Identification of Ten Mitosis Genes Associated with Tamoxifen Resistance in Breast Cancer

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Background: Endocrine therapy is the backbone therapy in estrogen receptor α (ER)-positive breast cancer, and tamoxifen resistance is a great challenge for endocrine therapy. Tamoxifen-resistant and sensitive samples from the international public repository, the Gene Expression Omnibus (GEO) database, were used to identify therapeutic biomarkers associated with tamoxifen resistance.

Materials and Methods: In this study, integrated analysis was used to identify tamoxifen resistance-associated genes. Differentially expressed genes (DEGs) were identified. Gene ontology and pathway analysis were then analyzed. Weighted correlation network analysis (WGCNA) was performed to find modules correlated with tamoxifen resistance. Protein-protein interaction (PPI) network was used to find hub genes. Genes of prognostic significance were further validated in another GEO dataset and cohort from Shanghai Ruijin Hospital using RT-PCR.

Results: A total of 441 genes were down-regulated and 123 genes were up-regulated in tamoxifen-resistant samples. Those up-regulated genes were mostly enriched in the cell cycle pathway. Then, WGCNA was performed, and the brown module was correlated with tamoxifen resistance. An overlap of 81 genes was identified between differentially expressed genes (DEGs) and genes in the brown module. These genes were also enriched in the cell cycle. Twelve hub genes were identified using PPI network, which were involved in the mitosis phase of the cell cycle. Finally, 10 of these 12 genes were validated to be up-regulated in tamoxifen-resistant patients and were associated with poor prognosis in ER-positive patients.

Conclusion: Our study suggested mitosis-related genes are mainly involved in tamoxifen resistance, and high expression of these genes could predict poor prognosis of patients receiving tamoxifen. These genes may be potential targets to improve efficacy of endocrine therapy in breast cancer, and inhibitors targeted these genes could be used in endocrine-resistant patients.

Keywords: breast cancer, tamoxifen resistance, WGCNA, PPI, mitosis

Introduction

As the most common cancer in the female, breast cancer is a great threat to world health.¹ It is the most common cause of cancer death in developing countries and second to lung cancer in more developed countries.² Breast cancer is a heterogeneous disease. Approximately 70% breast cancers are estrogen receptor α (ER)-positive.³ Endocrine therapy is used as the backbone therapy in ER-positive patients by blocking the ER pathway. Tamoxifen, a selective ER modulator, has dual agonistic/antagonistic effects on ER transcription, depending on its effect and location.⁴ Tamoxifen can cause cell cycle arrest in the G1 phase, inhibiting the proliferation and leading to apoptosis of breast cancer cells.⁵ In ER-positive

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patients, recurrence rates were reduced by almost 50% throughout the first 10 years, and the death rate was reduced by 25–30% after administration of 5-year tamoxifen.^{6,7} However, 30% patients who have taken tamoxifen for 5 years will have suffered from recurrence within 15 years. Therefore, finding new therapeutic biomarkers associated with tamoxifen resistance is important to overcome tamoxifen resistance.

Nowadays, gene sequencing has been widely used to identify biomarkers related to tumor biology.^{8,9} Large-scale sequencing made people have a better understanding of the heterogeneity, pathobiology and mechanism of cancers.³ As tamoxifen-resistant patient samples are difficult to obtain, no large-scale sequencing data about tamoxifen resistance have been systematically analyzed due to the limitation of sample size. However, high-throughput microarray and next-generation sequence datasets have been submitted by research groups to the international public repository, the Gene Expression Omnibus (GEO) database, and data in this database are freely available for integrated analysis.¹⁰ In our study, tamoxifen-resistant patients were derived from the GEO database in datasets GSE26971, GSE17705 and GSE45255, and integrated analysis was employed to have a better understanding of the mechanism of tamoxifen resistance.

Analysis of differentially expressed genes has mainly focused on the up-regulation and down-regulation of different genes, ignoring the interaction of different genes. Weighted gene co-expression network analysis (WGCNA) is a systems biology method that can be used to construct correlation networks and find modules of genes highly correlated to clinical traits.^{11,12} Candidate biomarkers or hub genes related to disease can be identified on the basis of correlation network.

In our study, 44 tamoxifen-resistant and 44 tamoxifen-sensitive patients who were matched with clinicopathological parameters were included. Differentially expressed genes (DEGs) were analyzed after normalization and gene ontology (GO) term enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed.^{13,14} Modules correlated to tamoxifen resistance were identified using WGCNA. An interaction of genes in module and DEGs were selected as candidate genes. The protein–protein interaction (PPI) network was constructed using candidate genes, and hub genes were identified according to the degree in the network.¹⁵ Genes with prognostic significance were considered as important genes involved in tamoxifen

resistance, and these genes were all involved in mitosis. This study sheds new light on the biological mechanisms of tamoxifen resistance and identifies new targets for tamoxifen-resistant patients.

Materials and Methods

Dataset

Microarray datasets GSE26971, GSE17705 and GSE45255 were downloaded from the National Center for Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/>). These microarrays were all generated using an Affymetrix Human Genome U133A microarray (HG-U133A). Dataset GSE6532 generated using the Affymetrix Human Genome U133 Plus 2.0 microarray (HG-U133_Plus_2) was used as a validation dataset. Patients who had taken tamoxifen after resection and suffered from distant metastasis within 2 years were defined as tamoxifen-resistant patients according to the International Consensus Guidelines for Advanced Breast Cancer (ABC). Patients who had no recurrence were defined as tamoxifen-sensitive patients in our study. All paired tamoxifen-sensitive and resistant patients had been administered tamoxifen in our analysis. Then tamoxifen-sensitive patients were 1:1 matched with tamoxifen-resistant patients according to their age, chemotherapy and tumor stage. A total of 17 patients from GSE26971, 19 patients from GSE17705 and 8 patients from GSE45255 were included in the tamoxifen-resistant group.

Processing of Microarray Data

The “getGEOSuppFiles” function in the “GEOquery” package of R was used to identify the raw data, and “cel” style files were downloaded from the GEO database using the “GEOquery” package of R. Raw data were converted to expression data using the affy package. Background adjustment and normalization were done using the *germa* package. Batch effect was removed using the *combat* function in the *sva* package. DEGs were calculated using the *limma* package, and statistical significance was defined as adjusted $P < 0.05$ and fold-change ≥ 1.5 .

GO and KEGG Analyses

Gene ontology (GO) was used to annotate biological processes, molecular functions and cellular components of genes, and Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to annotate the gene pathways. GO

functional analysis and KEGG pathway analysis were both performed separately in up-regulated and down-regulated DEGs. The cluster profiler was used to analyze the functional annotation. We also performed enrichment analysis using the DAVID website (<https://david.ncifcrf.gov/home.jsp>). Adjusted *P*-value <0.05 was considered as a significant enrichment.

WGCNA Analysis

Weighted correlation network analysis (WGCNA) was performed to find modules of highly correlated genes using the WGCNA package. The “goodSamplesGenes” function in WGCNA package was used to check for missing values, and hierarchical cluster analysis was used to detect outliers, with cut height of 140. After checking data for missing values and identification of outlier microarray samples, an appropriate soft power was selected to meet the requirements of a scale-free network. Genes with a threshold of variation coefficient of expression >0.25 was used to perform WGCNA analysis. One-step network construction was used to construct networks, and modules were identified. Eigengenes were correlated with external traits to identify modules that were significantly associated with the measured clinical traits. A scatterplot of gene significance (GS) vs module membership (MM) in the brown module was plotted to show the correlation of GS and MM.

PPI Network Construction and Hub Gene Identification

STRING (<http://string-db.org>) is a database of known and predicted protein–protein interactions, and it was used to construct a PPI network. The Cytoscape software and cytoHubba app were then employed to analyze the interactive relationship of the candidate proteins. The cytoHubba app in Cytoscape was used to identify hub genes ranked by degree method to rank the top 20 hub genes.

Candidate Genes Validation and RT-PCR Detection

Ten pairs of matched tamoxifen-resistant and sensitive breast cancer tissues were obtained between January 2009 and December 2011 at the Comprehensive Breast Health Center, Shanghai Ruijin Hospital. As these involved human participants they were reviewed and approved by the independent Ethical Committees of Ruijin Hospital, Shanghai Jiaotong University School of Medicine.

TRIZOL reagent (TaKaRa, Kusatsu, Japan) was used for the isolation of total RNA, and 1000 ng RNA was reverse-transcribed into cDNA using Primescript RT Reagent (TaKaRa, Japan). RT-PCR was performed using FastStart Universal SYBR Green Master (Roche, Switzerland) in a real-time PCR instrument (Applied Biosystems, USA), and β -actin was used as endogenous control. The primers are listed in [Supplementary Table 1](#).

Cell Culture

The MCF-7 cell line was obtained from the American Type Culture Collection (ATCC, USA) and was cultured in Roswell Park Memorial Institute 1640 (RPMI-1640, Thermo Fisher, USA), supplemented with 10% fetal bovine serum and 100 μ g/mL penicillin-streptomycin (Hyclone, USA).

Lentivirus Preparation and Infection

We used pLKO.1 (Addgene Plasmid, 10878) to generate lentiviral shRNA plasmids; shRNA plasmids targeting MELK, RACGAP1 and MAD2L1 were constructed. The sequence of shRNAs used were: MELK, 5'-GACAU CCUAUCUAGCUGCA-3'; MAD2L1, 5'-CUACUGAUC UUGAGCUCAU-3'; RACGAP1, 5'-CAACUAAGCGA GGAGCAAATT-3'. Lentivirus was generated by transfection of HEK293T cells with packaging vectors (pMD2.G and psPAX) and transducing vector and concentrated with PEG6000 (Sigma, USA). Forty-eight hours post-infection, puromycin (1 μ g/mL; 60210ES25, YEASEN) was used to select positively infected cells.

Colony Formation Assay

Cells transfected with scramble or shRNA were cultured into 6-well plates with 5000 cells and cultured for 15 days. After seeding cells for 24 hours, tamoxifen with concentration of 2 μ m/mL was added into every well for 15 days.

The colonies were fixed in 75% absolute ethanol for 10 minutes, washed twice with PBS and stained with Giemsa (Sigma, USA) for 15 min, then dried at room temperature. The colonies containing 50 or more cells in each well were counted.

Cell Viability Analysis

Cells were plated into 96-well in triplicates and then treated with tamoxifen of different concentrations. Cell viability was measured using Cell Titer-Glo 2.0 Assay (G9243, Promega), and data were collected on Synergy H4 Hybrid Reader (BioTek).

Survival Analysis

The key genes were identified as the intersecting genes of the brown module and DEGs. ER-positive patients treated with tamoxifen in three datasets with disease metastasis

survival within five years were divided into two groups according to the medium expression of key genes. Kaplan–Meier (K-M) plot was plotted using survival package.

Figure 1 Differentially expressed genes identified in tamoxifen-resistant and sensitive patients. **(A)** Heatmap and **(B)** volcano plot of differentially expressed genes in primary tamoxifen-resistant and sensitive patients. **(C)** KEGG pathway analysis of up-regulated and down-regulated DEGs. **(D)** GO functional analysis of up-regulated and down-regulated DEGs.

Results

Identification of DEGs in Tamoxifen-Resistant Patients

The DEGs of tamoxifen-resistant patients were analyzed, and a total of 564 DEGs were identified, including 441 down-regulated genes and 123 up-regulated genes compared to tamoxifen-sensitive patients (Figure 1A). DEGs are also listed in a volcano plot (Figure 1B). In KEGG pathway analysis (Figure 1C), the up-regulated genes are significantly enriched in pathways including cell cycle, cellular senescence and human T-cell leukemia virus 1 infection. The down-regulated genes are mainly enriched in complement and coagulation cascades, PPAR signaling pathway and arachidonic acid metabolism. In GO functional analysis (Figure 1D), these up-regulated genes are mainly enriched in terms including tubulin binding, microtubule binding and ATPase activity. The down-regulated DEGs are enriched in enzyme inhibitor activity and glycosaminoglycan binding.

Identification of Key Modules Using WGCNA Analysis

After excluding outliers, WGCNA analysis was performed to identify key modules correlated with tamoxifen resistance (Supplementary Figure 1). Soft threshold power was set to 6 to ensure a scale-free network (Figure 2). A total of 37 clusters were identified based on the criteria of a cut height = 0.25 and a minimum of 30 genes (Figure 3). To identify modules correlated to tamoxifen resistance, the module-trait relationship was analyzed, and the brown module with 576 genes was significantly related to tamoxifen resistance (Figure 4A). A scatterplot of module membership and gene significance indicates significant correlation between the brown module and tamoxifen resistance (Figure 4B).

Key Genes Identified with Tamoxifen Resistance

To identify genes mostly related to tamoxifen resistance, genes in the brown module and DEGs were overlapped

Figure 2 Soft threshold power identified in the weighted gene co-expression network analysis (WGCNA). **(A)** Analysis of scale-free fit index for various soft threshold powers. **(B)** Analysis of mean connectivity for various soft threshold powers.

Figure 3 Co-expression network modules for mRNA.

and a total of 81 genes were identified (Figure 5A). To explore the main function of these genes, KEGG pathway analysis was used, and pathways including cell cycle, oocyte meiosis and cellular senescence were enriched in these genes (Figure 5B). KEGG using DAVID was also calculated, and cell cycle was still the top pathway (Supplementary Figure 2). The enrichment analysis was similar to that in up-regulated genes of Figure 1C, indicating that those up-regulated genes in these pathways play an important role in tamoxifen resistance.

A PPI network was constructed to investigate the interaction between these 81 genes, which consists of 81 nodes and 1941 edges (Figure 5C). To explore hub genes in this network, cytoHubba was used and the top 20 genes were identified according to the degree in the network. AURKA, UBE2C, CCNA2, CDK1, KIF11, RRM2, TOP2A, BUB1B, CCNB2, MELK, BIRC5, NUSAP1, CDC20, KIF20A, KIF4A, MAD2L1, AURKB, DLGAP5, RACGAP1 and KIF23 were the

top 20 hub genes (Figure 5D), which were all up-regulated in tamoxifen-resistant patients (Table 1). Survival analysis found that high expression of 12 genes (AURKA, BIRC5, CCNA2, CCNB2, DLGAP5, KIF4A, KIF20A, KIF23, MELK, MAD2L1, RACGAP1, UBE2C) was associated with worse survival when patients were treated with tamoxifen (Figure 6). All these 12 mitotic genes have a prognostic role for ER-positive breast cancer using Kaplan–Meier plotter website (Supplementary Figures 3 and 4).

Furthermore, these 12 genes were validated in the GSE6532, and a total of 10 genes were up-regulated in tamoxifen-resistant patients (Figure 7A).

Validation of Candidate Genes for Tamoxifen Resistance

Ten of these genes (AURKA, CCNA2, CCNB2, DLGAP5, KIF4A, KIF20A, KIF23, MELK, MAD2L1, RACGAP1)

Figure 4 The brown module was identified with tamoxifen resistance. **(A)** Heatmap of trait correlation with tamoxifen resistance. The brown module was highly correlated with tamoxifen resistance. **(B)** A scatter plot of module membership in the brown module and gene significance.

were then further validated in 10 paired tamoxifen-resistant and sensitive breast cancer patients from the Ruijin cohort. Tamoxifen-resistant patients were all resisted, and their clinical characteristics were matched. Detailed characteristics for these patients are listed in [Supplementary Table 2](#). RT-PCR results demonstrated that these ten genes were all over-expressed in tamoxifen-resistant patients, and expression of these ten genes in tamoxifen-resistant

patients ranges from 2.2- to 4.7-fold that of tamoxifen-sensitive patients ($P < 0.05$, [Figure 7B](#)).

To validate the function of these genes in tamoxifen resistance, MELK, RACGAP1 and MAD2L1 were knocked down in MCF-7 cells because function of these genes in tamoxifen resistance was not reported while other genes were reported. As shown in colony formation assay and cell viability assay, knocking down these genes

Figure 5 Hub genes identified with tamoxifen resistance. **(A)** Venn plot of DEGs and genes in the brown module. A total of 81 genes overlapped. **(B)** KEGG pathway analysis of the 81 overlapped genes. **(C)** Protein-protein interaction network of the 81 overlapped genes. **(D)** Protein-protein interaction network of 21 hub genes. The intensity of red indicates the scores of degrees.

Table 1 Top 20 Genes in PPI Network Ranked by Degree Method and Foldchange in DEGs

Name	Degree	Foldchange
AURKA	68	1.656477
UBE2C	67	2.922333
CCNA2	66	1.77979
CDK1	66	2.047463
KIF11	65	1.954863
RRM2	65	2.411007
TOP2A	65	2.94478
BUB1B	65	1.765069
CCNB2	65	2.191142
MELK	64	2.212911
BIRC5	64	1.785661
NUSAP1	64	2.220914
CDC20	64	1.991213
KIF20A	64	1.809268
KIF4A	64	2.12466
MAD2L1	64	1.784906
AURKB	64	1.554373
DLGAP5	64	2.063802
RACGAP1	64	1.667147
KIF23	64	1.547577

Abbreviation: DEGs, differentially expressed genes.

sensitized cells to tamoxifen compared with control (Figure 7C and D). This validated the robustness of our analysis.

Discussion

In our study, we collected data from the GEO database, and an integrated analysis was used to identify genes associated with tamoxifen resistance in ER-positive breast cancer patients. Analysis of differentially expressed genes ignores correlation between different genes. In our study, we combined WGCNA with DEGs to identify differentially expressed genes with high correlation. The interaction of different proteins is an important mechanism to regulate cell biology. So, PPI network identified hub genes which were mostly engaged in tamoxifen resistance.

A total of 564 DEGs were identified, including 441 down-regulated genes and 123 up-regulated genes. The up-regulated genes were enriched in the cell cycle pathway, which is the most common pathway studied in tamoxifen resistance. The brown module with 576 genes was identified using WGCNA. An overlap of genes between DEGs and the brown module was also enriched in the cell cycle. A total of 10 mitosis genes with prognostic significance were identified as hub genes associated with tamoxifen resistance and were validated in the public database and in our cohort samples.

Studies of tamoxifen resistance mainly focus on acquired resistance due to limitation of sample size for tamoxifen resistance.^{16,17} According to the ESMO International Consensus Guidelines for Advanced Breast Cancer (ABC), acquired resistance was defined as patients who relapse while on adjuvant endocrine therapy (ET) but after the first 2 years, or relapse within 12 months of completing adjuvant ET, or have progression disease (PD) ≥ 6 months after initiating ET for ABC, while on ET. In our study, primary tamoxifen-resistant patients were studied using data from the public repository, and the mitosis phase of the cell cycle was identified as the main pathway. The most well-known mechanism of tamoxifen resistance is the mutation of ESR1.¹⁸ And recently, CDK4/6 inhibitors were combined with endocrine therapy to overcome endocrine resistance.^{19,20} In MONALEESA-7, 26.3% pre- and perimenopausal patients received tamoxifen. Patients receiving tamoxifen and ribociclib had longer median progression-free survival of 22.1 months (95% CI 16.6–24.7) than patients in the placebo group who had progression-free survival of 11.0 months (9.1–16.4) (HR 0.59, 95% CI 0.39–0.88). In our analysis, we illustrated the role of cell cycle-related genes in tamoxifen resistance, and these genes may serve as targets to overcome tamoxifen resistance. Except genes regulating G1/S transition like CCNE2 (Figure 5C), hub genes were all involved in mitosis. CCNE2 is a regulatory subunit of cyclin-dependent kinase 2. It interacts with CDK2 and forms a catalytically active kinase complex.²¹ This complex phosphorylates histone H1 and Rb and promotes the transition of G1/S. It has been reported that cell cycle and mitosis genes were involved in tamoxifen insensitivity and suppression of these genes could re-inhibit growth.^{22,23} Our study further indicated that therapy targeting mitosis is a potential strategy in overcoming tamoxifen resistance. As endocrine therapy is a standard adjuvant therapy of ER-positive breast cancer patients and there was no dataset

Figure 6 Survival analysis of 12 significant genes for disease-free survival. (A) K-M plot of AURKA for patients treated with tamoxifen. (B) K-M plot of BIRC5 for patients treated with tamoxifen. (C) K-M plot of CCNA2 for patients treated with tamoxifen. (D) K-M plot of CCNB2 for patients treated with tamoxifen. (E) K-M plot of DLGAP5 for patients treated with tamoxifen. (F) K-M plot of KIF4A for patients treated with tamoxifen. (G) K-M plot of KIF20 for patients treated with tamoxifen. (H) K-M plot of KIF23 for patients treated with tamoxifen. (I) K-M plot of MAD2L1 for patients treated with tamoxifen. (J) K-M plot of MELK for patients treated with tamoxifen. (K) K-M plot of RACGAP1 for patients treated with tamoxifen. (L) K-M plot of UBE2C for patients treated with tamoxifen.

Figure 7 Validation of ten genes in public database and samples from our own database. **(A)** Validation of significant genes in GSE6532. All genes are statistically significant. **(B)** Validation of significant genes in 10 pairs of patient samples in Ruijin cohort. **(C)** Colony formation assay for cells with knockdown of MELK, MAD2L1 and RACGAP1 treated with tamoxifen. **(D)** Cell viability assay for cells with knockdown of MELK, MAD2L1 and RACGAP1 treated with tamoxifen. * $P < 0.05$.

with patients both receiving and not receiving tamoxifen, we cannot evaluate the interaction of genes regarding prognosis and efficacy to tamoxifen. When we assessed the prognostic role of these mitotic genes in ER breast cancers, high expression of these genes indicated poor survival for ER breast cancers though the treatment information was unknown.

AURKA, Aurora kinase A, controls many processes of the G2/M transition.^{24–26} AURKA was identified as a marker for endocrine resistance in early estrogen receptor-positive breast cancer, and knockdown of AURKA made tamoxifen-resistant cells re-sensitized to tamoxifen treatment.²⁷ CCNA2 is highly expressed from S phase to early mitosis and binds to CDK1 during the transition from G2 to M phase.²⁸ It was overexpressed in tamoxifen-resistant cells. CCNB2 levels gradually increase during S and G2 phase and peak at mitosis.^{29,30} It was reported that ER-positive patients who have high expression of CCNA2 or CCNB2 have inferior survival.^{31,32} KIF4A, KIF20A and KIF23 are all mitotic kinesins and have

a highly conserved motor domain involving ATP-binding and microtubule-binding sequences.³³ They all promote the proliferation of breast cancers, and treatment with tamoxifen reduced the expression of these three proteins. Knockout of KIF4A re-sensitized cancer cells to tamoxifen.³⁴ Maternal embryonic leucine zipper kinase (MELK) is a member of both the sucrose-non-fermenting (snf)1 and the AMP-activated protein kinase (AMPK) families.³⁵ It has been reported that MELK is overexpressed in many cancers, and high expression of this gene correlated with a poor survival.^{35,36} MAD2L1 is a key protein in mediating spindle checkpoint activation.^{37,38} The disks large-associated protein 5 (DLGAP5), a microtubule-associated protein, is responsible for stabilizing and correct formation of microtubules, and the bipolar arrays of dynamic microtubules are critical in forming the mitotic spindle. It is overexpressed in many cancers and indicates a poor prognosis in these cancers.^{39–41} Rac GTPase-activating protein 1 (RACGAP1) links the mitotic spindle to the plasma

membrane to secure the final cut during cytokinesis in animal cells. RACGAP1 was identified as an oncogene in many cancers.^{42,43} The role of MAD2L1, DLGAP5 and RACGAP1 in tamoxifen resistance was validated in our study, and knockdown of these genes sensitizes breast cancer to tamoxifen.

Conclusion

In conclusion, this present study provides evidence that mitosis genes contributed to tamoxifen resistance. We concluded that ten mitosis genes showed high expression in tamoxifen-resistant patients and lead to poor analysis in patients receiving tamoxifen and in ER-positive breast cancer patients. Knockdown of three genes (MAD2L1, DLGAP5, RACGAP1) makes cells more sensitive to tamoxifen. Our results indicates that mitosis is an important biological process of tamoxifen resistance, and mitotic genes may serve as potential targets to overcome tamoxifen resistance.

Abbreviations

ER, estrogen receptor α ; GEO, Gene Expression Omnibus; DEGs, differentially expressed genes; WGCNA, weighted correlation network analysis; PPI, protein–protein interaction; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; HG-U133A, human genome U133A microarray; GS, gene significance; MM, module membership; K-M, Kaplan–Meier; AURKA, Aurora kinase A; MELK, maternal embryonic leucine zipper kinase; AMPK, AMP-activated protein kinase; DLGAP5, disks large-associated protein 5; RACGAP1, Rac GTPase-activating protein 1.

Ethics Approval and Informed Consent

The investigations involving human participants were reviewed and approved by the independent Ethical Committees of Ruijin Hospital, Shanghai Jiaotong University School of Medicine and all subjects gave written informed consent. The ethics number for our analysis is 2020-309. This study was conducted in accordance with the Declaration of Helsinki.

Author Contributions

XS participated in all experimental work and drafted the paper. XC and KS designed the article. SD, ZW and SL collected the data. All authors made substantial contributions to conception and design, acquisition of data, or

analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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