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Bioinformatics Analysis Uncovers the Importance of RTK-RAS-PI3K/Akt Regulation by Borneol in Overcoming Breast Cancer Resistance to Tamoxifen

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ABSTRACT

Currently, tamoxifen-based hormonal treatment remains the first line for luminal A (estrogen receptor [ER]-positive) subtype breast cancer, with a response of more than 30%. Chemoresistance was induced by the long-term use of tamoxifen therapy. Therefore, to prevent resistance and improve the effectiveness of tamoxifen, combined therapy is required. This study used bioinformatics to identify possible borneol target genes and their mechanism for overcoming tamoxifen resistance in breast cancer cells. We used data from the gene expression omnibus (GEO) collection to find differentially expressed genes (DEGs). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) site, version 6.8, was also used to undertake gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis of the DEGs. The STRING-DB site, version 11.0, was used to predict protein-protein interaction (PPI) study. The PPI analysis findings were visualized using the Cytoscape software, version 3.8.2. The hub gene was further calculated using the CytoHubba plugin. The genomic alterations from the hub gene were evaluated using cBioPortal, version 1.18.1. The potential target genes (PTGs) of borneol compounds are *ESR1, FGFR2, STAT3, ERBB4, PRKCA*, and RTK-RAS PI3K-Akt signaling as its prospective mechanism to overcome tamoxifen resistance in breast cancer cells. More studies are needed to confirm the potential of borneol to overcome tamoxifen resistance in breast cancer.

Keywords: Breast Cancer, Tamoxifen Resistance, Borneol, Bioinformatics

INTRODUCTION

Breast cancer primarily affects women, with 2.1 million new cases diagnosed each year and 6.8 million women living with the disease (WHO, 2019). The most frequent breast cancer subtype is estrogen receptor positive (ER+) or luminal A (Yersal & Barutca, 2014). The hormone-based treatment, tamoxifen, remains the treatment of luminal A breast cancer in more than 30% of cases (Fan *et al*., 2015). However, long-term tamoxifen monotherapy will result in tamoxifen resistance (Ali *et al*., 2016). As a result, combination therapy is required to overcome resistance and improve the efficacy of tamoxifen.

Borneol is a potential anticancer agent that can be used in combination with tamoxifen. In China, this compound is utilized as a guideline for traditional medicine (Zou *et al*., 2017). Borneol has also been found to decrease the proliferation of cancer cells, including the liver (Su *et al*., 2013), esophagus (Lee *et al*., 2020), and glioma (Lee *et al*., 2020; Wang *et al*., 2020). Borneol can overcome resistance when combined with another chemotherapeutic, including selenocysteine in liver cancer cells (Su *et al*., 2013), paclitaxel in ovarian cancer cells (Zou *et al*., 2017), temozolomide in brain cancer cells (Liu *et al*., 2018), and doxorubicin in lung cancer cells (Lai *et al*., 2020). Borneol is known to promote apoptosis in human glioma cells through the regulation of mTOR signaling (Wang *et al*., 2020). Borneol is widely used for increasing blood-brain barrier penetration of doxorubicin (Meng *et al*., 2019), carmustine (Guo *et al*., 2019) in glioblastoma, and gefitinib in lung cancer cells (Yuan *et al*., 2020) therapy. Recently, Borneol has increased the sensitivity of malignant glioma cells to radiotherapy by enhancing autophagy (Li *et al*.,

2021). However, the molecular mechanism of borneol for overcoming tamoxifen resistance remains unclear and requires further investigation.

This study uses a bioinformatics technique to uncover possible borneol target genes and biological processes expected to overcome tamoxifen resistance in MCF-7 breast cancer cells. We used microarray data from the gene expression omnibus (GEO) collection to find differentially expressed genes (DEGs). The examination of Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway through the Database for Annotation, Visualization, and Integrated Discovery (DAVID) website, version 6.8, is continued for DEGs results. The protein-protein interaction (PPI) network was then analyzed using the STRING-DB site, version 11.0. The PPI analysis findings were further analyzed using the Cytoscape software, version 3.8.2. Using the CytoHubba plugin of Cytoscape, the gene with the highest score was examined and chosen as the hub gene candidate. cBioPortal was used to study the gene change analysis of the hub gene to find potential borneol target genes to overcome tamoxifen resistance.

MATERIALS AND METHODS

Data collection and processing

The microarray data of tamoxifen-resistant MCF-7 cells were obtained from the GEO database, i.e., GSE67916 (Elias *et al.*, 2015). Two samples were selected consisting of tamoxifen-sensitive and tamoxifen-resistant MCF-7 cells. Borneol-treated MCF-7 microarrays were obtained from the GSE85871 (Lv *et al*., 2017). The microarray analysis of tamoxifen-sensitive and resistant MCF-7 cells and borneol-treated MCF-7 cells were processed using Affymetrix Human Genome U133A 2.0 microarray technology (Santa Clara, CA). Then, the data was analyzed using GEO2R, an online analysis site based on the R programming language, based on the previous study (Hermawan *et al*., 2020). The raw microarray data from GSE67916 and GSE85871 were distributed equally (Figure S1). Next, the DEGs screening was conducted on both data sets by setting the *p*-value to <0.05 and the Ilog Fold change to >1. The Venny site, version 2.1, was used to do the slice expression analysis of the two GSEs (Malo *et al*., 2020).

Analysis of the ontology gene (GO) and the KEGG pathway

The Venn diagram slices provide helpful information for future investigation.

Analysis of ontology genes (GO) and KEGG pathways was carried performed using the online website DAVID, version 6.8 (Yang *et al*., 2020). The analysis results were selected based on the reference *p*-value of <0.05.

Selection of hub gene and protein-protein interaction (PPI interaction)

PPI analysis was done using STRING-DB, version 11.0 (Szklarczyk *et al*., 2019), with a confidence level of more than 0.4. Then, the results of the analysis were displayed through the Cytoscape software, version 3.8.2 (Doncheva *et al*., 2019; Hermawan *et al*., 2020). Genes with a score of more than 5 were analyzed using the CytoHubba plugin and selected as hub gene candidates (Chin *et al*., 2014).

Analysis of genetic alterations of potential target genes (PTGs)

Genetic alterations of the hub gene were analyzed using cBioPortal (Wu *et al*., 2019). The highest score was chosen from the results of genetic alterations across breast cancer studies by considering a *p*-value of <0.05 (Hermawan *et al*., 2021). An analysis of the relationship between genetic alterations and the KEGG pathway was further performed.

RESULTS AND DISCUSSION

Data preparation

This study uses a bioinformatics approach to identify potential target genes and molecular mechanisms of borneol in overcoming tamoxifen resistance in breast cancer. Previously, there has been no study on borneol to overcome tamoxifen resistance in breast cancer therapy. The analysis of GSE67916 identified 1912 genes, consisting of 1048 upregulated and 863 downregulated genes (Figure 1B, Supplementary Table I). Meanwhile, GSE85871 has 1415 genes, with 388 being upregulated and 1026 downregulated (Figure 1B, Supplementary Table II). Then, the slice results from both GSE67916 and GSE85871 resulted in 173 DEGs were considered as potential target genes (PTGs) and were further analyzed by other databases (Figure 1B, Supplementary Table III).

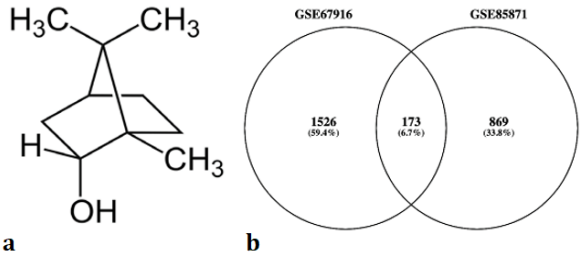

Figure 1. (A) The chemical structure of borneol. (B) A Venn diagram of tamoxifen-resistant and borneol-treated MCF-7 breast cancer cells.

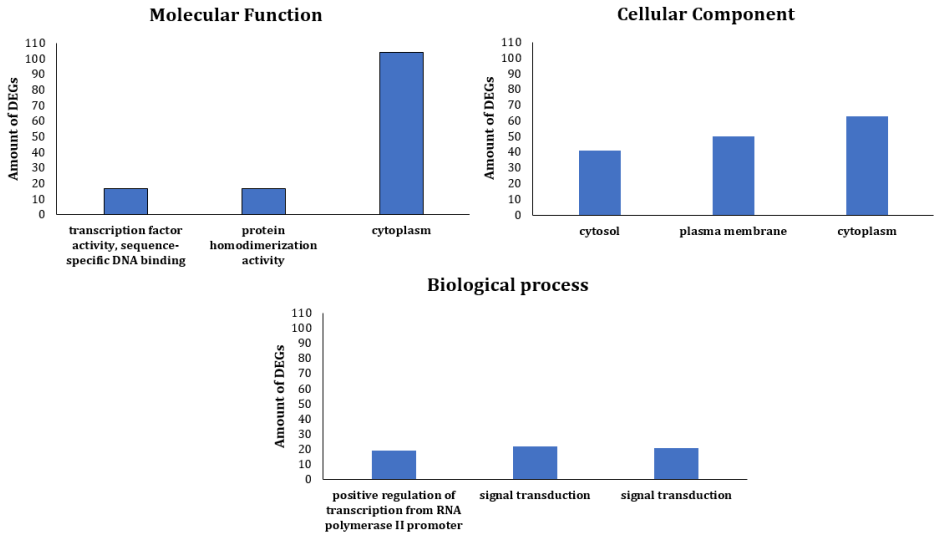

Figure 2. GO analysis of the DEGs using the DAVID database, version 6.8. (A). biological processes. (B) molecular functions. (C) cellular components.

Table I. KEGG pathways enrichment analysis of the DEGs analyzed using the DAVID database, version 6.8.

Term	p-value	Genes
hsa04151:PI3K-Akt signaling pathway	0.01323208	*ITGB4, PDGFC, COL4A3, MYB, COL4A5, PRKCA, FGFR4, SGK1, PRLR, FGFR2*
hsa04350:TGF-beta signaling pathway	0.01363875	*CDKN2B, BAMBI, BMP8B, LTBP1, SMAD5*

Analysis of gene ontology (GO) and KEGG Pathways

Several criteria were used to analyze the ontology gene (GO), including biological processes, cellular components, and molecular functions. DEGs influence three biological processes, signal transduction, positive regulation of DNA-templated transcription, and positive transcription control from the RNA polymerase II promoter **(Figure 2A,** Supplementary Table IV**).** Meanwhile, the molecular function study results revealed that DEGs affected protein binding functions **(Figure 2B,** Supplementary Table V**).** The results of the cellular component analysis showed that DEGs are part of the cytoplasm, plasma membrane, and cytosol **(Figure 2C,** Supplementary Table VI**).**

Fibroblast growth factor receptor 2 (FGFR2) and protein kinase C alpha type (PRKCA) are located on the endoplasmic reticulum and cell surface. FGFR2 and PRKCA are also involved in the transmembrane functions of protein kinase receptors and their ability to bind to enzymes.

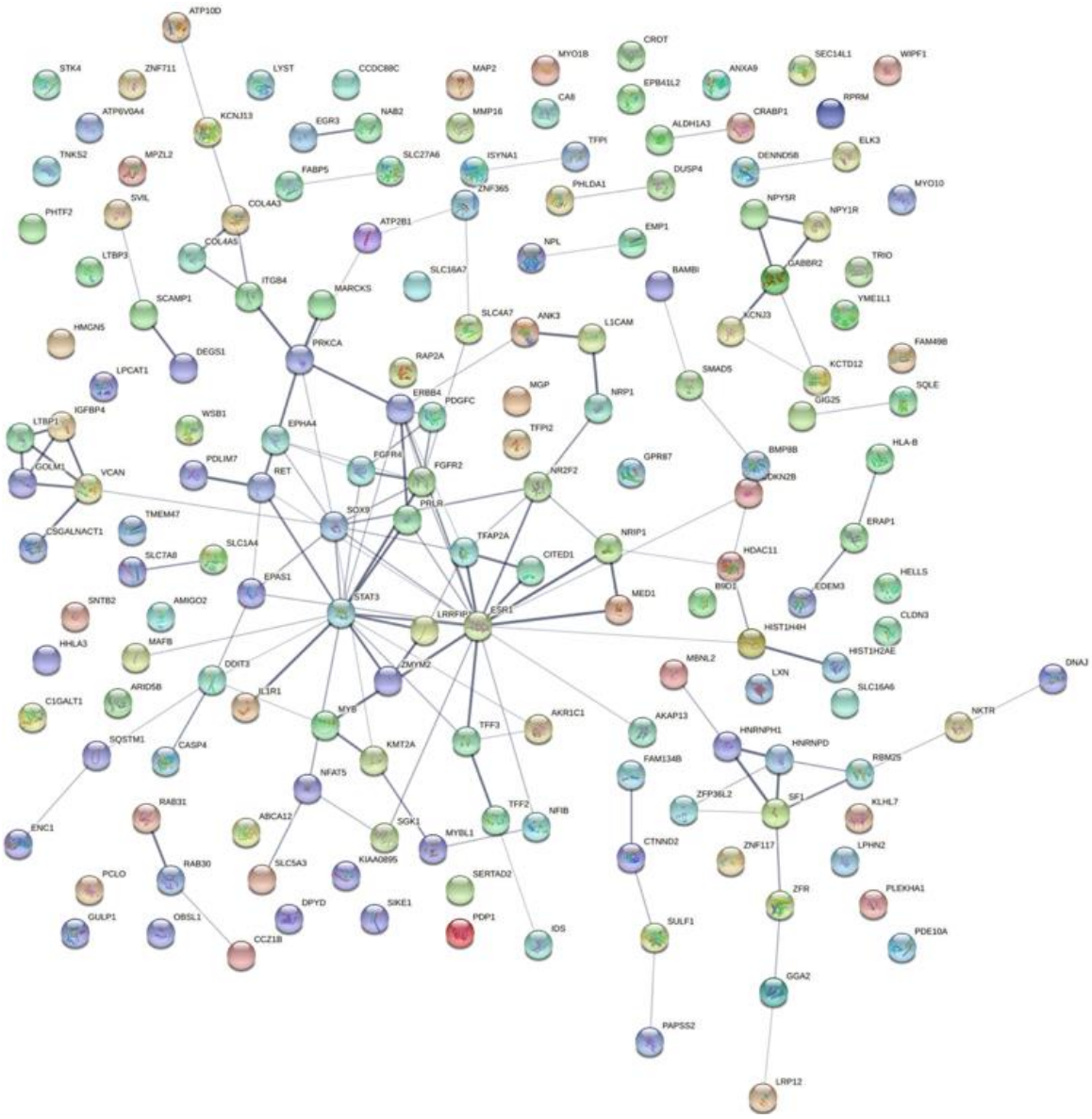

Figure 3. The protein-protein interaction (PPI) network of DEGs, as analyzed by STRING, version 11.0.

Table II. Seven genes with the highest degree scores from CytoHubba

No	Gene symbol	Gene name	Degree score
1	*ESR1*	Estrogen Receptor 1	46
2	*STAT3*	Signal Transducer and Activator of Transcription 3	32
3	*FGFR2*	Fibroblast growth factor receptor 2	23
4	*SOX9*	SRY-Box Transcription Factor 9	19
5	*ERBB4*	ERB-B2 Receptor Tyrosine Kinase 4	18
6	*TFAP2A*	Transcription Factor AP-2 Alpha	11
7	*NR2F2*	Nuclear Receptor Subfamily 2 Group F Member 2	9

According to the KEGG pathway study, DEGs regulate cancer mechanism pathways, such as the phosphatidylinositol 3 kinase-protein kinase B (PIK3-Akt) signal transduction pathway (Table I), Supplementary Table VII).

Protein-protein interaction (PPI) and the selection of hub genes

The interaction analysis between proteins was performed by identifying interactions between DEGs and then using CytoHubba as a plugin to explore the relationship between genes. A total of 173 proteins were arranged to form a protein network (with a CI index of 0.4), consisting of 165 nodes, 138 edges, a PPI enrichment value of 1.21e-4, and an average local clustering coefficient of 0.393 (Figure. 3). Moreover, *ESR1* (Estrogen Receptor 1), *STAT3* (Signal Transducer and Activator of Transcription 3), *FGFR2* (Fibroblast growth factor receptor 2), *SOX9* (SRY-Box Transcription Factor 9), *ERBB4* (ERBB2 Receptor Tyrosine Kinase 4), *TFAP2A* (Transcription Factor AP-2 Alpha), and *NR2F2* (Nuclear Receptor Subfamily 2 Group F Member 2) were chosen as the genes with the greatest degree scores (Figure 4, Table II).

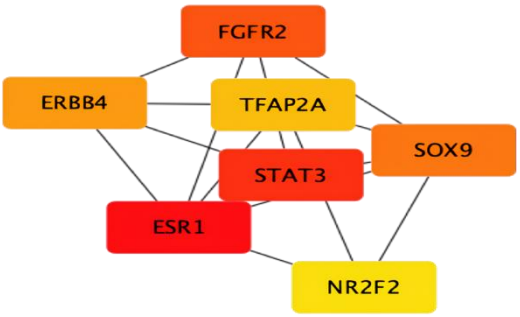

Figure 4. Hub genes, as selected by CytoHubba, are based on their highest degree scores.

Analysis of genetic alterations of selected PTGs

A total of five PTGs were selected, namely, *ESR1, STAT3, FGFR2, ERBB4,* and *PRKCA*, and were analyzed using cBioPortal to determine gene changes associated with breast cancer studies. *ESR1, STAT3, FGFR2*, and *ERBB4* were the genes selected from CytoHubba based on the highest degree scores. *PRKCA* was chosen among the DEGs from the KEGG pathway. The study named The Metastatic Breast Cancer Project (MBCP) Year 2020 was chosen because it is the latest study on breast cancer in cBioPortal. In addition, it was selected because cases of tamoxifen resistance also led to metastasis (Smyth *et al*., 2020) (Figure 5A). PTGs had alterations in their genes, including *STAT3* (4%), *FGFR2* (4%), *ERBB4* (5%), *ESR1* (10%), and *PRKCA* (14%) (Figure 5B). Furthermore, most of these gene alterations resulted from amplification and missense mutations (Figure 5B). Additional analysis showed that *ERBB2* was the nearby gene with the strongest connection in the gene network associated with PTGs (Figure 5C). We screened for surrounding genes and yielded two matched genes, i.e., ERBB4 and FGFR2, indicating that they may be used as therapeutic targets for borneol therapy. In addition, PTG may play a role in anticancer activity by regulating receptor tyrosine kinase (RTK) signaling (Table III).

Table III. The results of pathways related to alterations of selected PTGs, as analyzed by cBioPortal.

Pathway name	**RTK-RAS**
Score	2.00
Gene matched	*FGFR2, ERBB4*

RTK regulates cellular processes and plays a crucial role in the development of various tissue cancers. Mutations in this system cause constitutive activation of a cascade of signaling pathways, each of which plays a different function in protein overexpression that causes cancer (McDonell *et al*., 2015). Because RTKs are important in cancer progression, researchers must focus on oncogenic RTK signaling pathway driver mutations.

Proposed PTGs and mechanism of borneol in overcoming tamoxifen resistance

Estrogen binding to the estrogen receptor (ER) activates the PI3K pathway by inducing transcription factors, such as estrogen response elements, coactivators (CoA), and transcription factors. The estrogen-dependent signaling can be selectively inhibited by 4-hydroxy tamoxifen (4HT) (Okat, 2018). A mouse model study revealed that Chinese herbal formula containing borneol inhibited estrogen signaling in pre-cancerous breast tissue (Zhang *et al*., 2019).

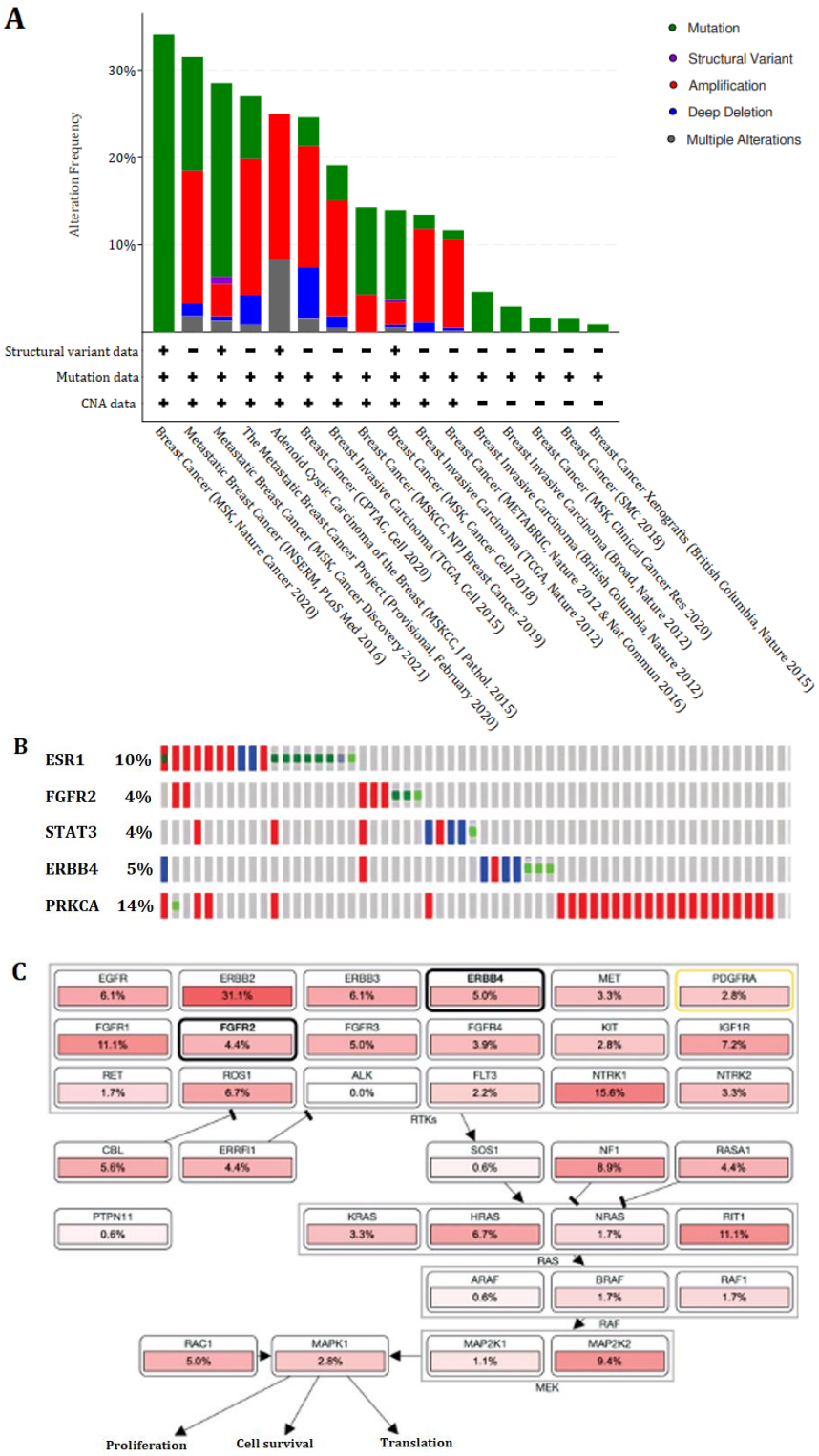

Figure 5. (A) Genetic alterations in *ESR1*, *STAT3*, *FGFR2*, *ERBB4*, and *PRKCA* in 18 breast cancer studies. (B) Percentage of genetic alterations in *ESR1*, *STAT3*, *FGFR2*, *ERBB4*, and *PRKCA* across breast cancer samples (based on the MBCP study 2020). (C) Neighboring gene connectivity of selected PTGs. All results were obtained from the cBioPortal database.

In this study, the KEGG pathway results revealed the molecular mechanism by which borneol compounds overcome tamoxifen resistance to breast cancer therapy. It is mediated via PI3K-Akt signaling. In addition, the investigation results using cBioPortal revealed that PTGs are involved in the RTK-RAS signaling pathway. The RTK pathway, which is upstream of both the PI3K-Akt and RAS signaling pathways (Mendoza *et al*., 2011), also plays a role in tamoxifen resistance by activating the estrogen-independent pathway.

RTK signaling begins when the ligand binds to one of the RTK families, such as ERBB4 or FGFR2, and then proceeds through dimerization, transphosphorylation, and activation (Mendoza *et al*., 2011). Intracellular signaling then produces PI3K transcriptional activator, which, in turn, produces RAS, RAF, p38MAPK, and p53 in the RTK-RAS signaling pathway (Castellano & Downward, 2011). Meanwhile, PDK1, Akt, and GSK-3 are activated by the PI3K-Akt pathway (Huang *et al*., 2018).

Several cellular functions, including proliferation, differentiation, and apoptosis, are controlled by the RAS, p38/MAPK, and p53 pathways (Yue *et al*., 2020). These three pathways are activated by binding to the proper ligand, dimerization, and phosphorylation to activate RAS (Maruyama, 2014). RAS then travels to the plasma membrane to phosphorylate and activate RAF and MEK (MAPK Kinase), which activates MAPK (Yue & Lopez, 2020). Tamoxifen resistance is linked to RAS, p38/MAPK, and p53 activation via estrogen receptor (ER) phosphorylation. The transcription of estrogen-regulated genes is then triggered by phosphorylation of serine 118 in the receptor domain AF-1 (Rani *et al*., 2019). As a result, the sensitivity of the ER to low estrogen concentrations increases in these three pathways, leading to tamoxifen resistance (Clarke *et al*., 2015). IGF stimulation can activate the RAS, p38/MAPK, and p53 pathways upstream, causing ER S118 phosphorylation and ER activation, increasing in the estradiol response (Viedma *et al*., 2014). According to reports, the upstream area of serine 282 can be phosphorylated by CK2, generating an increase in S282 phosphorylation, stabilizing the ER, and causing tamoxifen resistance (Viedma *et al*., 2014).

To date, scientists have been searching for compounds that target a serine 282 (S282) in tumor suppressor proteins to overcome tamoxifen resistance and trigger apoptosis. The dephosphorylation of S282, which activates the tumor suppressor gene p53 is occurred in apoptotic cells (Yang *et al*., 2019). P53 interacts with Bax and enters the mitochondrial membrane after being activated (Holley & Clair, 2009). The tumor suppressor gene p53 is activated, disrupting mitochondrial membrane permeability, and causing mitochondrial death (Elmore, 2007). Borneol targets oncogenes in the RAS pathway, p38/MAPK, and p53 in several recent investigations (Figure 6). Borneol is thought to protect against ischemic brain injury by suppressing the RAS system, which controls blood pressure and, therefore, reduces ischemic damage (Ma *et al*., 2021). Borneol also induced apoptosis by the upregulation of p38/MAPK (Yang *et al*., 2014). In addition, borneol inhibited glioma cancer cell proliferation by reducing p53 expression and Ki-67 labeling (Liu *et al*., 2018).

The activation of PI3K-Akt signaling stimulates downstream protein, for instance, GSK-3, which affects cell proliferation and survival, and metastasis (Kitagishi *et al*., 2012, Murwanti *et al*., 2020). In breast cancer cells, GSK-3 is observed when Akt is phosphorylated (McCubrey *et al*., 2008). Moreover, GSK-3 was upregulated in 35% patients with invasive ductal carcinomas and was linked to tumor progression (Prasad *et al*., 2009). In the process of skeletal development, there is a link between the control of GSK-3 and SOX9. GSK-3 phosphorylates SOX9 and type II collagen (COL2A1) (Itoh *et al*., 2012). SOX9 signaling induces cell proliferation and survival (Gao *et al*., 2015). GSK-3 kinase-dead overexpressing MCF-7 cells (KD) were more resistant to tamoxifen than the wild-type (Sokolosky *et al*. 2014). The anti-apoptotic protein Bcl-xl, which is inversely proportional to GSK-3 activity, as indicated by immunohistochemistry labeling of p-GSK-3 in breast cancer specimens, can affect GSK-3-mediated tamoxifen resistance (Ding *et al*., 2007). SOX9 accumulation was associated with the enhanced proliferation of invasive ductal carcinoma in breast cancer studies (Chakravarty *et al*., 2011). Furthermore, dose-response studies have revealed that SOX9 overexpression is linked

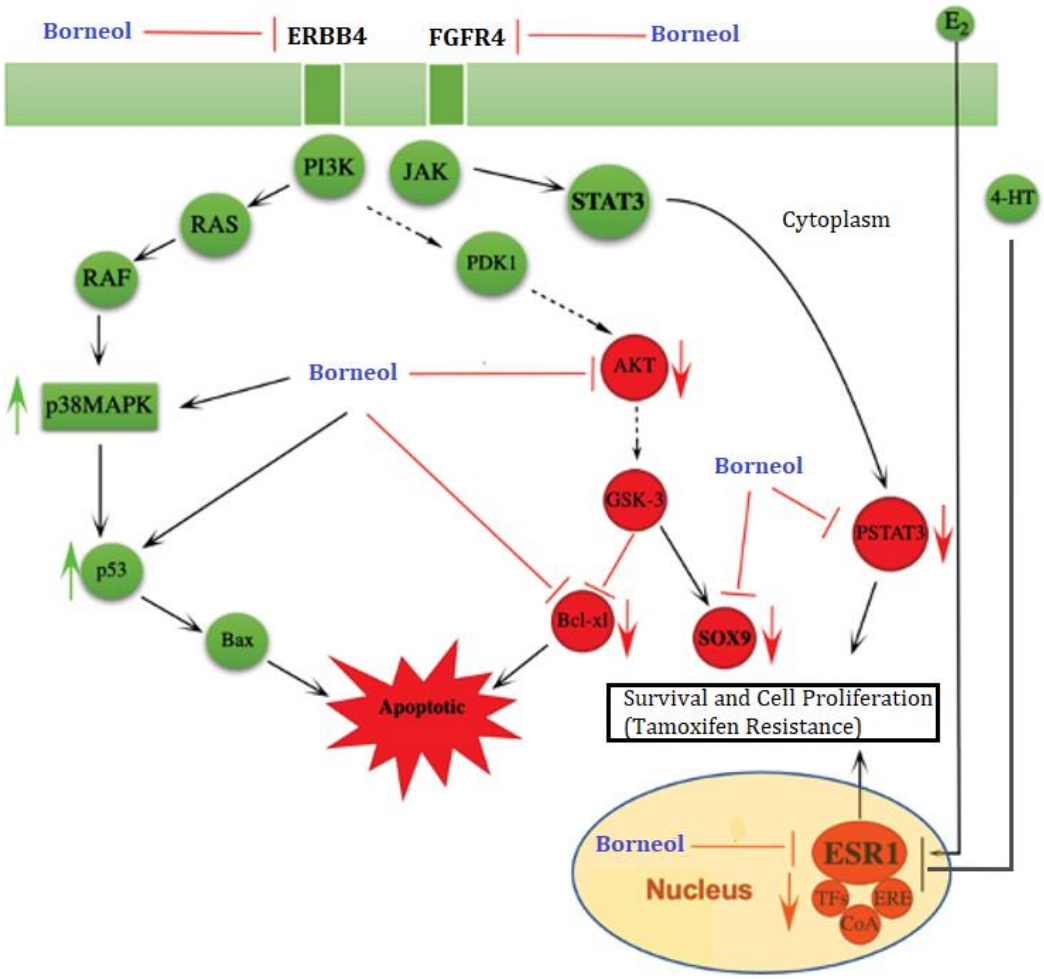

Figure 6. A proposed mechanism of borneol in overcoming tamoxifen resistance in breast cancer cells.

to a lower chance of survival in breast cancer patients (Gyorffy *et al*., 2010). By the immunofluorescence test, the overexpression of SOX9 causes an accumulation of these proteins in the cell nucleus, resulting in tamoxifen resistance (Xue *et al*., 2019). Borneol can also protect mice from cerebral ischemia by reducing proinflammatory cytokine generation by inhibiting SOX9 activity (Lei *et al*., 2017).

The JAK-STAT signaling pathway by activation of FGFR2 triggers oncogenic genes, including STAT3 and PSTAT3, resulting in cell proliferation and survival (Vainchenker & Constantinescu, 2013). In the cytoplasm, JAK recruits phosphorylated-STAT3 and activates the gene transcription process (Kiu & Nicholshon, 2012). An overexpression of STAT3 in ZR cells (ZR-75-30) resulted in tamoxifen resistance in another investigation. This overexpression is linked to elevated ZIP (ZRT, IRT-like Protein) expression in MCF-7 cells, leading to enhanced STAT3 activity and tamoxifen resistance (Zhu *et al*., 2020). Borneol also induces STAT3 activity in M2 macrophage cells (Zhang *et al*., 2017). However, these findings must be confirmed to know the potential of borneol in combating tamoxifen resistance.

Previously, borneol was shown to increase the sensitivity of human esophageal squamous cell carcinoma towards paclitaxel by inhibiting PI3K/Akt signaling (Meng, *et al*. 2018). Borneol also increases glioma cells sensitivity towards doxorubicin by inhibiting PI3K signaling and elevating reactive oxygen species (ROS) levels (Cao *et al*., 2019). Moreover, borneol induces G2/M cell cycle arrest and apoptosis of HepG2 hepatocellular carcinoma cells by increasing p53-dependent ROS

levels (Chen *et al*., 2015). Collectively, borneol is able to inhibit PI3K/Akt signaling pathway, however, the exploration of borneol as an agent to alleviate tamoxifen resistance in breast cancer by targeting PI3K/Akt pathway deserves further investigation. Borneol has to be validated by more research, such as molecular dynamics and docking. In addition, *in vivo* studies and clinical trials are needed to develop borneol to overcome tamoxifen resistance.

CONCLUSION

This study identified five possible borneol targets to overcome tamoxifen resistance, including *ESR1, FGFR2, STAT3, ERBB4,* and *PRKCA*. The proposed mechanism of borneol to overcome tamoxifen resistance in breast cancer cells is RTK-RAS and PI3K-Akt signaling. However, more studies are required to confirm the findings of this study.

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