Abstract. Triple-negative breast cancer (TNBC) is characterized by a deficiency in the estrogen receptor (ER), progesterone receptor (PR) and HER2/neu genes. Patients with TNBC have an increased likelihood of distant recurrence and mortality, compared with patients with other subtypes of breast cancer. The current study aimed to identify novel biomarkers for TNBC. Weighted gene co-expression network analysis (WGCNA) was applied to construct gene co-expression networks; these were used to explore the correlation between mRNA profiles and clinical data, thus identifying the most significant co-expression network associated with the American Joint Committee on Cancer-TNM stage of TNBC. Using RNAseq datasets from The Cancer Genome Atlas, downloaded from the University of California, Santa Cruz, WGCNA identified 23 modules via K-means clustering. The most significant module consisted of 248 genes, on which gene ontology analysis was subsequently performed. Differently Expressed Gene (DEG) analysis was then applied to determine the DEGs between normal and tumor tissues. A total of 42 genes were positioned in the overlap between DEGs and the most significant module. Following survival analysis, 5 genes [GIPC PDZ domain containing family member 1 (GIPC1), hes family bHLH transcription factor 6 (HES6), calmodulin-regulated spectrin-associated protein family member 3 (KIAA1543), MYLK2 and PPAN] were selected and their association with the American Joint Committee on Cancer-TNM diagnostic stage was investigated. The expression level of these genes in different pathological stages varied, but tended to increase in more advanced pathological stages. The expression of these 5 genes exhibited accurate capacity for the identification of tumor and normal tissues via receiver operating characteristic curve analysis. High expression of GIPC1, HES6, KIAA1543, MYLK2 and PPAN resulted in poor overall survival (OS) in patients with TNBC. In conclusion, via unsupervised clustering methods, a co-expressed gene network with high inter-connectivity was constructed, and 5 genes were identified as biomarkers for TNBC.

Introduction

Triple-negative breast cancers (TNBCs) are characterized by the lack of HER2/neu, progesterone receptor (PR) and estrogen receptor (1) (ER) gene expression. The TNBC subtype constitutes 10-15% of total breast tumors and 80% of basal-like breast cancers (1). Triple-negative and basal-like tumors commonly have a high histological grade (1). TNBCs also have a poor prognosis and tend to result in earlier relapse compared with other subtypes of breast cancer (1). Additionally, TNBCs exhibit increased chemosensitivity compared with other genotypes of breast cancer (2), thus, chemotherapeutic methods are currently the most prevalently used medical treatments (3). These include epidermal growth factor receptor (EGFR)-targeted therapies, multi-tyrosine kinase inhibitors, poly-ADP ribose polymerase (PARP) inhibitors and anti-angiogenic agents (4). However, patients with TNBCs usually exhibit poorer outcomes following chemotherapy, compared with patients with breast cancers of other subtypes (3). Hence, it would be beneficial to identify novel biomarkers associated with the progression of TNBCs, and identify new targets to improve their precise diagnosis and treatment.

Weighted-correlation network analysis (WGCNA) has previously been performed to construct non-scale co-expressed gene networks (5-8). In the present study, WGCNA was performed to identify the hub-module that contained genes showing a strong correlation with the pathological stage of TNBC. Subsequently, differentially expressed gene (DEG) analysis was performed on RNAsequencing (RNAseq) data of TNBC. The hub-genes were defined as all genes contained in the overlap between the DEGs and the hub-module. Gene
ontology (GO) and gene enrichment analyses were then employed, and identified several important terms in biological process, molecular function and cellular components. Survival analysis was also conducted, and 5 genes were selected from the hub-genes. Receiver operating characteristic (ROC) and Kaplan-Meier (KM) curves were plotted to indicate the capacity of these genes to differentiate tumor and para-tumor tissues, and to confirm the influence of these genes on the overall survival (OS) of patients with TNBC.

Materials and methods

Data processing and co-expression gene network construction using RNAseq data. A total of 1,240 RNAseq datasets from The Cancer Genome Atlas (TCGA) and clinical data for patients with breast cancer were downloaded from the University of California, Santa Cruz (UCSC) database using the Xena browser (https://xenabrowser.net/). The workflow is displayed in Fig. 1. The TNBC samples were filtered using the criteria of ‘not expressing genes for ER, PR and HER2/neu’. R software (version 3.5.2; http://www.r-project.org) was applied to perform WGCNA analysis. WGCNA was used to construct the gene co-expression network; co-expression similarity ($S_{i,j}$) was defined as the absolute value of the correlation coefficient between the mRNA expression profile of nodes $i$ and $j$:

$$s_{i,j} = |\text{cor}(X_i, X_j)|$$

Where $X_i$ and $X_j$ are mRNA expression values for genes $i$ and $j$, $S_{i,j}$ was calculated using the Pearson’s correlation coefficient between genes $i$ and $j$. Weighted-network adjacency was defined by raising the co-expression similarity to a power:

$$\alpha_{i,j} = S_{i,j}^\beta$$

The power of $\beta=4$ and scale free $R^2=0.95$ were selected as the soft-thresholding parameters to ensure a signed scale-free gene network.

By evaluating the correlation between the pathological stage of TNBC and the module membership with the ‘p. weighted’, a high-correlated module was identified. The tan modules which had the most significant adjusted P-values were selected. Genes involved in the tan modules were presented using Cytoscape v3.4.0 (https://cytoscape.org). The genes in the tan module were selected as the input for GO and KEGG analysis, which was performed using Metascape (http://metascape.org/gp/index.html).
Statistical analysis. Statistical analysis was performed using R (R Foundation for Statistical Computing; http://www.R-project.org/). The fold-change and Q-value (adjusted P-value) for para-tumor and tumor samples were calculated using the Limma package (9). A Q-value <0.05 was considered to be statistically significant. The overall survival analysis was conducted using the Survminer package (10), and the P-values in the KM curve were obtained using the log-rank test. The false discovery rate was set as 0.05 for analysis.

Results

WGCNA on RNAseq dataset of TNBC. In order to determine the co-expression network most highly associated with the
progress and prognosis of TNBC, TNBCTCGA RNAseq data downloaded from UCSC, was analyzed using WGCNA. The analysis showed TNBC clustering using the average linkage and Pearson's correlation methods. The scale-free network was constructed by raising the power of $\beta$ to 4 and by ensuring that the scale-free $R^2$ reached 0.95 (Fig. 2A and B). The clustering dendrogram of TNBC tissues is shown in Fig. 2C.

A total of 23 modules were found to be clustered, and this gene clustering is displayed as a dendrogram in Fig. 3A. The weighted network of all genes exhibited in a heat map, depicting the topological overlap matrix amongst the mRNA expression profiles (Fig. 3B). The tan module was determined using a trait-heat map to be the module with the strongest correlation with the pathological stage of TNBC (Fig. 3C). The correlation of genes with pathological stage, as well as module membership (the correlation of genes with clusters) in the tan module. The results revealed that genes, which had high a correlation with tan modules were also strongly associated with the pathological stage of TNBC. Based on the cut-off criteria (IGS>0.4), 129 genes with high connectivity were selected for the construction of the co-expression network. The inner connectivity in the tan module with the threshold (IGS>0.4) was plotted. This showed strong co-expression relationships in the tan module (Fig. 4).

**GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) Analysis.** The genes in the tan module were divided into three groups (biological process, cellular component, and molecular function), which were then analyzed using GO and KEGG analysis. In the biological process group, the most enriched genes concerned epigenetic regulation, cell metabolism, DNA repair and mRNA processing (Fig. 5A). The genes in the cellular component group that were most enriched
comprised ‘mitochondrial protein complex’, ‘endosomal part’ and ‘phagocytic cup’ (Fig. 5B). The most enriched genes in the molecular function group were in ‘cadherin binding’, ‘transcription corepressor activity’ and ‘SH3 domain binding’ (Fig. 5C). KEGG pathway analysis indicated that ‘autophagy’ and ‘AMPK signaling pathway’ were involved in the regulation of genes in the tan module (Fig. 5D).

**DEG analysis of TNBC tissues.** The DEGs were obtained from the analysis of RNAseq datasets, and a total of 2,169 significant genes were identified (Q-value<0.05; fold change >2) from the comparison of TNBT tissues and para-tumor tissues. The volcano plot indicates the fold-change and P-value of DEGs (Fig. 6A). The overlap between the genes discovered in DEG analysis and the genes in the tan module comprised 42 genes, which were selected as hub-genases. Among the 42 genes, 5 of which showed high correlation between expression level and survival probability, and were used for further analysis.

**Survival and expression of the 5 selected genes.** From the aforementioned hub-genases, 5 genes [GIPC PDZ domain containing family member 1 (GIPC1), hes family bHLH transcription factor 6 (HES6), calmodulin-regulated spectrin-associated protein family member 3 (KIAA1543), myosin light chain kinase 2 (MYLK2) and peter pan homolog (PPAN)] were selected to explore their association with the pathological progress. The unsupervised clustering results illustrate the co-expression of the 5 genes in the tan module and the extent of correlation between the 5 genes in TNBC was determined. Fig. 6B indicates high correlation between HES6 and GIPC1, and also between PPAN and GIPC1. The expression level of the genes in different pathological stages varied, and tended to be higher in more progressed pathological stages (Fig. 7). The expression levels of the 5 genes in tumor and para-tumor tissues were also plotted in Fig. 8, and showed significantly higher expression in tumor tissues (P<0.01). The ROC curves indicate that GIPC1, HES6, KIAA1543, MYLK2 and PPAN
all exhibited high diagnostic accuracy for the identification of para-tumor and tumor tissues (Fig. 9). The KM curves illustrate that the expression levels of the 5 genes were associated with the OS of patients with TNBC (Fig. 10). Furthermore, high-expression groups of KIAA1543, MYLK2 and PPAN were significantly associated with lower OS times when compared with low-expression of the same proteins (P<0.05).

**Discussion**

Breast cancer is an umbrella term summarizing carcinomas originating from the breast, and is one of the most prevalent cancer types worldwide. TNBCs are described as breast cancers that simultaneously lack expression of the ER, PR and HER2 genes. Triple-negative tumors represent 80% of basal-like molecular breast cancers (1). They have less favorable outcomes compared with other molecular subtypes of breast cancer, as they are commonly associated with a higher risk of early relapse and poor survival (11). Currently, no drug is available that specifically targets TNBC, and the results of chemotherapy are unsatisfactory. The molecular characteristics of TNBC include disruption of BRCA1 DNA repair associated (BRCA)-1 function (12,13). The low expression level of BRCA-1 and inhibitor of DNA binding 4, HLH protein (ID4) lead to the change from homologous repair (HR) to non-homologous end joining (NHEJ) or single-strand...
Figure 7. Expression of GIPC1, HES6, KIAA1543, MYLK2, and PPAN in different pathological stages of triple-negative breast cancer. (A) GIPC1. (B) HES6. (C) KIAA1543. (D) MYLK2. (E) PPAN. *P<0.05 and **P<0.005 indicated a significant difference between groups. Wilcoxon signed-rank test was used to evaluate the statistical significance of differences. GIPC1, GIPC PDZ domain containing family member 1; HES6, hes family bHLH transcription factor 6; KIAA1543, calmodulin-regulated spectrin-associated protein family member 3; MYLK2, myosin light chain kinase 2; PPAN, peter pan homolog.
annealing (SSA) pathways (14,15). HR is the most important DNA repair mechanism in healthy tissues and maintains genomic stability. However, the change to NHEJ (the alternative, more error-prone DNA-repair mechanism) can lead to
genetic instability in tumor tissues. The inhibitor olaparib, which targets PARP-1, can block the NHEJ of breast cancer types and ultimately inhibit DNA repair (16). Moreover, epigenetic dysregulation is well established as having a crucial role in cancer pathology and progression (17,18), and the current study identified enrichment of epigenetic regulation in TNBC.

However, the molecular characteristics of TNBC are not well understood. Exploring the mechanisms involved in the progress and prognosis of TNBC would be helpful for improving diag-
The present study determined a number of candidates using TGCA RNAseq datasets downloaded from UCSC database to identify promising biomarkers related to TNBC progression. WGCNA is a commonly used bioinformatics analysis tool used to identify the key modules and genes, which are associated with specific clinical traits. A study applied WGCNA analysis in breast cancer, identifying the association between cyclin B2 (CCNB2), F-box protein 5 (FBXO5), kinesin family member 4A (KIF4A), minichromosomal maintenance 10 replication initiation factor (MCM10) and TPX2 microtubule nucleation factor (TPX2) expression, and the survival of breast cancer patients (19). Another study used WGCNA to reveal the association between ATRX chromatin remodeler (ATRX) and TNBC (20). In the present study, WGCNA was performed to determine the association between American Joint Committee on Cancer-TNM stage and the gene co-expression module. By overlapping the genes from the tan module and DEG analysis,
42 hub genes were identified. Of these 42 genes, GIPC1, HES6, KIAA1543, MYLK2 and PPAN were selected to validate the strength of association with TNBC progression.

GIPC1 (also known as C19orf3) is a cytoplasmic protein that acts as an adaptor protein, linking receptor interactions to intracellular signaling pathways, including cell cycle regulation (21). GIPC1 protein is highly expressed both in cultured human breast cancer cells and in primary and metastatic tumor tissues (22). It is a cancer-associated immunogenic antigen in breast cancer which is also associated with bone metastasis development in breast cancer (23). The functions of GIPC1 include the regulation of apoptotic cell death, G2 cell-cycle arrest, modified cell adhesion and the migration of breast cancer cells (24). In the present analysis, GIPC1 was significantly upregulated in both triple-negative breast tumor tissue and a progressed pathological stage.

HES6 encodes a subfamily of basic helix-loop-helix transcription repressors with homology to the Drosophila enhancer of split genes (25). The overexpression of HES6 is found in metastatic carcinomas of different origins. Hes6 ectopic expression stimulates cell proliferation, not only in breast cancer T47D cells, but also in breast tumor growth in xenografts. The overexpression of HES6 also led to the induction of E2F transcription factor 1, a crucial target gene for the transcriptional repressor HES1 (26). Furthermore, the ER α-negative breast cancer cell lines MDA-MB-231 and SKBR3 express 4 to 10 times higher levels of Hes-6 mRNA, compared with ERα-positive T47D and MCF-7 cells. The aforementioned studies complement the present findings of an association between HES6 expression and estrogen in TNBC.

KIAA1543, also known as Nezha, was shown to bridge the minus ends of non-centrosome anchored microtubules with p120, which plays a crucial role in stabilizing cadherin-catenin mediated cell-cell adhesion complexes (27,28). KIAA1543 is also involved in epithelial-mesenchymal transition, and mediates the interaction between cadherin and microtubules. It is overexpressed in invasive lobular carcinomas but its role is poorly characterized (29,30). The underlying mechanisms of KIAA1543 on TNBCs need to be further established. The current study found KIAA1543 to be significantly upregulated in TNBC tissues and at advanced pathological stages. Moreover, high KIAA1543 expression was associated with poor OS in TNBC patients.

MYLK2 encodes a calcium/calmodulin-dependent serine/threonine kinase (31). A somatic mutation in, or amplification of, MYLK2 has been detected in several cancer tissues, compared with normal tissues (31). Moreover, proteomic analyses conducted on serum protein profiling results revealed the upregulation of MYLK2 in pancreatic cancer patients (32). The present study showed that in TNBC tissues, MYLK2 was overexpressed and was also associated with the poor patient OS. PPAN encodes an evolutionarily conserved protein similar to the Drosophila gene peter pan. A signature inferred from Drosophila mitotic genes revealed an association between PPAN expression and the survival of breast cancer patients (33). Tumor patients with high expression levels of PPAN have been shown to respond more favorably to treatment with anti-EGFR antibodies such as cetuximab (34). The data presented reveal a significant association between high PPAN expression and a poor OS in patients with TNBC.

Taken together, WGCNA and DEG analysis on RNAseq datasets from TNBC tissues revealed that the tan module was the most significantly associated with AJCC-TNM stage. The genes in the tan module showed high inter-connectivity with the co-expression network. Furthermore, GO and KEGG analysis revealed the enrichment of the genes in the related terms from GO. The overlap between the module and DEG analysis identified 42 genes, 5 of which were negatively associated with the OS of patients with TNBC. Therefore, the current study provides 5 novel biomarkers for TNBC, which exhibit potential as targets in the diagnosis and treatment of the disease.

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Availability of data and materials
The datasets generated and/or analyzed during the present study are available in the University of California, Santa Cruz repository, (https://xenabrowser.net/datapages/).

Authors' contributions
MB, JW, LB and KZ conceived the experiment design. LB, JW, KZ and TG performed the data analysis. JW and KZ revised the manuscript. LB, TG, JW, KZ and MB wrote the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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