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Identification of potential gene associated with berberine in overcoming tamoxifen resistance by functional network analysis

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ABSTRACT

Previously, berberine enhanced the sensitivity ofMichigan Cancer Foundation-7 (MCF-7)-resistant breast cancer cells toward tamoxifen; however, its molecular mechanism remains unclear. The purpose of this study is to identify the potential targets and molecular mechanisms of berberine in overcoming breast cancer resistance toward tamoxifen by using a bioinformatics approach for functional network analysis. The microarray data of tamoxifen-resistant and berberine-treated MCF-7 cells were obtained from GSE67916 and GSE85871, which resulted in differentially expressed genes (DEGs). The analysis of the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes pathway enrichment by using the Database for Annotation, Visualization, and Integrated Discovery revealed that several DEGs participated in the erbB tyrosine kinase signaling pathway. The analysis of protein–protein interaction network and hub gene selection by using STRING and Cytoscape identified the top 10 genes with the highest degree score. The analysis of genetic alterations by using cBioPortal demonstrated the genetic alterations of six potential target genes, including protein kinase C alpha type (*PRKCA*), epidermal growth factor receptor (*EGFR*), erb-b2 receptor tyrosine kinase 4 (*ERBB4*), amphiregulin (*AREG*), estrogen receptor 1 (*ESR1*), and *STAT1*. Moreover, importantly, the erbB signaling is a potential target for overcoming breast cancer resistance toward tamoxifen. Further studies are required to validate the results of this study.

INTRODUCTION

Breast cancer was one of the primary reasons for death among women worldwide in 2018 (Bray *et al*., 2018). Luminal A breast cancer, which expresses estrogen receptor (ER+) but does not express human epidermal growth factor receptor 2 (HER2-), has the highest incidence rate of 59% among breast cancer subtypes (Fallahpour *et al*., 2017). Endocrine therapy, including tamoxifen, has demonstrated the effectiveness of luminal A breast cancer treatment (Lindström *et al*., 2018). Despite its successful development as ER-targeted therapy, patients developed resistance toward tamoxifen, further leading to relapse and metastasis (Viedma-Rodriguez *et al*., 2014). Therefore, the development of combinational chemotherapy is essential to increase the effectiveness of tamoxifen therapy.

Berberine is a potential compound for a combinational therapy agent of tamoxifen. Berberine is an isoquinoline alkaloid isolated from the genus *Berberidaceae* (Spinozzi *et al*., 2014). The previous studies demonstrated that berberine exhibited an anticancer activity on various types of cancer including breast cancer (Tak *et al*., 2019), colorectal cancer (Palmieri *et al*., 2019), lung cancer (Zhu *et al*., 2015), ovarian cancer, prostate cancer, liver cancer, and cervical cancer (Liu *et al*., 2019). Previously, berberine showed cytotoxicity in Michigan Cancer Foundation-7 (MCF-7)-sensitive cells and enhanced the sensitivity of MCF-7-resistant cells to tamoxifen (Wen *et al*., 2016). However, the molecular mechanism of berberine in circumventing tamoxifen resistance remains unclear.

The purpose of this study is to identify the potential targets and molecular mechanisms of berberine in overcoming breast cancer resistance toward tamoxifen by using a bioinformatics approach for functional network analysis. The microarray data were obtained from the gene expresion omnibus (GEO) DataSets

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to generate the differentially expressed genes (DEGs). The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEGs revealed the mechanism of berberine in overcoming tamoxifen resistance. By using cBioPortal, the protein–protein interaction (PPI) network and genetic alteration analysis identified the potential targets of berberine in overcoming tamoxifen resistance.

MATERIALS AND METHODS

Data collection and processing

The microarray data of tamoxifen-resistant MCF-7 cells were obtained from GSE67916, entitled "Gene expression profiling identifies Src family tyrosine kinase as an important molecule in tamoxifen resistance and a predictor of early recurrence in patients treated with endocrine therapy" (Elias *et al*., 2015), which contained two samples from each MCF-7 tamoxifen-sensitive and resistant cells. The mRNA microarray data of berberine-treated MCF-7 cells were obtained from the public database GSE85871 entitled "The gene expression profiles in response to 102 traditional Chinese medicine (TCM) components: a general template for research on TCMs" (Lv *et al*., 2017), which contained two samples from each MCF-7 cells that were treated with 10 µM of berberine for 24 hours, and dimethyl sulfoxide was used as a control. The gene expression profiles in both gene series expressions were processed by using the microarray technology with Affymetrix Human Genome U133A 2.0 (Santa Clara, CA). There was a good distribution of value data for GSE67916 and GSE86871 (Supplementary Fig. S1). Data were processed by GEO2R, an online tool for GEO data analysis based on the R programming language. DEGs were screened from two data sets. The adjusted p-value < 0.05 and Ilog Fold change > 1 were used to select the significant DEGs.

Gene ontology and KEGG pathway enrichment analysis

The analysis of GO and KEGG pathway enrichment was conducted by the database for annotation, visualization, and integrated discovery (DAVID) v6.8 (Huang da *et al*., 2009). The value of $p < 0.05$ was selected as the cutoff value.

PPI network and hub gene selection

The analysis of the PPI network was constructed with STRING-DB v11.0 (Szklarczyk *et al*., 2015) with the confidence scores of greater than 0.4 and visualized by Cytoscape software (Shannon *et al*., 2003). Genes with a degree more than 5, which were analyzed by the CytoHubba plugin, were selected as the hub genes (Chin *et al*., 2014).

Analysis of genetic alterations of potential target genes (PTGs)

The genetic alterations of hub genes were analyzed by using cBioPortal (Cerami *et al*., 2012; Gao *et al*., 2013). The breast cancer study with the highest genetic alterations was chosen for a further connectivity analysis, and the value of $p < 0.05$ was considered as the cutoff value.

RESULTS AND DISCUSSION

Data collection and processing

The purpose of this study is to identify the potential targets and mechanisms of berberine in overcoming breast cancer resistance toward tamoxifen. The data from a GEO database have never been used for the discovery of berberine molecular targets and mechanisms in overcoming breast cancer resistance to tamoxifen. This study uses a bioinformatics-based functional network, including functional protein networks, mutual exclusivity mutations, gene neighbors, and drug-related gene networks, to find and identify the potential targets for berberine. In total, 1,030 DEGs were extracted from GSE67916, which consisted of 744 and 286 upregulated and downregulated genes, respectively (Supplementary Table S1 and Fig. S1). In all, 1,758 genes were retrieved from GSE85871, which consisted of 987 upregulated and 795 downregulated genes, respectively (Supplementary Table S2 and Fig. 1B). A total of 170 DEGs were retrieved from both GSE68916 and GSE86871 (Supplementary Table S3). The DEGs were further analyzed using public databases for functional network analysis.

Gene ontology and KEGG pathway enrichment analysis

The analysis of the GO and KEGG pathway enrichment was conducted to study the biological function and molecular mechanism of DEGs. The analysis of GO was conducted with DAVID based on three criteria: biological process, cellular component, and molecular function. Several DEGs participated in the biological process of positive regulation of transcription

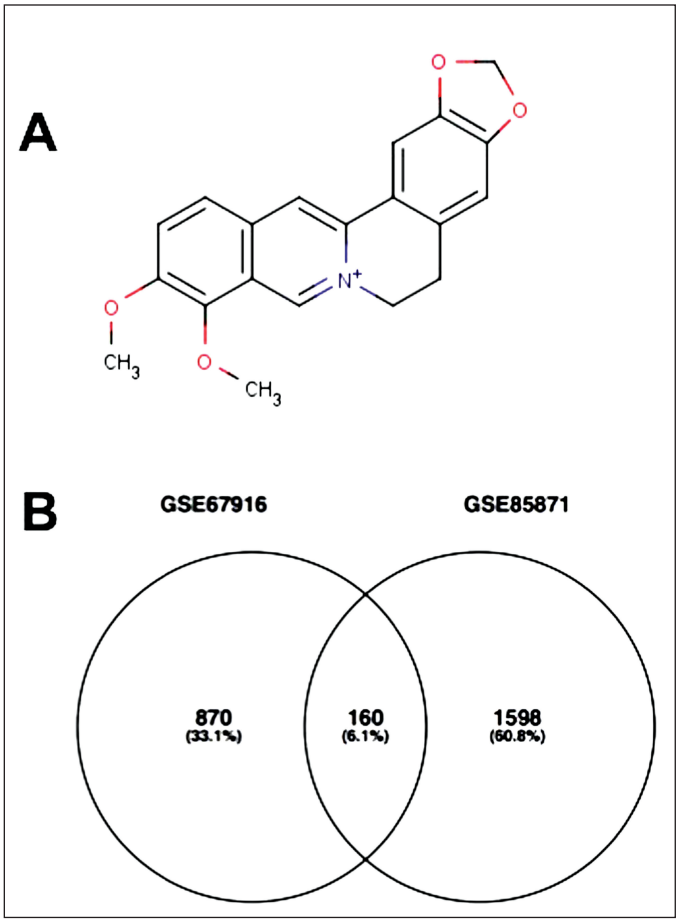

Figure 1. (A) Chemical structure of berberine. (B) A Venn diagram of tamoxifen-resistant and berberine-treated MCF-7 breast cancer cells.

and transmembrane receptor protein tyrosine kinase signaling pathway, for example, epidermal growth factor receptor (*EGFR*) and erb-b2 receptor tyrosine kinase 4 (*ERBB4*) (Supplementary Table S4). The DEGs are located in the endoplasmic reticulum and cell surface, including *EGFR*, Amphiregulin (*AREG*), and protein kinase C alpha type (*PRKCA*). The DEGs also play a molecular role in the transmembrane receptor protein tyrosine kinase activity and enzyme binding, for example, *EGFR, ERBB4, PRKCA,* estrogen receptor 1 (*ESR1*), and signal transducer and activator of transcription 1 (*STAT1)*. The KEGG pathway enrichment analysis showed that the DEGs regulate the pathways in cancer, mitogen-activated protein kinase 1 (MAPK) signaling pathway, and erbB signaling pathway, including *EGFR*, *ERBB4*, *AREG,* and *PRKCA* (Table 1).

PPI network and hub gene selection

The PPI network analysis was conducted to study the interaction among DEGs. Further analysis with CytoHubba was conducted to select the hub genes. In total, 160 proteins that were constructed for the protein network (confidence level of 0.4) consisted of 149 nodes, 173 edges, a PPI enrichment value of 2.55e–8, and an average local clustering coefficient of 0.391 (Fig. 2). The top 10 genes with the highest degree score were considered as hub genes and identified as *EGFR, ESR1, STAT1,* C-X-C motif chemokine 12 (*CXCL12*), *ERBB4,* Fibroblast growth factor receptor 2 (*FGFR2*), *S*RY-Box Transcription Factor 9 (*SOX9*), Insulin-like growth factor 1 receptor (*IGF1R*), MYB proto-oncogene (*MYB*), and *PRKCA* (Table 2).

Analysis of genetic alterations of PTGs

Six PTGs, including *PRKCA, EGFR, ERBB4, AREG, ESR1,* and *STAT1*, were analyzed by using cBioPortal to explore their genomic alterations across the breast cancer studies. *PRKCA, EGFR, ERBB4,* and *AREG* were selected among DEGs from KEGG pathway enrichment (erbB signaling pathway), whereas *ESR1* and *STAT1* were selected among DEGs based on the highest degree score using CytoHubba. A study, namely, the BRCA Institut National de la Santé et de la Recherche Médicale (INSERM) 2016 (Lefebvre *et al*., 2016), showed the highest genetic alterations among the breast cancer studies and was selected for further analysis (Fig. 3A). Genetic alterations for each target gene were found from 3% to 14%, including *STAT1* (3%), *PRKCA* (6%), *AREG* (6%), *EGFR* (7%), *ERBB4* (9%), and *ESR1* (14%) (Fig. 4). Moreover, most of the gene alterations belonged to amplification and missense mutation (Fig. 3B). Further analysis of mutual exclusivity showed that only two gene pairs (*ERBB4–AREG* and *ERBB4–STAT1*) exhibited a significant co-occurrence ($p < 0.05$) in a breast cancer study by the INSERM 2016 project (Table 3). These results indicated the pivotal role of *ERBB4, AREG,* and *STAT1* under the control of berberine treatment.

The analysis of gene networks connected to PTGs revealed that *TP53* is the gene neighbor with the highest connectivity (Fig. 4A). To reduce the network complexity, we screened the neighbors by 20% alteration, and the results only showed four query genes: *ERBB4,* Ret proto-oncogene (*RET*), *FGFR2,* and Fibronectin 1 (*FN1*). Moreover, *EGFR* and *ESR1* are the most druggable targets that highlighted the important roles of these genes in berberine treatment (Fig. 4B).

Proposed PTGs and the mechanism of berberine in overcoming breast cancer resistance to tamoxifen

The results of this study using functional network analysis highlighted potential therapeutic target genes PTGs, including *PRKCA*, *EGFR*, *AREG*, *ESR1*, and *STAT1*. Moreover, KEGG pathway enrichment analysis revealed the molecular mechanism of berberine in overcoming breast cancer resistance to tamoxifen, which is the erbB signaling pathway. The erbB signaling begins when the erbB receptor or the epidermal growth factor receptor binds to the ligand that leads to receptor dimerization, transphosphorylation, and activation of intracellular signaling including Janus kinase-signal transducer and activator of transcription, PI3K/AKT, MAPK, and protein kinase C (PKC) (Viedma-Rodriguez *et al*., 2014). The epidermal growth factor receptor, a transmembrane receptor protein tyrosine kinase family, for example, *EGFR* and HER2, is involved in the resistance of breast cancer cells toward tamoxifen (Choi *et al*., 2018; Yin and Wang, 2016). Moreover, the activation of MAPK signaling is involved in tamoxifen resistance in breast cancer cells (Yin *et al*., 2017). Taken together, the activation of erbB signaling is important for the mechanism of breast cancer resistance to tamoxifen and becomes a potential mechanism of berberine for overcoming the resistance phenomenon of tamoxifen.

In this section, we will also discuss the role of PTGs and its axis with erbB signaling and tamoxifen resistance. *PRKCA* encodes protein kinase C alpha (PKCα), which is a serine-threonine kinase that regulates several biological processes, including breast cancer progression (Pham and Tonetti, 2016). PKCα is known to regulate migration and invasion. Besides, it was found to be a poor

Table 1. KEGG pathway enrichment analysis of the DEGs.

Term	*p*-value	Genes
hsa05200:Pathways in cancer	0.001005133	*PRKCA*, *FGFR2*, *EGFR*, *IGF1R*, CCDC6, *RET*, BAX, BRCA2, FAS, *STAT1*, *CXCL12*, COL4A5
hsa05205:Proteoglycans in cancer	0.002638517	*PRKCA*, *EGFR*, *IGF1R*, *ERBB4*, ANK3, *ESR1*, FAS, ITPR1
hsa05168:Herpes simplex infection	0.028808731	SP100, SOCS3, C5, HLA-B, FAS, *STAT1*
hsa04917:Prolactin signaling pathway	0.029182478	PRLR, SOCS3, *ESR1*, *STAT1*
hsa04010:MAPK signaling pathway	0.031519967	*PRKCA*, *FGFR2*, *EGFR*, TAOK1, DUSP10, FAS, DDIT3
hsa04970:Salivary secretion	0.047331322	*PRKCA*, ATP2B1, ITPR1, MUC5B
hsa04012:ErbB signaling pathway	0.048700566	*PRKCA*, *EGFR*, *ERBB4*, AREG

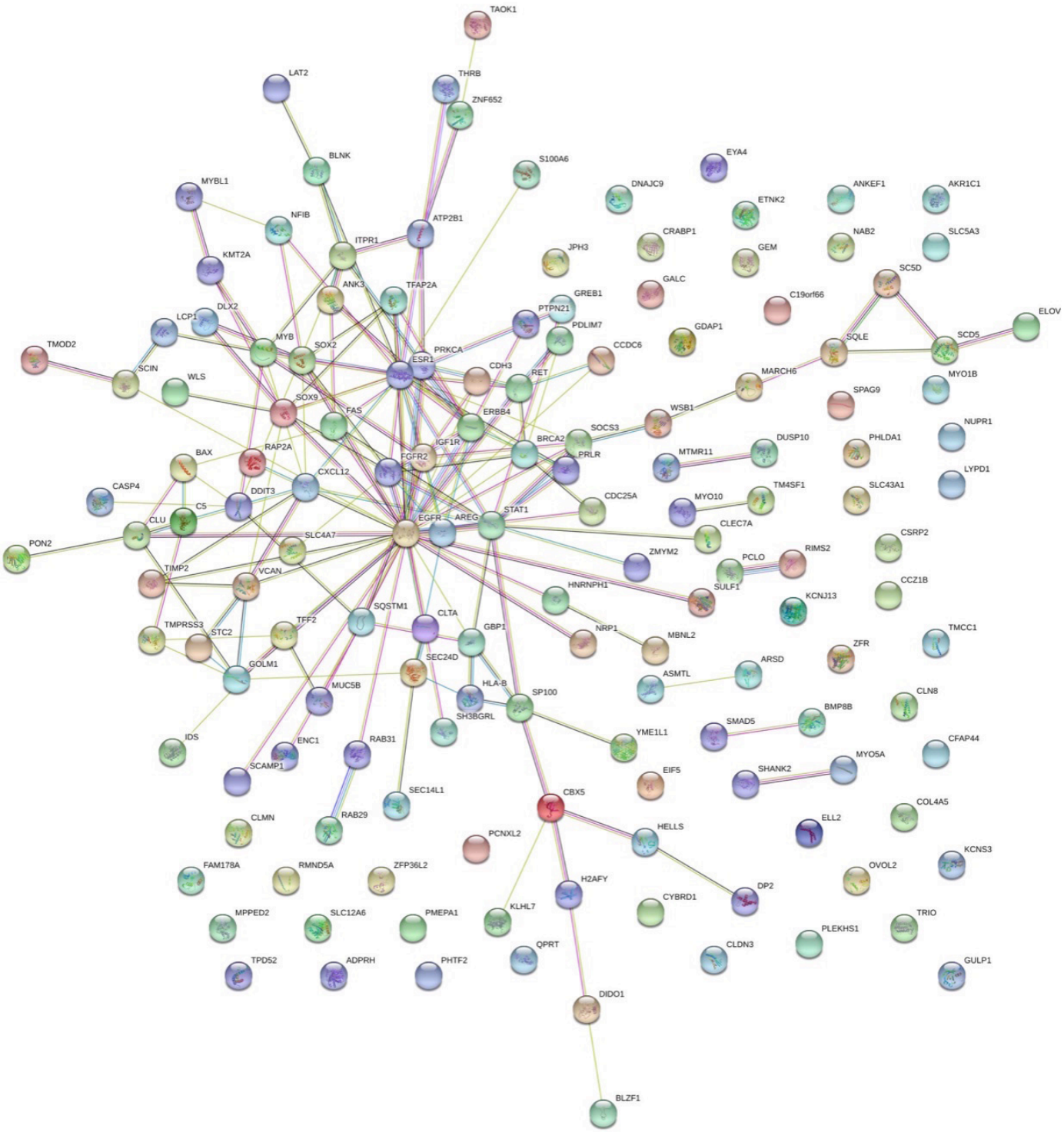

Figure 2. PPI network of protein related to overcoming tamoxifen resistance by berberine.

prognosis marker in breast cancer (Lonne *et al*., 2010). PKCα is highly expressed in the triple-negative breast cancer subtypes (Lin *et al*., 2017). Moreover, PKCα is also a predictive biomarker of poor response to endocrine therapy in ER-positive breast cancer and is a potential target in overcoming endocrine resistance in ER+ breast cancer (Pham and Tonetti, 2016; Pham *et al*., 2017). Berberine inhibits metastasis through the inhibition of PKCα signaling in breast cancer cells (Kim *et al*., 2012). PKC is involved in the ErbB receptor signaling in neuronal differentiation (García *et al*., 2013). However, the effect of berberine in tamoxifen-resistant breast cancer cells and erbB signaling remains elusive and needs to be clarified further.

Table 2. Top 10 hub genes based on degree score.

No	Gene symbol	Gene name	Degree score
1	*EGFR*	Epidermal growth factor receptor	34
2	*ESR1*	Estrogen receptor alpha	21
3	*STAT1*	Signal transducer and activator of transcription 1-alpha/beta	12
4	*CXCL12*	Stromal cell-derived factor 1	9
5	*ERBB4*	Receptor tyrosine-protein kinase erbB-4	9
6	*FGFR2*	Fibroblast growth factor receptor 2	9
7	SOX9	Transcription factor SOX-9	9
8	*IGF1R*	Insulin-like growth factor 1 receptor	7
9	MYB	Transcriptional activator Myb	7
10	*PRKCA*	Protein kinase C alpha type	7

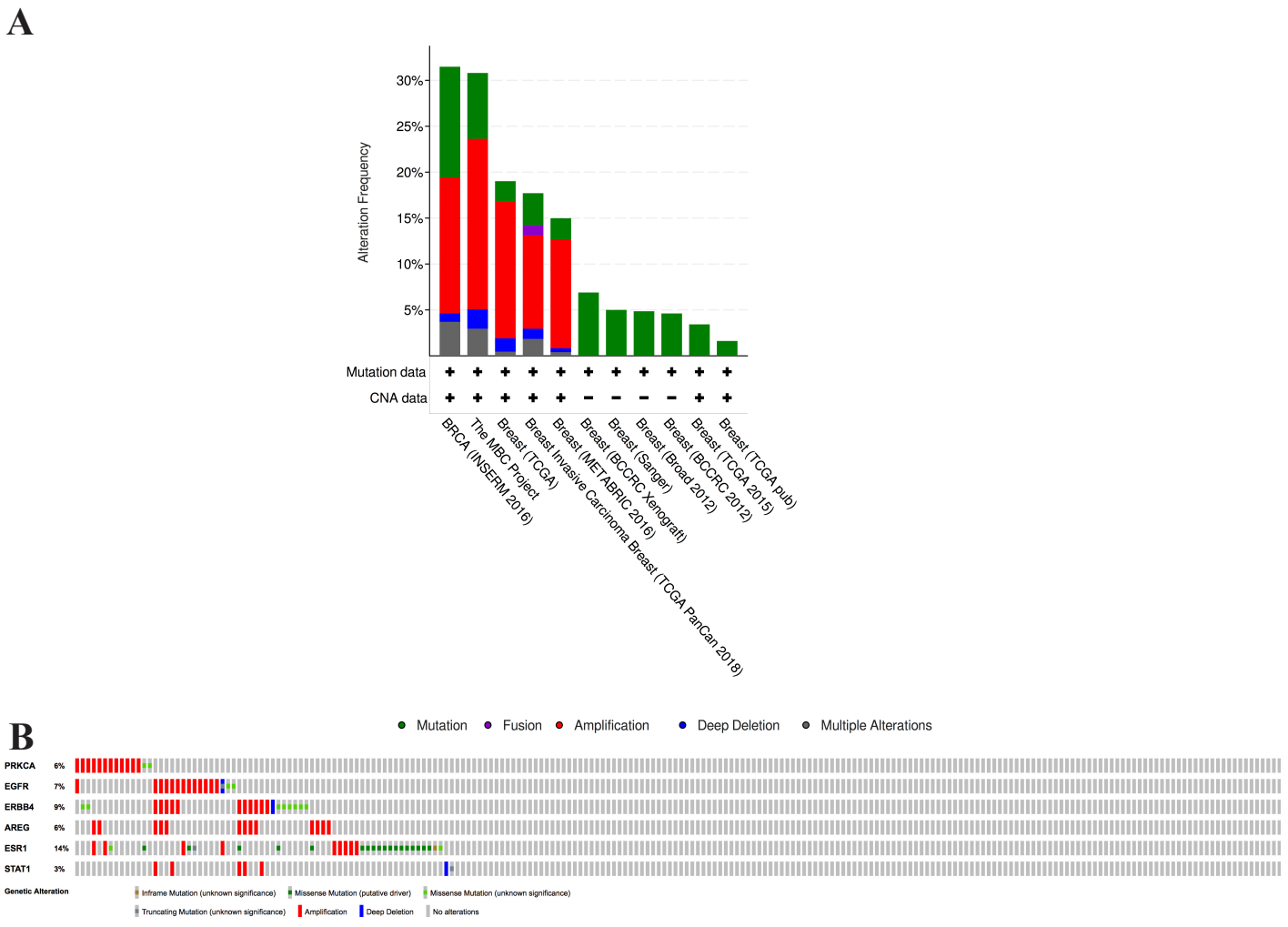

Figure 3. (A) Overview of changes in *PRKCA*, *EGFR*, *ERBB4*, *AREG*, *ESR1*, and *STAT1* in the genomics data set from 16 studies of breast cancer. (B) Summary alterations of *PRKCA*, *EGFR*, *ERBB4*, *AREG*, *ESR1*, and *STAT1* across breast cancer samples (based on a study by Lefebvre *et al*., 2016).

AREG encodes amphiregulin, a ligand of the epidermal growth factor receptor, which activates erbB signaling (Mao *et al*., 2018). Amphiregulin plays an essential role in the development of the mammary gland, the progression of ER-positive breast cancer, and proliferation and migration in the HER2-positive breast cancer cells (Schmucker *et al*., 2018). Amphiregulin regulates the breast cancer cell resistance to an aromatase inhibitor, exemestane, by modulating the autocrine loop (Wang *et al*., 2008). Moreover, amphiregulin is enriched in the ERα-positive breast cancer cells, which are required for the proliferation of estrogen-dependent breast cancer cells, and is downregulated in the endocrine-treated patients with breast cancer (Peterson *et al*., 2015). The downregulation of lipolysis-stimulated lipoprotein receptor (LSR) triggers the invasion of human endometrial cancer through the

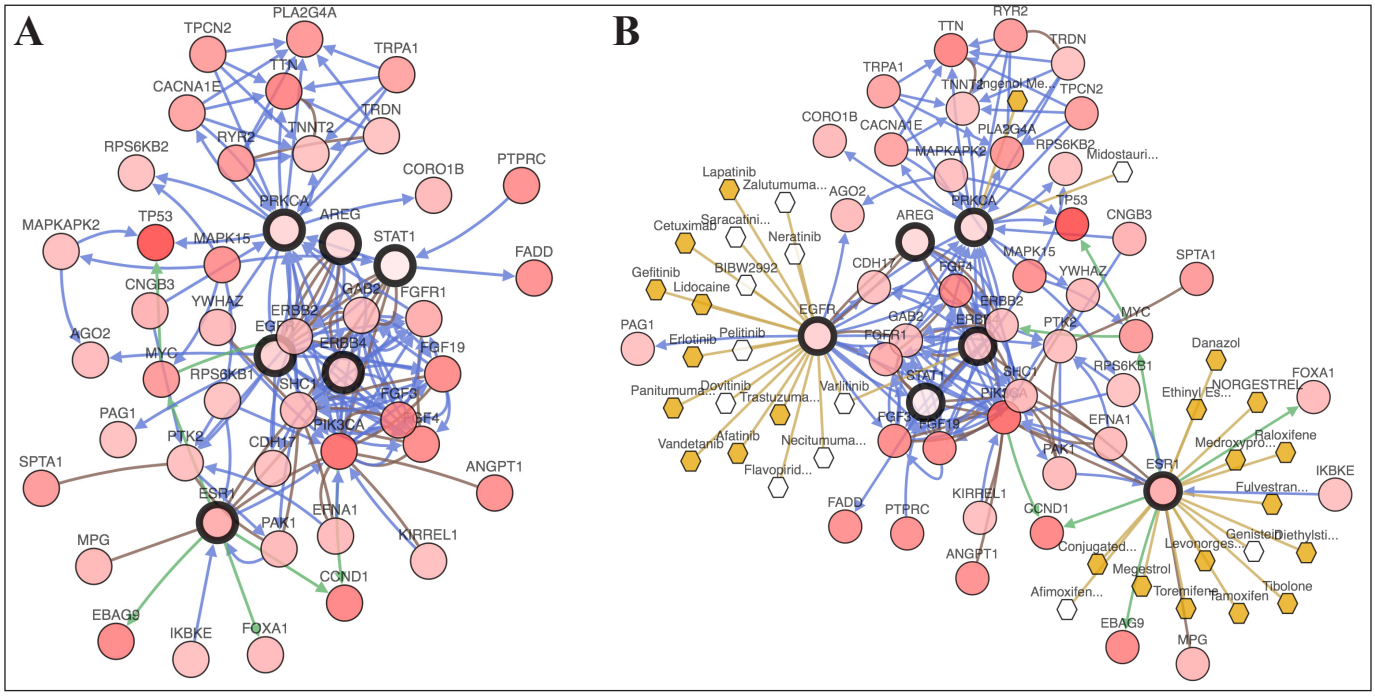

Figure 4. (A) Gene network and drug-gene network connected with *PRKCA*, *EGFR*, *ERBB4*, *AREG*, *ESR1*, and *STAT1* across breast cancer samples (based on a study by Lefebvre *et al*., 2016).

Table 3. Mutual exclusivity analysis of selected genes in metastatic breast cancer study.

A	B	*p*-value	Log2 odds ratio	Tendency
ERBB4	AREG	<0.001	>3	Co-occurrence
ERBB4	*STAT1*	<0.001	>3	Co-occurrence
AREG	*STAT1*	0.005	>3	Co-occurrence
EGFR	*ERBB4*	0.009	2.487	Co-occurrence
EGFR	AREG	0.06	2.132	Co-occurrence
EGFR	*STAT1*	0.087	2.478	Co-occurrence
PRKCA	*ESR1*	0.123	1.374	Co-occurrence
EGFR	*ESR1*	0.181	1.095	Co-occurrence
PRKCA	AREG	0.202	1.533	Co-occurrence
AREG	*ESR1*	0.281	0.907	Co-occurrence
PRKCA	*ERBB4*	0.379	0.769	Co-occurrence
ERBB4	*ESR1*	0.428	−0.644	Mutual exclusivity
PRKCA	*STAT1*	0.621	<−3	Mutual exclusivity
PRKCA	*EGFR*	0.722	−0.06	Mutual exclusivity
ESR1	*STAT1*	0.736	−0.008	Mutual exclusivity

upregulation of *AREG* (Kohno *et al*., 2019). Furthermore, the same author stated that berberine increases LSR, thereby inhibiting the invasion of endometrial cancer cells. Further studies of berberine in regulating amphiregulin in breast cancer resistance will help in revealing its role in overcoming tamoxifen resistance.

ERBB4 encodes human epidermal growth factor receptor 4 (HER4), which is a member of the human epidermal growth factor receptor family (Junttila *et al*., 2005). HER4 is a receptor tyrosine kinase that is crucial for the development of normal breast tissue (Sundvall *et al*., 2008). The activation of HER4 signaling is enhanced during breast cancer (Hollmén *et al*., 2012) and also plays a vital role in the HER2-positive breast cancer resistance toward HER2 inhibitor (Canfield *et al*., 2015). The increased expression of *ERBB4* is associated with a poor prognosis in patients with triple-negative breast cancer (Kim *et al*., 2016). Genetic alterations in *ERBB4*, namely, rs13423759, not only enhance breast cancer risk but are also associated with the increased metastasis in patients with breast cancer (Mansouri Bidkani *et al*., 2018). The interaction between HER4 and ER hinders the binding of tamoxifen to the ER, thereby inducing tamoxifen resistance (Wege *et al*., 2018). Moreover, the same author demonstrated that the overexpression of HER4 reduces the overall survival of postmenopausal women. However, targeting *ERBB4* signaling by berberine in overcoming tamoxifen resistance remains elusive.

EGFR encodes human epidermal growth factor receptor 1, a member of the epidermal growth factor receptor family, which is involved in the development of epithelial tissue as well as carcinogenesis in lung and breast cancers (Sigismund *et al*., 2018). Mutation and signaling activation of *EGFR* were found in a patient with triple-negative breast cancer (Sohn *et al*., 2014). The studies have shown that the inhibition of *EGFR* signaling sensitizes the triple-negative breast cancer cells to the *EGFR* inhibitor (Ali and Wendt, 2017; Foidart *et al*., 2019; Roncato *et al*., 2018). The inhibition of *EGFR* signaling by neratinib induces apoptosis in tamoxifen-resistant MCF-7 cells (Kim *et al*., 2015). The activation of *EGFR*/HER2 signaling and HER2 overexpression mediated the tamoxifen resistance in the ER-positive breast cancer cells by a mechanism involving a MAPK/AKT pathway (Massarweh *et al*., 2008). Berberine was found to induce senescence through the downregulation of signaling *EGFR* in the human glioblastoma cells (Liu *et al*., 2015). The studies also demonstrated that berberine inhibits *EGFR* signaling and increases the cytotoxicity

of *EGFR* inhibitors in the gastric cancer cells (Wang *et al*., 2016) and MCF-7 breast cancer cells (Jabbarzadeh Kaboli *et al*., 2019). Collectively, targeting *EGFR* with berberine in the tamoxifen-resistant breast cancer cells will be an interesting topic for further studies.

STAT1 encodes a transcription factor *STAT1* (Hix *et al*., 2013). The studies have shown that *STAT1* can play the role of an oncogene and tumor suppressor genes in any type of cancer (Meissl *et al*., 2017). *STAT1* is downregulated during the progression of ER-positive and negative breast cancer, thereby highlighting its role as a tumor suppressor gene (Chan *et al*., 2012). On the contrary, the activation of *STAT1* is involved in breast cancer resistance to endocrine (Huang *et al*., 2014). Berberine inhibits *STAT1* activation in autoimmune encephalomyelitis (Qin *et al*., 2010) and type 1 diabetic mice (Cui *et al*., 2009). The role of berberine in *STAT1* signaling in the tamoxifen-resistant breast cancer cells will help in understanding its role in overcoming tamoxifen resistance.

The analysis of gene networks connected to PTGs revealed that *TP53* and Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA) are the gene neighbors with the highest connectivity. The previous studies revealed the association between tamoxifen resistance and mutation in PIK3CA (Loi *et al*., 2010) and p53 (Elledge *et al*., 1995). Berberine demonstrated anticancer activity through p53-mediated apoptosis (Park *et al*., 2016) and PI3K/AKT signaling (Li *et al*., 2017). Moreover, *EGFR* and *ESR1* are the most druggable targets and, thus, highlighted the vital roles of these genes in berberine treatment. Recently, *EGFR* activation leads to breast cancer resistance toward tamoxifen by the downregulation of ER (Jeong *et al*., 2019). Taken together, in this study, the bioinformatics approach for functional network analysis helps us to direct the development of berberine as targeted therapy, especially the mechanism of tamoxifen resistance, which is erbB signaling and potential therapeutic target of berberine in circumventing tamoxifen resistance.

CONCLUSION

This study highlighted the six potential targets of berberine, including PRKCA, *EGFR*, *ERBB4*, *AREG*, *ESR1*, and *STAT1*, for overcoming breast cancer resistance to tamoxifen. More importantly, erbB signaling is a potential mechanism in overcoming the resistance of breast cancer cells toward tamoxifen. Further studies are required to validate the results of this study.

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CONFLICT OF INTEREST

Authors declare that they do not have any conflict of interest.

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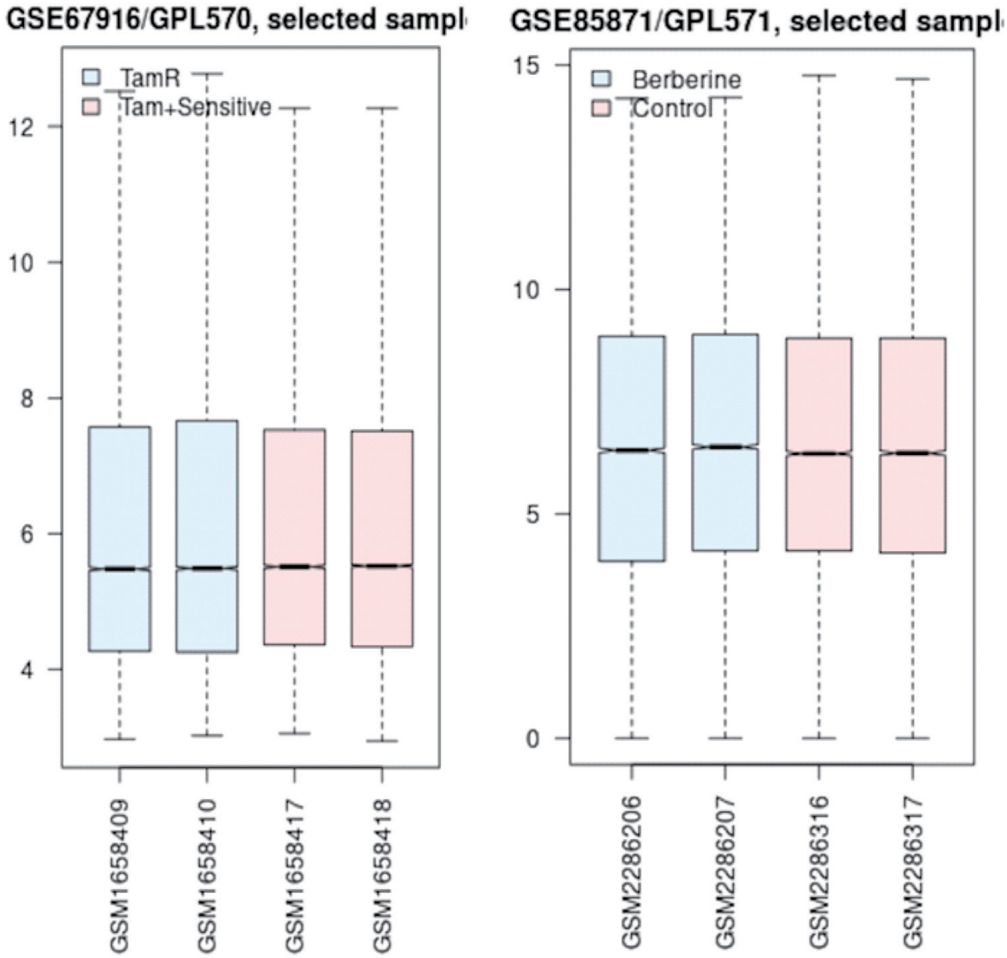
SUPPLEMENTARY MATERIAL



SupplementaryFigure S1. The distribution of value data for GSE67916 and GSE86871.