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 of Michigan 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 expression omnibus (GEO) DataSets.
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.
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), SRY-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 (PKCa), which is a serine-threonine kinase that regulates several biological processes, including breast cancer progression (Pham and Tonetti, 2016). PKCa 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, FGF1R, CCDC6, RET, BAX, BRCA2, FAS, STAT1, CXCL12, COL4A5 |
| hsa05168:Herpes simplex infection | 0.028808731   | PRKCA, EGFR, IGF1R, FGF1R, CCDC6, RET, BAX, BRCA2, FAS, STAT1, CXCL12, COL4A5 |
| hsa04310:Protein tyrosine kinase  | 0.029182478   | PRKCA, EGFR, IGF1R, FGF1R, CCDC6, RET, BAX, BRCA2, FAS, STAT1, CXCL12, COL4A5 |
| hsa04970:Salivary secretion       | 0.04731322    | PRKCA, EGFR, IGF1R, FGF1R, CCDC6, RET, BAX, BRCA2, FAS, STAT1, CXCL12, COL4A5 |
| hsa04981:Protein tyrosine kinase  | 0.048700566   | PRKCA, EGFR, IGF1R, FGF1R, CCDC6, RET, BAX, BRCA2, FAS, STAT1, CXCL12, COL4A5 |

Fig. 4 shows 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), SRY-Box Transcription Factor 9 (SOX9), Insulin-like growth factor 1 receptor (IGF1R), MYB proto-oncogene (MYB), and PRKCA (Table 2).
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 (Garcia 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.
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

| 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            |
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 Kabolli 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 Figure S1. The distribution of value data for GSE67916 and GSE86871.