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Original Article

Identification of therapeutic targets of breast cancer

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Abstract: Breast cancer is a heterogeneous disease. We aimed to identify more therapeutic targets with diagnostic and prognostic values in breast cancer treatment. Microarray data GSE6883 including 18 tumor samples and 6 normal control were downloaded from Gene Expression Omnibus based on two different platforms. Differentially expressed genes (DEGs) were identified using limma package in R language, followed by clustering analysis with Pearson correlation by hcluste package. Gene Ontology functional enrichment analysis was performed using Gene Set Enrichment Analysis and pathway enrichment analysis was carried out by database for annotation, visualization, and integrated discovery online tool. Protein-protein interaction (PPI) network was constructed using STRING database and sub-network was mined from PPI network by MCODE algorithm. A total of 1132 DEGs were screened from breast cancer samples compared with normal control. Up-regulated genes were mainly enriched in cell cycle pathway and down-regulated genes were significantly involved in organ development-related functions and glutathione metabolism pathway. In PPI network, *TOP2A*, *MKI67*, *PCNA* and *PRC1* were with higher degree. Sub-network with highest score included *PCNA* and *KLHL21* were mainly enriched in regulation of cell cycle. The five genes *TOP2A*, *MKI67*, *PCNA*, *PRC1* and *KLHL21* play important role in breast cancer progression through regulating tumor cell cycle and might be therapeutic targets in treatment of breast cancer.

Keywords: Breast cancer, differentially expressed genes, sub-network, gene ontology

Introduction

Breast cancer is one of the most popular malignancies occurred in women, with approximately 235,030 new cases diagnosed in the USA since the beginning of 2014 [1]. Breast cancer is a heterogeneous neoplasm and originated from the epithelial cells. Based on microarray gene expression profiling, breast cancers are classified into at least three molecular subgroups: basal like, human epidermal growth factor receptor 2 and luminal [2, 3].

According to the currently available evidence, the progression of breast cancer is believed to originate from non-neoplastic epithelium undergoing the following stages: usual epithelial hyperplasia, atypical ductal hyperplasia, carcinoma in situ and finally invasive [4]. Molecular markers and associated mechanisms can distinguish these stages and have the potential to be useful to identify patients with breast cancer. The up- and down-regulation, mutation of specific genes are major underlying mechanisms of progression and metastasis of breast cancer. Mucin is a family of O-glycosylated proteins which are differentially expressed in breast cancers [5] and broadly grouped into two main classed: secreted mucins and membrane-bound mucins. *MUC1* (mucin 1) has been suggested to be up-regulated and copy number of *MUC1* is increased in breast cancer cells [6]. Overexpression of *MUC1* in breast cancer cells has been showed to suppress apoptosis in response to DNA damage, oxidative stress and hypoxia, in parallel induce tumorigenicity [7, 8]. A growing body of information has suggested that mucins, especially MUC1 is involved in several signaling pathways in breast cancer. A current study has shown that MUC1 binds with fibroblast growth factor receptor which is involved in angiogenesis, tumor development and wound repair [9]. Phosphorylated MUC1 could prevent activation of apoptosis pathway and enhance the survival rate of breast cancer cells [10]. Moreover, previous studies have

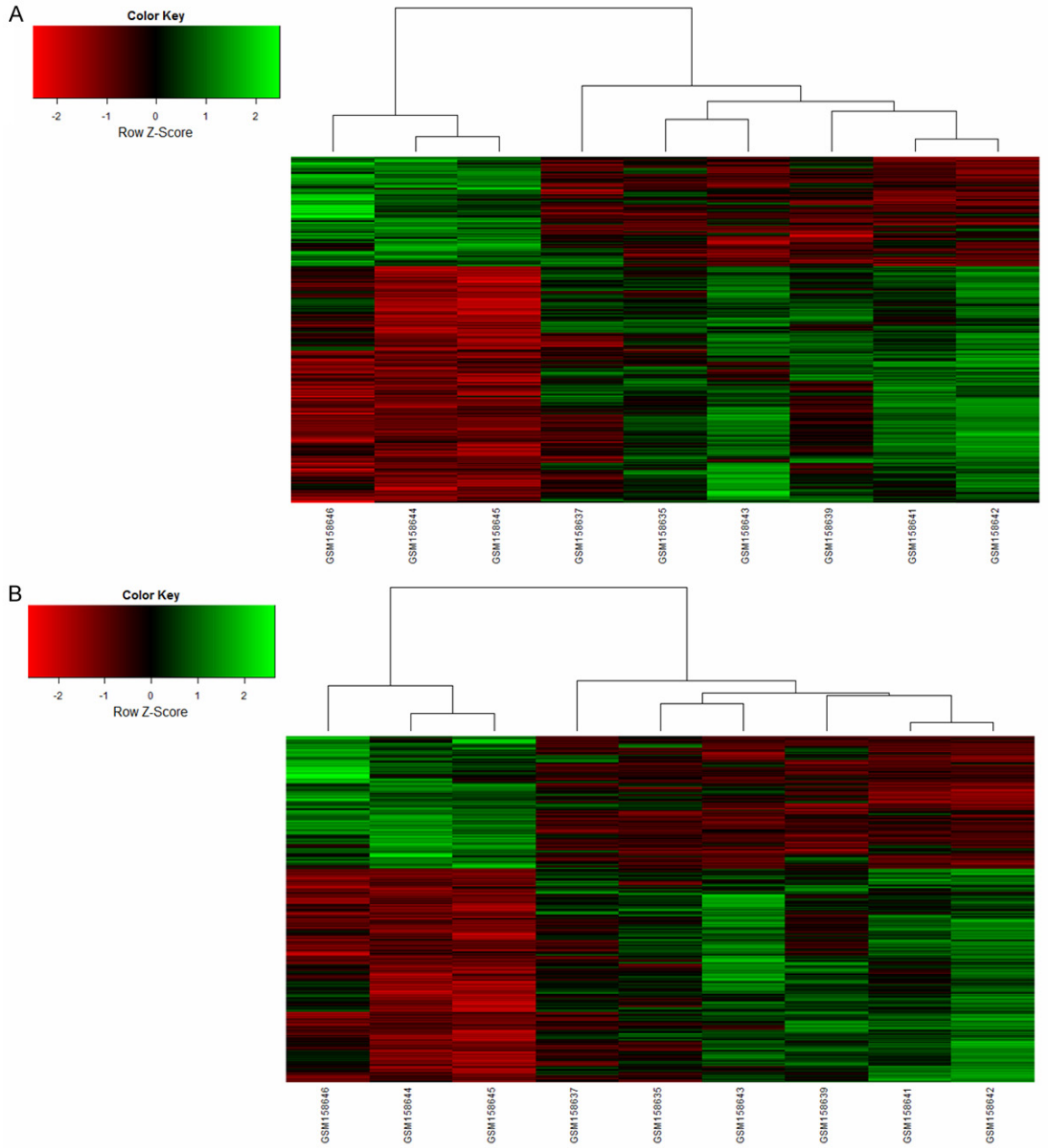

Figure 1. Hierarchical clustering of the differentially expressed genes (DEGs) based on platforms GPL96 (A) and GPL97 (B) between breast cancer samples and normal control with a color gradient for gene abundance ranks. The cluster of significant up-regulated DEGs is highlighted in green. The horizontal axis represents samples and vertical axis represents expression level of genes.

demonstrated that MUC1 can modulate cell proliferation of breast cancer cells via regulating cyclin D1 (CCND1) transcription [11]. *CCND1* is also a oncogene in breast cancer and has been determined to be overexpressed with a crucial role in cell cycle and proliferation [12]. In clinical treatment of breast cancer, *MUC1* and *CCND1* are with diagnostic and prognostic values, and thus, have emerged as promising targets for target therapy of breast cancer [13, 14]. Nevertheless, the molecular mechanism of breast cancer remains to be defined and more therapeutic targets needs to be detected in future study.

Previous studies focused on the gene expression profile GSE6883 have identified 186 differentially expressed genes (DEGs) between

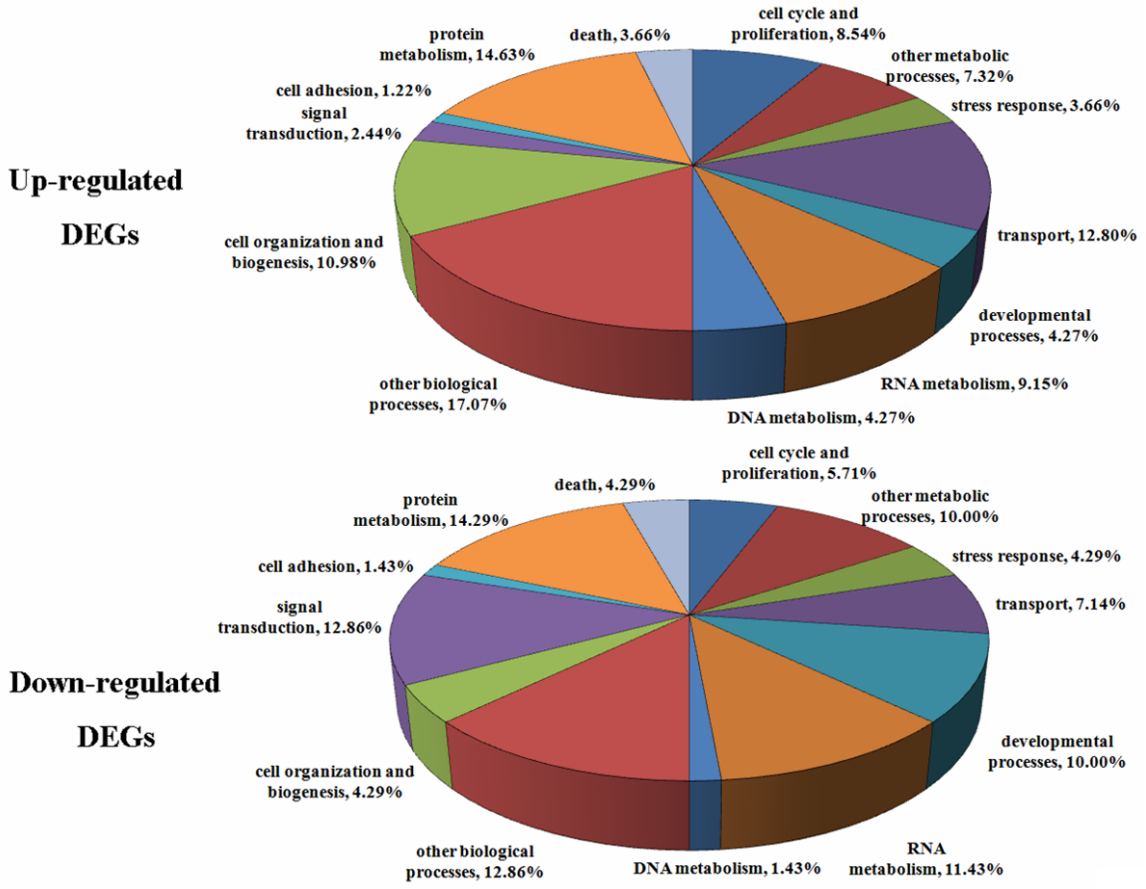

Figure 2. Functional categorization of up- and down-regulated differentially expressed genes (DEGs) between tumorigenic and normal cells. The significant DEGs were grouped into 13 categories according to their molecular functions, which include involvement in the cell cycle and proliferation, other metabolic processes, stress response, transport, developmental processes, RNA metabolism, DNA metabolism, other biological processes, cell organization and biogenesis, signal transduction, cell adhesion, protein metabolism and death.

breast cancer samples and normal control as a invasiveness gene signature [15], followed by survival analysis to study the association between the signature and survival in patients with breast cancer. However, the further functions of these genes in progression and metastasis of breast cancer are not identified.

With aim to fully elucidate the mechanism of breast cancer and detect novel therapeutic targets, we carried out functional and pathway enrichment analysis for identifying DEGs, followed by protein-protein interaction network construction and sub-network mining.

Materials and methods

Microarray data

Microarray data GSE6883 including 18 breast cancer samples and 6 normal control were derived from Gene Expression Omnibus based on the platforms of Aymetrix Human Genome U133A Array (GPL96) and Affymetrix Human Genome U133B Array (GPL97). Just 12 breast cancer samples and 6 normal control were selected in our analysis.

Data preprocessing

Raw data from two selected gene expression array (CEL files) were subjected to background adjustment, quantile normalization, finally summarization and log 2 transformation of expression value using Guanine Cytosine Robust Multi-Array Analysis (GCRMA) algorithm [16]. For one gene with several probes, mean value was calculated as expression value. On the contrary, one probe mapping to several genes was discarded.

Table 1. Gene Ontology functional enrichment analysis of differentially expressed genes using Gene Set Enrichment Analysis

Name	Count	FDR
Up-regulated		
CELL_CYCLE_PROCESS	38	0
MITOTIC_CELL_CYCLE	33	0
CELL_CYCLE_PHASE	31	0
CELL_CYCLE_GO_0007049	51	0
M_PHASE_OF_MITOTIC_CELL_CYCLE	22	0
M_PHASE	25	0
MITOSIS	20	0
REGULATION_OF_CELL_CYCLE	27	0.003559
CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS	24	0.055171
CELL_PROLIFERATION_GO_0008283	44	0.082137
ORGANELLE_ORGANIZATION_AND_BIOGENESIS	51	0.088707
DNA_METABOLIC_PROCESS	21	0.089897
CHROMOSOME_ORGANIZATION_AND_BIOGENESIS	16	0.086728
REGULATION_OF_CELL_PROLIFERATION	23	0.199194
Down-regulated		
MULTICELLULAR_ORGANISMAL_DEVELOPMENT	41	0.046278
RESPONSE_TO_CHEMICAL_STIMULUS	24	0.029657
ANATOMICAL_STRUCTURE_DEVELOPMENT	39	0.024736
SYSTEM_DEVELOPMENT	30	0.066295
RESPONSE_TO_EXTERNAL_STIMULUS	16	0.123577
ORGAN_DEVELOPMENT	22	0.130514
IMMUNE_SYSTEM_PROCESS	16	0.155127
ANATOMICAL_STRUCTURE_MORPHOGENESIS	20	0.222073
NEGATIVE_REGULATION_OF_CELLULAR_PROCESS	50	0.215456
NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS	52	0.206071
REGULATION_OF_CELLULAR_COMPONENT_ORGANIZATION_AND_BIOGENESIS	15	0.243838
CELL_SURFACE_RECEPTOR_LINKED_SIGNAL_TRANSDUCTION_GO_0007166	29	0.224713

Identification of DEGs in breast cancer

To identify DEGs in breast cancer samples compared with normal control, Limma package in R language [17] was used. Genes with the criteria of log 2 FC (fold change) ≥ 1 and *P* value calculated by Student's t-test less than 0.01 were considered to be differentially expressed in breast cancer.

Clustering analysis

A hierarchical clustering algorithm was applied to group up- and down-regulated DEGs based on the basis of expression value varied over all samples. Pearson correlation as a distance matrix was also performed using the R package hclust [18]. To visualize the results, expression level of each DEG was represented by green and red color.

Functional and pathway enrichment analysis of DEGs in breast cancer

To compare the difference of enriched functions for up- and down-regulated DEGs, identified genes were annotated within the Biological Process branch of the Gene Ontology (GO) [19]. To further study the functions of significant DEGs, GO functional enrichment analysis was performed using Gene Set Enrichment Analysis tool (GSEA) [20]. Kyoto Encyclopedia of Genes and Genomes(KEGG) [21] pathway enrichment analysis was conducted with DAVID (database for annotation, visualization, and integrated discovery) online tool [22]. The *P* value was adjusted for multiple testing by the false discovery rate (FDR) with Benjamini and Hochberg methods [23]. GO terms and pathway with FDR less than 0.05 were considered to be statistically significant.

Table 2. Pathway enrichment analysis of differentially expressed genes

Term	Count	FDR
Up-regulated		
hsa04110:Cell cycle	23	6.78E-09
hsa04120:Ubiquitin mediated proteolysis	17	1.76E-04
hsa04114:Oocyte meiosis	13	0.002081
hsa04914:Progesterone-mediated oocyte maturation	11	0.003032
hsa03050:Proteasome	8	0.003171
hsa00010:Glycolysis / Gluconeogenesis	9	0.003341
hsa04115:p53 signaling pathway	9	0.007241
hsa00520:Amino sugar and nucleotide sugar metabolism	7	0.009587
hsa00240:Pyrimidine metabolism	10	0.017792
hsa00230:Purine metabolism	13	0.026826
hsa04012:ErbB signaling pathway	9	0.029075
Down-regulated		
hsa00480:Glutathione metabolism	5	0.015701
hsa05210:Colorectal cancer	6	0.023242
hsa05200:Pathways in cancer	13	0.024496
hsa04710:Circadian rhythm	3	0.025454
hsa00982:Drug metabolism	5	0.031901
hsa04010:MAPK signaling pathway	11	0.033871

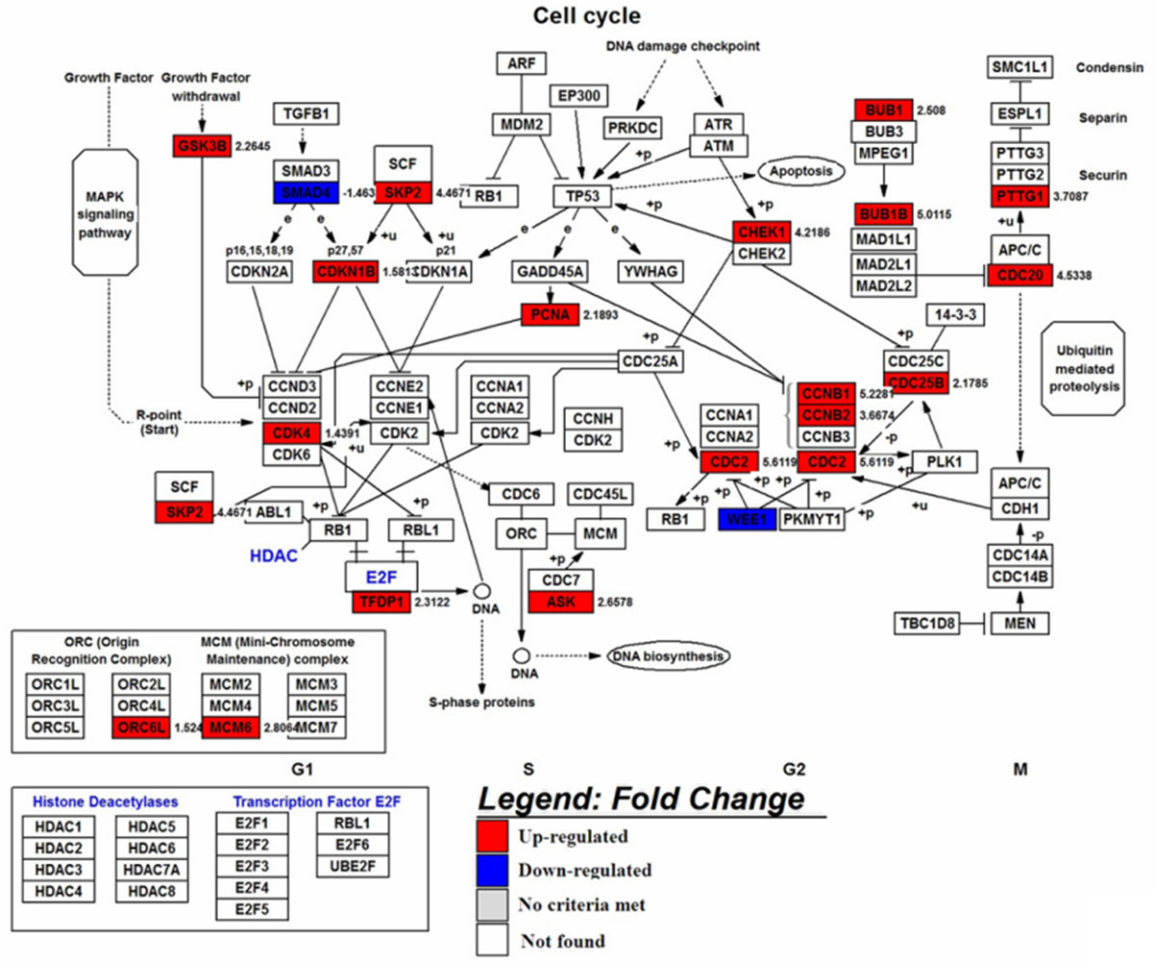

Figure 3. The cell cycle pathway is involved in the up-regulated differentially expressed genes (DEGs). Red rectangles represent the up-regulated DEGs in breast cancer samples compared with normal control. Blue rectangles represent the down-regulated DEGs in breast cancer samples compared with normal control.

Table 3. Oncogenic gene sets of differentially expressed genes in breast cancer

Gene Set	Count	FDR
Up-regulated		
RPS14_DN.V1_DN	21	0
CSR_LATE_UP.V1_UP	23	0
SRC_UP.V1_DN	19	0
GCNP_SHH_UP_LATE.V1_UP	27	2.50E-04
VEGF_A_UP.V1_DN	35	4.32E-04
PRC2_EZH2_UP.V1_UP	22	6.58E-04
MTOR_UP.V1_UP	26	0.005403
GCNP_SHH_UP_EARLY.V1_UP	23	0.008829
E2F1_UP.V1_UP	23	0.011365
ERB2_UP.V1_DN	40	0.019935
HOXA9_DN.V1_UP	23	0.025661
Down-regulated		
RAF_UP.V1_DN	24	0
STK33_DN	22	0.003033
STK33_SKM_DN	22	0.009735
RELA_DN.V1_UP	15	0.009917
PRC2_EZH2_UP.V1_DN	15	0.010151
ESC_V6.5_UP_LATE.V1_UP	15	0.012671
STK33_NOMO_UP	32	0.0151
TGFB_UP.V1_UP	15	0.025804
ATF2_UP.V1_DN	20	0.026568
STK33_NOMO_DN	18	0.027912

Oncogenic signature

To further characterize DEGs in breast cancer, GSEA was performed using all of the gene sets in Molecular Signature Database (MSigDB) C6 [24] to identify which gene sets were enriched in genes differentially expressed between breast cancer samples and control samples. The *P* value was adjusted for multiple testing by the FDR with Benjamini and Hochberg methods [23]. The threshold was FDR < 0.05.

PPI network construction

To construct PPI network of DEGs in breast cancer, identified genes were imported into STRING 9.1 software [25]. Cytoscape [26] was used to visualize the network and MCODE (Molecular Complex Detection) algorithm [27] was employed to mine sub-networks from PPI. To study the functions of DEGs in sub-network with highest clustering score, GO function analysis was carried out.

Results

Identification of DEGs in breast cancer and clustering analysis

According to the cutoff of $|\log FC| \geq 1$ and *P* value less than 0.05, we identified 1132 DEGs in breast cancer samples compared with normal control, including 739 up-regulated genes. Applying hierarchical clustering based on the identified DEGs between breast cancer samples and normal control (**Figure 1**), we observed that samples from patients with cancer were distinguished from samples from normal control.

Functional and pathway enrichment analysis of DEGs

To study the functions of DEGs in breast cancer, both up- and down-regulated DEGs were annotated within Biological Process branch of GO categories (**Figure 2**). In addition, significant functional and pathway enrichment analysis with the threshold *P* value less than 0.05 were also performed. As shown in **Figure 1**, both up- and down-regulated genes were mainly involved in Protein metabolism process. Additionally, up-regulated DEGs were significantly enriched in transport process but down-regulated DEGs in signal transduction. All genes with $|\log 2\ FC| \geq 1$ were ranked according to their log 2 FC and imported into GSEA to identify significant GO terms (**Table 1**). Up-regulated DEGs were mainly involved in cell cycle-associated functions and down-regulated genes were significantly enriched in organ development-related functions. Pathway enrichment analysis indicated that up-regulated DEGs were enriched in Cell cycle and Ubiquitin mediated proteolysis pathways, but Glutathione metabolism and Colorectal cancer pathways were screened in down-regulated DEGs (**Table 2**). The Cell cycle pathway was showed in **Figure 3**.

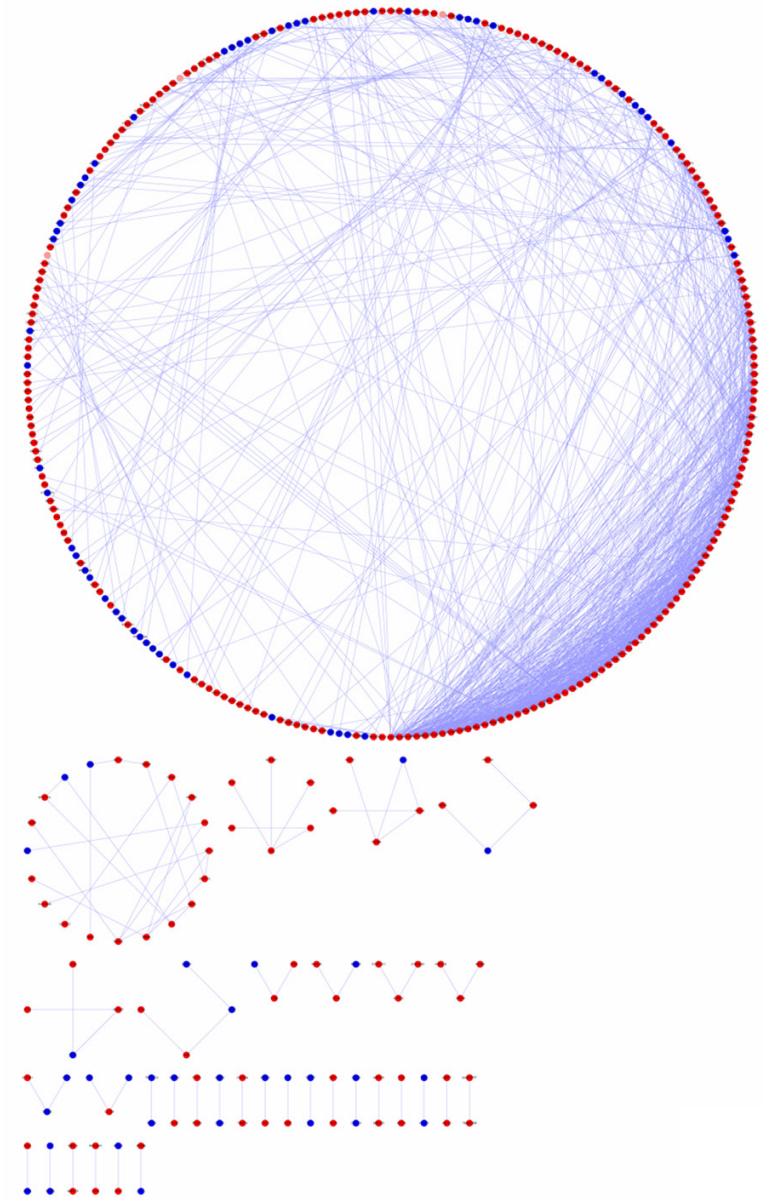

Figure 4. Protein-protein interaction network of differentially expressed genes (DEGs). Each edge between any two proteins (nodes) indicates an interaction. The red color indicates up-regulated DEGs and blue represents down-regulated genes.

Table 4. Differentially expressed genes with degree more than 10 in protein-protein interaction network

Node	Degree	Node	Degree	Node	Degree	Node	Degree
TOP2A	26	NEK2	17	CDKN3	14	RACGAP1	12
MKI67	22	ASPM	16	KIF20A	14	SGOL2	12
PCNA	22	CENPN	16	SHCBP1	14	SMC4	12
PRC1	20	DTL	16	CDC20	13	TYMS	12
KIF2C	19	KIF4A	16	CDC27	13	MELK	11
ZWINT	18	RRM2	16	UHRF1	13	PBK	11
CEP55	17	UBE2T	16	BUB1	12	CKAP5	10
DEPDC1	17	HMMR	15	CDK1	12	KIAA0101	10

Oncogenic gene sets in DEGs

To find oncogenic signatures from identified DEGs in breast cancer, GSEA analysis was performed using all of the gene sets from MSigDB-C6. In up-regulated genes, oncogenic gene sets included RPS-14_DN.V1_DN, CSR_LATE_UP.V1_UP and SRC_UP.V1_DN. In down-regulated DEGs, RAF_UP.V1_DN and STK33_DN were oncogenic gene sets (**Table 3**).

PPI network construction

The significant DEGs were mapped to STRING software and PPI network was constructed (**Figure 4**). In the network, 367 nodes and 1020 edges were included. DEGs *TOP2A* [topoisomerase (DNA) II alpha], *MKI67* (marker of proliferation Ki-67), *PCNA* (proliferating cell nuclear antigen) and *PRC1* (protein regulator of cytokinesis 1) were with higher degree more than 20. The nodes with degree more than 10 were listed in **Table 4**. Using MCODE we mined 12 sub-networks from the PPI network. The sub-network with highest cluster score 3.121 included 33 nodes and 103 edges (**Figure 5**), including *PCNA* and *KLHL21* (kelch-like family member 21). DEGs in the sub-network were mainly up-regulated and enriched in cell cycle-related functions.

Discussion

Breast cancer is the major cause of cancer-related deaths in women worldwide. Evidences are emerging suggest that breast cancer is a heterogeneous disease. To fully elucidate the molecular mechanism of breast cancer, we

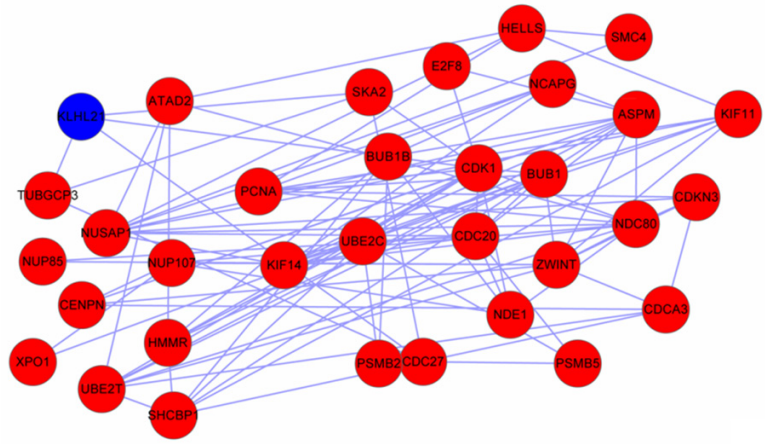

Figure 5. Sub-network mined from protein-protein interaction network with highest score. The red color indicates up-regulated genes and blue represents down-regulated genes. Each edge between any two proteins (nodes) indicates an interaction.

downloaded gene expression profile GSE6883 and screened significant DEGs between breast cancer samples and normal control. A total of 1132 DEGs were identified including 739 up-regulated genes. In PPI network, up-regulated *TOP2A*, *MKI67*, *PCNA* and *PRC1* were with higher degree more than 20. *PCNA* and down-regulated *KLHL21* were included in sub-network with highest cluster score which was mainly enriched in cell cycle-related function.

TOP2A encodes for the enzyme topoisomerase II α which catalyzes the breakage and reunion of double-stranded DNA. The enzymes play crucial roles in DNA replication, chromosome structure and transcription [28]. *TOP2A* is markedly up-regulated in proliferating cells and tumor cells functioning on catalyzing the ATP (Adenosine Triphosphate) dependent breakage and rejoining of double strand of DNA [29, 30]. More recently, the expression of *TOP2A* correlates with *MKI67* (marker of proliferation *Ki-67*) expression in breast cancers, the latter gene is expressed during all phases of the cell cycle except G0 [31]. Furthermore, amplification and deletion of *TOP2A* are predictive marker for the effect of adjuvant chemotherapy in patients with primary breast cancer, and interestingly, deleted cases had worse prognosis than amplified cases [32]. Notably, cancer is a disease of deregulated cell proliferation and survival in which cell cycle plays a vital role [33]. Additionally, emerging studies have shown that *TOP2A* is the target of several anticancer drugs to kill tumor cells by generating enzyme-mediated DNA damage and inducing apoptosis in breast cancer treatment [34].

As aforementioned above, *MKI67* is a nuclear marker of cell proliferation and associated with cell cycle. *MKI67* is overexpressed in breast cancer and has been indicated to be associated with prognosis and prediction in early breast cancer [35]. Interestingly, antiapoptotic protein B-cell CLL/lymphoma 2 and oncogene *p53* have been reported as being correlated with baseline *MKI67* [36, 37]. In addition, *PCNA* is also a proliferative marker and involved in the core DNA synthesis process, DNA damage and repair, as well as cell-cycle control [38, 39]. Phosphorylation of PCNA at Y211 by epidermal growth factor receptor enhances its association with c-Abl which is a non-receptor tyrosine kinase and regulates apoptosis and cell migration [40]. Previous study has demonstrated that deregulated c-Abl kinase leads to tumor development in breast cancers [41]. The two cell proliferation-associated markers were involved in functions of cell cycle and tumor progression.

PRC1 is a microtubule binding protein that is a part of the spindle midzone complex, and also the regulator of cytokinesis [42]. Emerging evidence has indicated that cytokinesis is the final step of the cell cycle and the physical separation of two daughter cell during cell division [43]. Failure to finish cytokinesis in mitosis has been reported to promote tumorigenesis [44]. PRC1 is phosphorylated by cyclin-dependent kinase 1 which regulates cell phage from G2 into M phage leading to chromosomal segregation [42]. Consistent with our results, *PRC1* has been showed to be overexpressed in both breast cancer cell line and clinical breast cancer tissues [45]. In addition, KLHL21 localizes to midzone microtubules during anaphase and is also necessary for completion of cytokinesis [46].

The five DEGs *TOP2A*, *MKI67*, *PCNA*, *PRC1* and *KLHL21* are identified from breast cancer samples and associated with cell-cycle function

and cell proliferation. They play vital role in progression and metastasis of breast cancer via regulating tumor cell cycle, and thus may be therapeutic targets for breast cancer.

Disclosure of conflict of interest

None.

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