Alterations in mRNA profiles of trastuzumab-resistant Her-2-positive breast cancer

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Abstract. Breast cancer is one of the most common malignancies in women. Neoadjuvant trastuzumab therapy improves the prognosis of certain Her-2-positive breast cancer patients, however around two-thirds of patients with Her-2-positive breast cancer do not benefit from Her-2-targeted therapy. To investigate the key mechanisms in trastuzumab resistance, potential biomarkers for neoadjuvant trastuzumab sensitivity were investigated using the gene expression omnibus (GEO) database for mRNA microarray data of Her-2-positive breast cancer patients who received neoadjuvant trastuzumab therapy. GEO profiles of 22 patients with a complete response and 48 patients with a partial response were identified in the GSE22358, GSE62327 and GSE66305 datasets. A total of 2,376, 1,000 and 1,152 differentially expressed genes in GSE22358, GSE62327 and GSE66305 datasets were demonstrated, respectively, utilizing GEO2R software. Furthermore, enriched gene ontology terms and Kyoto Encyclopedia of Genes and Genomes pathways were analyzed using the Database for Annotation, Visualization and Integrated Discovery software. Subsequently, a protein-protein interaction network was established using STRING software. The results demonstrated that low sex-determining region Y-box 11 and high Bcl-2 expression may be employed as markers for neoadjuvant trastuzumab therapy for Her-2-positive breast cancer. More importantly, phosphoinositide 3-kinase/Akt and angiogenesis pathways, which are known to be the key targets of trastuzumab, were activated at a lower level in the partial response patients, while the Wnt and estrogen receptor signaling pathways were activated in these patients. Therefore, combination therapy of trastuzumab and anti-Wnt or hormone therapy may be a promising treatment modality and should be tested in further studies.

Introduction

Breast cancer is one of the most common malignancies in women and demonstrates an increasing incident rate (1). Advances in neoadjuvant therapy have greatly altered the treatment of patients with breast cancer, particularly patients with Her-2-positive breast cancer. Neoadjuvant therapy increases the opportunity for breast-conserving surgery in patients that were originally candidates for mastectomy and allows a potentially more defined prognosis (2). Trastuzumab, a Her-2-targeting therapeutic antibody, has increased the survival of patients suffering from Her-2-positive breast cancer. Various randomized trials have demonstrated that trastuzumab significantly improved the efficacy of adjuvant and neoadjuvant chemotherapy (3-6). At present, trastuzumab is considered the standard primary therapy for Her-2-positive breast cancer in the National Comprehensive Cancer Network guidelines (https://www.nccn.org). Although the efficacy of trastuzumab in the neoadjuvant settings for Her-2-positive breast cancer is remarkable, the drug resistance limits its potential. Around two-thirds of patients with Her-2-positive breast cancer cannot benefit from Her-2-targeted therapy (7). Trastuzumab resistance may lead to delays in treatment as well as unnecessary costs and trastuzumab-associated side effects, therefore, it is imperative to identify which patients are unlikely to benefit from trastuzumab treatment. In the last decade, a number of studies have aimed to investigate the mechanisms of resistance to Her-2-targeted therapies and to identify molecular targets for resistance-conferring factors.

The most commonly recognized anti-cancer mechanism of trastuzumab is the targeting of the extracellular domain of the Her-2 receptor and the inhibition of the downstream phosphoinositide 3-kinase (PI3K)/Akt pathway; therefore, PIK3CA, a mutation of the PI3K gene, was considered to be an important reason for trastuzumab resistance (8), while several other studies demonstrated that the PIK3CA gene is common in Her-2-positive breast cancer but demonstrated no significant association between the PIK3CA mutation and trastuzumab resistance (9-11). Various studies have also investigated the transcriptome of trastuzumab-resistant Her-2-positive breast

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cancer, but these studies were based on limited samples and demonstrated great heterogeneity (12-14).

In the present study, the microarray transcriptome data of trastuzumab-resistant breast cancer and trastuzumab-sensitive breast cancer in previously reported neoadjuvant therapy studies were compared to identify potential biomarkers for trastuzumab-sensitive Her-2-positive breast cancer and investigate the potential molecular mechanisms of trastuzumab resistance.

Materials and methods

Microarray data. The Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) was searched for mRNA expression microarrays of Her-2-positive breast cancer patients who received trastuzumab-based neoadjuvant chemotherapy with the key terms 'breast cancer AND (trastuzumab OR herceptin)', and 24 GEO series were identified. Nine were excluded for not reporting neoadjuvant trastuzumab therapy, five were excluded for not reporting data of human samples, three were excluded for poor data quality, two were excluded for not reporting mRNA microarray data and two were excluded for reporting no complete response patients. As a result, three gene expression profiles (GSE22358, GSE62327 and GSE66305) were finally obtained from the GEO database. Microarray data of GSE22358 included 10 breast cancers with complete response and 13 breast cancers with no complete response (12,14). GSE62327 consisted of 6 breast cancers with complete response and 18 breast cancers with no complete response (14). GSE66305 included 6 breast cancers with complete response and 17 breast cancers with no complete response (13).

Data processing. The GEO database archives a large number of high-throughput functional genomic studies that contain data that are processed and normalized using various methods. GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r/) was applied to screen differentially expressed mRNAs and genes between breast cancer with partial response to trastuzumab-based neoadjuvant chemotherapy and breast cancer with pathological complete response. P<0.05 was set as the cut-off criteria for differently expressed genes (DEGs). DEGs yielded by at least two datasets were selected for further analysis. DEGs with P<0.05 and fold-change (|FC|)>1.5 in all three datasets were selected as biomarkers of trastuzumab resistance. Venn Diagrams were created using the Venn Diagrams software (http://bioinformatics. psb.ugent.be/webtools/Venn/) to display the overlap of DEGs between the three datasets.

Functional and pathway enrichment analysis. Upregulated and downregulated genes were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.8; http://david. abcc.ncifcrf.gov/) software. GO and KEGG pathway enrichment analysis were performed for identified DEGs using the DAVID database. P<0.05 was set as the cut-off criterion.

Protein-protein interaction (PPI) network construction and module selection. The identification of functional interactions
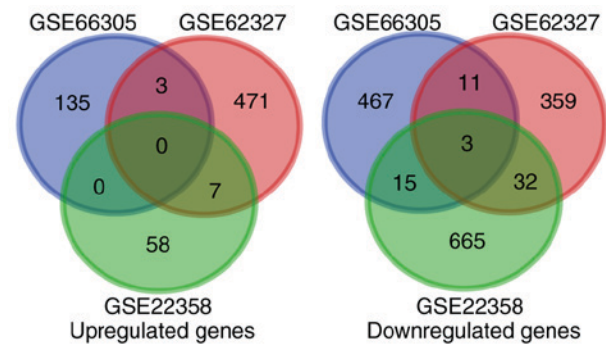

Figure 1. Upregulated and downregulated DEGs in breast cancer cases with a partial response to trastuzumab. Common DEGs among the three datasets were identified using Venn diagrams. Three genes, including sex-determining region Y-box 11, ATPase phospholipid transporting 8B2 and outer dense fiber of sperm tails 2-like, were reported to be downregulated in the partial response group in all three datasets. A total of 58 genes were reported to be downregulated in the partial response group in two datasets and 10 genes were reported to be upregulated in the partial response group in two datasets. DEGs, differentially expressed genes.

between proteins among the DEGs can provide context in the molecular mechanism of cellular processing. In the present study, a PPI network of DEGs was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING; http://string.embl.de/) database. The cut-off criterion for PPI scores was set at 0.4. Subsequently, the PPI network was analyzed using the Molecular Complete Detection (MCODE, version 1.4.2) plug-in of the Cytoscape software (version 3.4.0) (15) And enrichment analysis were performed in the PPI network using the DAVID software.

Results

Identification of DEGs. Preliminary screening yielded a total of 2,376, 1,000 and 1,152 DEGs in the GSE22358, GSE62327 and GSE66305 datasets, respectively (1,305, 546 and 398 upregulated genes, and 1,071, 454 and 754 downregulated genes, respectively). Among them, phosphate cytidylyltransferase 2 ethanolamine, estrogen receptor 1 (ESR1) and synaptogyrin 1 were upregulated in all three datasets, and sex-determining region Y-box 11 (SOX11), ATPase phospholipid transporting 8B2 (ATP8B2) and outer dense fiber of sperm tails 2-like (ODF2L) were downregulated in all three datasets. Furthermore, when P<0.05 and |FC|>1.5 were used as the criteria to further screen the DEGs with the most significant difference of expression, a total of 981, 972 and 555 DEGs were identified from the GSE22358, GSE62327 and GSE66305 datasets, respectively (488, 548 and 105 upregulated genes, and 493, 424 and 450 downregulated genes, respectively). Among them, SOX11, ATP8B2 and ODF2L were downregulated with >1.5-fold alterations in all three datasets (Fig. 1).

GO and pathway analysis. To assess the function of the DEGs, GO and KEGG pathway enrichment analyses were performed using DAVID software. The upregulated genes in partial response patients were primarily enriched in 'intracellular organelle lumen', 'transcription activator activity', 'cytoskeletal protein binding', 'tissue morphogenesis' and 'response to extracellular stimulus'. Downregulated genes in

Table I. Top 10 GO terms enriched in DEGs in Her-2-positive breast cancer with partial response to trastuzumab.

A, Upregulated DEGs

GO term	Gene function description	Count	P-value
GO:0070013	Intracellular organelle lumen	11	0.026
GO:0016563	Transcription activator activity	5	0.026
GO:0008092	Cytoskeletal protein binding	5	0.050
GO:0048729	Tissue morphogenesis	4	0.016
GO:0009991	Response to extracellular stimulus	4	0.027
GO:0033273	Response to vitamin	3	0.016
GO:0060562	Epithelial tube morphogenesis	3	0.017
GO:0046661	Male sex differentiation	3	0.020
GO:0043583	Ear development	3	0.032
GO:0043627	Response to estrogen stimulus	3	0.038

B, Downregulated DEGs

GO term	Gene function description	Count	P-value
GO:0016021	Integral component of membrane	32	0.004
GO:0005886	Plasma membrane	26	0.011
GO:0070062	Extracellular exosome	21	0.004
GO:0005887	Integral component of plasma membrane	11	0.047
GO:0007165	Signal transduction	10	0.037
GO:0043066	Negative regulation of apoptotic process	7	0.009
GO:0005925	Focal adhesion	6	0.018
GO:0030424	Axon	5	0.011
GO:0005913	Cell-cell adherens junction	5	0.037
GO:0010628	Positive regulation of gene expression	5	0.020

GO, Gene ontology; DEGs, differentially expressed genes.

partial response patients were primarily enriched in 'integral component of membrane', 'plasma membrane', 'extracellular exosome', 'signal transduction' and 'negative regulation of apoptotic process' (Table I). The upregulated KEGG pathways in the partial response group were 'pathways in cancer', 'basal cell carcinoma', 'melanogenesis' and 'tight junction'. Downregulated pathways in the partial response group were 'PI3K-Akt signaling pathway', 'FoxO signaling pathway', 'Rap1 signaling pathway', 'dopaminergic synapse' and 'cytokine-cytokine receptor interactions' (Table II).

PPI network construction. Upregulated DEGs in the partial response group were mapped with the STRING database. With a PPI score >0.4, a PPI network with 96 nodes and 134 edges was constructed, as presented in Fig. 2A. Two modules were obtained from a PPI network of DEGs using MCODE, one with 32 nodes and 60 edges, and the other with 6 nodes and 6 edges (Fig. 2B). GO term and KEGG pathway enrichment analysis revealed that genes in module 1 were associated with 'protein binding' and 'cellular response to vascular endothelial growth factor stimulus' GO terms, while genes in module 2 were associated with 'frizzled binding', 'canonical Wnt signaling pathway' and 'neuron differentiation GO terms,

and 'basal cell carcinoma', 'melanogenesis', 'Wnt signaling pathway' and 'signaling pathways regulating pluripotency of stem cells' KEGG pathways (Table III).

Discussion

Utilization of trastuzumab has greatly improved the prognosis of patients with Her-2-positive breast cancer. Neoadjuvant regimens, including trastuzumab, have demonstrated improved complete response rates compared with the previous neoadjuvant regimens; however, given the high rate of trastuzumab resistance and the high treatment expense, the effects of these regimens are far from satisfying. The combined regimens of trastuzumab and other targeted drugs are currently being tested (16) and further knowledge of the detailed mechanism of trastuzumab resistance is required. Several studies have examined the gene expression profiles of patients receiving neoadjuvant trastuzumab therapy using microarray gene chips (12,13), but the majority of these studies were performed with a small sample size and the results varied between studies. In the present study, microarray data from three GEO datasets were analyzed, which collectively included the gene expression data of 22 complete response tumors and 48 partial

Table II. KEGG pathways enriched in DEGs in Her-2 positive breast cancer with partial response to trastuzumab.

A, Upregulated DEGs

KEGG term	Description	Count	P-value	Genes
hsa05200	Pathways in cancer	5	0.008	DVL3, WNT4, WNT3, BCL2, RARA
hsa05217	Basal cell carcinoma	3	0.008	DVL3, WNT4, WNT3
hsa04916	Melanogenesis	3	0.025	DVL3, WNT4, WNT3
hsa04530	Tight junction	3	0.044	EPB41L1, MAGI1, MYH14

B, Downregulated DEGs

KEGG term	Description	Count	P-value	Genes
hsa04151	PI3K-Akt signaling pathway	8	0.004	FLT1, OSMR, VEGFA, CREB3L2, ITGB4, LPAR3, PRKAA1, BCL2L11
hsa04068	FoxO signaling pathway	5	0.008	CDKN2B, MAPK14, PRKAA1, STK4, BCL2L11
hsa04015	Rap1 signaling pathway	5	0.035	FLT1, MAPK14, VEGFA, LPAR3, CALML5
hsa04728	Dopaminergic synapse	4	0.040	DDC, MAPK14, CREB3L2, CALML5
hsa04060	Cytokine-cytokine receptor interaction	5	0.046	TNFRSF21, FLT1, OSMR, VEGFA, IL22RA2

KEGG, Kyoto encyclopedia of genes and genomes; DEGs, differentially expressed genes.

response tumors, in order to cast light on the mechanism of trastuzumab resistance in Her-2-positive breast cancer.

Several molecular mechanisms have been proposed to explain the action of the trastuzumab on Her-2, which are divided into the following categories: Inhibiting the downstream PI3K/Akt signaling pathway of Her-2 (17); promoting the phosphorylation of phosphatase and tensin homolog (18); reducing cancer cell proliferation by reducing the expression of the cyclin D1 protein, leading to G1 arrest; inhibiting angiogenesis by reducing the production of the vascular endothelial growth factor (VEGF); inhibiting Her-2 ectodomain cleavage (19); and binding the Fc-γ receptor III on immune effector cells and inducing antibody dependent cell mediated cytotoxicity (20). In the present study, it was demonstrated that several targeted pathways of trastuzumab exhibited a relatively lower activity in the partial response group, which may have a role in trastuzumab resistance.

Sustained excessive proliferative signaling is one of the most fundamental hallmarks of cancer (21). It is reported that overexpression of Her-2 may lead to the activation of the PI3K/Akt pathway, and the excessively activated PI3K-Akt signaling pathway serves a crucial role in the proliferation of breast cancer (22). Okutur *et al* (23) reported that 96% of Her-2-positive breast cancer exhibited overexpression of the PI3K protein and 70.4% exhibited overexpression of the Akt protein. Inhibition of the PI3K/Akt pathway through targeting Her-2 is considered to be one of the key mechanisms underlying the anti-tumor effects of trastuzumab (24); however, in the present study, it was demonstrated that among the patients receiving trastuzumab treatment, patients with partial responses tended to have lower PI3K-Akt pathway activity prior to treatment, compared with the patients with a complete response. In addition, the present

study revealed that genes associated with the activation of the Wnt signaling pathway, including Wnt family member 3 (WNT3), WNT4 and disheveled segment polarity protein 3, were excessively expressed in the partial response group. The expression of WNT3 was reported to activate the Wnt/β-catenin pathway and promote a epithelial-mesenchymal transition-like phenotype in trastuzumab-resistant Her-2-overexpressing breast cancer cells, resulting in an increase in cell invasion and proliferation (25). The results of the current study indicated that a proportion of Her-2-positive breast cancers may acquire trastuzumab resistance by downregulation of the PI3K/Akt pathway and may maintain proliferative signaling by the upregulation of the Wnt pathway. Therefore, combining Wnt-targeted therapy with trastuzumab may help to enhance the complete response rate of Her-2-targeted therapy.

Cancer cells have been demonstrated to acquire the oxygen and nutrients required for their rapid proliferation by inducing angiogenesis, which is also a key target of trastuzumab (26). In the present study, trastuzumab-resistant breast cancers exhibited downregulated cytokine-cytokine receptor interaction pathways, including the downregulation of VEGF and its receptor, Fms-related tyrosine kinase 1. In addition, the PPI analysis demonstrated the downregulation of a module primarily associated with the cellular response to VEGF stimulus in the partial response group. These results indicate that trastuzumab-resistant breast cancers may be independent of tumor angiogenesis and therefore be resistant to the antiangiogenic effect of trastuzumab. These results may also suggest that trastuzumab-resistant breast cancers also tend to be poorly vascularized even prior to neoadjuvant chemotherapy, which may lead to low regional trastuzumab concentration in the tumor foci.
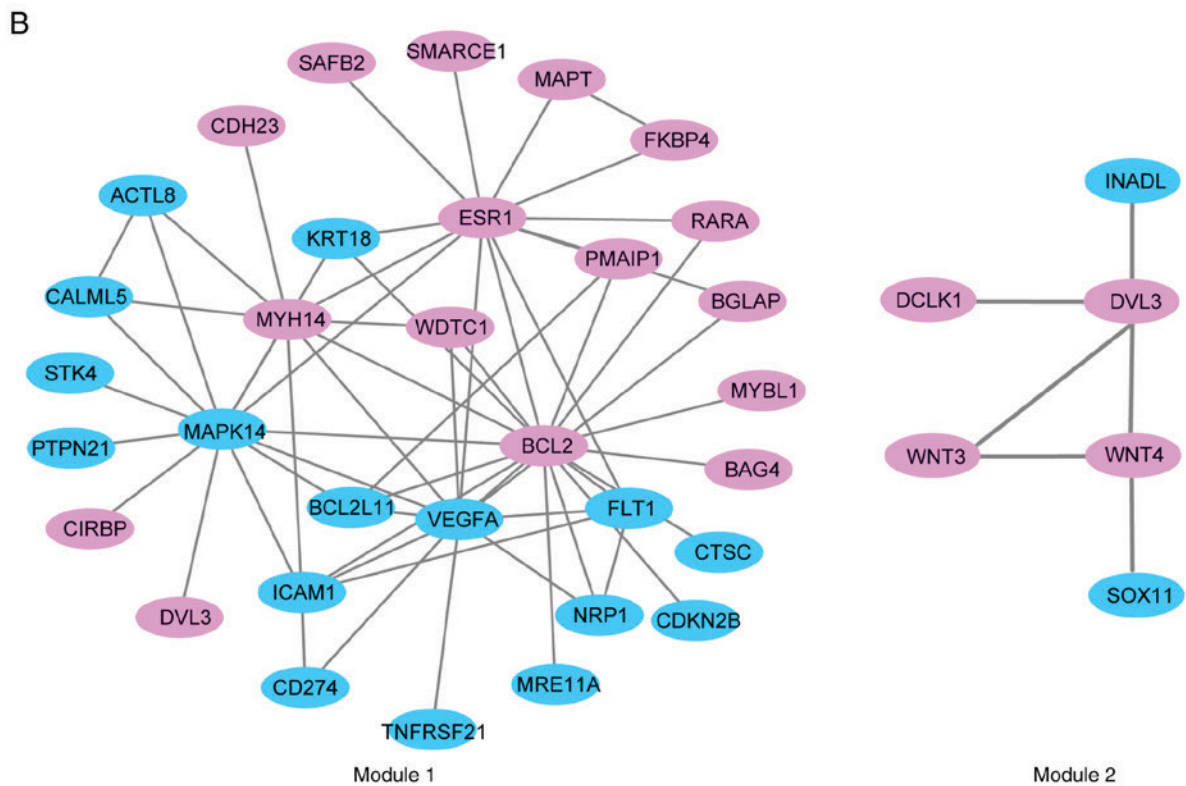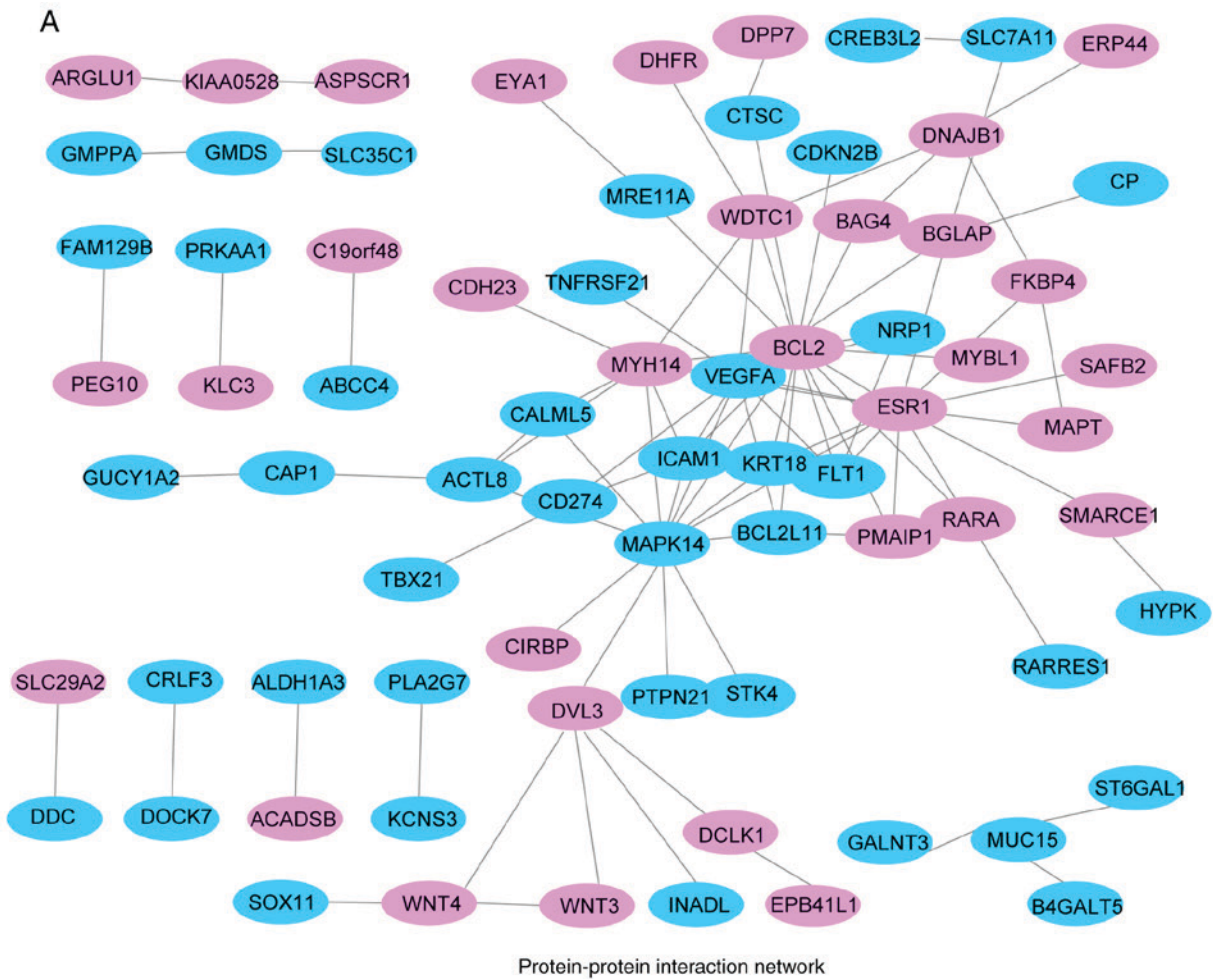
Figure 2. PPI network of DEGs identified in breast cancer cases with a partial response to trastuzumab. (A) DEGs identified in at least two datasets were used to construct the PPI network. Pink nodes indicate upregulated genes, while blue nodes indicate downregulated genes. The lines between nodes represent the interactions between genes. (B) Two PPI modules were extracted from the PPI network using MCODE in Cytoscape. PPI, protein-protein interaction; DEGs, differentially expressed genes.

Table III. Enriched GO terms and KEGG pathways in the two protein-protein interaction network modules.

A, Module 1

Category	GO/KEGG ID	Description	P-value
GOTERM_MF_DIRECT	GO:0005515	Protein binding	7.13×10^{-5}
GOTERM_BP_DIRECT	GO:0035924	Cellular response to vascular endothelial growth factor stimulus	8.90×10^{-6}

B, Module 2

Category	GO/KEGG ID	Description	P-value
GOTERM_MF_DIRECT	GO:0005109	Frizzled binding	2.65×10^{-5}
GOTERM_BP_DIRECT	GO:0060070	Canonical Wnt signaling pathway	1.44×10^{-4}
GOTERM_BP_DIRECT	GO:0030182	Neuron differentiation	1.89×10^{-4}
KEGG_PATHWAY	hsa05217	Basal cell carcinoma	6.22×10^{-5}
KEGG_PATHWAY	hsa04916	Melanogenesis	2.07×10^{-4}
KEGG_PATHWAY	hsa04310	Wnt signaling pathway	3.96×10^{-4}
KEGG_PATHWAY	hsa04550	Signaling pathways regulating pluripotency of stem cells	4.08×10^{-4}

GO, Gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; FDR, false discovery rate.

In the present study, SOX11, ATP8B2 and ODF2L were demonstrated to be downregulated in breast cancer with a partial response to trastuzumab with >1.5 FC compared with the complete response breast cancer cases. Enrichment and network analysis was used in combination with DEGs to reduce the risk of type I errors in the search for biomarkers. Although ATP8B2 and ODF2L were demonstrated to be differentially expressed between the partial response and complete response groups in all three datasets, the pathways and GO terms associated with the two genes were not enriched and no interactions were demonstrated between these two genes and the other genes in the PPI network. SOX11 belongs to the subgroup C of the SOX gene family, which encode transcription factors with important roles in embryonic development and cell differentiation, and may contribute to the development and progression of central nervous system malignancies, solid tumors and aggressive mantle cell lymphoma (27). SOX11 has been demonstrated to directly activate the PI3K/Akt signaling pathway and suppress the Wnt signaling pathway (27,28), and promote tumor angiogenesis by regulation of platelet-derived growth factor (27). As mentioned above, the PI3K/Akt pathway and tumor angiogenesis were demonstrated to be suppressed, and the Wnt pathway was activated, in trastuzumab-resistant breast cancer. These results are consistent with the function of SOX11, which indicates that downregulation of SOX11 may serve a critical role in the acquirement of trastuzumab resistance. Therefore, SOX11 may be used as a potential biomarker for trastuzumab sensitivity and, more importantly, it may also serve as a potential therapeutic target of trastuzumab resistance in breast cancer.

The results of the current study demonstrated that ESR1 was upregulated in the partial response group in all three datasets, and DEGs upregulated in the partial response group were enriched in the 'response to estrogen stimulus' GO term. In addition, PPI analysis also highlighted an ER-associated PPI network; ESR1, B-cell lymphoma 2 (BCL2), DnaJ homolog subfamily B member 1, WD and tetratricopeptide repeats 1, and myosin heavy chain 14, were the top 5 nodes in this network based on the centrality degree. ESR1 protein, also termed ER, has been extensively investigated in breast cancer and is one of the defining features in classifying tumor subtype and assigning therapeutic strategies in breast cancer (29). ER was considered to be an alternative pathway to Her-2 blockade due to its ability to activate certain Her-2 signaling members, including transforming growth factor-a (30). ER-negative breast cancers have been reported to exhibit an enhanced complete response rate to trastuzumab-based chemotherapy in several clinical studies, regardless of the treatment regimen (31,32). Patients with ER positive and Her-2 positive breast cancer demonstrated worse trastuzumab response compared with ER negative and Her-2 positive patients, while hormone therapy targeting ER following trastuzumab and chemotherapy resulted in increased progression-free survival (33). Therefore, Her-2 blockade combined with endocrine therapy may also be a reasonable neoadjuvant regimen for Her-2-positive ER-positive breast cancer.

Additionally, in the present study, the expression of BCL2, an estrogen-associated protein, was demonstrated to be increased in the partial response group (34). BCL2 has been demonstrated to prevent apoptosis and inhibit proliferation (35). Paradoxical results have been reported regarding the effect of BCL2 expression on the treatment of breast cancer. Several studies reported that increased expression of BCL2 may predict a good prognosis in breast cancer (36,37),

while other studies demonstrated that the prognostic effect of BCL2 may differ between different molecular subtypes of breast cancer (36,38,39). Giuliano *et al* (40) described the parallel upregulation of BCL2 as a mechanism of survival entirely dependent on ER activity and potentially leading to anti-Her-2 resistance (40,41). In addition, an *in vitro* experiment performed on BT474 cells, a Her-2-overexpressing breast cancer cell line, demonstrated that increased BCL2 expression contributed to trastuzumab resistance (39). These results indicate that Her-2-positive breast cancer may acquire trastuzumab resistance via the ESR1/BCL2 pathway. Therefore, BCL2 may be used as a novel molecular target for improving the response of breast cancer to trastuzumab.

In conclusion, the present study demonstrated that Her-2-positive breast cancer with trastuzumab resistance exhibited low PI3K/Akt pathway, low tumor angiogenesis and high ER pathway activity. Trastuzumab-resistant breast cancers may acquire proliferation signaling by upregulating the Wnt signaling pathway. Therefore, combination therapy consisting of trastuzumab and anti-Wnt or hormone therapy may be a promising treatment modality and should be investigated in further studies. Furthermore, low SOX11 and high BCL2 expression may be employed as biomarkers for neoadjuvant trastuzumab therapy of Her-2-positive breast cancer.

Availability of data and materials

Microarray data used in this article can be downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo) with the accession number GSE22358, GSE62327 and GSE66305.

Authors' contributions

Data analysis was performed by BZ, HN, LL and YZ. Manuscript was drafted by BZ and YZ. YS, LS and DH performed the GEO and reference search. HN was in charge of language editing.

Ethics approval and consent to participate

All data used in this study comes from the GEO database and no clinical trial or animal experiment was included in this study. This study was granted an exemption from requiring ethics approval by the ethics committee of Qilu Hospital (Qingdao, China).

Consent for publication

All data used in this study came from the GEO database, and the data submitters have declared that their studies comply with the NIH genomic data sharing policy and have the appropriate consent/permission to submit the data to a public database and these information does not compromise participant privacy: (https://www. ncbi.nlm.nih.gov/geo/info/faq.html#patient).

Competing interests

The authors declare that they have no competing interests.

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