in various solid tumors, the use of proper adjuvants is important to effectively treat tumor patients. Adjuvants used in radiotherapy should not only increase the sensitivity of tumors to radiation but also induce optimal cell death. In particular, the induction of regulated cell death (RCD) by adjuvants is imperative in host immunity during radiotherapy. In this review, the effects of mTOR inhibition on tumor radiosensitivity were discussed. Tumor cells can be resistant to ROS through mTOR-mediated antioxidant defense. Thus, the inhibition of mTOR signaling in these tumor types could attenuate the expression of antioxidant enzymes and make tumors sensitive to ROS. In addition, the inhibition of mTOR might induce RCD, such as autophagy and ferroptosis, in tumors during irradiation. There is a lack of validation of the regulatory effects of mTOR inhibition on antioxidant mechanisms during radiotherapy. Thus, further studies on mTOR inhibitors are required for efficient radiotherapy. In this review, the attenuation of the KEAP1-NRF2 pathway through inhibiting mTOR signaling has been suggested as an approach to enhance radiosensitivity.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Whole-Exome Sequencing Characterized the Landscape of Somatic Mutations and Pathways in Colorectal Cancer Liver Metastasis

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Purpose. Liver metastasis remains the leading cause of cancer-related mortality in colorectal cancer. The mechanism of occurrence and development of liver metastasis from colorectal cancer is unclear. *Methods*. The primary tumor tissues and blood samples of 8 patients with liver metastasis of colorectal cancer were collected, followed by nucleic acid extraction and library construction. Whole-exome sequencing was performed to detect the genomic variations. Bioinformatics was used to comprehensively analyze the sequencing data of these samples, including the differences of tumor mutation burden, the characteristics of gene mutations, and signaling pathways. *Results*. The results showed that the top three genes with the highest mutation frequency were *TP53*, *APC*, and *KRAS*. Tumor mutation burden of this study, with a median of 8.34 mutations per MB, was significantly different with The Cancer Genome Atlas databases. Analysis of molecular function and signaling pathways showed that the mutated genes could be classified into five major categories and 39 signaling pathways, involving in Wnt, angiogenesis, P53, Alzheimer disease-presenilin pathway, notch, and cadherin signaling pathway. *Conclusions*. In conclusion, we identified the extensive landscape of altered genes and pathways in colorectal cancer liver metastasis, which will be useful to design clinical therapy for personalized medicine.

1. Introduction

Colorectal cancer (CRC) is the third most common type of malignancy and leading cause of cancer-related death worldwide [1]. Metastasis is still the main cause of cancer-related morbidity, and mortality of colorectal cancer due to liver metastasis accounts for about 25% [2]. Although, early detection and prevention or surgical resection of primary and metastatic lesions can reduce the risk of CRC and improve survival of CRC [3–5], metastatic CRC is still the leading cause of cancer-related deaths, and treatment options are not as selective.

A previous study suggested that the frequency rates of mutations such as *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* in CRC differ among population [6]. *AMER1* is a frequently mutated gene in CRC comprising 553 samples [7]. *TMEM9*, as a novel human transmembrane protein, transactivated by β-catenin functions as a positive feedback regulator of WNT signaling in CRC and mTOR signaling, has been suggested to be an important factor involved in tumorigenesis [8, 9]. Therefore, a better understanding of the biological and phenotypic evolution of CRC and its molecular and genetic mechanisms during the transfer process is crucial.

To further investigate the genetic characteristics of colorectal cancer liver metastasis (CRLM), we performed whole-exome sequencing (WES) in 8 patients with CRLM. Somatic mutations, tumor mutation burden (TMB), molecular functions of mutational genes, and signaling pathways were analyzed. It is expected to provide clinical help for the treatment of patients with liver metastasis from colorectal cancer.

2. Patients and Methods

2.1. Patient Specimen Acquisition. Blood and primary CRC tumor tissue samples were collected from 8 patients with CRLM in the Oncology Department of the First Affiliated Hospital of Xiamen University during the period May 2018 to November 2018. Informed written consent was obtained from all patients before inclusion in the study. Respective tumor tissue samples which were a histologically confirmed adenocarcinoma by two molecular pathologists matched with the inclusion criteria. The study was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Committee of the First Affiliated Hospital of Xiamen University [10].

2.2. DNA Extraction. DNA was extracted from serial sections cut from tumor samples and matched peripheral blood leukocytes as germline DNA control. The cases with tumor cell populations were estimated by pathologists to ensure more than 70% of cells were tumor cells. The DNA was isolated from the FFPE and blood samples using the DNeasy Blood and Tissue Kit (69504, QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. DNA quantity was assessed by using Agilent's Bioanalyzer (USA).

2.3. Whole-Exome Sequencing and Data Processing. The targeted capture pulldown and exon-wide libraries from genomic DNA were generated through the xGen® Exome Research Panel (Integrated DNA Technologies, Inc., Illinois, USA) and the TruePrep DNA Library Prep Kit V2 for Illumina (#TD501, Vazyme, Nanjing, China). The sequences of captured libraries were performed as pair-end reads on sequences on the Illumina HiSeq 2500 platform. Sequencing reads were processed and mapped to the reference GRCh37/hg19 human genome assembly and to the identified germline variations. Further local rearrangements were performed to improve the alignment of individual reads [11].

2.4. Variant Annotation and Mutation Signature Analysis. Somatic mutations identification and indels were annotated through Mutect [12] and Somatic Indel Detector [13]. The variant data were annotated using ANNOVAR [14] and Oncotator [15] and converted to MAF files by maf tools [16]. The cancer driver genes were analyzed using Intogen [17], including Oncodrive FM and Oncodrive CLUST. Both tools detect signals of positive selection, which appear in genes whose mutations are selected during tumor development and are therefore likely drivers. The landscape of top driver mutation spectrum predicted by Intogen for tumors was visulized via R Script, including mutation rate and mutation subclass/subtypes (filtering cutoff, ONCODRIVE FM P value ≤ 0.1).

Table 1: Patient characteristics.

Characteristic	No. of cases	Proportion (%)
Total number	$n = 8$	
Age, years (mean)	66.6 (46–83)	
Sex		
Male	5	62.5
Female	3	37.5
Smoking history		
Smoker	1	12.5
Nonsmoker	7	87.5
Drinking	1	12.5
Metastsis	7	87.5
Anatomical classification		
Right hemicolon	2	25.0
Left hemicolon	6	75.0
Stage		
IV	8	100
Chemotherapy	8	100

2.5. Statistical Analyses. All the correlate clinical and biological variables were employed using the SPSS Statistics 22.0 package and ggpubr package [18] in R [19] by means of Fisher's test or a nonparametric test when necessary. The Kruskal–Wallis test was used to analyze whether TMB differ between different data sets.

3. Results

3.1. Patient Characteristics. We collected tumor tissue and matched blood from 8 patients with CRLM at the time of diagnosis, including 5 males and 3 females, with an average age of 66.6 years (range, 46–83 years). One of the patients was a former smoker, and the other seven were nonsmokers. Additionally, one male patient was also alcoholic. According to the anatomical classification system, 75.0% (6/8) of samples were classified as left hemicolon carcinoma, and the other 2 patients were right hemicolon carcinoma. All the patients were in stage IV and treated with chemotherapy. 37.5% (3/8) of the patients had a history of chronic disease, including diabetes, hypertension, coronary heart disease, and hyperuricemia. No patients received radiation therapy before surgery. The detailed clinical characteristics of the patients are shown in Table 1 and Supplemental Table 1.

3.2. Whole-Exome Sequencing and Identification of Somatic Mutations. We performed WES on DNA from 8 tumor tissues along with blood matched and then analyzed successfully with a mean depth of 244x. Somatic mutations were identified by comparing significant changes in nonreference alleles in the tumor and control groups. Overall, 1151 nonsynonymous single nucleotide variants (SNV) were

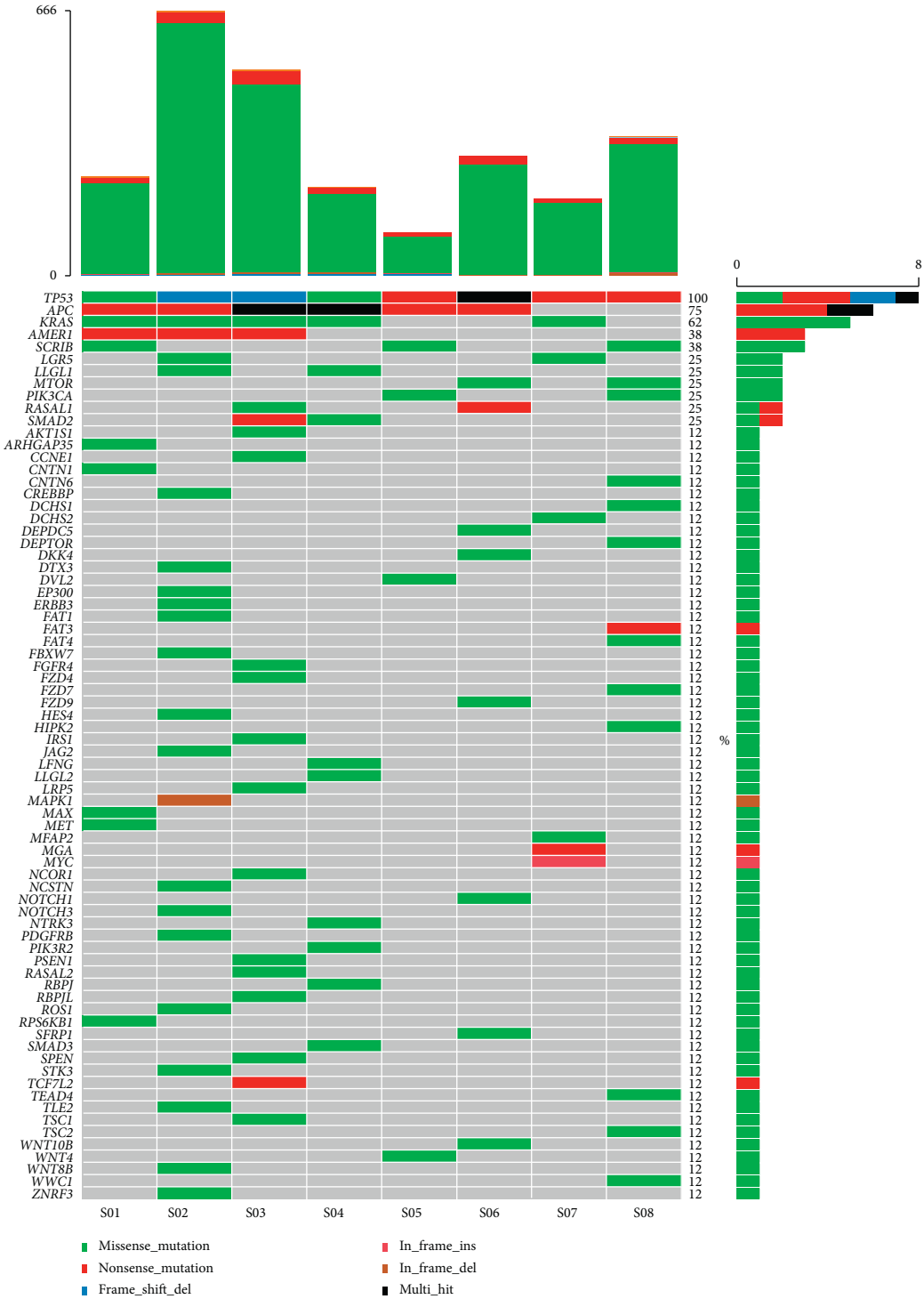

Figure 1: Landscape of somatic mutations in CRLM. The different colored tables represent different types of mutations (middle bars). We also calculated somatic mutations SNV using only somatic nonsynonymous mutations sequenced with WES for each sample (top bars), and the right bars represent the absolute number of mutations observed per gene across all samples.

identified (Supplemental Table 2). An overview of the whole-exome sequencing results and the algorithm-generated arm-length copy number alterations are shown in Figure 1. Each gene with a nonsynonymous SNV was reviewed against known mutations identified in prior studies and subjected to Mutsig analysis. As shown in Figure 1, S02 have the most SNVs, following S03. We listed the top 75 genes based on the frequency of mutations. Among them, *TP53* (100%), *APC*

(75%), and *KRAS* (62%) were the genes with the highest mutation rates. Missense mutation was the most common type of mutation, along with frame shift del, in frame ins, frame del, and so on (Figure 1).

We also calculated TMB using only somatic nonsynonymous mutations sequenced with WES. On the whole, we found that the TMBs of different samples were significantly different, with a median of 8.34 mutations per MB (range, 2.79–17.04 mutations/MB) (Figure 2).

In order to compare the differences in TMB between CRLM and TCGA database (COAD and READ), we used the Kruskal–Wallis nonparametric test to test the anova of multiple groups of data after homogeneity of variance test (therefore, anova cannot be used) and found significant difference between multiple database cohorts ($P = 5.9e - 05$) (Figure 3).

3.3. The Landscape of Mutational Signatures. In principle, all types of mutations (such as substitutions, indels, and rearrangements) and any accessory mutation characteristic, for example, the sequence context of the mutation or the transcriptional chain where the mutation occurred, can be incorporated into the set of features by which a mutational signature is defined.

We extracted mutational signatures using base substitutions, and six classes of substitutions (C > A, C > G, C > T, T > C, T > A, and T > G) were referred to by the pyrimidine of the mutated Watson–Crick base pair. In this study, the six mutation types were compared with the TCGA database, and it was found that the proportion of these six mutation replacement types was roughly the same. The mutation percent of C > T was the highest in all substitutions, and this study has no significant difference with COAD and READ in this substitution. T > G substitution have significant difference between CRLM with COAD and READ (Figures 4(a) and 4(b)).

3.4. CRLM-Related Gene Molecular Function and Pathway Analyses. In order to further characterize the functions of mutational genes and their involved regulatory pathways, we used PANTHER classification system [20], an Ontology-Based Pathway Database Coupled with Data Analysis Tools. The results showed that molecular functions were divided into five categories, namely, binding, catalytic activity, molecular function regulator, molecular transducer activity, and transcription regulator activity. Of these, the category of binding (40) and catalytic activity (32) have the most function hits (Figure 5).

Through the PANTHER classification system pathway analysis, it was found that 74 pathway-related genes were involved in a total of 39 primarily signaling pathways, among which the pathways with higher frequency were Wnt signaling pathway (P00057), angiogenesis (P00005), P53 pathway (P00059), Alzheimer disease-presenilin pathway (P00004), notch signaling pathway (P00045), and cadherin signaling pathway (P00012). The other involved pathways and the genes involved in each pathway were referred to Figure 6 and Supplemental Table 3.

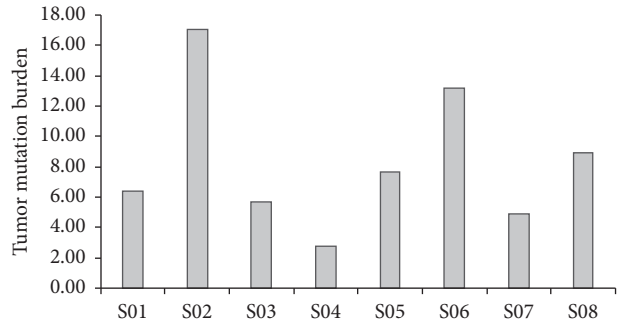

Figure 2: TMB analysis in CRLM patients.

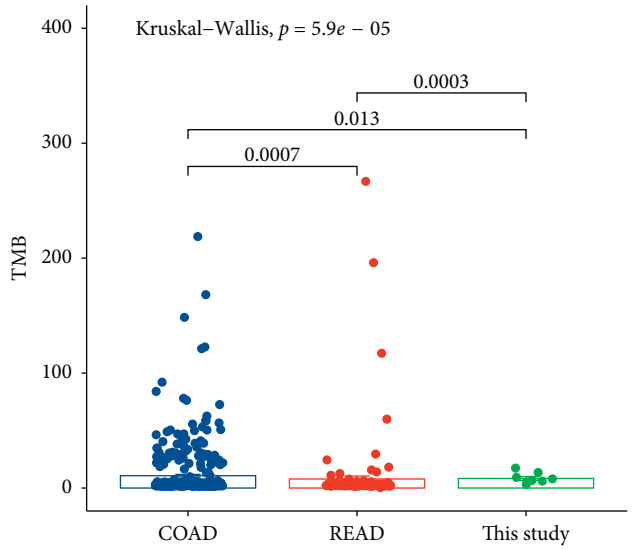

Figure 3: Comparison of TMB between the date in this study and COAD with READ by thwe Kruskal–Wallis test. The primary sites of COAD and READ are colon and rectum.

4. Discussion

CRC is the third most common malignancy in many countries and the second leading cause of cancer death. It develops from benign adenomatous polyp to invasive cancer, and nearly 50% of CRC patients develop into CRLM [21]. Without treatment, the median survival period of patients with colorectal liver metastasis is only 5–10 months, and the survival rate of over 5 years is less than 0.5% [22].

The molecular pathogenesis of CRC is related to a variety of genetic changes that result in abnormal activation of proto-oncogenes and inactivation of tumor suppressor genes [23]. We briefly described the characteristics of the CRLM by WES and had important insights into the genes and mechanisms of cancer occurrence and development. We found 1151 SNVs and the prevailing mutations were *APC*, *KRAS*, and *TP53* (Figure 1, Supplemental Table 2), which is in accordance with data reported by The Cancer Genome Atlas Network [24]. Currently, there are dozens of biomarkers related to checkpoint inhibitors, among which TMB, PD-L1, and MSI/dMMR have been verified by phase III clinical trials and are widely used in clinical practice.

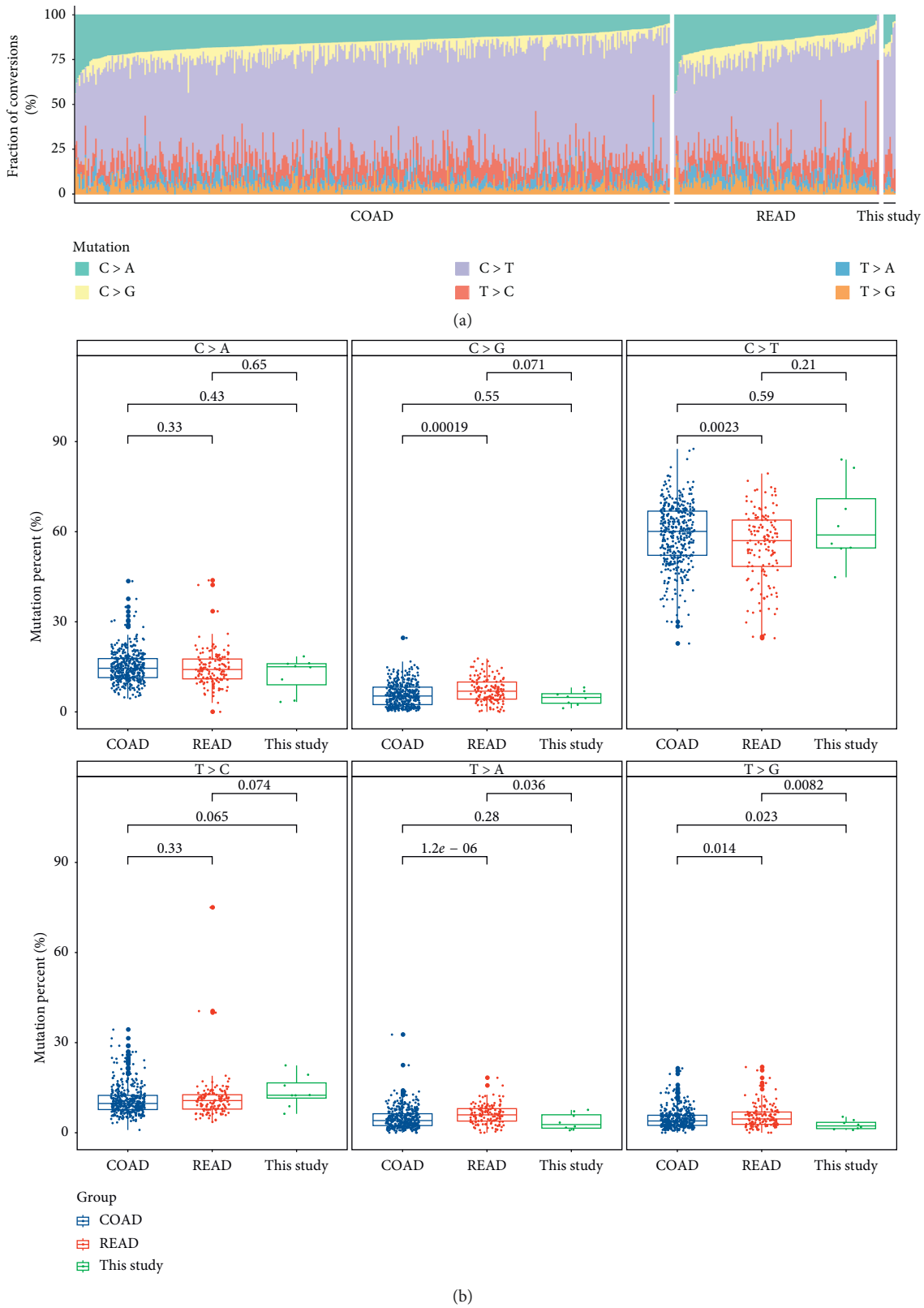

Figure 4: Mutational signature difference in multiple groups. (a) Transition and transversion proportions for six nucleotide changes. The stacked proportion bar chart is sorted by increasing the transition/transversion fraction. (b) Transition/transversion (Ti/Tv) ratios.

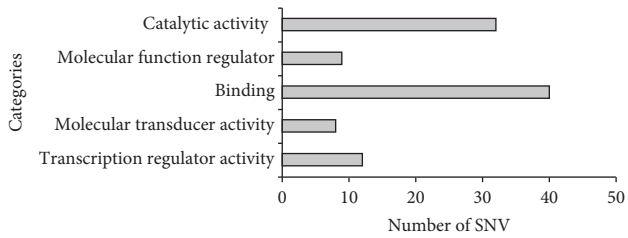

Figure 5: Go-slim molecular function by PANTHER for single nucleotide variants.

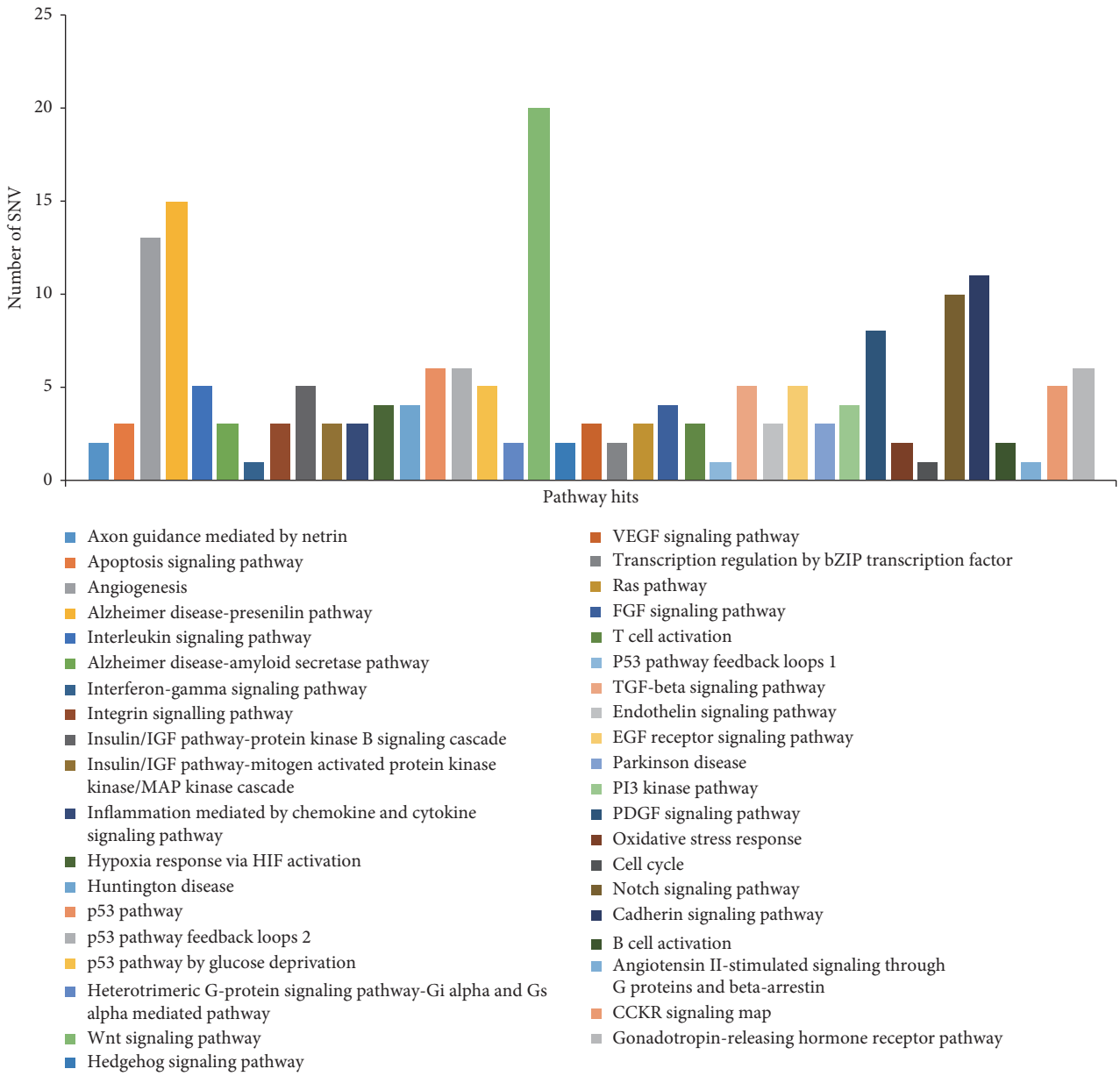

Figure 6: Analysis of pathways involving nonsynonymous genes in CRLM.

Tumor mutation load (TMB) is a new biomarker for predicting PD-1/PD-L1 immune response [25]. Even though it has been reported that TMB ≥20 mutation/Mb (TMB-H) alone is not suitable for predicting the immunotherapy effect of each solid tumor type [26], we found that there was a significant difference in TMB between CRLM and colon and rectum, but the TMB did not exceed 20 mutations per MB (mean 8.34) (Figures 2 and 3). For different cancer types, the

setting of high TMB threshold may need more clinical studies and a large number of patient information statistics.

The signatures can be understood as different mutation processes often generate different combinations of mutation types. Thousands of somatic mutations can be identified in a single cancer sample, making it possible to decipher the mutant signature, even if several mutations are operative [27]. The C > A mutational signature, is associated with smoking and chewing tobacco. Six classes of substitutions were extracted, and there was no significant difference in mutation percent between CRLM and colon with rectum cohorts (Figure 4). The genetic characteristics of liver metastasis may be more similar to that of the primary tumor, and the treatment strategy should be more similar to that of the primary tumor colorectal cancer.

Through pathway analysis, we found that oncogenes represented by *KRAS*, *PIK3CA*, *AKT1*, *PIK3R*, and tumor suppressor genes represented by *TP53*, *APC*, *EP300*, *CREBBP*, and *PIK3R1* were mutated, which may lead to changes in angiogenesis, TGF-β, Wnt signaling pathway, notch signaling pathway, and other pathways (Figure 6, Supplemental Table 3). The pathway is complex, mainly reflected in the fact that one mutation gene is involved in multiple pathways [28], and various pathways are also cross-regulated, such as angiogenesis and notch signaling pathway.

5. Conclusion

Our study identified changes in driver gene mutations, TMB, and base Ti/Tv ratios in CRC with liver metastasis compared with rectal or colon cancer, although our study has some limitations, such as small sample size and lack of matched CRC liver metastasis samples. In conclusion, the current findings help define the genomic landscape of CRLM and identify specific pathways that are frequently altered, providing direction for research of targeted therapies against these tumors.

Data Availability

All the related software and scripts used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors have declared no conflicts of interest.

Authors' Contributions

Liuxing Feng, Shifu Hong, and Jin Gao contributed equally to this work. Liuxing Feng, Shifu Hong, Jin Gao, and Jiayi Li designed the project and devised the experiments. Liuxing Feng, Shifu Hong, and Jin Gao performed the experiments and provided tumor samples and clinical information. Jiayi Li dealt with the figures and prepared the main manuscript. All authors contributed to the discussions and manuscript preparation.

Acknowledgments

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Supplementary Materials

Supplemental Table 1: clinical characteristics of the patients. Supplemental Table 2: the number of SNV in the CRLM samples. Supplemental Table 3: list of pathway genes in CRLM. (*Supplementary Materials*)

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