Original Article

DNAJB4 identified as a potential breast cancer marker: evidence from bioinformatics analysis and basic experiments

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Background: Breast cancer (BC) is the leading cause of tumor-related death in women worldwide, but its pathogenesis is not clear. The efficient screening of new therapeutic targets for BC through bioinformatics and biological experimental techniques has become a hot topic in BC research.

Methods: The bioinformatics method was used to analyze the gene chips and obtain the hub genes, playing an important role in the development of BC. The biological processes (BP) involved in the hub genes were analyzed by Bingo, and the impact of each hub gene on disease-free survival (DFS) and overall survival (OS) in BC patients was evaluated in the Kaplan-Meier Plotter database. The expression of *DNAJB4*, the hub gene with the greatest degree and having an effect on the prognosis of BC patients, was detected in BC cell lines and clinicopathological specimens. And *DNAJB4* was selected for further biological experiments and clinical prognosis verification.

Results: Ten hub genes including *DNAJB4*, the greatest degree genes, were found by bioinformatics analysis of BC gene chips. *DNAJB4* expressions in both BC cell lines and clinicopathological specimens were detected and the results showed that *DNAJB4* was significantly down-regulated in BC cell lines and tissues. After interfering with the expression of *DNAJB4*, it was found that the invasion and migration ability of MDA-MB-231 cell line was significantly enhanced *in vitro*. The clinical survival data of BC patients showed that patients with high *DNAJB4* expression had longer DFS.

Conclusions: *DNAJB4* may be a tumor suppressor gene in BC as it could regulate invasion and migration of BC cells and its expression level is related to the prognosis of BC patients. Nevertheless, further researches are still necessary to verify its role in BC so as to provide evidences for clinical guidance regarding diagnosis and treatment.

Keywords: Breast cancer (BC); *DNAJB4*; bioinformatics; prognosis

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Introduction

Breast cancer (BC) is the most common malignant tumor in women worldwide (1,2). Although some studies have pointed out that BC can be prevented (3-6), its morbidity and mortality have rapidly climbed to the top of the list of female tumors in recent years (2). In 1894, Halsted put forward the classical radical mastectomy, which emphasized the complete resection of the tumor (7). But the success rate of BC treatment was still worrying (8). With the deepening of research, Fisher, an American scholar, first proposed that BC was a systemic disease in the 1970s (9-11). This theory provided a theoretical basis for the application of anthracyclines and taxanes in postoperative adjuvant chemotherapy for BC, and laid the foundation for modern comprehensive treatment of BC. At present, the standard process of clinical diagnosis and treatment of BC is the combination of surgery, chemotherapy, radiotherapy, endocrine therapy and targeted therapy after medical imaging detection, tumor marker detection and pathological biopsy (12). Compared with the treatment of BC in the 1980s, the current treatment regimen has improved the prognosis of patients with BC to some extent (2). This method is effective in the treatment of early BC, but it is not effective in patients with advanced BC or distant metastasis (13). Since most of the patients diagnosed with BC for the first time are advanced BC (14-16), the above-mentioned schemes have been difficult to meet the current needs of BC diagnosis and treatment. Targeted therapy as an important part of BC treatment, a large number of studies have shown that HER-2 overexpression of BC patients with HER-2 targeted drugs trastuzumab and pertuzumab can significantly improve the prognosis of patients (17,18). However, HER-2 overexpression BC accounts for only 14% of clinical BC patients (2). Finding new and effective targets for BC therapy has become an urgent problem to be solved in the process of clinical diagnosis and treatment of BC.

In recent years, with the rapid development of gene sequencing technology and the establishment of a large number of tumor database, using bioinformatics technology to screen potential tumor diagnosis and treatment targets has become an important branch of contemporary medicine (19-21). Through bioinformatics techniques, Ge *et al.* (22) found that ZGRF1 may be associated with the poor prognosis of triple negative BC. Li *et al.* (23) reported that mitochondrial proteins interacting with ZW10 can be used as prognostic biomarkers of human BC. In this study, differentially expressed genes (DEGs) including *DNAJB4* were screened by analyzing the gene chip downloaded from GEO data. Because *DNAJB4* was at the core of the PPI network we built, and it was valued by the research team. However, due to the limitations of contemporary sequencing technology and the great heterogeneity of patients in different data sets, the current bioinformatics analysis results still need to be verified by laboratory data. Although *DNAJB4* has been reported in previous studies on BC, the studies were mostly limited to the relationship between *DNAJB4* expression level and clinical prognosis or tumor drug resistance (24,25). There were few studies on the biological behavior and related signal pathways of *DNAJB4* in BC cells. Therefore, this study intends to use the combination of bioinformatics technology and basic experimental technology to identify the biological processes (BP) in BC, in which DNAJ4 was involved, and predicted its related signaling pathways, so as to provide new ideas for the clinical diagnosis and treatment of BC. We present the following article in accordance with the REMARK reporting checklist (available at http://dx.doi.org/10.21037/gs-20-431).

Methods

Data sources

Chip data

Gene Expression Omnibus database (GEO), is a gene expression database created and maintained by The National Center for Biotechnology Information (NCBI). Founded in 2000, it contains high-throughput gene expression data submitted by research institutions around the world, and the chip data of all database are open, free and shared to researchers all over the world. We downloaded two microarrays including GSE15852 (26) from HG-U133A Affymetrix Human Genome U133A Array (Platform GPL96) and GSE42568 (27) from HG-U133_Plus_2 Affymetrix Human Genome U133 Plus 2.0 Array (Platform GPL 570) of GEO. In the GSE15852 data set, we selected 86 gene expression profiles, including 43 normal breast tissue samples and 43 primary tumor samples from BC patients. In the GSE42568 data set, we selected 121 gene expression profiles, including 17 normal breast tissue samples and 104 primary tumor samples from BC patients.

Characteristics of patients and specimens

This study adopted the purposive sampling method to choose the patient, who were treated in the First Affiliated Hospital of Wannan Medical College. All patients were admitted and

treated according to Guidelines of Chinese society of Clinical Oncology (BC). The inclusion criteria were established as follows: (I) no neoadjuvant chemotherapy or neoadjuvant endocrine therapy before operation; (II) the surgical method accepted by patients was modified radical mastectomy or breast-conserving surgery; (III) methylene blue lymph node tracing method was used during the operation; and the scope of axillary lymph nodes were dissected on the affected side included at least area I and II. (IV) The routine pathology report confirmed that it was invasive BC. (V) The clinical staging of BC is in stage I-II, according to the AJCC staging guidelines. (VI) The postoperative chemotherapy and radiotherapy were completed on time according to the postoperative BC pathological classification. Exclusion criteria: (I) incomplete basic information; (II) bilateral BC; (III) recurrent secondary operation of BC; (VI) complicated with other malignant tumors; (V) history of exposure to radiation and carcinogenic chemicals.

We obtained the BC specimens and adjacent tissues (3 cm from the edge of the cancer edge) of the BC patients included in this study from the specimen library of the Pathology Department of the First Affiliated Hospital of Wannan Medical College. All specimens were fixed with 10% formalin during routine resection and routinely dehydrated and preserved by paraffin embedding. The paraffin-embedded tissue block is stored in the normal temperature paraffin-embedded specimen library by the pathology specialist.

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of the First Affiliated Hospital of Wannan Medical College (No. 2019070, the registration number of ethics board) and informed consent was taken from all the patients.

BC cell lines

MCF10A, MBA-MD-231 and MDA-MB-436 BC cell lines were purchased from Precell Biotechnology Co., Ltd. (https://www.procell.com.cn/). MCF10A human normal breast cells were cultured in DMEM (11995065, Gibco, USA) +5% Horse Serum (16050-122, Gibco, USA) +20 ng/mL EGF (PHG0311, Gibco, USA) +0.5 μg/mL hydrocortisone (C-130, Sigma, USA) +10 μg/mL insulin (91077C-100MG, Sigma, USA) +1% NEAA (0823, ScienCell, USA) +1% P/S (SV30010, Hyclone, USA) medium. MBA-MD-231 human BC cell lines were cultured in Leibovitz's L-15 (11415-064, Gibco, USA) +10% FBS (10099-141, Gibco, USA) +1% P/S (SV30010, Hyclone, USA) medium. The culture condition of MDA-MB-436 BC cell line was Leibovitz's L-15 (11415-064, Gibco, USA) +10 μg/mL insulin +16 μg/mL glutathione (16100, Thermo, USA) +15% FBS (10099-141, Gibco, USA) +1% P/S (SV30010, Hyclone, USA). Except for the identity provided by Procell Biotechnology Co., Ltd., the cell line has not been independently certified. All cells were cultured in 5% CO_2 humidifier at 37 ℃, and mycoplasma contamination was detected in all cell lines.

Experimental method

Screening of DEGs

GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) is an interactive network analysis tool officially released by GEO database, which relies on two R packages, GEOquery (28) and LIMMA (29). It is mainly used to analyze and obtain biological information contained in gene chips of GEO database. In this study, GEO2R was used to screen DEGs between normal breast tissue and BC. The P value was adjusted by Benjamini and Hochberg [false discovery rate (FDR)] method, and the adj. P value was used as the basis to distinguish statistically significant and false positive genes. In the study, when a gene symbol has a unique probe set, it is considered to be statistically significant. On the contrary, probe sets lacking of corresponding gene symbols or genes with not only one probe set were removed or averaged, respectively. In the present study, when $|Log_2FC| > 1$ and adj. P value <0.01 is considered to be statistically significant.

KEGG and GO enrichment analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/) (30) is a bioinformatics database with annotations and information links as its main functions. The database quotes Gene Ontology (GO) (31) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (32) established by the Kanhisa Laboratory of the Bioinformatics Center of Kyoto University, Japan. DAVID database also integrates analysis tools, so this tool can find out the most significantly enriched biological annotations from thousands of related annotations at the statistical level, which are large-scale lists of genes or proteins, and provide comprehensive biological function annotations for corresponding genes to help users extract biological information.

Construction of protein-protein interaction (PPI) network

The interaction between the most significant module

and hub genes network proteins in cells can reveal the function of proteins at the molecular level, which is essential for the understanding of cell development, differentiation and apoptosis, as well as other biological regulatory mechanisms and life activities. It provides an important theoretical basis for exploring the pathogenesis of major diseases, the treatment and prevention of diseases and the development of new drugs. In this study, the PPI network was constructed using the Search Tool for the Retrieval of Interacting Gene (STRING) database (https://string-db.org) (33), based on the DEGs determined by the researchers to identify relationships between these DEGs and the functional groups. Cytoscape (version 3.7.2) (34), an open software, was used to visualize the PPI networks. Molecular Complex Detection (MCODE) (version 1.6) (35), a plug-in of Cytoscape, was used to filter out the most significant module from the PPI network with degree cut-off =2, node score cut-off =0.2 and K-Core =2.

Selection and analysis of HUB gene

The free Cytoscape plug-in CytoHubba (36) is used to identify central genes from the most significant module. The BP of HUB genes were analyzed and visualized using Biological Networks Gene Oncology tool (37) (Bingo) (version 3.0.3) plug-in of Cytoscape. The overall survival (OS) and disease-free survival (DFS) of Hub gene were analyzed by Kaplan-Meier method in Kaplan-Meier Plotter (38) (http://kmplot.com/analysis/index.php?p).

Immunohistochemistry

The breast carcinomas and their adjacent normal tissues were fixed with formalin (10%), embedded in paraffin and cut into thick sections of 4 μm. According to the manufacturer's instructions, tissue sections were stained with *DNAJB4* antibody (1:50, Abcam, USA), secondary antibody kit (SP-9000, ZSGB-BIO, China), DAB kit (ZLI-9017, ZSGB-BIO, China). Two experienced pathologists examined the immunostaining results and divided them into five grades according to the percentage of positive cells (PP), that is, ≤25% for 1 score, 26–50% for 2 scores, 51–75% for 3 scores, and 76–100% for 4 scores. According to the staining intensity (SI) of nucleus or cytoplasm, it was divided into four grades: 0 score for no staining, 1 scores for weak staining, 2 scores for moderate staining and 3 scores for strong staining. Finally, immunohistochemical staining is considered positive when SI and PP take more than 3 scores (39).

Production and transfection of lentiviral vector

Three lentiviral vectors of DNAJB4-shRNA were designed to knock down the expression of DNAJB4 in MDA-MB-231 and MDA-MB-436 cells. The sequences of DNAJB4 specific siRNA1 (siDNAJB4-1) are: 5'-GGGAATTGAGAAAGGAGCTTCAGAT-3'; siRNA2 (siDNAJB4-2) are: 5'-GAGGCATTGTGTGGCTGCTCAATTA-3'; siRNA3 (siDNAJB4-3) are: 5'-TGAGGTGTCCTTCCCAGATACTATA-3'; control virus vector siRNA is: 5'TTCTCCGAACGTGTCACGTAA-3'. And the most effective vectors are selected by RT-qPCR (Figure S1). The non-targeted shRNA lentivirus vector is used as the negative control, and the blank control refers to the non-vector. The shRNA lentivirus vector was purchased from Hanbio Biotechnology Company (Shanghai, China) (Figure S2, Table S1).

RT-qPCR

The total RNA of these collected cells was extracted using TRIzol reagent (15596026, Invitrogen, USA). Samples were treated with chloroform (20%), vortexed briefly, and incubated at room temperature for 15 min. Samples were then centrifuged at high speed at 4 ℃ for 15 min. The aqueous phase was moved to a new tube and an equal volume of isopropanol was added. Samples were incubated at room temperature for 10 min, followed by centrifugation at high speed at 4 ℃ for 10 min. Pellets were washed in 95% ethanol, dried and resuspended in nuclease-free water. RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo, USA) was used to synthesize cDNA, according to the manufacturer's instructions. Using DNAJB4 and β-actin specific primers made by Sangon Biotech (Shanghai, China), qPCR was performed in LightCycler96 (Roche, USA) according to TB Green® Premix Ex Taq™ II (RR820A, Takara, Japan) instructions. The fold change in the expression of DNAJB4 was calculated by the $2^{-\Delta\Delta CT}$ formula, and the GAPDH mRNA level was used as a control. The sense primer of DNAJB4 is 5'-CCAGCAGACATTGATTTTTATCATT-3'. The antisense primer of DNAJB4 is 5'-CCATCCAGTGTTGGTACATTAATT-3'. The sense primer of β-actin is 5'-AGCACTGTGTTGGCGTACAG-3', and antisense primer of β-actin is 5'-TCCCTGGAGAAGAGCTACGA-3' (Figure S2, Table S1).

Western bolting

Using lysate (PMFS: RIPA =1:100) (BOSTER Biological Technology, China) to separate total protein of the collected

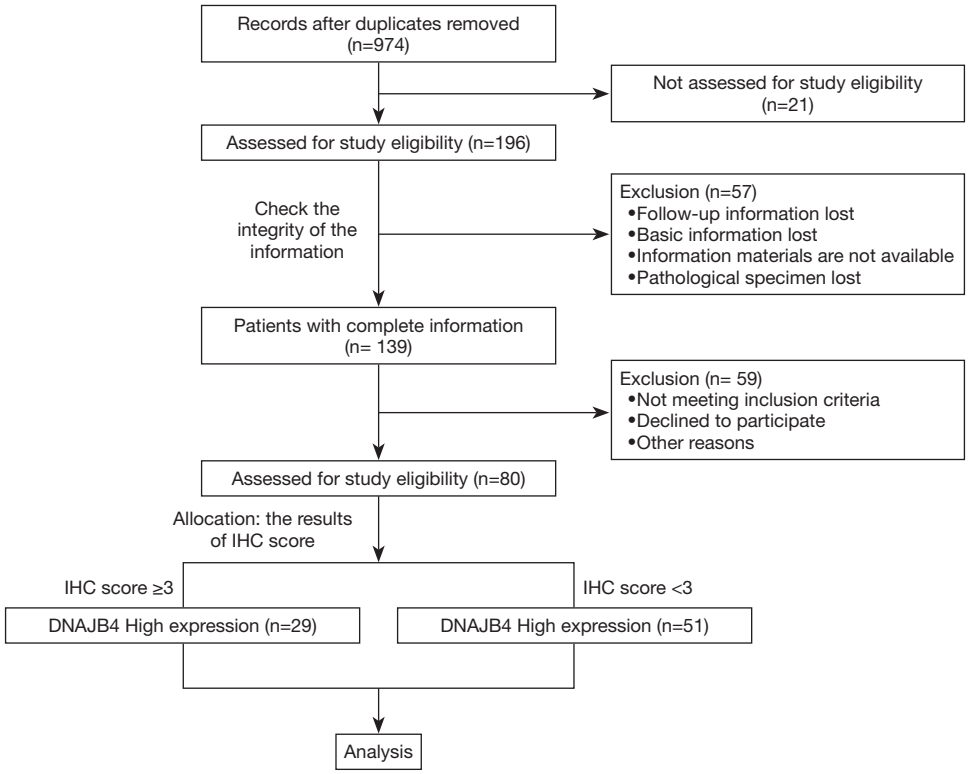


Figure 1 The flow of patients through the study.

cells. The protein samples were separated by SDS-PAGE (8%) and transferred to a PVDF membrane (IPVH00010, millipore, USA). Membranes were blocked in 5% non-fat dry milk (1706404, BioRad, USA) dissolved in TBS-T for 1 h and incubated overnight at 4℃ with primary antibodies. The membranes incubated with the corresponding horseradish peroxidase (HRP) (abs20002A, absin, China) coupled with the secondary antibody. ECL reagent (WBKLS0100, Millipore, USA) was used to detect immune response bands.

Transwell assay

The transwell invasion assay used 24 transwell chambers (MCEP24H48, Millipore, USA). First, the upper ventricle surface of the basement membrane of the Transwell chamber was coated with Matrigel (354230, Corning, USA). Then, 3×10^4 cells were suspended in 0.2 mL serum-free medium and added to the insert. 0.5 mL medium containing 20% fetal bovine serum was added into the lower chamber as a chemotactic inducer. After incubating at 37 ℃ for 48 hours, the cells on the upper surface of the membrane were carefully removed with cotton swabs. The cells on the lower surface were fixed with 100% methanol, and then stained with 0.1% crystal violet (C8470, Solarbio, China). Five random visual fields were selected, each insert was magnified 200×, and the number of cells was counted under an optical microscope (Leica, Germany).

Wound healing experiment

The cells were seeded in a 6-well plate at an appropriate density and cultured until 100% confluence. The yellow straw tip was used to make a straight scratch to simulate the wound. Microscope-based photos were taken at 0 or 36 hours after wounding.

Design of clinical study

This study is a retrospective study, and the patients included in the study were not stratified or matched. The first patient included in this study on January 1, 2012, and the follow-up of the last case ended on November 23, 2019. BC local recurrence or distant metastasis is considered to be the research endpoint. The flow of patients through the study was described in *Figure 1*.

Statistical analysis

The experimental data statistics of this project were carried

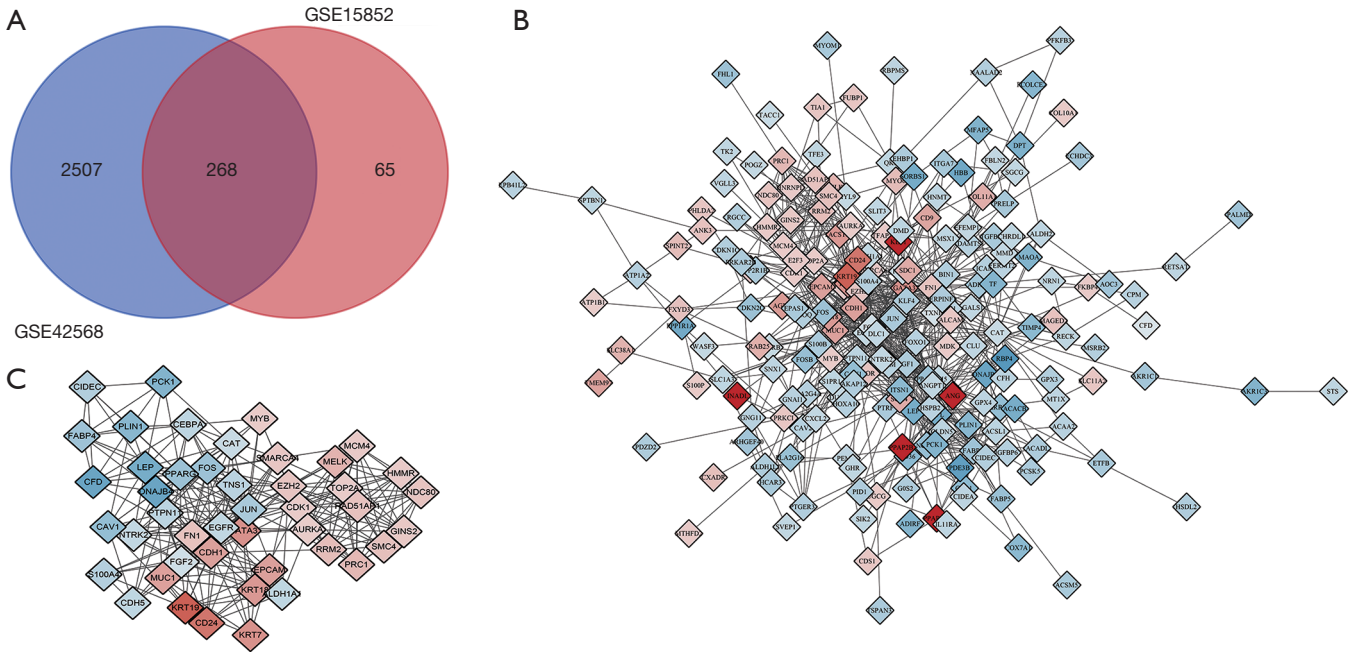


Figure 2 Venn diagram, PPI network and the most significant module of DEGs. (A) DEGs were selected with a log2|FC|change >1 and adj.P value <0.01 among the mRNA expression profiling sets GSE42568 and GSE15852. The 2 datasets showed an overlap of 268 genes. (B) The PPI network of DEGs was constructed by STRING and visualized by cytoscape. (C) The most significant module was obtained from PPI network with 45 nodes and 299 edges. Upregulated genes are marked in red; downregulated genes are marked in blue. PPI network, protein-protein interaction network; DEGs: differentially expressed genes; FC, fold charge.

out in SPSS 24.0 for Windows. The measurement data were shown as mean ± SD. The measurement data were analyzed by two-tailed one-way ANOVA, and the LSD method was used for pairwise comparison among groups. The survival curve was drawn by Kaplan-Meier method, and the difference of survival data was tested by Logrank test. In the study, the test level α is set to 0.05, a value of P<0.05 was considered as statistically significant.

Results

Identification of DEGs in BC

In the GSE15852 and GSE42568 data sets, compared with normal breast samples, GEO2R analysis was used to screen for DEGs in BC samples meeting the criteria that adjP<0.01 and |logFC| ≥1 (2,775 genes were selected in GSE42568 data set and 333 genes were selected in GSE15852 data set). And 268 DEGs were overlapped in the two data sets, as demonstrated in the Wayne diagram (*Figure 2A*), which includes 73 up-regulated genes and 195 down-regulated genes.

Gene ontology and KEGG pathway analysis of DEGs in breast

The GO and KEGG pathway analysis of DEGs in was carried out by using DAVID online annotation tool. The results of GO analysis showed that the changes in the BP of DEGs were obviously enriched in negative regulation of cell proliferation, lipid metabolic process, cellular response to hormone stimulus, response to drug, positive regulation of protein kinase B signaling, positive regulation of transcription and DNA-templated, response to estrogen. The changes of molecular function (MF) were mainly concentrated in oxidoreductase activity, heparin binding, nucleic acid binding transcription factor activity and ATP binding. The changes of cell composition (CC) were mainly concentrated in membrane raft, cell surface, lateral plasma membrane, bicellular tight junction, apical plasma membrane and membrane. KEGG pathway analysis showed that down-regulated DEGs were mainly enriched in PPAR signaling pathway, AMPK signaling pathway, pathways in cancer; and the up-regulated DEGs were primarily enriched in ECM-receptor interaction; protein digestion

Table 1 GO and KEGG pathway enrichment analysis of in BC samples

Term	Description	Count	adj. P value
Downregulated			
GO:0008285	Negative regulation of cell proliferation	17	3.24E–06
GO:0006629	Lipid metabolic process	11	5.01E–06
GO:0032870	Cellular response to hormone stimulus	7	6.19E–06
GO:0042493	Response to drug	13	7.13E–05
GO:0051897	Positive regulation of protein kinase B signaling	7	2.22E–04
GO:0016491	Oxidoreductase activity	9	7.93E–04
GO:0008201	Heparin binding	8	0.001001847
GO:0045121	Membrane raft	16	1.40E–09
GO:0009986	Cell surface	17	7.02E–05
GO:0005925	Focal adhesion	14	1.07E–04
GO:0005737	Cytoplasm	72	4.51E–04
hsa03320	PPAR signaling pathway	11	3.67E–08
hsa04152	AMPK signaling pathway	9	4.19E–04
hsa05200	Pathways in cancer	13	0.01207368
Upregulated			
GO:0045893	Positive regulation of transcription, DNA-templated	7	6.74E–04
GO:0043627	Response to estrogen	3	0.003787
GO:0001071	Nucleic acid binding transcription factor activity	2	0.008079
GO:0005524	ATP binding	12	0.008835
GO:0016328	Lateral plasma membrane	4	3.85E–04
GO:0005923	Bicellular tight junction	4	0.004091
GO:0016324	Apical plasma membrane	5	0.004375
GO:0016020	Membrane	10	0.010443
bta04512	ECM-receptor interaction	4	0.004793
bta04974	Protein digestion and absorption	3	0.043512

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BC, breast cancer.

and absorption (*Table 1*).

PPI network construction and module analysis

The network construction of DEGs was carried out in the online tool of STRING, and the results were visualized in Cytoscape (*Figure 2B*). The MCODE plug-in in Cytoscape software was used to grab the most significant module (*Figure 2C*) in the network constructed by DEGs. The genes contained in the most significant module in the PPI network were analyzed by GO and KEGG in the DAVID online tool. The results indicate that the genes in this module were mainly enriched in response to estrogen, mitotic spindle organization, RNA polymerase II transcription factor activity (ligand-activated) (*Table 2*).

Hub gene selection and analysis

The cytoHubba plug-in in Cytoscape software was used

Table 2 GO and KEGG pathway enrichment analysis of DEGs in the most significant in BC samples

Term	Description	Count	adj. p value
GO:0043627	Response to estrogen	3	4.07E–04
GO:0007052	Mitotic spindle organization	2	0.031162761
GO:0004879	RNA polymerase II transcription factor activity, ligand-activated sequence-specific DNA binding	2	0.022515
GO:0005524	ATP binding	7	0.006149481
GO:0003682	Chromatin binding	4	0.00944271
GO:0000979	RNA polymerase II core promoter sequence-specific DNA binding	2	0.063353758
GO:0005829	Cytosol	5	0.016557
GO:0005811	Lipid particle	2	0.047888
GO:0070938	Contractile ring	2	0.00992
GO:0045120	Pronucleus	2	0.012737
fca03320	PPAR signaling pathway	5	1.26E–05
fca05200	Pathways in cancer	7	1.11E–04
fca04152	AMPK signaling pathway	5	1.31E–04
fca04010	MAPK signaling pathway	5	0.001618

GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; BC, breast cancer.

to capture the genes in the most significant module in PPI network which ranked the top 10 and node degree >10 as Hub genes. The gene symbols, full names and functions related to these captured Hub genes were listed in *Table 3*, in which *DNAJB4* has the highest degree of 29. The BP of HUB gene was analyzed and visualized (*Figure 3*) by the plug-in of Bingo, a biological network gene oncology tool of Cytoscape APPs. The analysis results suggested that the biological functions of the hub genes were mainly focused on regulation of cell cycle process, regulation of nuclear division, cell cycle process, cell cycle, cell division. The analysis of hub genes in the Kaplan-Meier Plotter online tool showed that there was a significant difference in OS between BC patients with *CDH1*, *CDK1*, *PPARG*, *LEP*, *DNAJB4*, *FGF2* and *EZH2* altered and the iterated (*Figure 4A*); and there is also a significant difference in DFS between patients with and without changes in *CDH1*, *CDK1*, *PPARG*, *LEP*, *DNAJB4*, *FGF2*, and *EZH2* (*Figure 4B*).

Expression of DNAJB4 in BC cell lines and cancer tissues

Western Bolt was used to detect the expression of *DNAJB4* in normal breast cell line MCF10A and BC cell lines. The results showed that the expression of *DNAJB4* in MDA-MB-231 and MDA-MB-436 cell lines was significantly lower than that in MCF10A cell line, and the difference was statistically significant (*Figure 5A*,*B*). The expression of *DNAJB4* was detected by immunohistochemistry on cancer tissues and the adjacent tissues of 80 BC patients (basic demographic characteristics can be found in Table S2). The results revealed that the immunohistochemical staining scores of *DNAJB4* in BC was significantly lower than those in adjacent tissues (*Figure 5C*,*D*).

Effect of down-regulation of DNAJB4 on migration and invasion of BC cell line

Wound healing assay was used to detect the migration ability of BC cells. The results manifested that 36 hours after the establishment of the wound model, the migration ability of *DNAJB4* knockdown MDA-MB-231 and MDA-MB-436 BC cells increased significantly (P<0.05) (*Figure 6*). The results of Transwell chamber assay found that after interfering with the expression of *DNAJB4* in MDA-MB-231 and MDA-MB-436, the number of cells invaded through the chamber was significantly increased compared with the control group, and the difference was statistically significant (P<0.05) (*Figure 7*).

Table 3 Functional of hub genes with degree ≥10

No.	Gene symbol	Full name	Function
1	*CDH1*	Cadherin 1	Pathways: oas05200:Pathways in cancer; oas05218:Melanoma; GO:0035635 entry of bacterium into host cell; GO:0099576 regulation of protein catabolic process at postsynapse, modulating synaptic transmission; GO:0071681 cellular response to indole-3-methanol
2	*PPARG*	Peroxisome proliferator activated receptor gamma	Pathways: oas05200:Pathways in cancer; fca03320:PPAR signaling pathway; GO:0060694 regulation of cholesterol transporter activity; GO:2000230 negative regulation of pancreatic stellate cell proliferation; GO:0071306 cellular response to vitamin E
3	*EZH2*	Enhancer of zeste 2 polycomb repressive complex 2 subunit	Pathways: fca04152:AMPK signaling pathway; fca04068:FoxO signaling pathway; GO:0098532 histone H3-K27 trimethylation; GO:0014834 skeletal muscle satellite cell maintenance involved in skeletal muscle regeneration; GO:0036333 hepatocyte homeostasis
4	*DNAJB4*	Dnaj heat shock protein family (Hsp40) member B4	Pathways: fca04068:FoxO signaling pathway; fca05205:Proteoglycans in cancer; GO:0051085 chaperone cofactor-dependent protein refolding; GO:0032781 positive regulation of ATPase activity; GO:0051084 'de novo' posttranslational protein folding
5	*LEP*	Leptin	Pathways: fca04920:Adipocytokine signaling pathway; GO:2000486 negative regulation of glutamine transport;GO:0033686 positive regulation of luteinizing hormone secretion;GO:0046881 positive regulation of follicle-stimulating hormone secretion
6	*FN1*	Fibronectin 1	Pathways: oas05200:Pathways in cancer; oas05205:Proteoglycans in cancer; GO:2001202 negative regulation of transforming growth factor-beta secretion;GO:1904237 positive regulation of substrate-dependent cell migration, cell attachment to substrate;GO:2001201 regulation of transforming growth factor-beta secretion
7	*EGFR*	Epidermal growth factor receptor	Pathways: oas05200:Pathways in cancer; oas05205:Proteoglycans in cancer; GO:0043006 activation of phospholipase A2 activity by calcium-mediated signaling;GO:0032431 activation of phospholipase A2 activity;GO:1902722 positive regulation of prolactin secretion
8	*CDK1*	Cyclin dependent kinase 1	Pathways: oas05203:Viral carcinogenesis; oas05168:Herpes simplex infection; GO:1905448 positive regulation of mitochondrial ATP synthesis coupled electron transport;GO:0006977 DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest;GO:1905446 regulation of mitochondrial ATP synthesis coupled electron transport
9	*FGF2*	Fibroblast growth factor 2	Pathways: oas05205:Proteoglycans in cancer;oas04015:Rap1 signaling pathway; GO:0014843 growth factor dependent regulation of skeletal muscle satellite cell proliferation;GO:2000546 positive regulation of endothelial cell chemotaxis to fibroblast growth factor;GO:0001658 branching involved in ureteric bud morphogenesis
10	*JUN*	Jun proto-oncogene, AP-1 transcription factor subunit	Pathways: oas05200:Pathways in cancer;oas04510:Focal adhesion; GO:0045657 positive regulation of monocyte differentiation;GO:0043922 negative regulation by host of viral transcription;GO:1990441 negative regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress

The relationship between the expression of DNAJB4 and the prognosis of BC patients

The survival analysis was performed on the collected follow-up data, and the correlation between the expression level of *DNAJB4* protein and the prognosis of BC patients was analyzed by Kaplan-Meier method. Comparison of DFS between the two groups demonstrated that the median follow-up was 64 months (4–68 months), and the total number of events were 25 cases (DFS: 25/80 =31.5%), of which 5 were in the *DNAJB4* high expression group (2 lung metastasis, 1 bone metastasis and 2 chest wall recurrence; DFS: 5/29 =17.24%). 20 cases progressed in *DNAJB4* low expression group (7 cases of lung metastasis, 3 cases of bone metastasis, 6 cases of liver metastasis, 4 cases of chest wall recurrence; DFS: 20/51 =39.22%). The median survival time of DFS in patients with high expression of *DNAJB4* was higher than that in patients with low expression of *DNAJB4* (66.45 *vs.* 54.27 months). Logrank test showed that there was significant difference in DFS between the

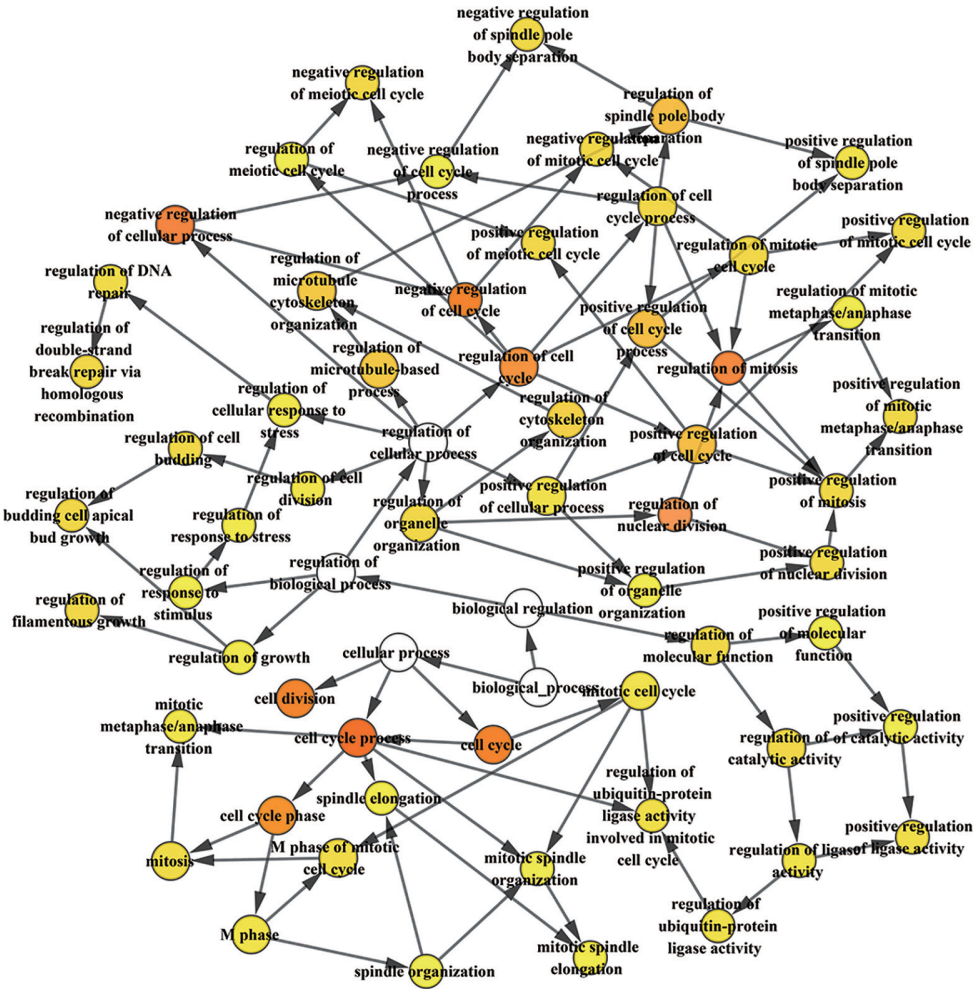
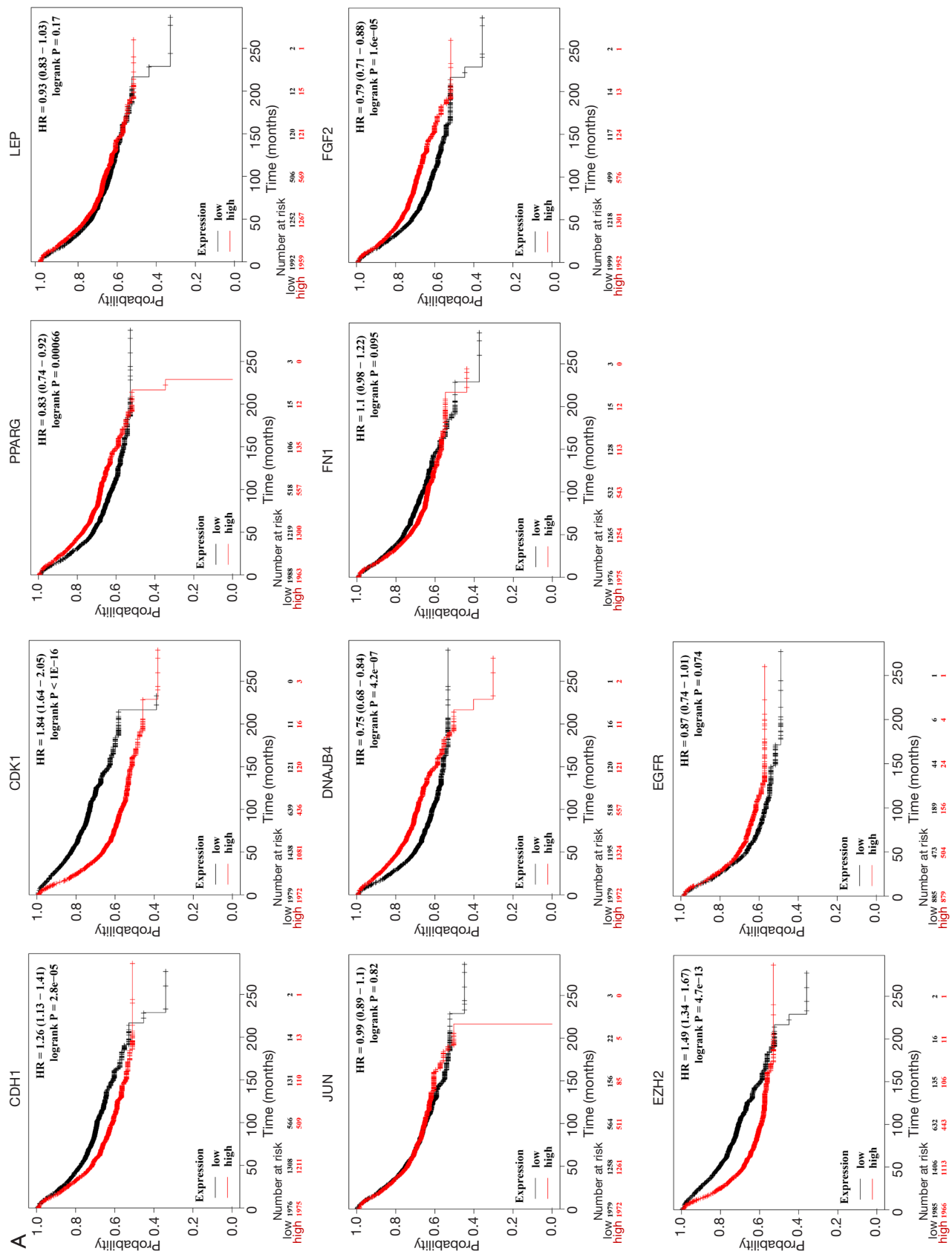


Figure 3 Interaction network and biological process analysis of the hub genes. The biological process analysis of hub genes was constructed using BiNGO. The color depth of nodes refers to the corrected P value of ontologies. The size of nodes refers to the numbers of genes that are involved in the ontologies. P<0.01 was considered statistically significant.

two groups (HR =2.944, 95% CI: 1.101–5.48, P=0.024) (*Figure 8*). BC patients with high expression of *DNAJB4* had a relatively good prognosis.

Discussion

BC is the leading cause of tumor-related death in women worldwide (2). In recent years, although some progress has been made in the prevention, diagnosis and treatment of BC with increasing researches (6,40,41). However, the pathogenesis of BC is not clear. Besides, the morbidity and mortality continue to rise and the age of patients' trend to become younger and younger (2,42). BC has the common characteristics of most solid tumors, that is, abnormal genetic and epigenetic changes in cancer occurrence and development (43-45). The concept of molecular subtypes of BC was put forward by Perou's team (46) in 2000, and it is pointed out that patients with different molecular types of BC have different clinical characteristics and prognosis. With the development of genomics, transcriptome, proteomics and bioinformatics, the conclusion of Perou's team has been confirmed by most studies. BC-related molecules have also been reported that the overexpression of *ERBB2* is high risk of postoperative recurrence and poor prognosis (47,48); and *BRCA1/2* gene mutation has a higher risk of BC (49,50). Therefore, the screening and identification of BC-related genes and signal pathways is of great significance for the clinical prevention and treatment



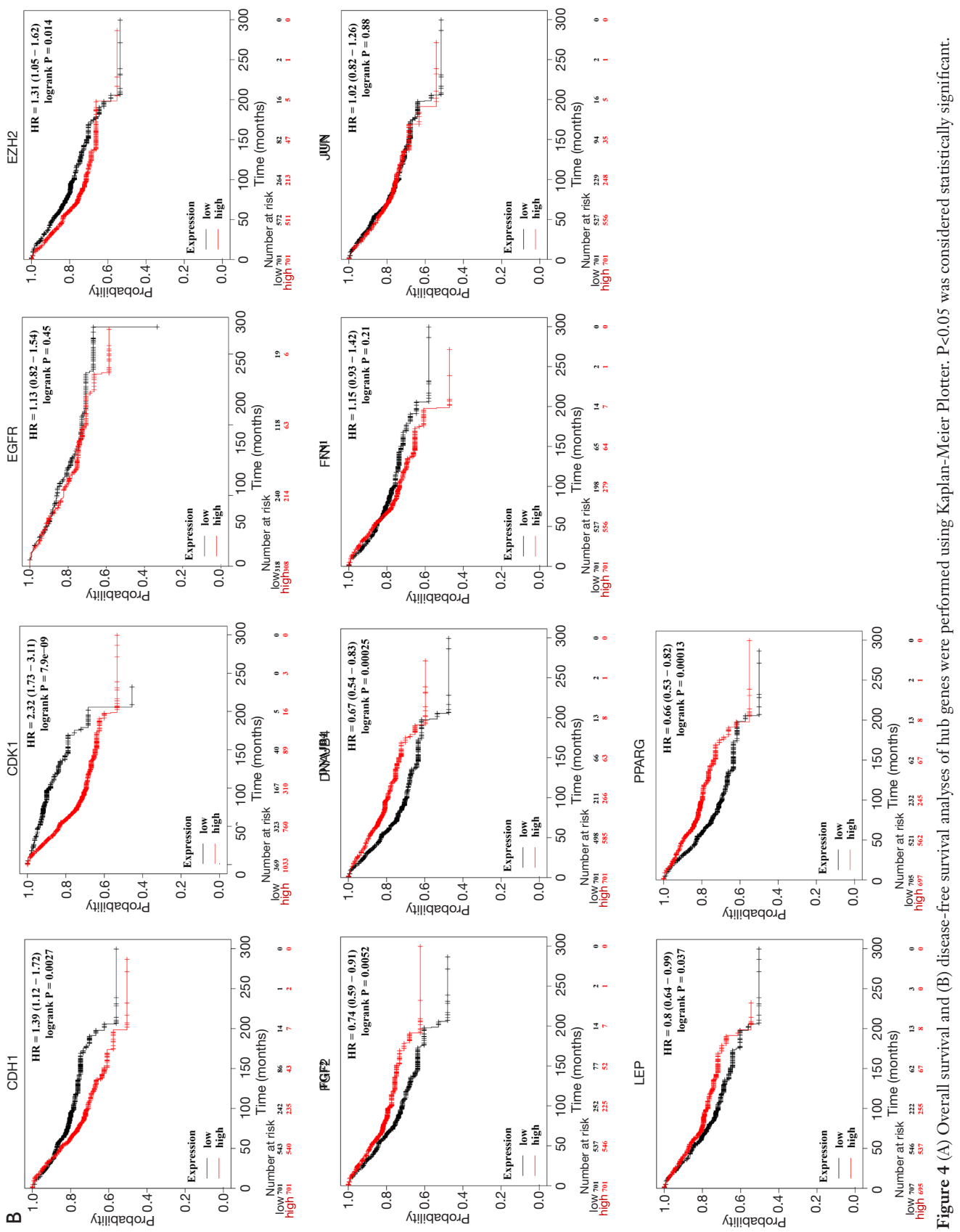


Figure 4 (A) Overall survival and (B) disease-free survival analyses of hub genes were performed using Kaplan-Meier Plotter. P<0.05 was considered statistically significant.

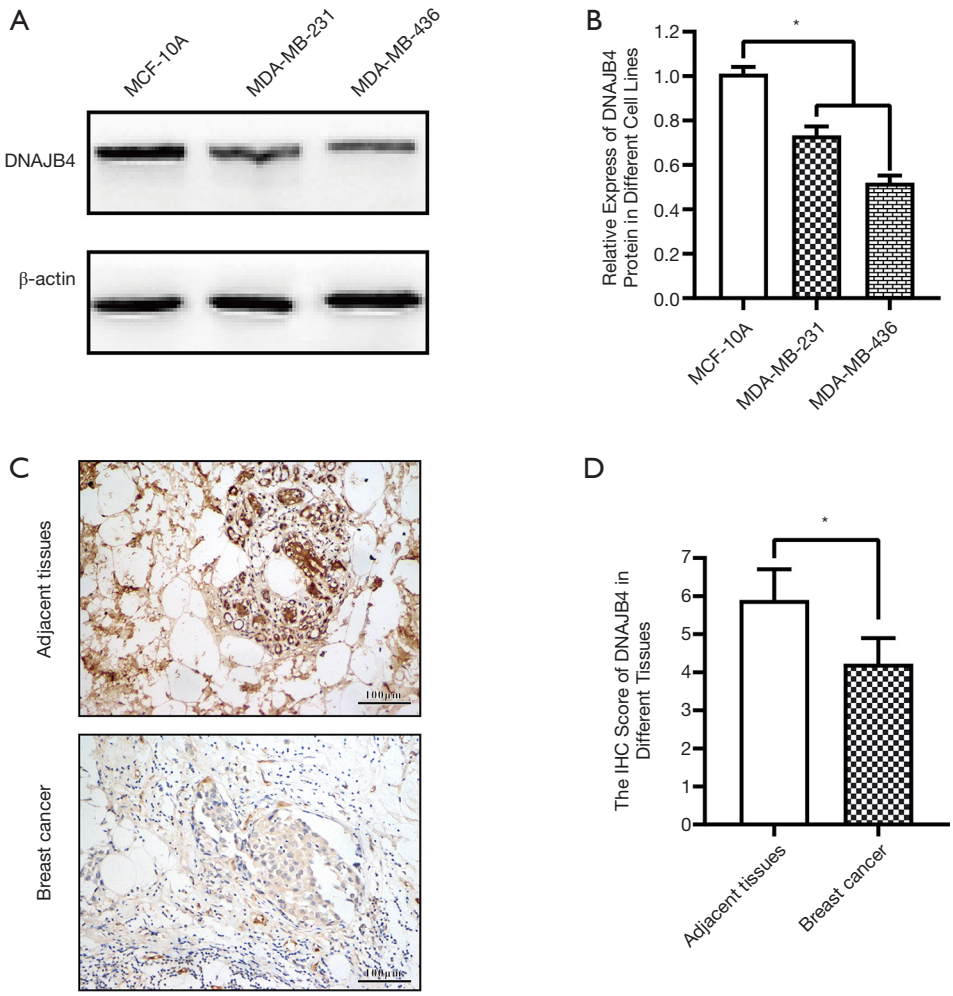


Figure 5 *DNAJB4* expression in breast cancer cell lines and breast cancer tissues. (A) Western Blot was used to detect the expression level of *DNAJB4* in MCF10A, MDA-MB-231, MDA-MB-436 cell lines. (B) Image J quantitatively analyzes the expression level of *DNAJB4* in cell lines, and uses LSD method to analyze the direct expression difference of MDA-MB-231, MDA-MB-436 cell line and MCF10A, *, P<0.05; Immunohistochemical staining showed the expression of *DNAJB4* in adjacent tissues and breast cancer tissues. (D) Immunohistochemical staining scores, and univariate analysis of variance was used to compare expression differences between the two groups, *, P<0.05.

of BC. Based on the results of high-throughput sequencing, bioinformatics technology, and its efficiency in the selection of tumor-related candidate genes has made it a popular technology in modern oncology research. However, due to the limitations of contemporary sequencing techniques, the results still need to be verified by biological experiments.

In this study, we identified 268 DEGs from two mRNA chip datasets (GSE15852 and GSE42568) in the GEO database, including 73 up-regulated genes and 195 down-regulated genes. The annotation results of DAVID database reveal that the DEGs of BC are enriched in the BP of negative regulation of cell proliferation, lipid metabolic process, cellular response to hormone stimulus, response to drug, positive regulation of protein kinase B signaling, positive regulation of transcription and DNA-templated, response to estrogen in the perspective of membrane raft, cell surface, lateral plasma membrane, bicellular tight junction, apical plasma membrane and membrane perspectives. Instead, the annotation results of KEGG pathway suggest that the DEGs were enriched in PPAR signaling pathway, AMPK signaling pathway, pathways in cancer etc. Existing studies have shown that PPAR signaling pathway, AMPK signaling pathway and others all play important roles in tumorigenesis and development (51,52),

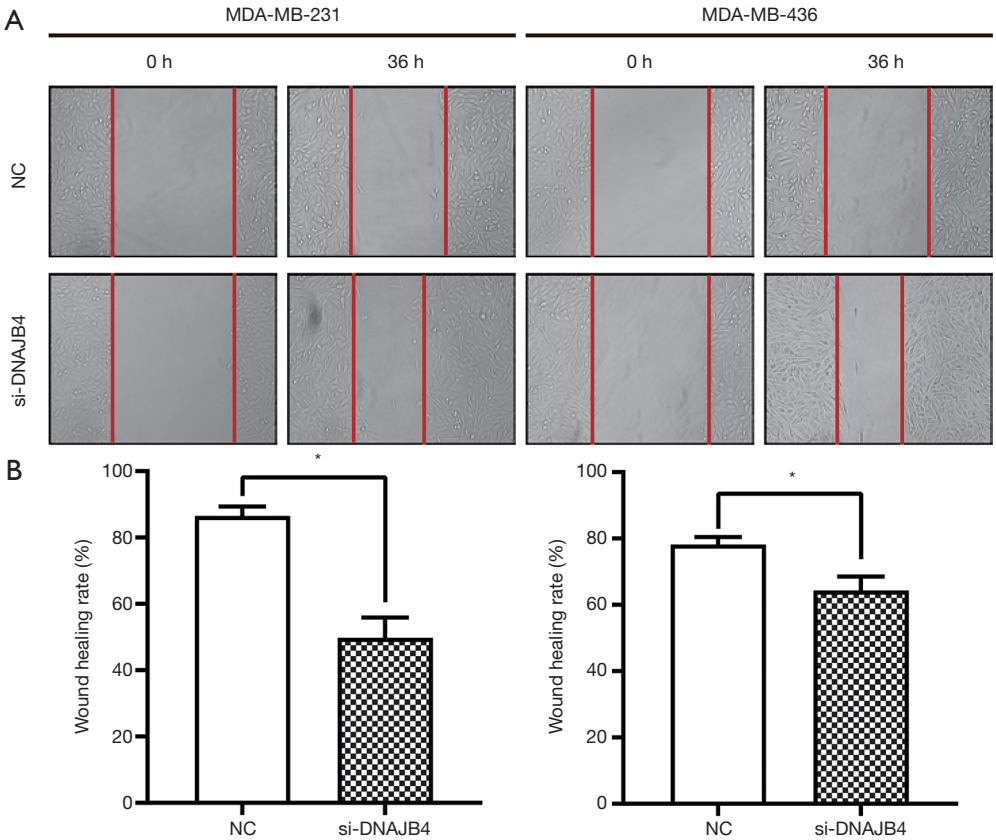


Figure 6 Knocking down *DNAJB4* enhances motility. (A) MDA-MB-231 and MDA-MB-436 cells were transfected with NC and siDNAJB4, respectively. Wound healing assays were performed 36h recovery period. (B) Quantification of motility from (A). *, P<0.05. NC, negative control.

and the results of this study are consistent with it. The screened DEGs were used to construct a PPI network in the STRING database. The construction results were visualized in Cytoscape software, and the most significant module of DEGs was found out through Cytoscape's MCODE plugin. The functional annotation of the most significant module showed that its related signaling pathways were mainly concentrated in PPAR signaling pathway, pathways in cancer, AMPK signaling pathway and MAPK signaling pathway. The CytoHubba plug-in was used to grab the genes ranked in the top 10 of the most significant modules as Hub genes, including *CDH1*, *PPARG*, *EZH2*, *DNAJB4*, *LEP*, *FN1*, *EGFR*, *CDK1*, *FGF2*, and *JUN*. HUB genes were analyzed for GO and KEGG enrichment through the Bingo plug-in in Cytoscape. The results suggest that the BP of HUB genes are mainly enriched in regulation of cell cycle process, regulation of nuclear division, cell cycle process, cell cycle, cell division. In order to clarify the correlation between the expression level of each Hub gene and the prognosis of patients, all HUB genes were placed in the Kaplan-Meier Plotter online tool for survival analysis. The analysis found that there is a significant difference in OS between BC patients with *CDH1*, *CDK1*, *PPARG*, *LEP*, *DNAJB4*, *FGF2* and *EZH2* changed and the unchanged; and there is also a significant difference in DFS between patients with and without changes in *CDH1*, *CDK1*, *PPARG*, *LEP*, *DNAJB4*, *FGF2*, and *EZH2*.

Among the 10 hub genes, the degree of *DNAJB4* was the highest, which was 29. In the survival analysis of Kaplan-Meier Plotter database, it was found that the differential expression of *DNAJB4* had a significant effect on both DFS and OS. These results suggest that *DNAJB4* may play an important role in the occurrence and development of BC. In order to determine the expression level and role of *DNAJB4* in BC, the expression level of *DNAJB4* was detected in 80 cases of BC and BC cell lines. The results

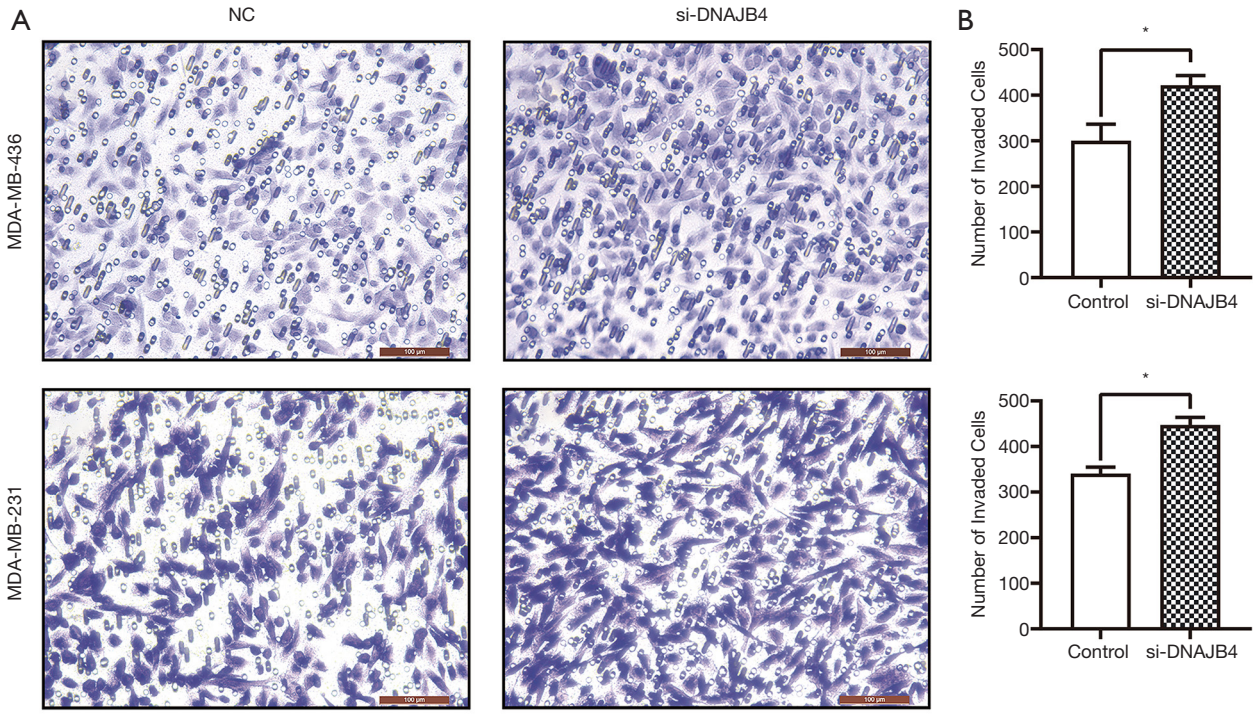


Figure 7 Knockdown of *DNAJB4* enhance breast cancer cell invasion. (A) MDA-MB-231 and MDA-MB-436 cells transfected with si-*DNAJB4* were more invasive as compared to control NC cells. Crystal Violet Staining Solution was used for cell staining in transwells experiment. (B) Quantification of invasion from (A). *, P<0.05. NC, negative control.

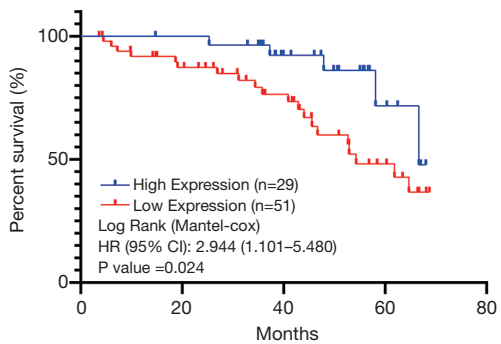


Figure 8 Disease-free survival curves according to *DNAJB4* expression level for all patients. Kaplan Meier curves of two groups, *DNAJB4* high-expression group (blue line), and *DNAJB4* low-expression group (red line) were generated and compared with the log rank test. High-expression group showed significantly better prognosis in all of the DFS. DFS, disease-free survival.

demonstrate that *DNAJB4* was low expressed in both BC and BC cell lines. Further functional experiments of BC cells showed that after down-regulating the expression of *DNAJB4* in MDA-MB-231 and MDA-MB-436 BC cell lines by gene interference technique, the invasion and migration ability of MDA-MB-231 and MDA-MB-436 BC cells was significantly enhanced. The DFS data of 80 cases of BC included in this study were tested by Logrank test. It was found that the risk of recurrence of BC patients with low expression of *DNAJB4* was 2.944 times higher than that of patients with high expression of BC, and the difference was statistically significant. It should be pointed out that in this study, there is no further study on the specific mechanism of how *DNAJB4* inhibits the invasion and metastasis of BC cells, and there is a lack of relevant *in vivo* experiments, and the relevant conclusions still need to be verified by future studies.

Conclusions

In summary, this study found that *DNAJB4* may be a tumor suppressor gene, which is low expressed in BC. Preliminary experimental results indicate that *DNAJB4* may be involved in the regulation of invasion and migration of BC cells, and its expression level is related to the prognosis of BC patients. Therefore, further study on the BP and molecular mechanism of *DNAJB4* could provide new ideas for the diagnosis and treatment of BC.

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Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at http://dx.doi.org/10.21037/gs-20-431

Data Sharing Statement: Available at http://dx.doi.org/10.21037/gs-20-431

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by institutional ethics board of the First Affiliated Hospital of Wannan Medical College (No. 2019070, the registration number of ethics board) and informed consents were taken from all the patients.

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Supplementary

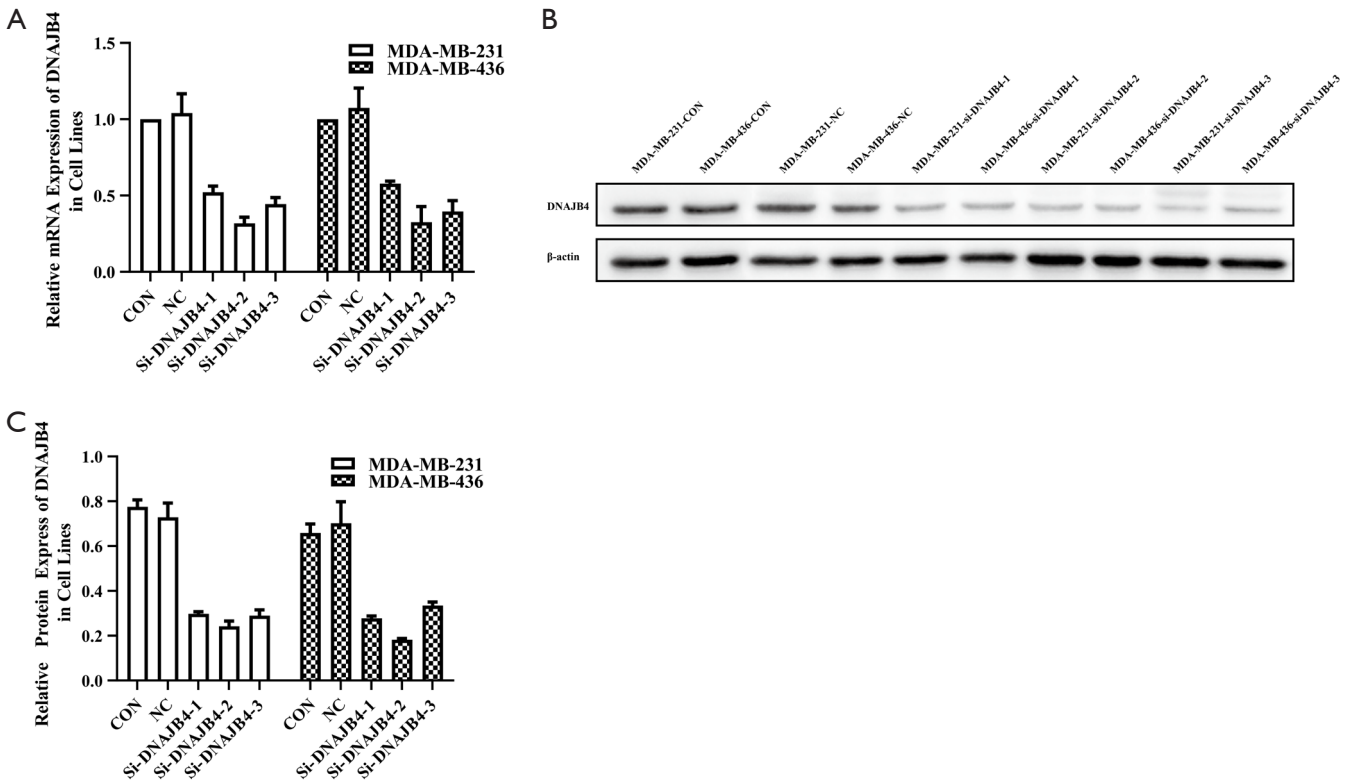

Figure S1 Establishment and screening of DNAJB4 knockdown stable transgenic cell lines in breast cancer cell lines. (A) qPCR is used to detect the interference efficiency of different siRNAs on DNAJB4 gene expression. (B) After different siRNA interferes with cells, western blot is used to detect the expression level of DNAJB4 protein in cell lines. (C) Image J quantitatively analyzes the Western blot results. CON, control; NC, negative control; qPCR, real-time polymerase chain reaction.

The siRNA sequence and shRNA sequence of the control viral vector are as follows:
The siRNA sequence: TTCTCCGAACGTGTCACGTAA
The shRNA sequence:

Top strand:
GATCCGTTCTCCGAACGTGTCACGTAATTCAAGAGATTACGTGACACGTTCGGAGAATTTTTTC

Bottom strand:
AATTGAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGAATTACGTGACACGTTCGGAGAACG

Target gene siRNA sequence and shRNA sequence:
The siRNA sequence 1: GGGAATTGAGAAAGGAGCTTCAGAT
The shRNA sequence 1:

Top strand:
GATCCGGGAATTGAGAAAGGAGCTTCAGATTTCAAGAGAATCTGAAGCTCCTTTCTCAATTCCCTTTTTTG

Bottom strand:
AATTCAAAAAAGGGAATTGAGAAAGGAGCTTCAGATTCTCTTGAAATCTGAAGCTCCTTTCTCAATTCCCG

The siRNA sequence 2: GAGGCATTGTGTGGCTGCTCAATTA
The shRNA sequence 2:

Top strand:
GATCCGAGGCATTGTGTGGCTGCTCAATTATTCAAGAGATAATTGAGCAGCCACACAATGCCTCTTTTTTG

Bottom strand:
AATTCAAAAAAGAGGCATTGTGTGGCTGCTCAATTATCTCTTGAATAATTGAGCAGCCACACAATGCCTCG

The siRNA sequence 3: TGAGGTGTCCTTCCCAGATACTATA
The shRNA sequence 3:

Top strand:
GATCCGTGAGGTGTCCTTCCCAGATACTATATTCAAGAGATATAGTATCTGGGAAGGACACCTCATTTTTTG

Bottom strand:
AATTCAAAAAATGAGGTGTCCTTCCCAGATACTATATCTCTTGAATATAGTATCTGGGAAGGACACCTCACG

Figure S2 Interference target design and primer synthesis.

http://dx.doi.org/10.21037/gs-20-431

Table S1 The primer sequence used in qPCR

Primers	Sequence (5' to 3')
DNAJB4	
Sense primer	5'-CCAGCAGACATTGATTTTTATCATT-3'
Antisense primer	5'-CCATCCAGTGTTGGTACATTAATT-3'
β-actin	
Sense primer	5'-AGCACTGTGTTGGCGTACAG-3'
Antisense primer	5'-TCCCTGGAGAAGAGCTACGA-3'

qPCR, real-time polymerase chain reaction.

Table S2 Basic information sheet for 80 breast cancer patients included in the study

Item	Mean ± SD/n
Age (years)	56.18±14.40
Gender	
Male	0
Female	80
BMI (kg/m^2)	20.82±2.31
Menopause	
No	26
Yes	54
Follow time (months)	40.70±2.06
Grand	
I	18
II	36
III	26
T	
1	23
2	39
3	18
N	
0	21
1	27
2	26
3	6
Recurrence	
No	55
Yes	25

BMI, body mass index; SD, standard deviation.

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