MYLK, CNN1, TAGLN and LMOD1 identified as potential prognostic biomarkers for bladder cancer using bioinformatics analysis

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Abstract

Background: Bladder cancer (BCa) is a challenge carcinoma that occurs on the bladder mucosa, which is the most common malignant neoplasm of the urinary system. Great efforts have been made to elucidate its pathogenesis. However, the molecular mechanisms involved in BCa remain unclear. Therefore, there is an urgent need to identify effective biomarkers to accurately predict the progression and prognosis of BCa.

Material and methods: To investigate potential prognostic biomarkers of BCa, we download the GSE23732 expression profile from Gene Expression Omnibus (GEO) database. The GEO2R analysis tool was performed to identify the DEGs between BCa and normal bladder mucosae tissue. Gene Ontology (GO) functional annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed for the screened DEGs by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) online tool. We employed the Search Tool for the Retrieval of Interacting Genes (STRING) database to construct the protein-protein interaction (PPI) network of DEGs. Subsequently, the PPI network's information was visualized by Cytoscape software. The Gene Expression Profiling Interactive Analysis (GEPIA) resource was used to describe the OS and DFS outcomes in bladder cancer patients based on the hub genes expression levels.

Results: A total of 396 DEGs comprising 344 upregulated genes and 52 downregulated genes were screened. The results of the GO analysis showed that DEG was mainly enriched in proteinaceous extracellular matrix, extracellular matrix, heparin binding and extracellular matrix organization. In addition, KEGG pathway analysis showed that DEGs were mainly enriched in PI3K-Akt signaling pathway, Focal adhesion, MAPK signaling pathway. A PPI network was constructed using the 396 DEGs, 10 hub genes were selected and 4 of them including MYLK, CNN1, TAGLN and LMOD1 were associated with overall survival and disease-free survival.

Conclusion: MYLK, CNN1, TAGLN and LMOD1 may represent promising prognostic biomarkers and potential therapeutic option for BCa.

Introduction

Bladder cancer (BCa) is a tumor that occurs on the bladder mucosa, which is the most common malignant neoplasm of the urinary system [1]. It is the fifth most common type of cancer in the world, with an estimated 81,400 new cases of bladder cancer each year and more than 17,000 deaths in the USA in 2020 [2]. Urothelial cancer is the most common type, accounting for about 90% of all bladder cancers [3]. Depending on the degree of muscle invasion, BCa can be divided into non-muscle-invasive bladder cancer (NMIBC), characterized by a high recurrence rate, and muscle invasive bladder cancer (MIBC), which has a <50% of 5-year overall survival according to biological characteristics [4]. Around 75% of patients suffered from NMIBC at the time of first diagnosis, although they have received various treatments, about 10% to 30% of patients will become MIBC [5]. Therefore, in order to understand the

molecular mechanisms involved in the carcinogenesis, proliferation and recurrence of BCa, we urgently need an effective diagnostic molecular marker that can accurately predict the progress and prognosis of BCa.

Currently, with the rapid development of high-throughput technologies and gene chips, many research institutions have identified key genes related to the occurrence and development of tumors through bioinformatic analysis. Gene Expression Omnibus (GEO) database played a significant role in bioinformatic analysis, it can provide sufficient data and reliable evidence for us to discover potential biomarkers of the tumor. Screening differentially expressed genes (DEGs) through bioinformatics analysis is of great significance to the diagnosis, treatment and prognosis of diseases.

For this purpose, we downloaded the GSE23732 dataset from GEO and screened DEGs using the GEO2R online tool. The volcano plot of DEGs was generated using the R software package ggplot2. Gene Ontology (GO) functional annotation analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed for the screened DEGs. Then, we established a protein-protein interaction (PPI) network to identify hub genes related to BCa. In addition, an online website called Gene Expression Analysis Interaction Analysis (GEPIA) was used to perform survival analysis of these pivotal genes. Expression levels of these genes and Pearson correlation analysis were used to visualize potential relationships between genes as well as to provide new insights about potential therapeutic targets for BCa patients.

Materials And Methods

Microarray Data

The original gene expression profiles were downloaded from the National Center of Biotechnology Information (NCBI) GEO database (http://www.ncbi.nlm.nih.gov/geo/). A total of 1,126 series about human bladder cancer were retrieved from the GEO database. After careful review, the GSE23732 expression profile was selected. The GSE23732, based on GPL6244 Affymetrix Human Gene 1.0 ST Array, was submitted by Singh PK et al. Totally seven bladder cancer tissues and one normal bladder mucosa tissue was enrolled in GSE23732.

Data Processing and DEGs Screening

The GEO2R online analysis tool (https://www.ncbi.nlm.nih.gov/geo/geo2r/) was performed to determine the DEGs between the primary BCa and normal bladder mucosae samples, adjusted P-value<0.05 and $|\log2FC| \geq 2.0$ were considered as the threshold to identify DEGs. The volcano plot was generated using the R software package ggplot2.

Enrichment Analysis of GO and KEGG Pathway

GO analysis is a common method to study gene function enrichment. Gene functions are classified into three types: biological process (BP), molecular function (MF), and cellular component (CC) [6]. KEGG is a

database that integrates a large amount of information about genomes, diseases, biological pathways, and system functions [7]. In this study, GO annotation analysis and KEGG pathway enrichment were performed by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tools(https://david.ncifcrf.gov/) [8]. P<0.01 and gene count≥10 were considered statistically significant.

PPI network construction and hub genes Selection

The Search Tool for the Retrieval of Interacting Genes (STRING) website is an online database that was used to evaluate the interactions between different proteins(http://string-db.org/) [9]. We constructed a protein-protein interaction (PPI) network of DEGs using the STRING database. The interaction with a confidence score of≥0.4 was considered significant and retained. Subsequently, the PPI network's information was visualized by Cytoscape software (www.cytoscape.org/) [10]. CytoHubba which is a Cytoscape plugin was used to calculate the degree of each protein node. In our study, the top ten genes were then selected as the hub genes using the MCC method in cytoHubba.

Survival analysis, expression levels, and correlations of hub genes

The online Gene Expression Profiling Interactive Analysis (GEPIA) resource (http://gepia.cancer-pku.cn/) was used to characterize the overall survival (OS) and disease-free survival (DFS) outcomes based on the hub genes expression levels [11]. Based on Pearson correlation analysis, genes associated with OS and DFS were identified as potential prognostic molecular markers. Expression levels of these genes and Pearson correlation analysis were used to visualize potential relationships between genes as well as to provide new insights about potential therapeutic targets for BCa patients.

Results

Identification of DEGs

We processed the GSE23732 dataset utilizing GEO2R and the lggplot2 R package, adjusted P-value<0.05 and |log2FC|≥2.0 were considered to be the threshold for identifying DEGs, a total of 396 DEGs containing 344 upregulated genes and 52 downregulated genes were screened. A volcano plot of the DEGs was shown in Fig. 1.

Enrichment Analysis of GO and KEGG Pathway

GO annotation analysis and KEGG pathway enrichment were performed by DAVID online analysis tools, which were shown in Table 1 and Table 2, respectively. Gene functions can be divided into three types: BP, MF, and CC ontologies. the GO analysis results showed that DEGs were mainly enriched in CC, including Proteinaceous extracellular matrix, Extracellular matrix, Extracellular space, Z disc, Extracellular exosome, Extracellular region, Sarcolemma, Plasma membrane, Cell surface, Focal adhesion, and Membrane raft. MF analysis showed that the DEGs were significantly enriched in Heparin binding, Actin binding, Actin filament binding, and Calcium ion binding. For BP, the DEGs were enriched in Extracellular matrix organization, Cell adhesion, Positive regulation of peptidyl-tyrosine phosphorylation, and Muscle

contraction. These results suggest that most DEGs are enriched in intercellular interactions and extracellular regions. which are involved in the proliferation and migration. In addition, KEGG pathway analysis showed that DEGs were mainly enriched in PI3K-Akt signaling pathway, Focal adhesion, MAPK signaling pathway, Hypertrophic cardiomyopathy, Cell adhesion molecules, ECM-receptor interaction, Vascular smooth muscle contraction, and cGMP-PKG signaling pathway. The results indicate that DEGs have common pathways in metabolic and adhesion molecule signaling pathways.

PPI network construction and hub genes Selection

We employed the STRING database to construct the PPI network of DEGs. A total of 371 nodes and 1,098 edges were involved in the PPI network, as presented in Fig. 2. Subsequently, the top 10 hub genes were identified among the 396 DEGs using MCC method in cytoHubba, which were Myosin heavy chain 11 (MYH11), Myosin light chain 9 (MYL9), Myosin light chain kinase family member 4 (MYLK), Transgelin (TAGLN), Sodium channel epithelial 1 gamma subunit (CNN1), Leiomodin 1 (LMOD1), Smoothelin (SMTN), Tropomyosin 2 (TPM2), Actin 2 (ACTG2), and Actin 1 (ACTC1), the network of these hub genes were shown in Fig. 3.

Survival analysis, expression levels, and correlations of hub genes

The GEPIA database was used to perform the prognosis of BCa and association analysis of mRNA expression. Among the 10 hub genes, only four genes were found to be associated with both OS and DFS: MYLK, CNN1, TAGLN and LMOD1 (all $P<0.05$), as presented in Figure 4. The expression levels of these four genes were shown in Fig. 4, where all of them were low in tumor tissues but high in normal tissues, and they were significantly different between the normal and BCa tissues. Moreover, their low levels of expression were associated with a better prognosis. The Pearson correlation coefficients between the gene expression levels are also shown in Fig. 5 (all $R>0.9$).

Discussion

BCa is one of the most prevalent malignancies of the genitourinary system, which has been identified as the fourth and tenth leading cause of cancer-related deaths in males and females, respectively [12]. The pathogenesis of BCa is complex and involves several factors at multiple steps, including intrinsic genetic factors and extrinsic environmental factors, as with other cancers. Such as smoking, chemical pollution, genetic mutation, and single nucleotide polymorphism, etc [13-15]. Currently, with the rapid development of molecular biology techniques, the mechanism of BCa research and medical treatment have been greatly improved. However, the critical pathogenesis in BCa is incompletely understood, the long-term prognosis of BCa remains poor, patients with BCa usually have no specific symptoms in the early stage, most of them were already at an advanced stage when they are detected [16]. Clinicians are largely faced with advanced and metastatic disease for which few interventions are available. Thus, in order to reduce the risk of death in BCa, highly specific and sensitive biomarkers are urgently needed as they can help in gaining knowledge on the pathogenesis of the disease and determining individualized treatment.

Bioinformatics is a multidisciplinary research area, which is specifically used to identify candidate genes to help understand the genetic basis of diseases and provide new insights for the study of molecular mechanisms. In the present study, we extracted microarray data from the GSE23732 dataset to identify DEGs between cancerous and normal specimens, a total of 396 DEGs comprising 344 upregulated genes and 52 downregulated genes were screened using bioinformatics analysis. KEGG and GO enrichment analyses were used to gain a deeper understanding of potential biological functions and pathways associated with bladder tumorigenesis. GO enrichment results showed that DEGs were mainly enriched in proteinaceous extracellular matrix, extracellular matrix, and extracellular space. These results suggest that most DEGs are enriched in extracellular regions, which are involved in the proliferation and migration. Additionally, for KEGG pathway analysis, the pathways associated with DEG were primarily related to the PI3K-Akt pathway, focal adhesion, and MAPK signaling pathway. The PI3K-Akt signaling pathway plays a crucial role in malignant tumorigenesis and progression. Overactivation of the PI3K/Akt signaling pathway promotes malignant transformation of cells by regulating cell proliferation, apoptosis, migration, immune evasion and drug resistance [17-20]. whereas inhibition of the PI3K/Akt signaling pathway can inhibit the growth cycle of bladder cancer cells [21]. Focal adherence is a membrane related macromolecule assembly that links the actin cytoskeleton and integrin to the extracellular matrix. Previous studies indicated that it plays an important role in the regulation of cell proliferation, migration, and invasion, and is closely associated with the development of various malignant tumors [22-24]. MAPK is an important signaling pathway that mediates extracellular signals to intracellular. Four major MAPK pathways have been identified in mammals: namely p38, c-jun, ERK and ERK5 signaling pathway [25]. The activation of MAPK signaling pathway affects not only tumorigenesis and progression, but also metastasis, invasion and drug resistance [26, 27].

In addition, a PPI network was constructed to investigate the interrelationship of the DEGs using MCC method in cytoHubba, and the ten hub genes were identified, which were MYH11, MYL9, MYLK, TAGLN, CNN1, LMOD1, SMTN, TPM2, ACTG2, and ACTC1. However, only MYLK, CNN1, TAGLN and LMOD1 had relationships with both OS and DFS, so they were subjected to further analysis, where they all had low expression levels in tumor tissues but high expression levels in normal tissues. In addition, their low expression levels were associated with a better prognosis. From these results, we assume that MYLK, CNN1, TAGLN and LMOD1 may function as oncogenes.

MYLK, also referenced as MLCK, is a calmodulin-dependent threonine/serine kinase found on chromosome 3 (3q21.1) which is part of the immunoglobulin gene superfamily and is widely distributed in various eukaryotic and non-muscle cells [28]. MYLK consists of four regions, including the N-terminal actin-binding region, the central kinase region, the calmodulin-binding region and the C-terminal myosin-binding region. The function of MYLK is to enhance the activity of myosin, which in turn promotes the contraction of myosin and the adhesion of stress fibers [29]. MYLK regulates myosin activity through phosphorylation and dephosphorylation of myosin light chains and plays an important role in many biological processes such as proliferation, differentiation and metastasis. Previous studies have shown that overexpression of MYLK is a negative prognostic factor for carcinogenesis and prognosis in different neoplasms, including hepatocellular, gastric, prostate and breast carcinomas [30-32]. Current findings

demonstrated that MYLK expressed itself differently in cancerous and normal tissues and was identified as a pivot gene in the PPI network. Patients with high MYLK expression had shorter OS and DFS, these results suggest that MYLK may be a potential oncogene.

CNN1, also known as calmodulin 1, is one of three calmodulin isoforms. The gene encoding this protein is located on human chromosome 19 (19q13.2), and is a marker for differentiation of cardiac and smooth muscle [33]. CNN1 plays an important role in the development of blood vessels through the stabilization of actin and inhibition of cellular motility. For example, it has been pointed out that downregulation of CNN1 may inhibit the development of ovarian cancer [34]. However, our results showed that higher expression of CNN1 correlated with poorer prognosis of BCa. We hypothesized that CNN1 could function as a tumor suppressor gene in the human body, however, with the development and progression of BCa, the hub gene can be captured by tumor cells and transformed into harmful genes, so tumor cell protection becomes a major role of CNN1. From another perspective, it is known that the stromal microenvironment in tumor tissue is different from the stroma of the corresponding normal tissue in many human cancers. Given that studies have focused on CNN1 in BCa, the underlying biological function of CNN1 requires further investigation.

TAGLN, also called SM22, is a part of the calponin family of actin-binding proteins. TAGLN demonstrates great potential to alter motility via its interactions with actin. As an actin-binding protein, aberrant expression of TAGLN has been shown to be associated with other cancers, including, pulmonary adenocarcinoma, and pancreatic cancer [35-37]. Chen Z et al. demonstrated that TAGLN upregulation can promote the migration and invasion of BCa cells via invadopodia formation and the induction of epithelial-mesenchymal transition [38]. This finding is consistent with our research, and we suggest that TAGLN may serve as a potential prognostic biomarker for BCa.

The last hub gene is LMOD1, also known as Leiomodin-1, could be activated by serum response factor (SRF) or myocardin (MYOCD) and functions in smooth muscle cell differentiation [39]. Previous studies reported that aberrant upregulation of LMOD1 as a poor prognostic marker in a variety of tumors, including colorectal cancer, Leiomyosarcoma and prostate cancer [40-42]. The relationship between LMOD1 and the pathogenesis of BCa remain unclear. However, considering the significant role of LMOD1 in tumors and combined with the results of our analysis, we suggest that LODM1 may be a potential biomarker in BCa.

Taken together, the purpose of this study was to detect DEGs involved in tumorigenesis BCa through bioinformatics analysis. A total of 396 DEGs were identified. Subsequently, the key nodes identified in the PPI network constructed with these DEGs and the genes involved in the significant module, including MYLK, CNN1, TAGLN, and LMOD1, may play a major role in the development of BCa, and can function as potential biomarkers for BCa. However, our research contained certain shortcomings. First of all, the data were obtained from public databases, and the quality of the data was not evaluated. Secondly, our research has limited itself to selecting candidate biomarkers associated with pathogenesis and prognosis, which may lead to the neglect of certain information. Last but not least, the results were fully

based on the use of publicly available databases, so biological experiments were necessary to validate our findings, such as qPCR, western blot, and subcutaneous tumor model of nude mice, which would be carried out in our future studies.

Conclusion

Our bioinformatics analysis screened 396 DEGs between BCa and normal bladder tissues based on the gene expression profiles obtained from public database. Among them, four hub genes including MYLK, CNN1, TAGLN and LMOD1 may be the potential biomarkers for diagnosis and therapeutic targets in BCa. This finding can provide a deeper and more comprehensive understanding of the occurrence and development of BCa.

Declarations

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Availability of data and materials

The datasets used in the current study are downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23732).

Authors' contributions

ZYT and XHQ participated in the design of this study, and they both performed the statistical analysis and drafted the manuscript. SF, ZXD, and YGZ carried out the study and collected important background information and data. XRZ, HFW, and JSW helped to revise the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Abbreviations

BCa, bladder cancer; BP, biological process; CC, cellular component; MF, molecular function; DEG, differentially expressed gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate.

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Tables

Table 1 Significantly enriched GO terms of DEGs in BCa

Category	Term	Description	Count	PValue	FDR
CC term	GO:0005578	Proteinaceous extracellular matrix	34	3.24E-17	9.81E-15
CC term	GO:0031012	Extracellular matrix	33	5.01E-15	7.60E-13
MF term	GO:0008201	Heparin binding	21	3.25E-11	1.58E-08
BP term	GO:0030198	Extracellular matrix organization	23	8.06E-11	1.67E-07
CC term	GO:0005615	Extracellular space	63	2.63E-10	2.66E-08
BP term	GO:0007155	Cell adhesion	33	1.20E-09	1.24E-06
CC term	GO:0030018	Z disc	15	8.44E-08	6.39E-06
CC term	GO:0070062	Extracellular exosome	94	1.58E-07	9.60E-06
CC term	GO:0005576	Extracellular region	63	2.20E-07	1.11E-05
CC term	GO:0042383	Sarcolemma	12	8.63E-07	3.74E-05
CC term	GO:0005886	Plasma membrane	121	1.45E-06	5.51E-05
MF term	GO:0003779	Actin binding	19	7.51E-06	0.001820234
CC term	GO:0009986	Cell surface	28	1.05E-05	3.34E-04
CC term	GO:0005925	Focal adhesion	23	1.10E-05	3.34E-04
BP term	GO:0050731	Positive regulation of peptidyl-tyrosine phosphorylation	10	4.11E-05	0.021226834
CC term	GO:0045121	Membrane raft	15	6.16E-05	0.001695569
BP term	GO:0006936	Muscle contraction	11	6.26E-05	0.025873385
MF term	GO:0051015	Actin filament binding	11	2.33E-04	0.022615488
MF term	GO:0005509	Calcium ion binding	29	3.07E-04	0.02484058

Table 2 Significantly enriched KEGG pathway of DEGs in BCa

Category	Term	Description	Count	PValue	Genes
KEGG pathway	hsa04151	PI3K-Akt signaling pathway	19	4.76E-04	FLT1, CSF1, ITGB3, IGF1, THBS2, GHR, FGF7, IL6, TCL1A, RELN, COL4A2, COL4A1, COL6A2, COL6A1, ITGA7, COL6A6, ITGA5, FGF10, FGFR1
KEGG pathway	hsa04510	Focal adhesion	18	2.08E-06	FLT1, PRKCB, ITGB3, CAV1, IGF1, THBS2, MYLK, RELN, COL4A2, COL4A1, COL6A2, COL6A1, ITGA7, COL6A6, FYN, FLNC, ITGA5, MYL9
KEGG pathway	hsa05414	MAPK signaling pathway	14	2.47E-08	CACNB2, RPS6KA6, FGF7, GADD45B, TGFB3, PRKCB, CACNA2D1, CACNA1C, HSPA2, FLNC, FGFR1, FGF10
KEGG pathway	hsa05410	Hypertrophic cardiomyopathy (HCM)	13	9.37E-08	TGFB3, TPM2, ITGB3, CACNA2D1, CACNA1C, IGF1, CACNB2, IL6, ACTC1, DES, ITGA7, DMD, ITGA5
KEGG pathway	hsa04514	Cell adhesion molecules (CAMs)	12	2.52E-04	NLGN1, NEGR1, CDH1, CLDN7, CNTN1, NCAM1, NCAM2, CD34, CD22, HLA-DQA1, JAM2, JAM3
KEGG pathway	hsa04512	ECM-receptor interaction	11	1.71E-05	RELN, COL4A2, SV2B, COL4A1, ITGB3, COL6A2, COL6A1, ITGA7, COL6A6, ITGA5, THBS2
KEGG pathway	hsa04270	Vascular smooth muscle contraction	11	2.18E-04	PRKCB, KCNMA1, PLA2G2A, MRVI1, CACNA1C, AVPR1A, MYL9, ACTG2, PRKG1, MYLK, ADCY5
KEGG pathway	hsa04022	cGMP-PKG signaling pathway	11	0.002282707	PLN, PDE2A, KCNMA1, PDE3A, MRVI1, CACNA1C, MYL9, ADRA2A, PRKG1, MYLK, ADCY5

Figures

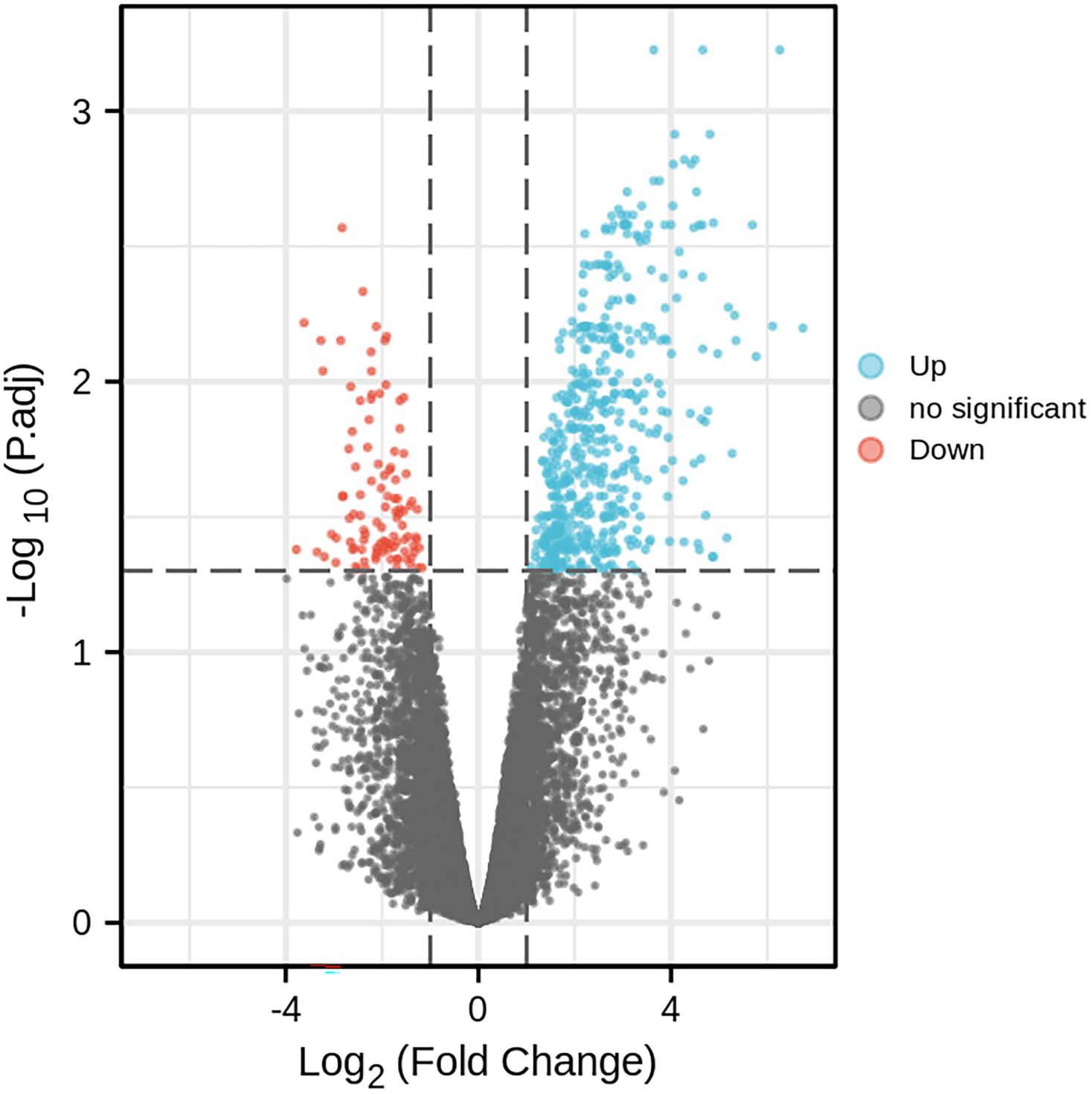

Figure 1

Volcano plot of GSE23732 microarray. Blue nodes indicate up-regulated genes based on an adjusted P-value<0.05 and log2FC>2.0, red nodes indicate down-regulated genes based on an adjusted P-value<0.05 and log2FC<-2, gray indicates genes with unchanged expression

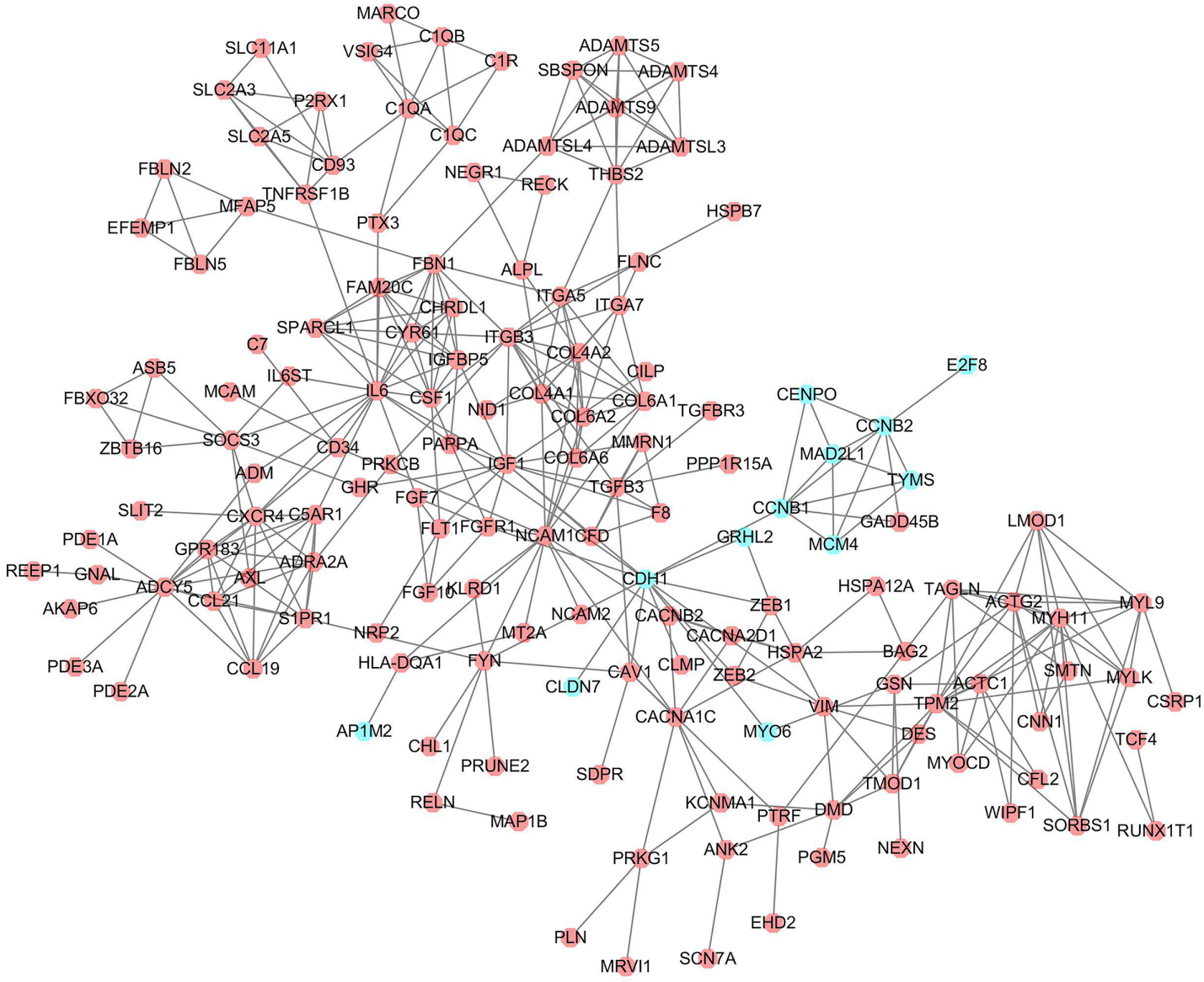

Figure 2

Protein-protein interaction network (PPI) constructed with the differentially expressed genes (DEGs) using STRING online database. Red color represents upregulated genes, and blue color represents downregulated genes

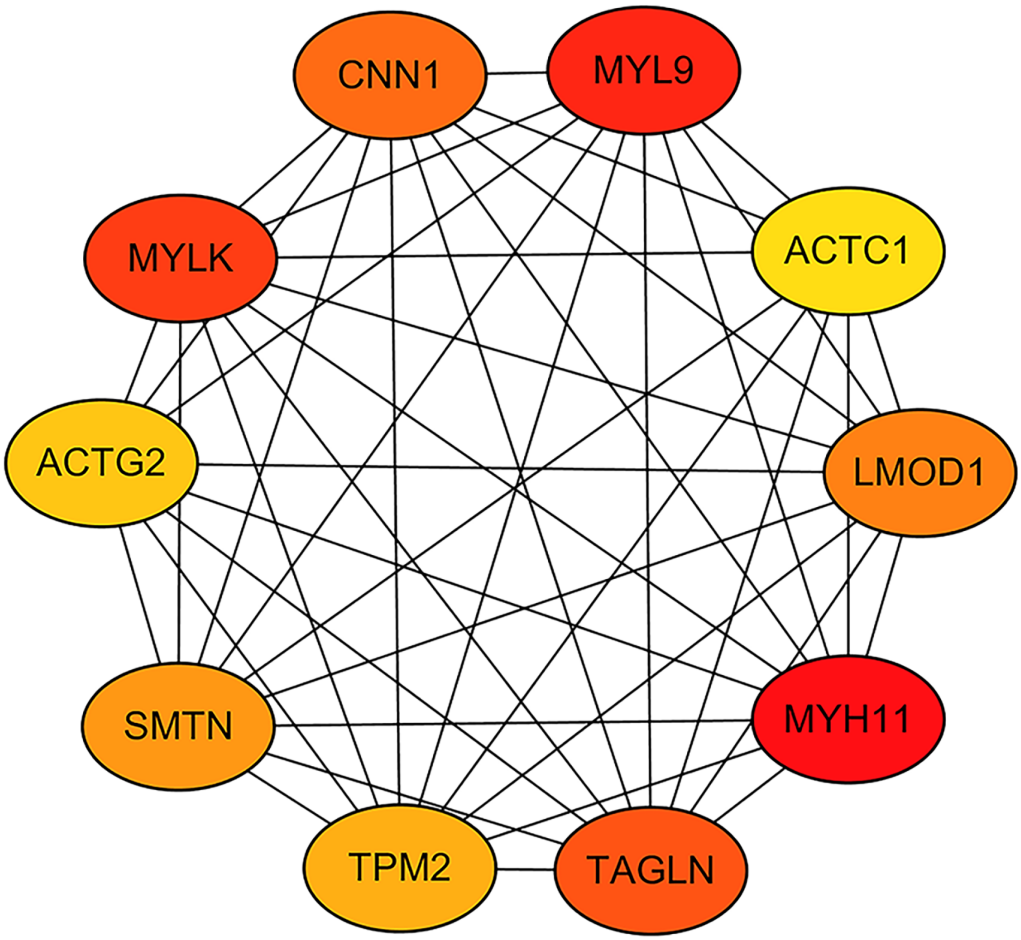

Figure 3

A meaningful module obtained from the protein-protein interaction network

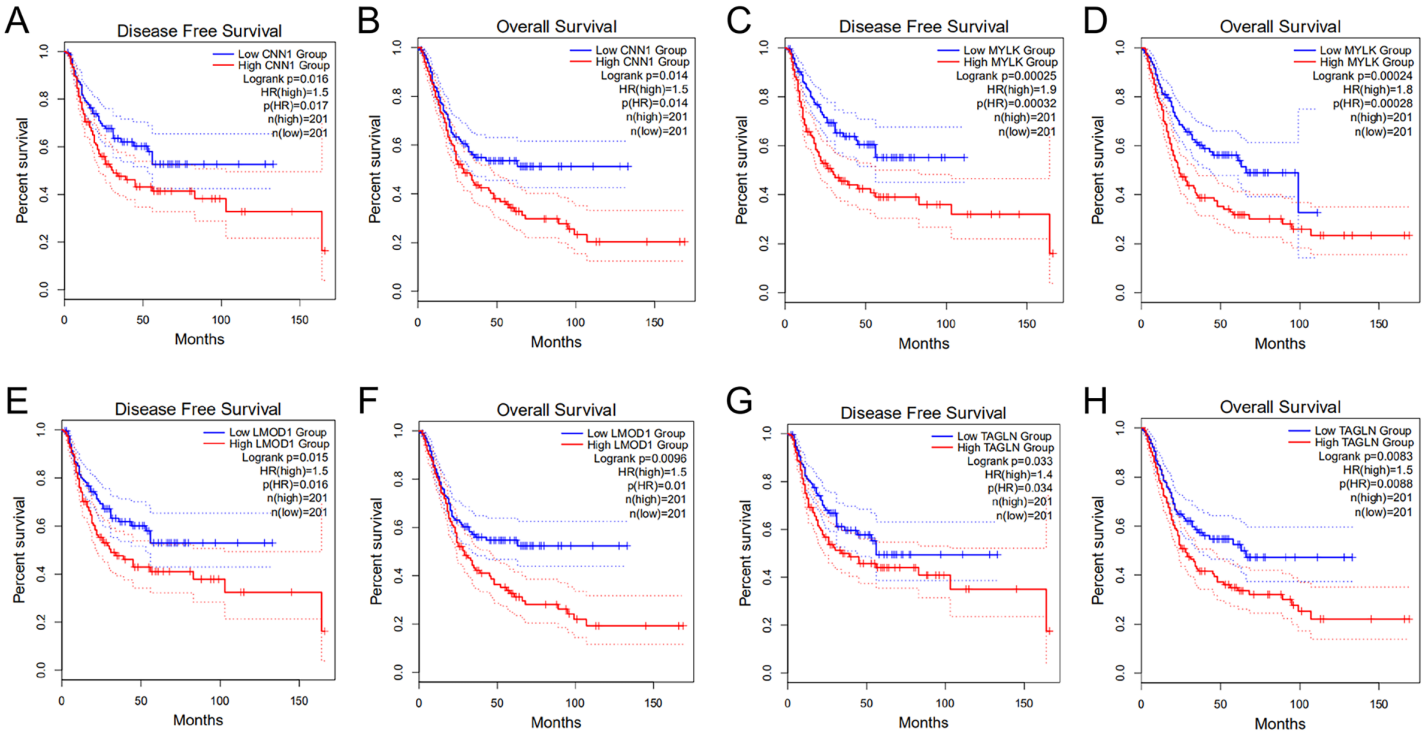

Figure 4

Prognostic analysis of overall survival and disease-free survival. Four core genes were associated with the prognosis of patients with bladder cancer. CNN1 (A and B); MYLK (C and D); LMOD1 (E and F); TAGLN (G and H)

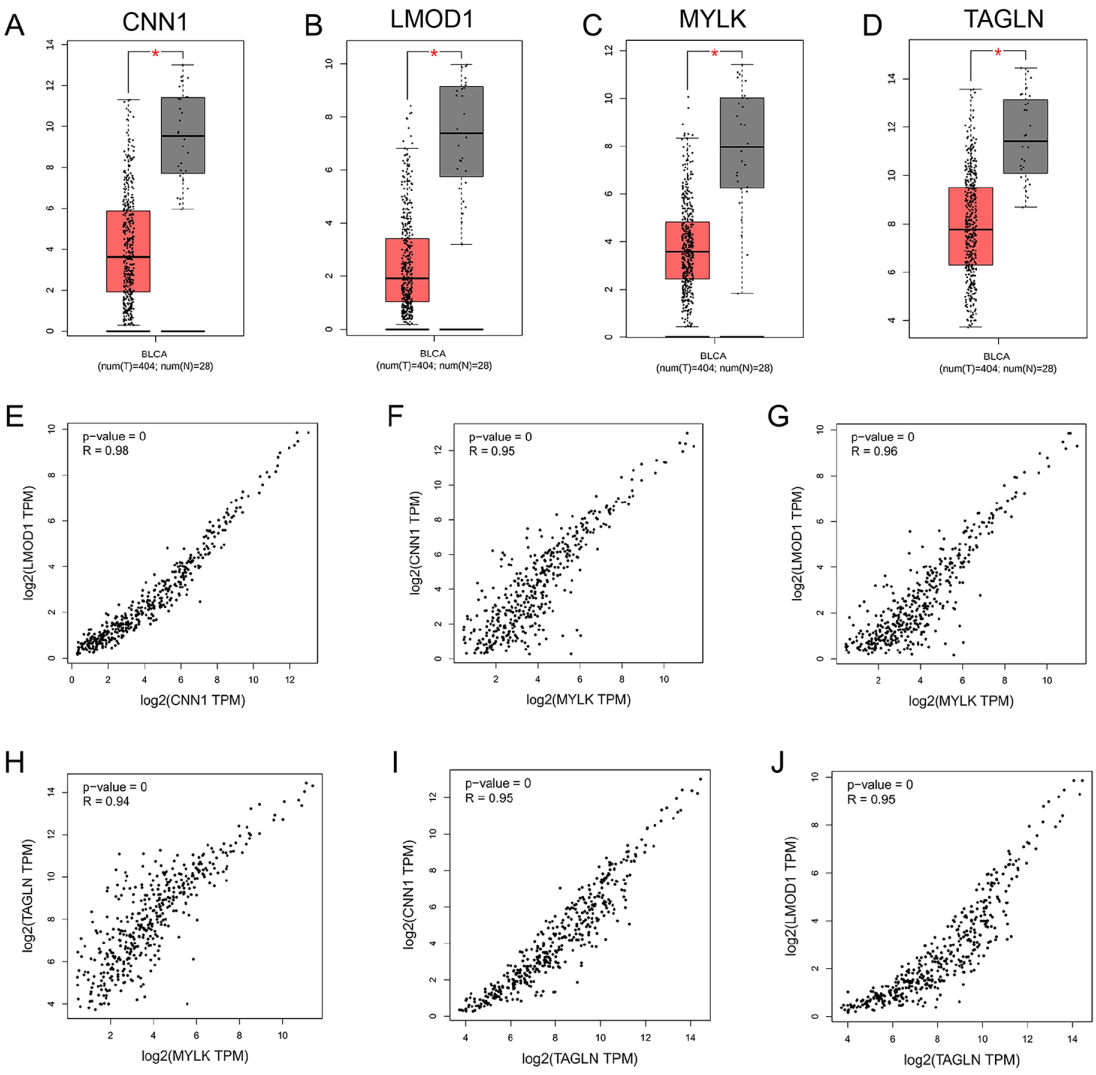

Figure 5

Expression levels of mRNA and Pearson correlation analysis of MYLK, CNN1, TAGLN and LMOD1 genes