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# Molecular-level effects of eribulin and paclitaxel on breast cancer based on differential co-expression network analysis

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**ABSTRACT.** We investigated the effects of eribulin and paclitaxel on breast cancer (BC) by exploring molecular biomarkers and pathways. Co-expression networks were constructed by differentially co-expressed genes and links, and centralities were analyzed to explore the hub genes. Pathway-enrichment analysis was performed. The hub genes were validated using the polymerase chain reaction and western blotting. A total of 132 and 153 differentially expressed genes were identified in BC cell lines treated with eribulin and paclitaxel, respectively. Six hub genes were identified in two co-expression networks. The spliceosome pathway was the mutually significant pathway. The validation analysis was basically consistent with the bioinformatics. We successfully identified several hub genes and pathways relevant to the effects of eribulin and paclitaxel on BC based on the network analysis.

**Key words:** Breast cancer; Eribulin; Paclitaxel; Co-expression network; Hub genes; Pathway-enrichment analysis

 

## INTRODUCTION

Breast cancer (BC), the most frequently diagnosed carcinoma in females and the second leading cause of cancer death in women, is a heterogeneous disease with various pathological entities (Siegel et al., 2013). The rate of progression of BC depends on various factors, such as the histological type of the tumor, the woman's age, the hormonal conditions, the tumor microenvironment, the status of the receptors, and the genetic material (Reeder and Vogel, 2008). Despite the efficacy of many anti-cancer agents and the improved disease-free survival and overall survival of breast cancer patients, some patients still succumb to this disease (Jemal et al., 2011). Chemotherapy increases the 15-year survival rate by 10% in women with breast cancer who are younger than 50; in older women, the increase is 3% (EBCTCG, 2005). Despite new diagnostic and treatment options, roughly 30% of early-stage patients progress to metastatic disease (Dawood et al., 2010). Chemotherapy is predominantly used for cases of BC. Paclitaxel is a microtubule-stabilizing mediator medication that is used to treat a number of cancer types. It alters microtubule dynamics that are essential for maintaining cellular structure and play an important role in cellular functions, for example, the cell cycle (Sève and Dumontet, 2008; Perez, 2009). Eribulin (full name, eribulin mesylate; trade name, Halaven) was formally known as E7389 or ER-086526. It is also a microtubule-targeting modulator and has been used effectively to treat cancer (Towle et al., 2001). Currently, eribulin and paclitaxel are approved for the treatment of metastatic breast cancer (Martín, 2015; Mukai et al., 2015). However, the biological mechanisms underlying the chemotherapeutic effects of these drugs are still unclear.

Over the past decade, high-throughput technologies have brought unprecedented opportunities for the large-scale analysis of disease-related genes. They can be used to make sense of data and ascertain the key molecular mechanisms of biological phenomena. Complex diseases are usually characterized by diverse etiology, activation of multiple-signal transduction pathways, and various gene mutations. Network-based analysis has become an important and powerful approach to the elucidation of the biological implications underlying complex diseases (Baranzini et al., 2009; Sun et al., 2010; Jia et al., 2011).

We attempted to attain a system-wide understanding of the biological mechanisms underlying the curative effects of two drugs on BC. After screening the differentially expressed genes (DEGs) in the BC treatment groups (eribulin and paclitaxel) and the untreated BC subjects, we developed co-expression networks for BC treated with eribulin and paclitaxel by differentially co-expressed genes and links (DCGL). In addition, we explored the hub genes of these complex networks based on degree centrality analysis. Ultimately, the hub genes were validated in BC tissues treated with eribulin and paclitaxel using reverse-transcription polymerase chain reaction (RT-PCR) and western blotting. The study predicted the underlying molecular biomarkers relevant to the effects of eribulin and paclitaxel on breast cancer, which might reveal the mechanisms of eribulin and paclitaxel in BC treatment.

## MATERIAL AND METHODS

### Data collection and preprocessing

Based on the Affymetrix GeneChip Human Genome U133 Plus 2.0 [HG-U133_Plus_2] platform and the E-GEOD-50811 gene expression dataset (Dezső et al., 2014)

untreated BC samples and treated BC cases were recruited from the ArrayExpress Archive of Functional Genomics Data (http://www.ebi.ac.uk/arrayexpress/). The ArrayExpress Archive is an international functional genomic database at the European Bioinformatics Institute (EMBL-EBI), and is recommended by most journals as a repository for data supporting peer-reviewed publications (Kolesnikov et al., 2015). The E-GEOD-50811 gene expression dataset included 162 treated BC cases, comprising 81 eribulin-treated and 81 paclitaxel-treated BC cell lines, and 79 untreated cell lines, with three technical replicates as controls.

Before analysis, we conducted data preprocessing of the E-GEOD-50811 expression profile data using the expresso function in the Affy package (Gautier et al., 2004). To reduce the influence of nonspecific dataset factors, we performed background adjustment using the robust multichip average method (Ma et al., 2006) and data normalization using the quantile-based algorithm (Rifai and Ridker, 2001). PerfectMatch and mismatch match values were revised and selected using the MicroArray Suite 5.0 (MAS 5.0) algorithm (Pepper et al., 2007) and the median method, respectively. The AffyBatch data were converted to a gene expression dataset structure. The data were then screened using the featureFilter method of the genefilter package. Ultimately, each probe was mapped to one gene by getSYMBOL, where the probe is discarded if it does not match any genes.

## Detection of the DEGs

To select key genes in the eribulin- or paclitaxel-treated BC cell lines, the DEGs in the untreated controls and treated BC cell lines were screened using the Significance Analysis of Microarrays (SAM) package, which correlates a large number of features (for example genes) with an outcome variable, such as a group indicator, quantitative variable, or survival time (Tibshirani et al., 2011). SAM assigns a score to each gene on the basis of a change in gene expression relative to the standard deviation of repeated measurements. Genes with scores greater than a threshold are deemed potentially significant. The percentage of such genes identified by chance is the false-discovery rate (FDR). The tables of thresholds, cutoff points, and the corresponding FDRs for SAM analysis were calculated using the functions of SAMR. compute.delta.table (Tusher et al., 2001). The significant gene table was computed starting with the samr object "samr.obj" and the delta.table object "delta.table" (Tusher et al., 2001).

The "relative difference" *d (i)* in gene expression is:

$$d(i) = \frac{\bar{x}_I(i) - \bar{x}_U(i)}{s(i) + s_0} \qquad \text{(Equation 1)}$$

where $\bar{x}_I(i)$ and $\bar{x}_U(i)$ are defined as the average levels of expression for gene ($i$) in states I and U, respectively. The "gene-specific scatter" $s(i)$ is the standard deviation of repeated expression measurements. Genes were ranked to find significant changes in gene expression by the magnitude of their $d(i)$ values. To determine the number of falsely significant genes generated by SAM, horizontal cutoffs were defined as the smallest $d(i)$ among the genes and called significantly induced, and the least negative *d(i)* among the genes were called significantly repressed. The number of falsely significant genes corresponding to each permutation was computed by counting the number of genes that exceeded the horizontal cutoffs for induced and repressed genes. The DEGs in eribulin- and paclitaxel-treated BC cell lines were selected based on delta value of 1.2745 and 1.2307, respectively.

## Construction of differential co-expression networks

DCGL 2.0 (Yang et al., 2013) is an R package for identifying differentially co-expressed genes and links (DCGs and DCLs, respectively) from gene expression microarray data. It examines the expression correlation based on the exact co-expression changes of gene pairs between two conditions, and thus can distinguish between significant co-expression changes and relatively trivial ones (Yu et al., 2011). It has four functional modules: gene filtration, link filtration, differential co-expression analysis (DCEA), and differential regulation analysis. The differential co-expression profile (DCp) (Liu et al., 2010; Yu et al., 2011) and differential co-expression enrichment (DCe) (Liu et al., 2010; Yu et al., 2011) are involved in the DCEA module for extracting DCGs and DCLs.

DCp was used on the filtered set of gene co-expression value pairs. We measured differential co-expression (dC) of the co-expression value pairs related to a particular gene using a length-normalized Euclidean distance. We then performed a permutation test to assess the significance of dC, and a large number of permutation dC statistics formed an empirical null distribution. The P value for each gene could then be estimated.

DCe was also used to identify DCGs and DCLs based on the 'Limit Fold-Change' (LFC) model. First, we divided correlation pairs into three parts according to the pairing of signs of co-expression values and the multitude of co-expression values: pairs with the same signs, pairs with different signs, and pairs with differently signed high co-expression values. The first two parts were separately processed using the 'LFC' model to produce two subsets of DCLs, whereas the third part was directly added to the set of DCLs. The differential co-expression networks of eribulin- and paclitaxel-treated BC cell lines were determined from all the gene links. In our study, we defined the differential co-expression networks of eribulin and paclitaxel as network N1 and network N2, respectively.

## Centrality analysis

To further identify key players in biological processes in the BC cell lines, we conducted a centrality analysis based on the number of nodes in the different networks (Scardoni and Laudanna, 2012). Centrality measures mainly comprise degree centrality, closeness centrality, and shortest path between centrality, in which the degree of the equivalent number of nodes directly adjacent to a given node v (indicating the degree the vertex) is the simplest topological index. Calculation of the degree allows the determination of the "degree distribution" P (k), which gives the probability that a selected node has exactly k links. Nodes with a high degree (highly connected) are called "hubs" and interact with several other genes, suggesting a central role in the interaction network. An obvious order of the vertices of a graph can be established by sorting them according to their degree (Koschützki and Schreiber, 2008). The degree $C(v)$ of a node $v$ was defined as:

$$C(v) = \sum_{j} a_{vj} \qquad \text{(Equation 2)}$$

Genes with degrees >20 were defined as hub genes in this study.

## Pathway-enrichment analysis

To further investigate the enriched pathways of the DEGs that were relevant to eribulin-

or paclitaxel-treated BC cell lines, a pathway analysis was performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (www.genome.jp/kegg/), which is widely used for the comprehensive inference pathway mapping of genes. We submitted the DEGs to the online tool of the Database for Annotation, Visualization and Integrated Discovery (Huang et al., 2009) (DAVID, http://david.abcc.ncifcrf.gov), and obtained all the pathways these genes enriched, using an empirical P value threshold of 0.01.

## Validation of the hub genes

### *Materials*

We selected primary BC cell lines to perform our validation analysis. The control group comprised primary BC cell lines before treatment and the treatment groups comprised BC cell lines that had been successfully treated with drugs. We divided the treatment group into eribulin- and paclitaxel-treated groups.

### *RT-PCR analysis*

Total RNA was used in first-strand synthesis with an oligo (dT18) primer, and was treated with 2 μL RNasin (40 μ/μL), 8.0 μL 5X reverse transcriptase buffer, 8.0 μL dNTPs, and 2 μL AMV reverse transcriptase (5 μ/μL) according to the manufacturer instructions. The RT-PCR primer sequences for the hub genes in networks N1 and N2 are listed in Table 1. The PCR system comprised: 10 μL 10X PCR buffer, 1 μL Taq DNA polymerase, 3 μL each forward and reverse primer, and 8 μL dNTPs. The PCR conditions are given in Table 2. Complementary DNA was used as a template, and β-actin was used as the internal reference. The experiment was repeated three times.

**Table 1.** Primer sequences and product lengths of the 12 hub genes in networks N1 and N2.

| Gene | Primer sequences (5'-3') | Length (bp) |
|---|---|---|
| *DUSP8* | F: TCATCTGCGAGAGCCGCTTCAT<br>R: AGCCAGACAGTGGACGATGACT | 140 |
| *FSTL3* | F: ACATTGACACCGCCTGGTCCAA<br>R: ACTCCACGCCGTCGCACGAAT | 114 |
| *TUBA1C* | F: CGGGCAGTGTTTGTAGACTTGG<br>R: CTCCTTGCCAATGGTGTAGTGC | 150 |
| *KLF6* | F: AACCAGGCACTTCCGAAAGCAC<br>R: CTCAGAGGTGCCTCTTCATGTG | 113 |
| *EIF3B* | F: ACAAGCAGCAGGCGAACACCAT<br>R: TCCACAAACGCTAAGGCACCGT | 97 |
| *UBR2* | F: TCTTTCAGCAGACATTAGAACTGG<br>R: TCAGGAACCTGAGTTTGTGCGG | 115 |
| *KIF20A* | F: CAAGAGGCAGACTTTGCGGCTA<br>R: GCTCTGGTTCTTACGACCCACT | 130 |
| *PTPRK* | F: CACAGCCATCAATGTCACCACC<br>R: CACCTTTGGCTTGTGCTGGTCT | 128 |
| *ZSCAN20* | F: CCTGGCAAACATGCTGAGAAGG<br>R: TCTGGTCCTTGCTCTTTCTCGG | 125 |
| *DEPDC1* | F: CTCGTAGAACTCCTAAAAGGCATG<br>R: CAACATCTTCCTGGCTTAGTTCTC | 129 |
| *UNG* | F: CCACACCAAGTCTTCACCTGGA<br>R: CCGTGAGCTTGATTAGGTCCATG | 101 |
| *AURKA* | F: GCAACCAGTGTACCTCATCCTG<br>R: AAGTCTTCCAAAGCCCACTGCC | 158 |
| *ACTB* | F: CTCCATCCTGGCCTCGCTGT<br>R: GCTGTCACCTTCACCGTTCC | 268 |

**Table 2.** Polymerase chain reaction (PCR) amplification reaction conditions for the 12 hub genes.

| Gene | Reaction conditions |
|---|---|
| *DUSP8* | 94°C 5 min; 35 cycles of 94°C 30 s, 58°C 30 s, 72°C 40 s; 72°C 10 min |
| *FSTL3* | 95°C 5 min; 40 cycles of 95°C 30 s, 60°C 30 s, 72°C 60 s; 72°C 10 min |
| *TUBA1C* | 95°C 2 min; 40 cycles of 95°C 15 s, 60°C 40 s, 72°C 20 s; 72°C 10 min |
| *KLF6* | 94°C 2 min; 45 cycles of 95°C 10 s, 55°C 15 s,72°C 10 s; 72°C 10 min |
| *EIF3B* | 95°C 5 min; 45 cycles of 95°C 10 s, 62°C 20 s, 72°C 15 s; 72°C 10 min |
| *UBR2* | 94°C 2 min; 40 cycles of 95°C 10 s, 60°C 30 s, 60°C 30 s; 72°C 10 min |
| *KIF20A* | 95°C 10 min; 40 cycles of 95°C 15 s, 60°C 30 s, 72°C 10 s; 72°C 10 min |
| *PTPRK* | 95°C 10 min; 40 cycles of 95°C 15 s, 62°C 60 s; 72°C 10 min |
| *ZSCAN20* | 94°C 10 min; 35 cycles of 94°C 15 s, 60°C 20 s,72°C 10 s; 72°C 10 min |
| *DEPDC1* | 95°C 5 min; 40 cycles of 95°C 10 s, 60°C 30 s, 72°C 15 s; 72°C 10 min |
| *UNG* | 95°C 10 min; 40 cycles of 95°C 15 s, 60°C 60 s; 72°C 10 min |
| *AURKA* | 95°C 5 min; 35 cycles of 95°C 30 s, 60°C 30 s, 72°C 40 s; 72°C 7 min |
| *ACTB* | 95°C 2 min; 40 cycles of 94°C 30 s, 58°C 30 s, 72°C 30 s; 72°C 10 min |

## Western blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (12%) was conducted using 10 μg protein, and the protein was electrotransferred (4°C, 300 mA, 2 h) to a nitrocellulose membrane (NC). The NC membrane was then sealed with TBST (a mixture of Tris-buffered saline and Tween 20) containing 5% skimmed milk powder at 37°C for 2 h. Rabbit anti-human antibody (diluted by 1:10,000 blocking solution) was applied to the NC membrane and incubated at 37°C for 2 h. Unbound antibody was washed away with TBST (three times), and horseradish peroxidase-labeled sheep anti-rabbit IgG secondary antibody (1:5000) was incubated with the NC again at 37°C for 2 h. After flushing with TBST, the substrate was applied to the NC for 3 min and exposed in the dark. The experiment was repeated ten times.

## Statistical analysis

The products of the PCR experiment were analyzed by 1.5% agarose gel electrophoresis and the Quantity One gel imaging software (Bio-Rad, Hercules, CA, USA), and the results are reported as the content of the purpose gene relative to the β-actin bands. Analysis of the gray values from the western blotting experiment target bands was conducted using the ImageJ software (Invitrogen, Carlsbad, CA, USA), and the results are reported as the content of the purpose protein relative to the glyceraldehyde 3-phosphate dehydrogenase bands.

# RESULTS

## Detection of DEGs

A total of 241 samples associated with BC from the dataset were preprocessed to identify DEGs using the SAM package. Finally, 132 eribulin-treated and 153 paclitaxel-treated DEGs were identified in the treated BC cell lines.

## Construction of differential co-expression networks and centrality analysis

The differential co-expression network N1 comprised 132 nodes and 216 edges (Figure 1), and N2 comprised 153 nodes and 212 edges (Figure 2).

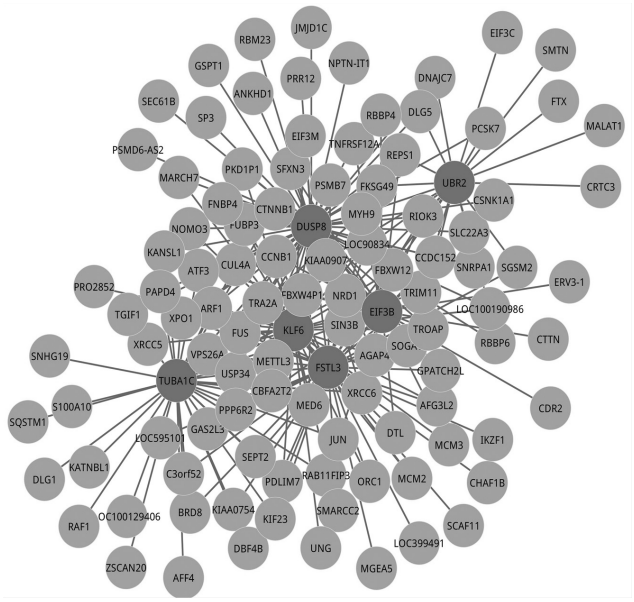


**Figure 1.** Differential co-expression networks of breast cancer cell lines treated with eribulin based on 132 differentially expressed genes. There were 132 nodes and 216 edges in the co-expression network; nodes refer to genes and edges between nodes indicate the interactions between genes in the network. The six hub genes are represented in dark gray.

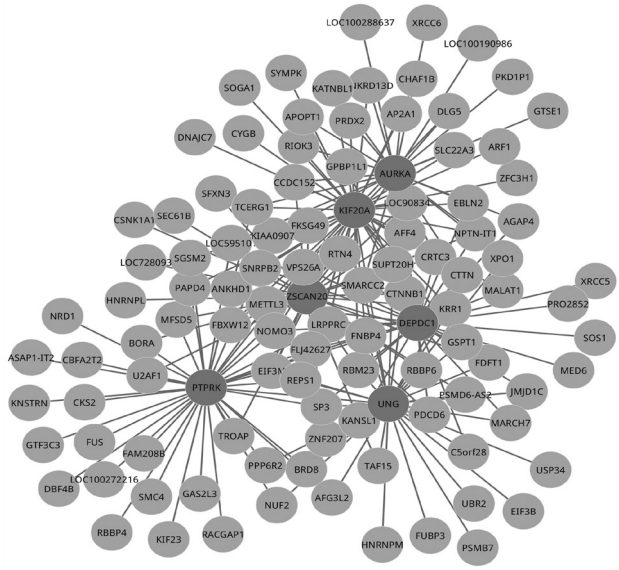


**Figure 2.** Differential co-expression networks of breast cancer cell lines treated with paclitaxel based on 153 differentially expressed genes. There were 153 nodes and 212 edges in the co-expression network; nodes refer to genes and edges between nodes indicate interactions between genes in the network. The six hub genes are represented in dark gray.

By accessing degree centrality analysis under the threshold value degree >20 in descending order, we obtained six hub genes in co-expression networks N1 and network N2. There was no similarity between the hub genes in networks N1 and N2. The results are shown in Table 3.

**Table 3.** Hub genes of networks N1 and N2.

| Network N1 | | Network N2 | |
|---|---|---|---|
| Genes | Degree | Genes | Degree |
| *DUSP8* | 58 | *KIF20A* | 45 |
| *FSTL3* | 42 | *PTPRK* | 41 |
| *TUBA1C* | 40 | *ZSCAN20* | 36 |
| *KLF6* | 34 | *DEPDC1* | 33 |
| *EIF3B* | 27 | *UNG* | 32 |
| *UBR2* | 22 | *AURKA* | 28 |

## Pathway-enrichment analysis

Pathway analysis based on the KEGG database showed that the DEGs in the BC cell lines treated with eribulin were significantly enriched in three terms: pathogenic *Escherichia coli* infection (P = 0.0032), spliceosome (P = 0.0041), and colorectal cancer (P = 0.0096). The DEGs for the BC cell lines treated with paclitaxel were significantly enriched in spliceosome (P = 0.0075). Therefore, we conclude that the spliceosome pathway was the mutually significant term for the DEGs in the BC cell lines treated with eribulin or paclitaxel.

## Validation of the hub genes

We verified the mRNA and protein expression levels of key genes (*DUSP8*, *FSTL3*, *TUBA1C*, *KLF6*, *EIF3B*, and *UBR2* in network N1; and *KIF20A*, *PTPRK*, *ZSCAN20*, *DEPDC1*, *UNG*, and *AURKA* in network N2) using RT-PCR and western blotting. The results of the relative expression levels of the hub genes in N1 and N2 by RT-PCR and western blotting are shown in Figures 3 and 4, respectively.

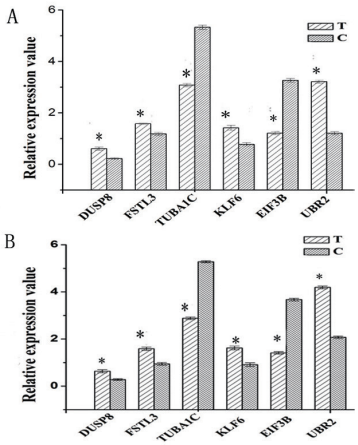


**Figure 3.** Relative expression of hub genes in breast cancer (BC) cell lines treated with eribulin. **A.** Polymerase chain reaction results and **B.** results of western blotting analysis. T stands for the BC cell line samples treated with eribulin, and C represents the control samples. *P < 0.05.

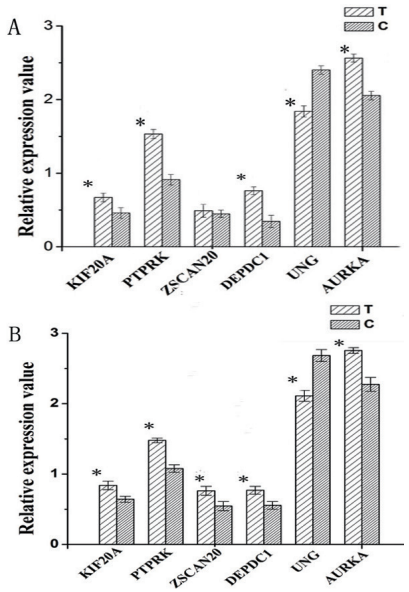


**Figure 4.** Relative expression of hub genes in breast cancer (BC) cell lines treated with paclitaxel. **A.** Polymerase chain reaction results and **B.** results of western blotting analysis. T stands for the BC cell line samples treated with paclitaxel, and C represents the control samples. *P < 0.05.

We found that all the hub genes in N1 and N2 were significantly differentially expressed in the BC cell lines that had been treated with eribulin and paclitaxel, relative to the control group ($P < 0.05$), except for *ZSCAN20* in network N2 ($P > 0.05$). The proteins corresponding to all the hub genes in N1 and N2 were significantly differentially expressed between the eribulin- or paclitaxel-treated BC cell lines relative to the controls ($P < 0.05$). In addition, we noticed that the relative expression levels of *DUSP8*, *FSTL3*, *KLF6*, and *UBR2* were upregulated, and the relative expression level of *TUBA1C* was downregulated in N1, which was consistent with the bioinformatic results, while the relative expression level of *EIF3B* contradicted the bioinformatics in the eribulin-treated BC cell lines. In the N2 network, *KIF20A*, *PTPRK*, *DEPDC1*, and *AURKA* were upregulated, and *UNG* was downregulated, which was consistent with the bioinformatic results, while the relative gene expression level of *ZSCAN20* was not consistent with the bioinformatics.

## DISCUSSION

We selected key genes in eribulin- or paclitaxel-treated BC cell lines and screened the DEGs in the untreated controls and treated cases using the SAM package. We then constructed differential co-expression networks for eribulin and paclitaxel as network N1 and network N2, respectively. To further identify hub genes and pathways for BC in biological processes, we used centrality analysis based on the node degrees in networks N1 and N2 and pathway-enrichment analysis based on the KEGG database. We identified several molecular biomarkers and relevant pathways in networks N1 and N2. Finally, we validated the hub genes; the results were consistent with the bioinformatic results.

Based on the centrality analysis for the co-expression networks N1 and N2, we identified six hub genes in both N1 and N2 (*DUSP8*, *FSTL3*, *TUBA1C*, *KLF6*, *EIF3B*, and *UBR2* in network N1; and *KIF20A*, *PTPRK*, *ZSCAN20*, *DEPDC1*, *UNG*, and *AURKA* in network N2). *DUSP8* and *KIF20A* in particular had the highest degrees in N1 and N2, respectively, which may be important for BC therapy. DUSP is a phosphatase that can act upon tyrosine or serine/threonine residues; it is well documented that *DUSP8* promotes hypermethylation (Lim et al., 2007). Several observations suggest that *DUSP8* affects the activation of the mitogen-activated protein kinase pathway in pancreatic cancer (Furukawa, 2015), and it serves as novel therapeutic targets for hepatocellular carcinoma (Fan et al., 2009). Additionally, it has been reported that the identification of candidate causal single nucleotide polymorphisms and genome-wide association study data have identified the candidate gene *DUSP8* that might contribute to BC susceptibility (Lee et al., 2014a). In humans, the kinesin-like protein KIF20A is encoded by the *KIF20A* gene. It has been reported that KIF20A is related to the emergence of several types of cancer. It is a novel and promising candidate target for immunotherapeutic anti-cancer pancreatic cancer therapy (Imai et al., 2011), and it may be a potential molecular target for drug intervention in gastric cancer (Yan et al., 2012) and prostate cancer (Waltering et al., 2009). In this study, we postulated that KIF20A may be a molecular biomarker that influences breast cancer paclitaxel resistance. Moreover, the result was consistent with the research by Khongkow et al. (2015).

Pathway analysis showed that the functional pathways were inconsistent between the two groups. There were three enriched terms for the eribulin-treated BC cell lines and one enriched term for the paclitaxel-treated BC cell lines ($P \leq 0.01$). We noticed that the spliceosome pathway was the mutually significant term. A spliceosome is a large and complex molecular machine found primarily within the splicing speckles of the cell nucleus of eukaryotic cells. The spliceosome is assembled from RNAs and protein complexes. Alterations in the expression of a spliceosome protein can impact both specific splicing events and tumor cell motility in BC (Lee et al., 2008). Lee et al. (2014b) have suggested that splicing regulator interactions can suppress the metastatic progression of BC by altering the transcriptome. What is more, the spliceosome has been identified as an attractive therapeutic target in cancer therapy (Quidville et al., 2013).

In conclusion, our results identified several hub genes and pathways associated with the treatment of BC. Moreover, the validation results of most of the hub genes were consistent with the bioinformatic results. Therefore, those genes might be underlying biomarkers for eribulin and paclitaxel treatment associated with BC. Further validation and studies are needed to elucidate the effects of eribulin and paclitaxel on BC.

## Conflicts of interest

The authors declare no conflict of interest.

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