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Original Article

Identification of ECM and EMT relevant genes involved in the progression of bladder cancer through bioinformatics analysis

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Abstract: Background: Bladder cancer (BC) is very common among cancers of urinary system. It was usually categorized into two types: non-muscle invasive bladder cancer (NMIBC) and muscle invasive bladder cancer (MIBC). NMIBC and MIBC groupings are heterogeneous and have different characteristics. Objectives: The study was aimed to find some hub genes and related signal pathways which might be engaged in the progression of BC and to investigate the relationship with clinical stages and its prognostic significance. Methods: GSE37317 datasets were acquired from Gene Expression Omnibus (GEO) database. GEO2R on-line tool was selected to screen the differentially expressed genes (DEGs) of the two different types of BC. Then, Gene Ontology (GO) enrichment and KOBAS-Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of these DEGs were conducted. A protein-protein interaction (PPI) network was employed to help us screen hub genes and find significant modules. Finally, we made analysis of gene expression and survival curve by GEPIA and Kaplan-Meier plotter database. Results: 224 DEGs were screened in total, with 110 showing increased expression and 114 demonstrating decreased expression. GO and KEGG pathway enrichment analysis showed that DEGs were mostly involved in collagen fibril organization, extracellular matrix (ECM) structural constituent, bHLH transcription factor binding, AGE-RAGE signaling pathway and TGF-beta signaling pathway. Only 3 hub genes (DCN, JUN, THBS1) displayed significantly higher expression compared to those in the healthy controls. These hub genes were also strongly related to clinical stages as well as overall survival (OS) of BC patients. Conclusions: Taken together, most of hub genes involved in the progression of BC were related to ECM and EMT. In addition, 3 hub genes (DCN, JUN, THBS1) were strongly related with clinical stages and OS of BC patients. This study can enhance our comprehension of the progression of NMIBC and identify novel potential targets for MIBC.

Keywords: Muscle invasive bladder cancer, non-muscle invasive bladder cancer, differentially expressed genes, clinical stages, bioinformatics analysis

Introduction

Bladder cancer (BC), one of the most popular malignant tumors of the urinary tract, causes 3.1 deaths per 100,000 males and 0.8 deaths per 100,000 females annually [1]. There are usually two common types of BC, one is non-muscle invasive bladder cancer (NMIBC) and the other is muscle-invasive bladder cancer (MIBC) [2]. The standard treatment of NMIBC was transurethral resection of bladder tumor (TURBT), which usually could achieve good efficacy. However, one thorough surgery such as radical cystectomy is required to control the development of the tumor for MIBC patients, which can have a bad effect on BC patients [3]. In addition, MIBC also poses a serious threat to the clinical prognosis of patients [4]. Although TURBT and intravesical therapy were implemented for NMIBC patients, the risk that tumors progressed into MIBC was still high [5]. NMIBC and MIBC are both types of bladder cancer, but they have different pathological and molecular characteristics [6]. Therefore, identifying the probability of NMIBC progressing to MIBC is crucial, which will help doctors take more measures for these patients.

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To date, there were many theories about the molecular mechanism of the progression of NMIBC. Some molecules, such as FGFR3, PIK3CA, TERT, and p53, would change during the progression of NMIBC [7]. The immune response was also an key factor in the progression of NMIBC [8]. The extracellular matrix (ECM) is the extracellular microenvironment composed of proteins and polysaccharides secreted by cells. It was involved in the process of the progression and metastasis of BC [9]. However, the mechanism how ECM promotes the progression of BC remains unclear. Epithelial-mesenchymal transition (EMT) was closely linked to the progression of tumors and proved to have a certain effect on the growth of BC [10]. Although genetic changes are crucial during the progression of NMIBC, the interactions among these genes have not been well studied through comprehensive analysis. And there are limited studies on the relationship between genes expression and clinical stages, as well as the value of genes in predicting clinical prognosis.

This study aims to find candidate hub genes and related pathways which may be relevant to the progression of NMIBC through bioinformatics methods, thereby providing us new diagnostic and treatment strategies for BC patients.

Materials and methods

Microarray dataset

The GSE37317 dataset was acquired from GEO database. This dataset, which was based on the GPL96 platform, was comprised of 11 MIBC samples and 8 NMIBC samples [11].

DEGs analysis

GEO2R online tool was used to help us screen DEGs between the two different types of BC samples [12]. $P < 0.05$ and $|logFC| \geq 1.0$ were regarded as statistically significant. DEGs with logFC > 1 were considered to be upregulated genes, and those with logFC < -1 were considered to be downregulated genes.

GO functional and KEGG pathway enrichment analysis

With the help of the DAVID database (https://david.ncifcrf.gov/), we conducted GO enrichment and KEGG pathway enrichment analysis for the identified DEGs. Volcano plot was drawn by an online platform for data analysis and visualization [13].

PPI network establishment and module analysis

Search Tool for the Retrieval of Interacting Genes (STRING) database (http://string-db.org/) was used to construct the PPI network [14]. The combined score > 0.4 was selected as the cut-off. Cytoscape was applied to visualize the PPI network [15]. MCODE plugin was applied to establish the significant module. The parameters were: MCODE scores > 5, degree cut-off = 2, node score cut-off = 0.2, k-core = 2 and max. depth = 100 [16].

Hub genes selection

Through utilizing the CytoHubba application, hub genes were screened by their degree scores [17]. The top 10 genes with highest degree scores were judged the hub genes.

Expression and survival analysis

GEPIA was a convenient and useful analysis tool that had a variety of functions. It could help us analyze the gene expression along with its relationship with clinical stages of cancer [18]. Survival analysis was conducted by the Kaplan-Meier plotter database (http://kmplot.com/).

Statistical analysis

Gene expressions in BC tissues was analyzed by Student's t-test. Kaplan-Meier survival curves were constructed by Kaplan-Meier plotter database. The OS was analyzed by Kaplan-Meier method with log-rank test. $P < 0.05$ was deemed as statistically significant.

Results

Screening of DEGs

There were 224 DEGs between MIBC and NMIBC samples, with 110 showing increased expression and 114 demonstrating decreased expression (**Figure 1**).

GO terms analysis

GO terms analysis of these 224 DEGs was performed to investigate their functions. GO enrich-

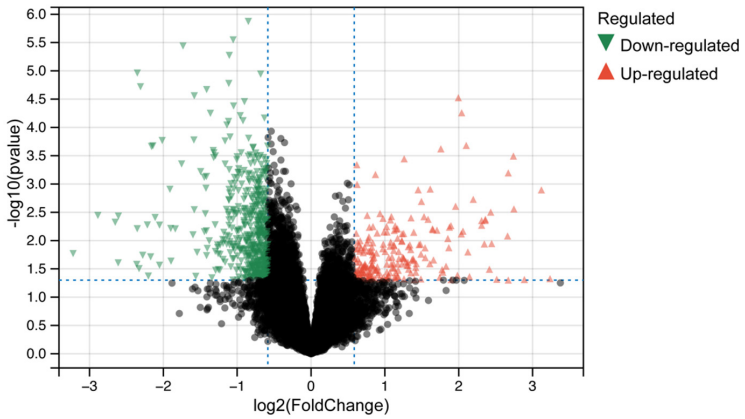

Figure 1. DEG analysis of the GSE37317 data set. DEGs were screened using GEO2R analysis. Green represents down-regulated genes, red represents up-regulated genes and black represents genes with unchanged expression. DEG, differentially expressed gene.

ment usually included three aspects, namely biological process (BP), cellular component (CC) and molecular function (MF). In terms of BP, up-regulated DEGs were mostly involved in collagen fibril, cell adhesion and ECM; while down-regulated DEGs were mostly enriched in circadian regulation, TGF-β pathway and positive regulation of endothelial cell chemotaxis. As far as CC was concerned, the identified up-regulated DEGs were engaged in extracellular space and region and endoplasmic reticulum lumen; while the down-regulated DEGs were mainly involved in basolateral plasma membrane, cytoplasm and extracellular exosome. In MF group, it was revealed that the up-regulated DEGs were mostly involved in ECM structural constituent, ECM structural constituent conferring tensile strength and protease binding; whereas the down-regulated DEGs were involved in bHLH, protein dimerization activity and RNA polymerase II sequence-specific DNA binding transcription factor binding (**Figure 2**).

KEGG pathway analysis

As can be seen from **Figure 3**, up-regulated DEGs were mainly enriched in ECM-receptor interaction, AGE-RAGE pathway, focal adhesion, proteoglycans in cancer and protein digestion and absorption, while the down-regulated DEGs were mainly associated with pathways regulating pluripotency of stem cells, TGF-β pathway, chemical carcinogenesis reactive oxygen species, fatty acid metabolism and alcoholic liver disease (Table S1).

PPI network establishment and hub genes analysis

When these identified DEGs were typed into STRING database, the PPI network was quickly constructed, which could be further visualized by Cytoscape (**Figure 4A**). MCODE was applied to establish the gene cluster with highest score, which contained 21 nodes and 171 edges (**Figure 4B**). The top 10 hub genes were FN1, COL1A1, JUN, THBS1, COL1A2, THBS2, SMAD3, COL3A1, POSTN and DCN (**Figure 4C**; **Table 1**).

Expression level and survival analysis

GEPIA analysis revealed that 3 hub genes (DCN, JUN, and THBS1) were significantly upregulated ($P < 0.05$) (**Figure 5**), while other 7 hub genes showed no significant differences (Figure S1). Then, the 3 chosen hub genes were further investigated by GEPIA to explore the relationship with the clinical stages. It was suggested that the 3 hub genes were strongly correlated with clinical stages of BC (**Figure 6**). The survival curve indicated that compared to higher expression group, lower expression group of DCN, JUN and THBS1 had a better prognosis ($P < 0.05$) (**Figure 7**). Apart from the 3 hub genes, FN1 and THSB2 were also found to have a noticeable impact on the OS, though they showed no obvious expression difference in cancer tissues (**Table 2**).

Discussion

Bladder cancer is very common among cancers of urinary system [19]. About 75% BC patients may have NMIBC at the time of initial diagnosis [20]. BC mostly occurs latently, lacks specific clinical symptoms in the early stage, and is prone to progress to MIBC, seriously affecting the prognosis of patients [21]. The main treatment for NMIBC is TURBT followed by intravesical therapy [22]. However, 30%-80% of tumors will recur within 5 years, and 1%-45% of tumors

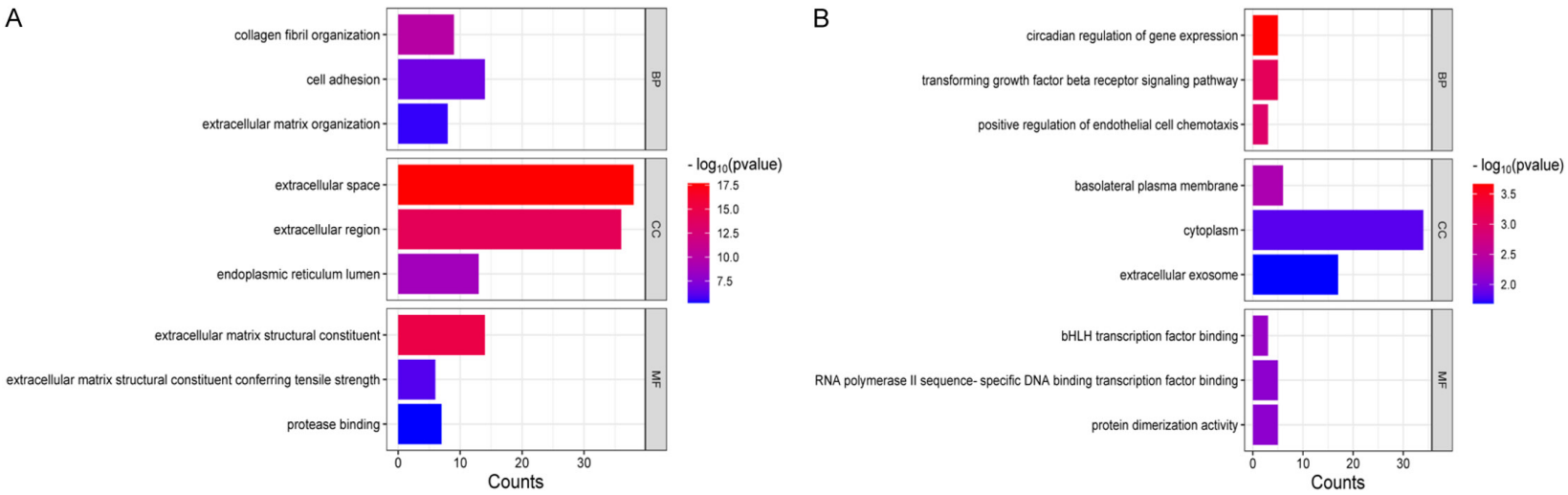

Figure 2. GO enrichment analysis of DEGs using the DAVID database. A. Up-regulated genes. B. Down-regulated genes.

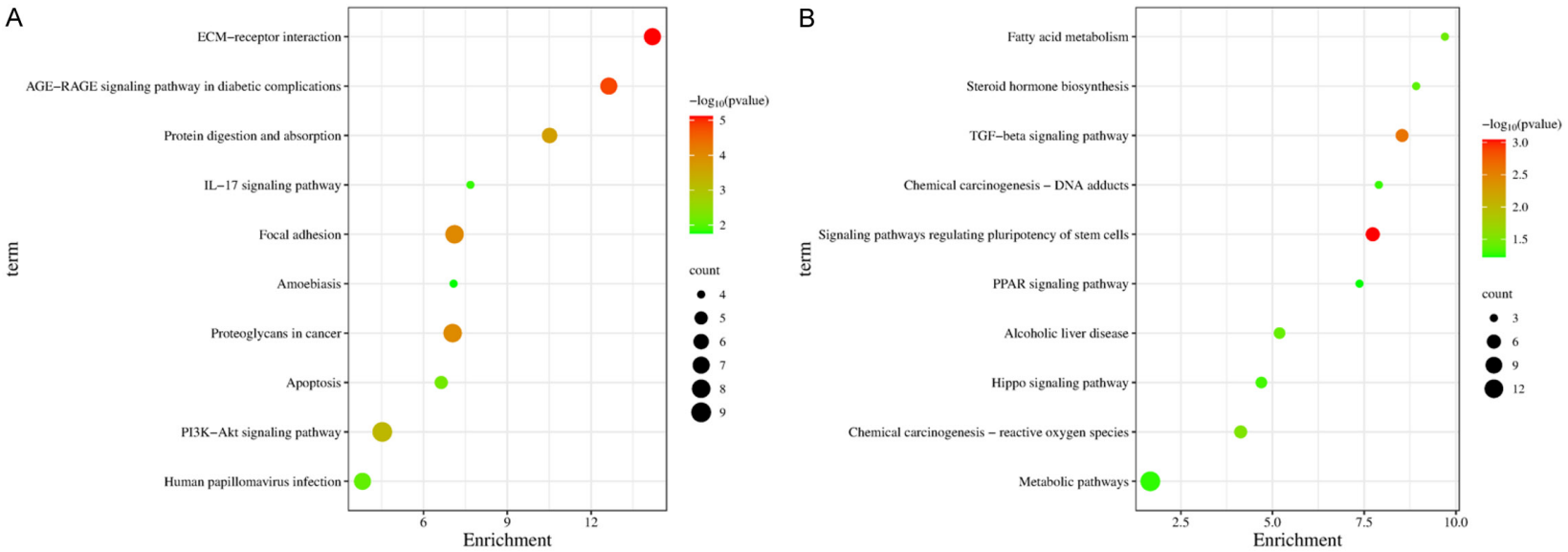

Figure 3. KEGG pathway analysis of DEGs using the DAVID database. A. Up-regulated genes. B. Down-regulated genes.

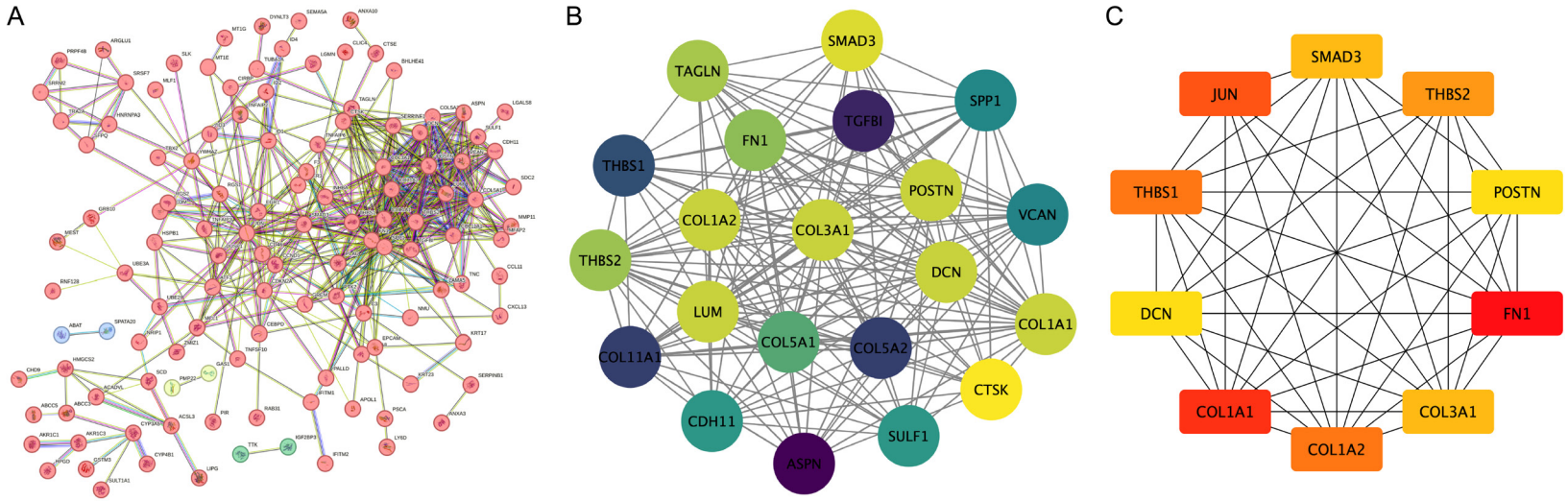

Figure 4. PPI network analysis and identification of hub genes. A. Protein-protein interactions of DEGs were analyzed using the STRING database. B. The most significant module in the PPI network was identified using MCODE plugin. C. The hub genes were screened using the CytoHubba plugin in Cytoscape software.

Table 1. Hub genes screened in the key module

Rank	Genes	Degree
1	FN1	42
2	COL1A1	35
3	JUN	34
4	THBS1	30
5	COL1A2	30
6	THBS2	28
7	SMAD3	25
8	COL3A1	25
9	POSTN	24
10	DCN	24

will progress into MIBC within 5 years [23]. Previous studies showed that many differences existed in gene expression profiles of NMIBC and MIBC [24]. Hence, it is vital for us to investigate the biological mechanisms of progression of NMIBC. We may find some predictive biomarkers to help us distinguish between progressive NMIBC and non-progressive NMIBC. Benefited from gene chip technology, a large amount of research data has been uploaded to public databases, which we can use for further study [25].

In this study, GSE37317 datasets of two types of BC samples were analyzed. 224 DEGs were screened, with 110 DEGs showing increased expression and 114 DEGs demonstrating decreased expression. From GO enrichment analysis, we could see that DEGs were mostly enriched in ECM, TGF-β pathway, bHLH transcription factor binding and so on. ECM, an important component of multicellular organisms was proved to be involved in the regulation of various tumor phenotypes [26]. However, the mechanism how ECM regulated the progression of tumors is still unclear. Collagen is an ingredient of ECM, and many studies proved that it was closely related to tumor metastasis [27-29]. In addition, ECM also determines the location of tumor metastasis [30]. More and more evidence suggested that ECM had a big influence on the progression of BC. The mechanism of ECM regulating the progression of BC involves multiple pathways [31, 32]. The stiffness of ECM can also promote EMT through biomechanical signals [33]. TGF-β falls into the transforming growth factor family [34]. On top of regulating the expression of oncogenes, TGF-β can also regulate the tumor suppressor genes simultaneously [35]. In the late stages of cancer, TGF-β could induce EMT, thereby promoting the progression of tumors [36]. In addition, TGF-β is also closely related to ECM and EMT. It can enhance the migration of tumor cells by regulating ECM remodeling and inducing EMT [37, 38]. bHLH factors can regulate the expression of key genes which are important for cell proliferation, apoptosis, and angiogenesis [39]. Several studies revealed that bHLH factors were also involved in the process of EMT [40]. bHLH factors could inhibit tumor progression by interrupting the PI3K/AKT and MAPK signal pathway [41, 42]. A recent study found that polyphyllin II might inhibit the progression of BC by regulating bHLH transcription factor [43].

To further investigate the possible molecular mechanisms and metabolism in the process of the progression of NMIBC, KEGG enrichment analysis was then performed. The results reflected that TGF-β pathway, ECM-receptor interaction and AGE-RAGE pathway were involved. After binding to AGE receptors (RAGEs), AGEs can trigger inflammation and immunosuppression [44]. The AGE-RAGE pathway was proved to be an important factor in tumor progression [45]. Some studies reported that AGE-RAGE pathway could enhance the advancement of prostate cancer by PI3K/AKT pathway [46]. The RAGE/TLR4 pathway could be activated by AGEs as well, leading to the increased expression of MMP9 which could degrade ECM, thereby promoting the progression of tumors [47].

Then, the PPI network was established to explore the interactions and functional connections of proteins. Through Cytoscape software, we could further visualize the network clearly and acquire the significant module and hub genes. It was found that most hub genes were related to collagen. As mentioned before, collagen was a component of ECM and had a strong relationship with the progression of cancers. Next, GEPIA was used to compare the status of gene expression between tumor and normal tissues. JUN, DCN, and THBS1 turned out to increase significantly in BC tissues. Prior studies showed that JUN was markedly elevated in tumor tissues [48]. Inhibition the expression of JUN could significantly prevent cancer

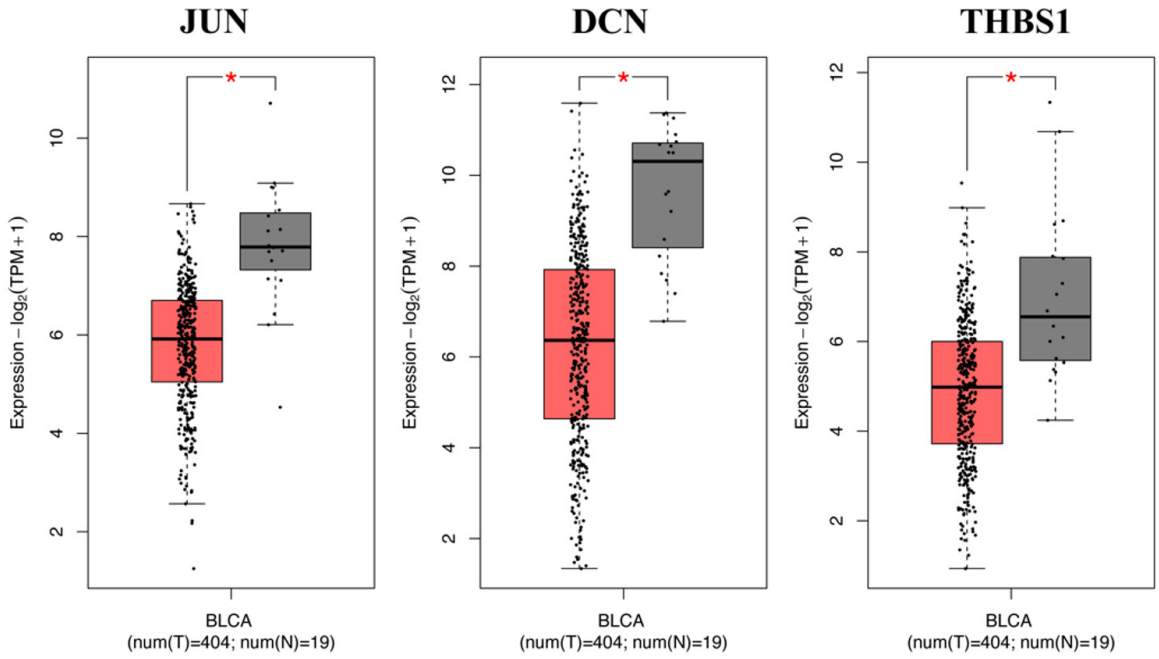

Figure 5. Validation of the 3 hub genes overexpression in BC tissues using the GEPIA database. *P < 0.05, unpaired Student's t-test.

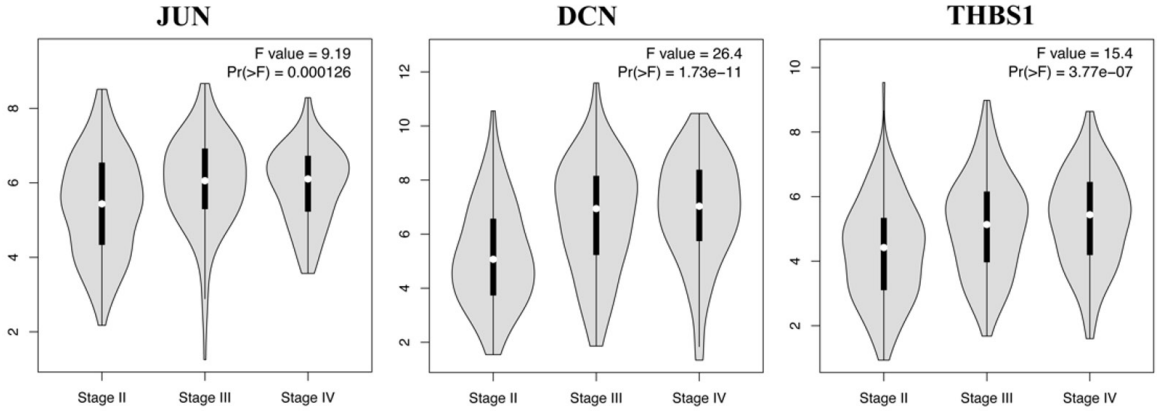

Figure 6. Validation of the 3 hub genes associated with clinical stages using the GEPIA database.

from progression [49]. DCN belongs to ECM, which is the ligand of various cytokines and crucial for the progression of tumors [50, 51]. THBS1 is also an ingredient of ECM and a series of biological processes could be modulated by this protein, such as cell adhesion, migration, and angiogenesis. THBS1 can accelerate the progression of different tumors through different mechanisms [52, 53]. Therefore, we selected these 3 hub genes for further study of their relationship with clinical stages. It was found that the expression intensity of these 3 hub genes was different at different clinical stages. Finally, the survival analysis suggested that these 3 hub genes also had a great impact on the OS of BC patients. Aside from the 3 hub genes, FN1 and THSB2 also had a vital impact on BC patients' prognosis.

In conclusion, we found potential hub genes and pathways that might engage in the progression of BC by bioinformatics analysis. It was worth noting that most genes were related to ECM and EMT. In addition, this study also found that the 3 genes (DCN, JUN, THBS1) were closely related to clinical stages and OS of BC

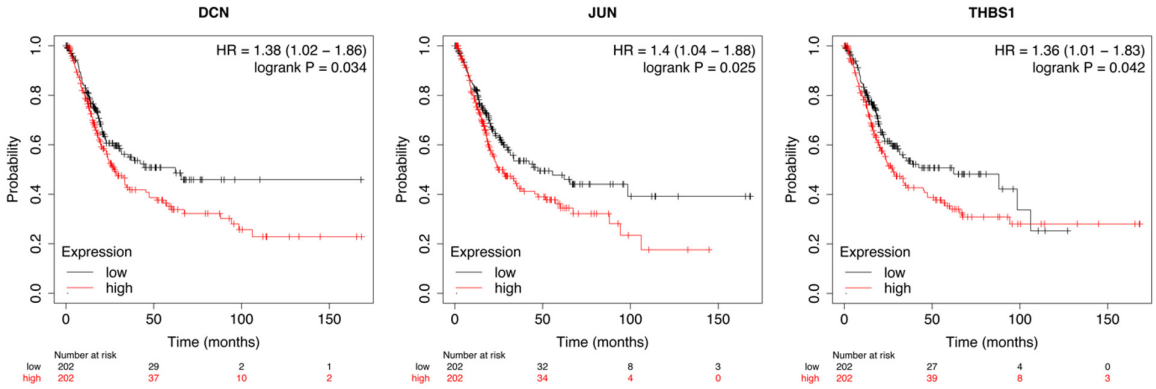

Figure 7. Kaplan Meier curves for overall survival analysis of the 3 hub genes in BC patients. Red line represented the high expression group, black line represented the low expression group.

Table 2. The relationship between expression levels of hub genes and the overall survival of BC patients

Gene name	*P*-value	HR
FN1	0.0045*	1.54
COL1A1	0.14	1.25
JUN	0.025*	1.4
COL1A2	0.086	1.3
THBS1	0.042*	1.36
THBS2	0.0098*	1.48
COL3A1	0.063	1.33
SMAD3	0.23	0.84
DCN	0.034*	1.38
POSTN	0.19	1.22

The relationship between expression levels of hub genes and the overall survival of BC patients (using the KMplot database). P < 0.05 was used as the threshold. *considered to have significant differences. HR, hazard ratio.

patients. In the future, further studies such as polymerase chain reaction and western blot, still need to be done to confirm the expression of these hub genes.

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Disclosure of conflict of interest

None.

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Table S1. The top 5 enriched KEGG pathways of up-regulated DEGs and down-regulated DEGs

Term	Enrichment	*p*-value	count
Up-regulated DEGs			
ECM-receptor interaction	14.19335206	7.60E-06	7
AGE-RAGE signaling pathway in diabetic complications	12.63208333	1.49E-05	7
Focal adhesion	7.111658456	9.94E-05	8
Proteoglycans in cancer	7.042276423	1.06E-04	8
Protein digestion and absorption	10.51213592	2.23E-04	6
Down-regulated DEGs			
Signaling pathways regulating pluripotency of stem cells	7.73277786	9.09E-04	6
TGF-beta signaling pathway	8.532308905	0.00249373	5
Chemical carcinogenesis - reactive oxygen species	4.13223929	0.03002197	5
Fatty acid metabolism	9.699888018	0.03660419	3
Alcoholic liver disease	5.191489362	0.03922968	4

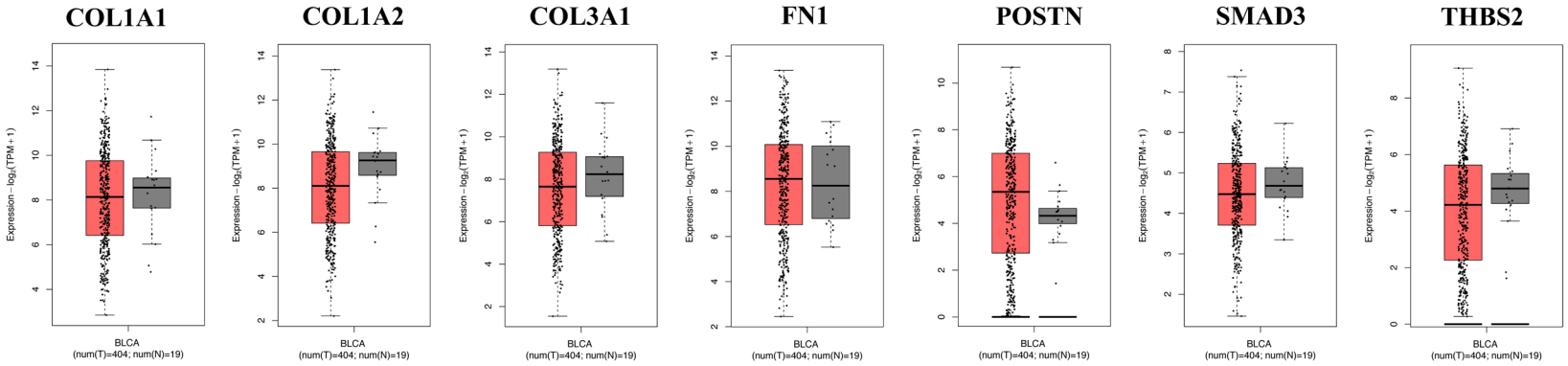

Figure S1. Validation of the 7 hub genes expression in BC tissues using the GEPIA database.