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ORIGINAL ARTICLE

Identification of invasive key genes in breast cancer by bioinformatics analysis

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Summary

Purpose: *Of all breast cancers, triple-negative and HER-2 positive are the most aggressive breast cancer subtypes with a high risk of recurrence and worse prognosis. The study's purpose was to further assess the molecular mechanisms underlying aggression of breast cancer.*

Methods: *The microarray gene expression datasets of GSE29431 and GSE53752 were obtained from the GEO (Gene Expression Omnibus) database, which include HER-2 positive breast cancer, triple-negative breast cancer (TNBC) and normal breast tissue samples. Differentially expressed genes (DEGs) were determined using the LIMMA package of R software and subsequently functional enrichment analysis was performed by the ClusterProfiler package in the R platform. The STRING database was used to construct a protein-protein interaction (PPI) network. The most significant module and key genes were identified by Cytoscape software. Utilizing the Kaplan-Meier plotter and UALCAN database, we defined the key genes associated with prognotic values and molecular subtypes as invasive genes.*

Results: *In total, 428 common DEGs were identified, including 143 upregulated and 285 downregulated. GO and KEGG pathway enrichment analysis indicated that the upregulated genes were associated with mitotic nuclear division and cell cycle, whereas the downregulated genes were significantly associated with response to peptide and PPAR signaling pathway, respectively. A PPI network with 57 nodes and 335 edges was established, from which one most significant module was identified. Moreover, 12 key genes selected from the module with high degree centrality = 21 were highly associated with high clinical aggressiveness and worse overall survival rate.*

Conclusions: *Our studies could enhance the understanding of the molecular mechanism of breast cancer aggressiveness, and the identification of invasive key genes promoted the individualized and comprehensive treatment.*

Key words: *HER-2 positive breast cancer, TNBC, DEGs, bioinformatics, invasive genes, GEO*

Introduction

Breast cancer is the most common type of malignancy affecting seriously the quality of life of patients and has an increasing incidence worldwide [1,2]. Breast cancer is a highly heterogeneous disease with 4 molecular subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER-2)-enriched, and basal-like subtypes, which exhibit distinct molecular characteristics and clinical behaviors [3,4]. Because of HER-2 gene amplification or protein over-expression, HER-2 positive breast cancer is closely associated with aggressive clinical behaviour [5,6], while triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype, exhibiting increased recurrence and decreased survival [7]. Therefore, they represent highly invasive biological characteristics. To improve the chance of survival, it is necessary to further understand the molecular mechanisms of aggressiveness in breast cancer.

A large number of microarrays expression datasets are publicly available in the Gene Expression Omnibus (GEO) database(https://www.ncbi.nlm.nih.

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gov/geo/), and data-mining the deposited datasets using bioinformatics methods may advance the understanding of invasiveness.

In this study, two gene expression datasets, namely GSE29431 and GSE53752, were downloaded from the GEO database. DEGs between TNBC, HER-2 positive breast cancer, and normal breast tissues were identified respectively using the LIMMA package of R software (R version 3.6.2). Subsequently, Gene ontology function and KEGG pathway enrichment analysis were performed for DEGs by the clusterProfiler package in R software. The Search Tool for the Retrieval of Interacting Genes (STRING) database and protein-protein interaction (PPI) database were used to identify the significant module and key genes. The expression levels and prognostic values of key genes were assessed using the online Kaplan-Meier (KM) plotter database(kmplot.com/analysis) and UVALCAN database (http://ualcan.path.uab.edu/index.html).

Methods

Microarray data

The gene expression profiles analyzed in this study were obtained from the GEO database. GSE29431 was based on platform GPL570 ([HG-U133_Plus_2]Affymetrix Human Genome U133 Plus 2.0 Array), including 12 HER-2 positive breast cancer samples and 28 normal breast tissue samples, while GSE53752 was based on the Agilent GPL7264 (Agilent-012097 Human 1A Microarray(V2)G4110B), including 51 TNBC samples and 25 normal breast tissue samples. The characteristics of the selected 2 datasets are summarized in Table 1.

Identification of DEGs

To avoid false-positive results, corrections were made using the Bonferroni method. DEGs were determined using LIMMA package of R software [8,9] with a criterion of an adjust p value cut-off of <0.05 along with at least two-fold change. The common DEGs were identified by the Draw Venn Diagram tool(http://bioinformatics.psb.ugent.be/webtools/Venn/).

GO enrichment and KEGG pathway analyses of DEGs

Gene ontology terms for the Biological Process (BP), Cellular Component (CC) and Molecular Function (MF) were analyzed. To identify GO enrichment and KEGG pathway of DEGs, the functional enrichment analysis was performed using R package [10], clusterProfiler(v3.12.0), with adjust p value <0.05 as a cut-off.

Construction of protein-protein interaction network and modules analysis

A protein-protein interaction (PPI) network for DEGs was constructed using STRING database (https://string-db.org/cgi/)with the combined score≥0.9, and was visualized using Cytoscape software (http://cytoscape.org). Subsequently, the most significant module was identified by MCODE [11] plugin in cytoscape software, while the key genes were screened by the node degree in the Cytoscape plugin, cytoHubba [12].

Expression analysis of key genes in different breast subtypes

UALCAN [13] is a user-friendly, interactive website for analyzing cancer transcriptome data, and provides a silicon-based platform for validation of target genes and identification of tumor subpopulation-specific candidate biomarkers. To further screen invasive-related genes, we evaluated the expression of key genes in different subtypes of breast cancer samples by the UALCAN database.

Survival analysis of invasive key genes

The Kaplan-Meier plotter [14,15] (http://kmplot.com/analysis/) is a comprehensive online platform that can assess the effect of 54,675 genes on survival, based on 10,293 cancer samples. We conducted overall survival analysis of invasive key genes in breast cancer patients from the TCGA-BRCA database by using the Kaplan-Meier plotter, with prognosis considered significant if a log rank p value was < 0.05.

Results

Identification of DEGs

GSE29431 and GSE53752 were selected and underwent differentially expressed genes (DEGs) analysis using LIMMA package, with a cutoff of adjust p value < 0.05 and fold-change ≥ 2. In total, 838 genes were up-regulated and 1417 genes were down-regulated in GSE29431, while 469 genes were up-regulated and 776 genes down-regulated in GSE53752. The volcano plot showed the differentially expressed genes (Figure 1). The VENN plot showed the common 428 differentially expressed genes in two datasets (Figure 2).

Table 1. Basic characteristics of two datasets

Datasets	*Sample of breast cancer*		*Platform*
GSE29431	HER-2 Positive BC	12	GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus2.0 Array
	Normal	28	
GSE53752	TNBC	51	GPL7264 Agilent-012097 Human 1A Microarray (V2)G4110B
	Normal	25	

GO enrichment and KEGG pathway analyzes of DEGs

GO function and pathway analyses were performed on common upregulated and downregulated DEGs by clusterProfiler separately. We analyzed three parts of GO enrichment, including biological process (BP), cellular component (CC) and molecular function (MF). For the biological process (BP), upregulated genes were significantly enriched in the mitotic nuclear division and nuclear division, while downregulated genes were apparently enriched in the response to peptide and response to peptide hormone. For the cellular component (CC), upregulated genes were enriched in the spindle and chromosomal region, while downregulated genes were apparently enriched in the extracellular matrix and collagen-containing extracellular matrix. For the molecular function (MF), upregulated genes were enriched in the protein C-terminus binding, while downregulated genes were apparently enriched in the heparin binding and extracellular matrix structural constituent.

Furthermore, KEGG pathway analysis indicated that upregulated DEGs were mainly enriched in tht Cell cycle and Oocyte meiosis, while downregulated DEGs were involed in the PPAR signaling pathway and Tyrosine metabolism. The results of GO and KEGG pathway analysis are listed in Figure 3 and Figure 4.

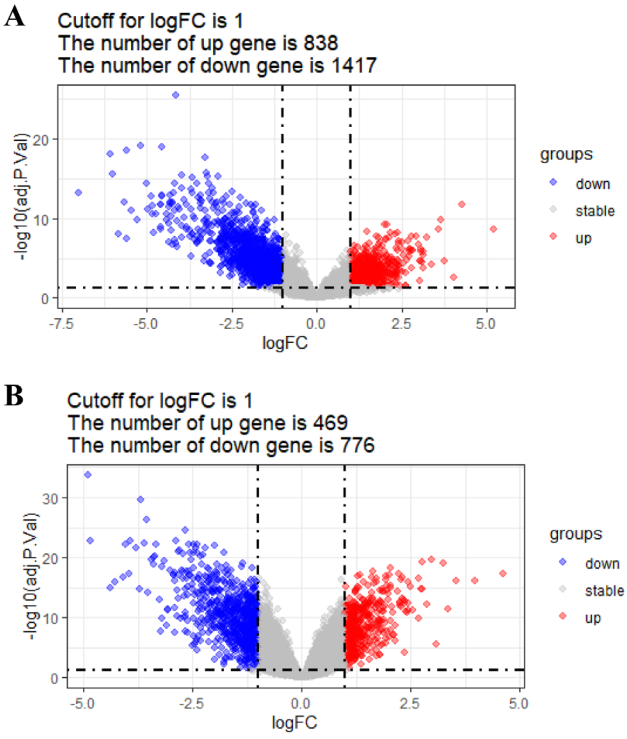

Figure 1. The volcano plot of DEGs in two datasets. **A:** showed the volcano plot of DEGs for dataset GSE29431; **B:** showed the volcano plot of DEGs for dataset GSE53752. Red presented upregulated genes with log2FC>1 and adj p-value <0.05, while blue presented downregulated genes with log2FC<1 and adj p-value <0.05. Grey presented genes with no significant difference. FC: fold change; adj p-value: adjusted p-value; DEGs: differentially expressed genes.

Construction of protein-protein interaction network and modules analysis

The 428 differentially expressed genes were input into STRING database for PPI network analysis which was visualized by Cytoscape software, and achieved a PPI network of 57 nodes and 335 edges, with the combined score=0.9 (Figure 5A). The most important cluster 1 which contained 22 nodes and 221 edges was identified using the plug-in Molecu-

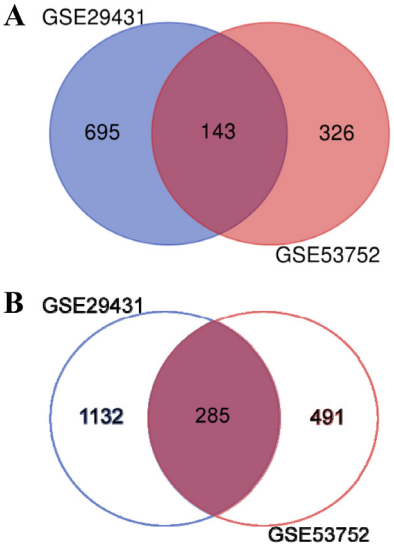

Figure 2. Venn diagram of common DEGs from the two datasets; **A:** Common upregulated genes; **B:** Common downregulated genes.

Table 2. The thirteen key genes with higher degree in Cluster 1

Gene symbol	*Degree*	*Expression*
CDK1	21	up
NUSAP1	21	up
BUB1	21	up
KIF20A	21	up
CENPF	21	up
DLGAP5	21	up
CDCA8	21	up
UBE2C	21	up
SPAG5	21	up
BIRC5	21	up
CCNA2	21	up
KIF2C	21	up
CDC20	21	up

lar Complex Detection (MCODE) tool in Cytoscape (Figure 5B). In total, 13 key genes are shown in Table 2 and were selected by a criterion of degree = 21 in the network.

Expression analysis of key genes and identification of invasive genes

We analyzed the expression levels of 13 key genes across different molecular subtypes in

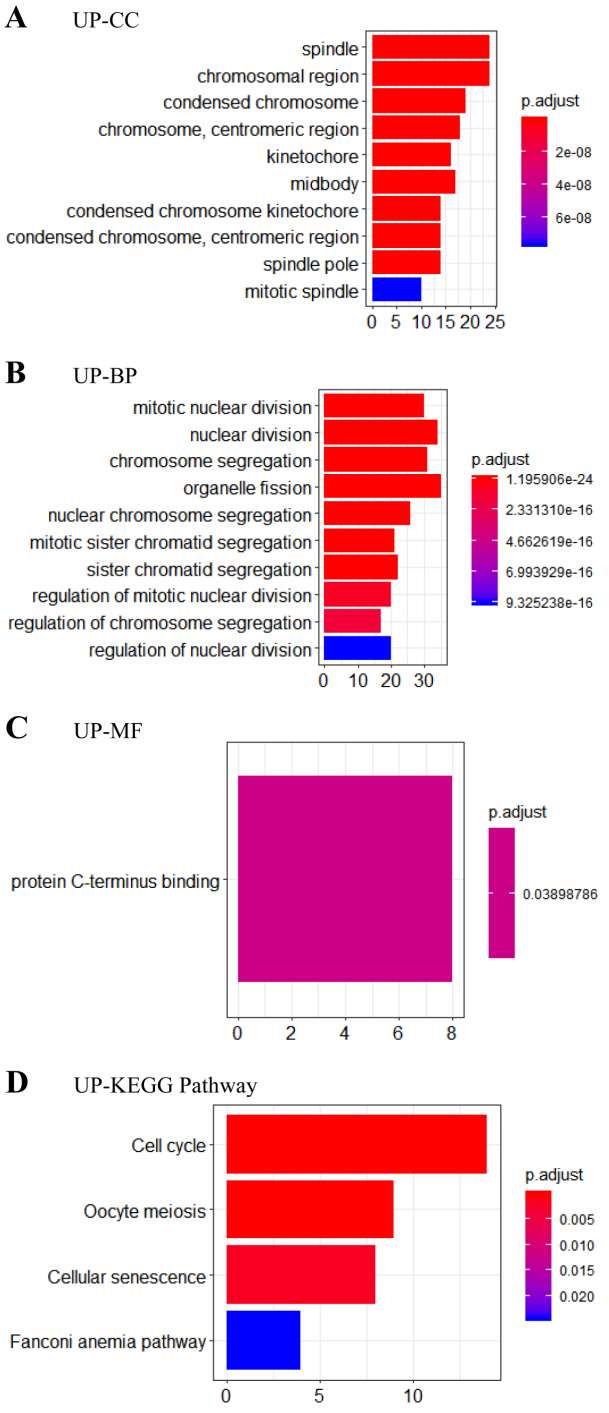

Figure 3. GO and KEGG pathway functional enrichment analysis of common upregulated DEGs from the GSE29431 and GSE53752 datasets. **A:** enrichment of cell component; **B:** enrichment of biological process; **C:** enrichment of molecular function; **D:** analysis of KEGG pathway; adj p-value <0.05 was considered as cutoff value of significant difference; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: differentially expressed genes; adj p-value: adjusted p-value.

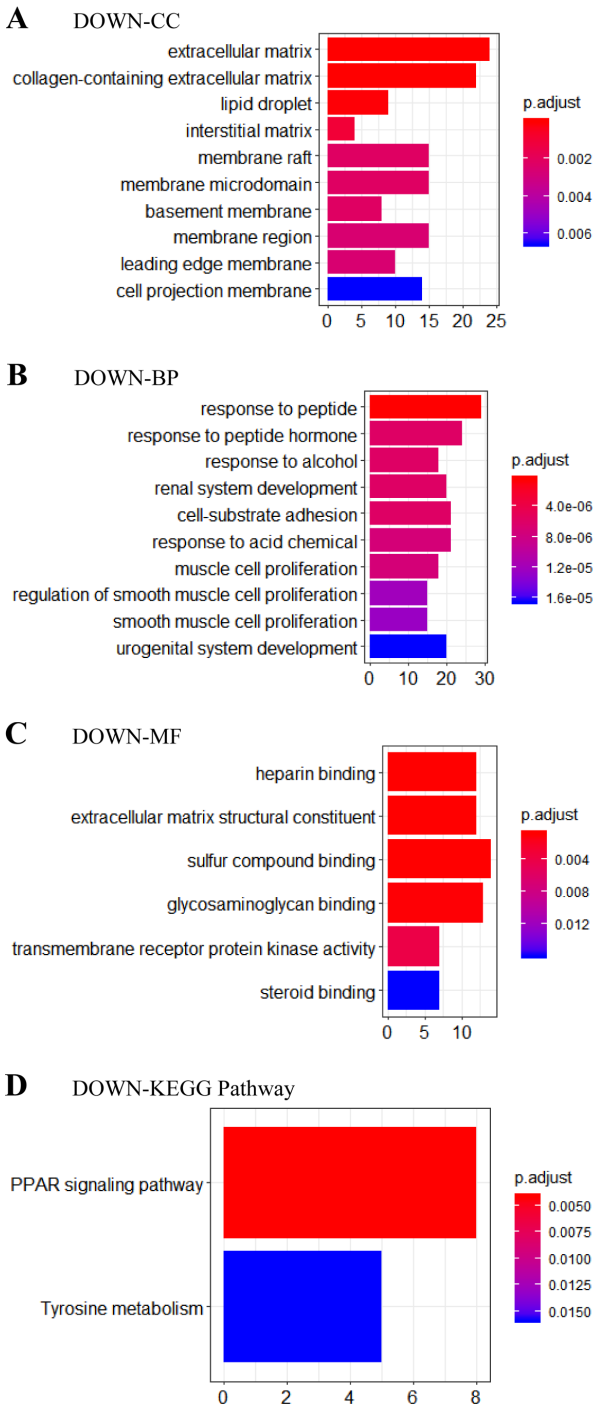

Figure 4. GO and KEGG pathway functional enrichment analysis of common downregulated DEGs from the GSE29431 and GSE53752 datasets. **A:** enrichment of cell component; **B:** enrichment of biological process; **C:** enrichment of molecular function. **D:** analysis of KEGG pathway; adj p-value <0.05 was considered as cutoff values of significant difference;GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; DEGs: differentially expressed genes; adj p-value: adjusted p-value.

breast cancer by the UVALCAN database. As it is shown in Figure 6, 12 key genes were closely associated with different molecular subtypes, including CDK1, BIRC5, BUB1, CCNA2, CDC20, CDCA8, DLGcAP5, KF2C, KIF20A, NUSAP1, SPAG5 and UBE2C, with statistical significance of differences ($p<0.05$). Compared with triple negative breast cancer, the expression levels of 12 invasive genes were significantly lower in luminal breast carcinoma. As a result, 12 genes served as invasive key genes.

Survival analysis of invasive key genes

To determine if invasive key genes expression was related to patient prognosis, we performed survival analysis using online Kaplan-Meier plotter to evaluate the correlation between invasive key genes expression levels and overall survival rates in breast cancer patients. Using median expression level as the cutoff point, the 12 invasive key genes were categorized into high-expression group and low-expression group. Kaplan-Meier survival

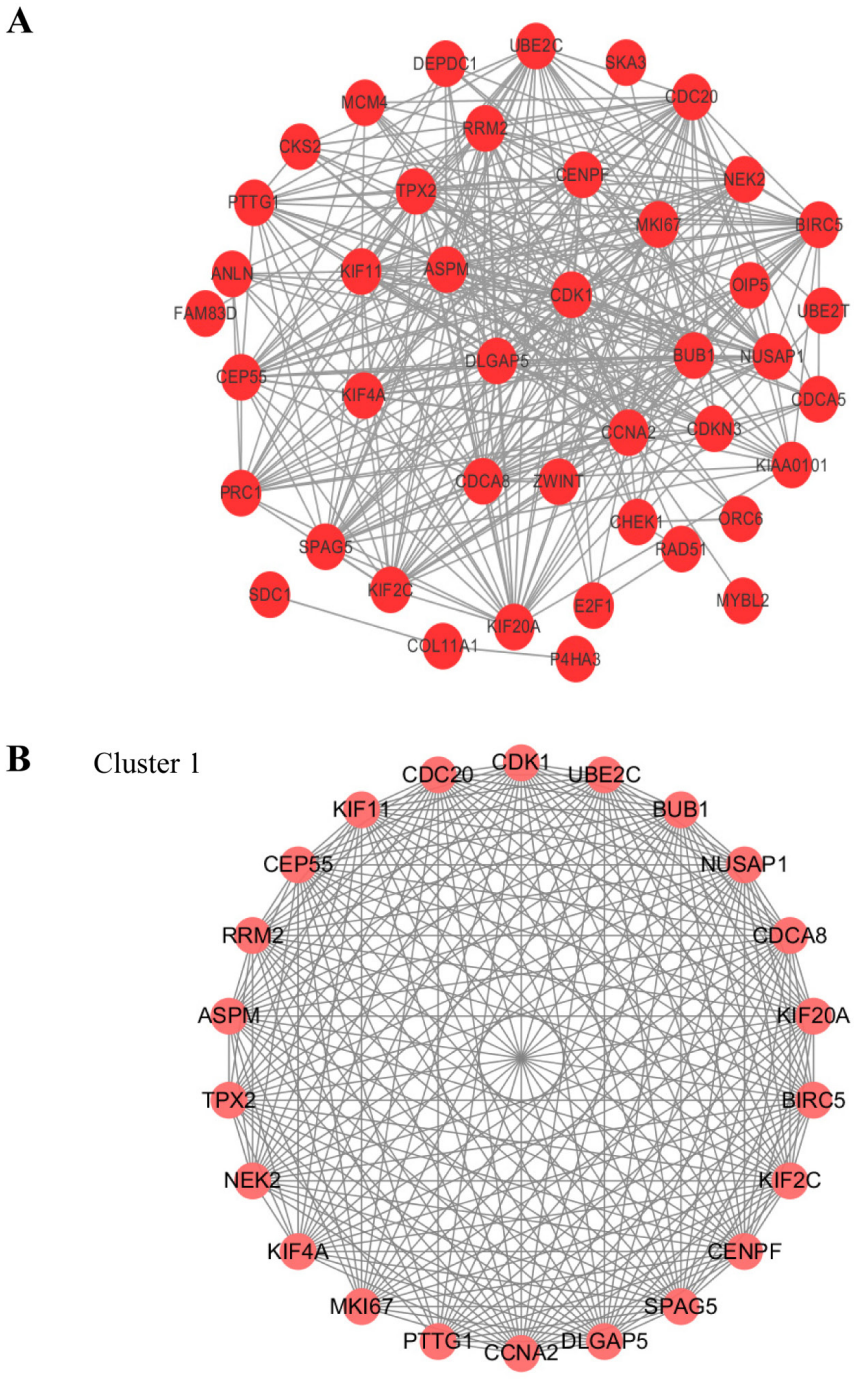

Figure 5. A: PPI network was constructed from common differentially expressed genes; red represented upregulated genes; **B:** The most significant module was identified from the PPI network; nodes indicated genes, while edges indicated protein-protein interaction; PPI: Protein-Protein interaction.

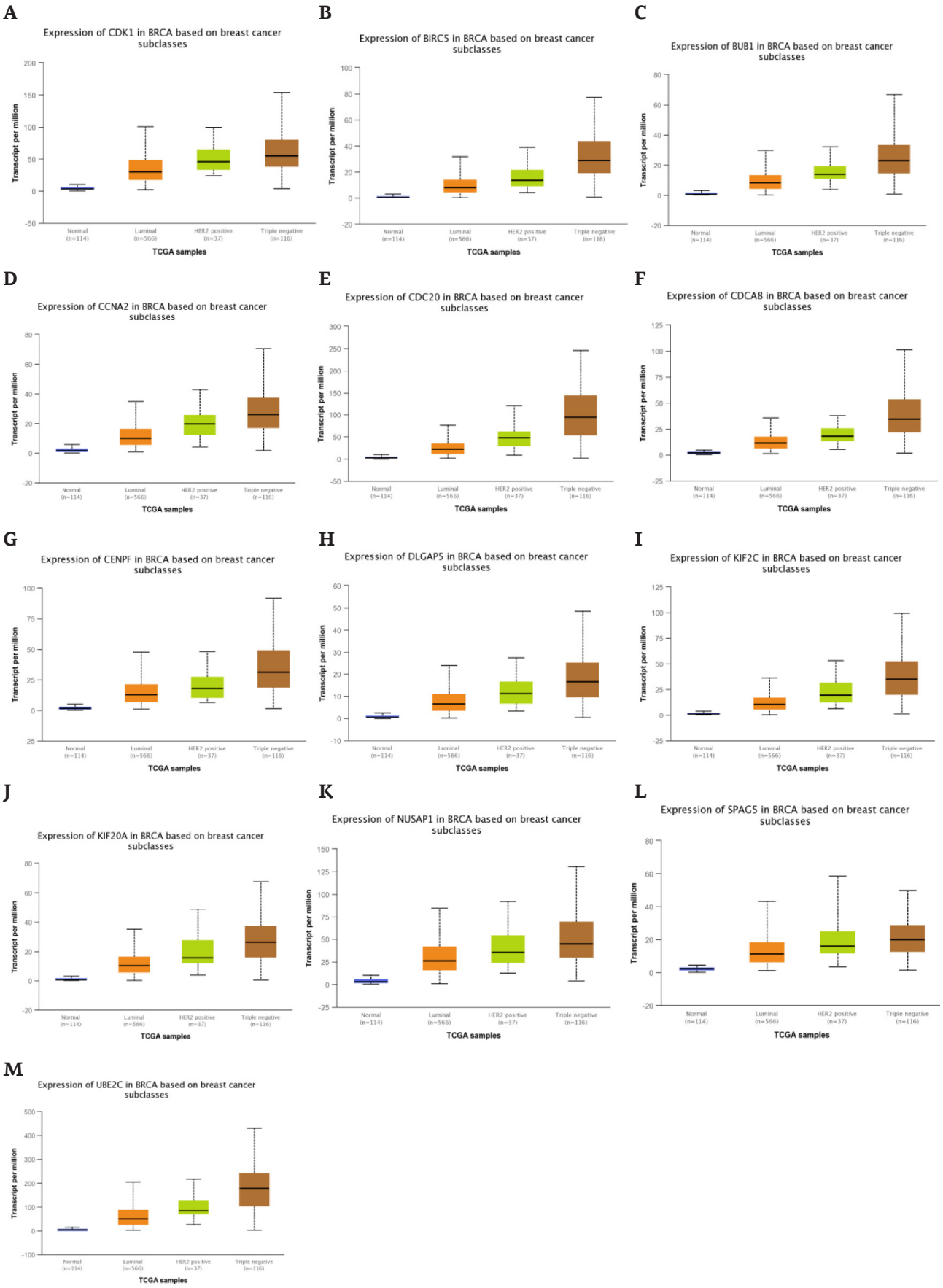

Figure 6. The expressions of invasive key genes among molecular subtypes of breast cancer; **A:** CDK1; **B:** BIRC5; **C:** BUB1; **D:** CCNA2; **E:** CDC20; **F:** CDCA8; **G:** CENPF without statistical significance; **H:** DLGAP5; **I:** KIF2C; **J:** KIF20A; **K:** NUSAP1; **L:** SPAG5; **M:** UBE2C.

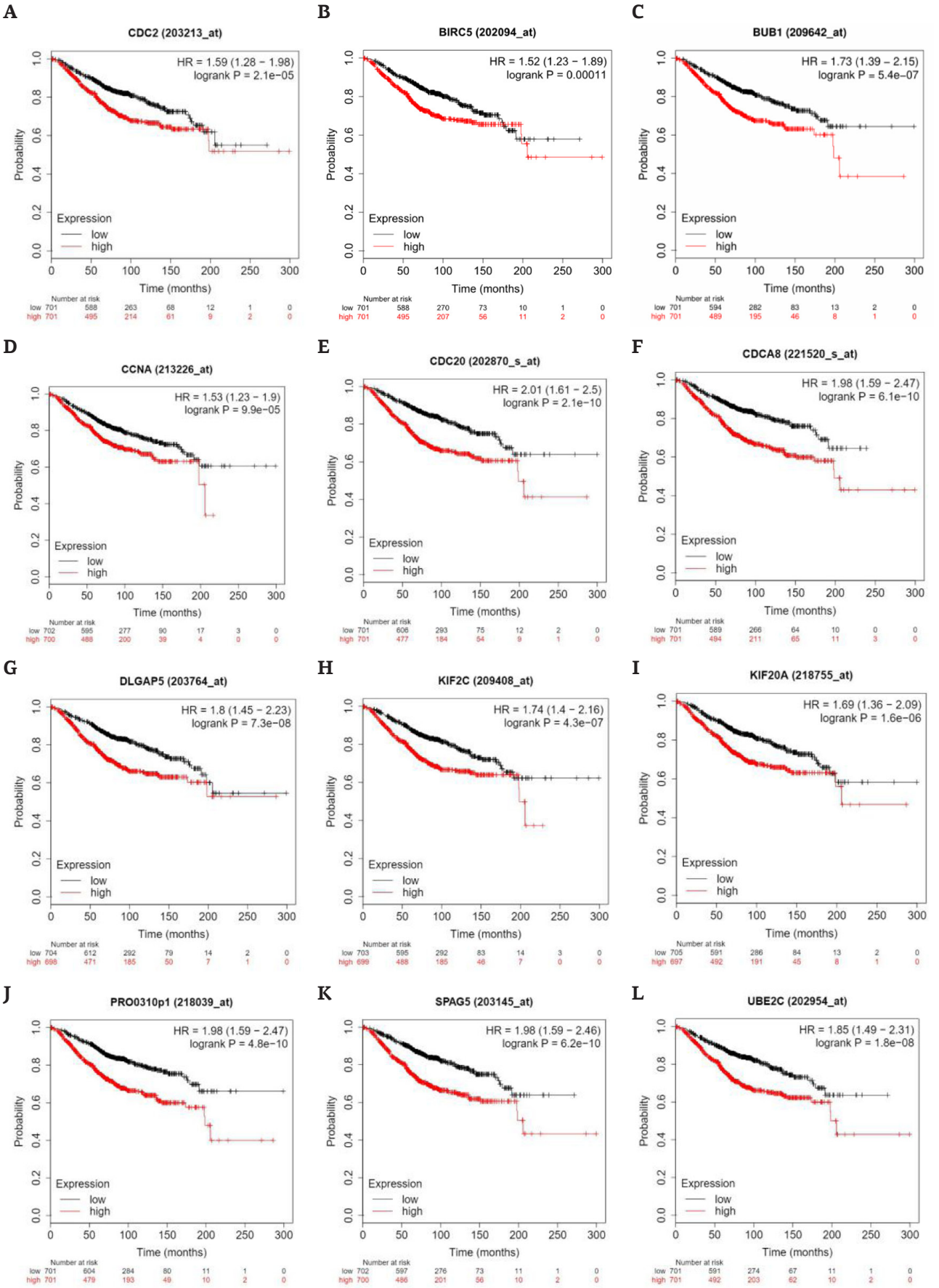

Figure 7. Kaplan-Meier survival curves of 12 invasive genes in breast cancer; Overall survival curves for **(A)** CDK1, known as CDC2; **(B)** BIRC5; **(C)** BUB1; **(D)** CCNA2, known as CCNA; **(E)** CDC20; **(F)** CDCA8; **(G)** DLGAP5; **(H)** KIF2C; **(I)** KIF20A; **(J)** NUSAP1, known as PRO0310p1; **(K)** SPAG5; **(L)** UBE2C.

analysis showed that high-expression group of invasive key genes were associated with a worse overall survival of breast cancer patients (log rank $p<0.05$). The results of survival analysis are shown in the Figure 7.

Discussion

Breast cancer is a highly heterogeneous disease whose aggressiveness apparently differs among different subtypes [16-18]. Because of high aggressiveness, HER-2 positive and TNBC are characteristic of high lymph node metastasis, high recurrence rate, and high mortality [19-21]. Until now, the molecular mechanisms of invasiveness of breast cancer are unclear. In the present study, GSE29431 and GSE53752 were deeply analyzed by LIMMA package to identify 428 commonly changed DEGs, including 143 upregulated and 285 downregulated genes in TNBC and HER-2 positive breast cancer compared to normal breast cancer samples.

GO was performed, which showed that the DEGs were mainly enriched in biological process. Upregulated genes were primarily associated with mitotic nuclear division and nuclear division, while downregulated genes were located in the response to peptide and response to peptide hormone. Upregulated DEGs showed an enrichment of KEGG pathway related to cell cycle and Oocyte meiosis, which are associated with development of tumors [22]. Downregulated DEGs were mostly associated with signaling pathway and tyrosine metabolism.

Next, based on the analysis in the STRING database and Cytoscape software, PPIs that contained 57 nodes and 335 edges were obtained and visualized, with the combined score=0.9. The most significant genes were selected, with a cutoff of degree = 21. By VALCAN database, we got 12 invasive key genes which their expression levels differred in different molecular subtypes in breast cancer, including CDK1, BIRC5, BUB1, CCNA2, CDC20, CDCA8, DLGAP5, KIF2C, KIF20A, NUSAP1, SPAG5 and UBE2C. Throungh Kaplan-Meier-plotter database, 12 invasive key genes were related with worse prognosis in breast cancer patients.

Based on the enrichment of GO and KEGG pathway, these upregulated DEGs generally may serve an important role in tumorigenesis and tumor proliferation. Aberrant mitosis often leads to tumor occurrence [23]. Molecular studies have shown that tumor development is closely associated with the cell cycle. Therefore, regulation of tumor cell cycle is an important strategy and target of tumor therapy [24-26]. Then, downregulated DEGs increased the aggressiveness of breast cancer through decreasing the hormone receptor expression [27-28].

CDK1 is one of the most important functions for regulating cell cycle progression in the majority of mammalian cells [29]. Several studies have shown that CDK1-dysregulation leads to robust tumor growth and high proliferation rate of cancer cells [30]. BIRC5 has been shown to play vital roles in carcinogenesis by influencing cell division and proliferation by inhibiting apoptosis [31]. BIRC5 repression was able to decrease the proliferation of breast cancer cells, implying that BIRC5 acts like a tumor driver [32]. BUB1 is well-known as a key component of mitotic checkpoint, and plays important roles in the proliferation and progression of the breast carcinoma [33,34]. CCNA2 (also known as CyclinA2) belongs to the highly conserved cyclin family and is expressed in most tissues in the human body [35]. It was reported that CCNA2 may be involved in the processes of epithelial-mesenchymal transitions (EMT) and metastasis [36]. Cell division cycle 20 (CDC20) is critical in cell cycle progression and indicates an aggressive course of disease risk [37]. The human cell division cycle associated 8 (CDCA8) gene is a member of the chromosomal passenger complex (CPC) and is indispensable for segregation of the chromosome during cell division [38]. Overexpression and nuclear accumulation of CDCA8 are linked to poor prognosis for cancer patients and are important for growth, survival and the malignant nature of cancer [39]. DLGAP5 is a novel cell cycle-regulated gene that can inhibit the proliferation and invasion of hepatocellular carcinoma cells [40], but its function in breast cancer cells is not clear. KIF2C, the mitotic centromere associated kinesin 271 (MCAK), is the most representative member of Kine-272-sin-13, and is correlated with lymph node metastasis and tumor stage [41]. KIF20A was localized to the Golgi apparatus and consisted of 890 amino acids [42]. Recent studies have shown that KIF20A, associated with breast caner, is a significant downstream target gene of Hedgehog (Hh) signaling, which was related to cancer cell proliferation, invasion, metastasis, and autophagy [43]. NUSAP1, identified as an overexpression marker gene in invasive carcinomas, is a 55-KD vertebrate protein that plays a key role in spindle assembly and normal cell cycle progression [44,45]. However, the role of NUSAP1 in invasive breast cancer has not yet been reported. Sperm-associated antigen 5 (SPAG5, also named DEEPEST, MAP126 or hMAP126), located on chromosome 17q11.2, was up-regulated in M-phase cells and played a vital role in cell mitosis and cell cycle checkpoint regulation [46]. In the recent studies, SPAG5 contributed to disease progression

in ER+ breast cancer subtypes [48]. UBE2C plays an important role in the ubiquitin-proteasome system. The ubiquitin-proteasome system precisely regulates the cell cycle through proteasome-mediated protein degradation pathways in eukaryotes. High UBE2C expression is associated with a high grade of malignancy, low differentiation, high metastatic tendency, and poor patient survival in a wide range of solid tumors including breast cancer [47-49].

In the present study, we explored the molecular mechanisms and biomarkers about aggressiveness in breast cancer by comprehensive bioinformatics analysis. 12 invasive key genes which were closely associated with prognosis were identified, including CDK1, BIRC5, BUB1, CCNA2, CDC20, CDCA8, DLGAP5, KIF2C, KIF20A, NUSAP1, SPAG5 and UBE2C. These findings provided new insights into the study of breast cancer and promoted individual treatments.

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Conflict of interests

The authors declare no conflict of interests.

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