# Identification and functional analysis of risk-related microRNAs for the prognosis of patients with bladder urothelial carcinoma

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**Abstract.** The aim of the present study was to investigate risk-related microRNAs (miRs) for bladder urothelial carcinoma (BUC) prognosis. Clinical and microRNA expression data downloaded from the Cancer Genome Atlas were utilized for survival analysis. Risk factor estimation was performed using Cox's proportional regression analysis. A microRNA-regulated target gene network was constructed and presented using Cytoscape. In addition, the Database for Annotation, Visualization and Integrated Discovery was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway enrichment, followed by protein-protein interaction (PPI) network analysis. Finally, the K-clique method was applied to analyze sub-pathways. A total of 16 significant microRNAs, including hsa-miR-3622a and hsa-miR-29a, were identified (P<0.05). Following Cox's proportional regression analysis, hsa-miR-29a was screened as a prognostic marker of BUC risk (P=0.0449). A regulation network of hsa-miR-29a comprising 417 target genes was constructed. These target genes were primarily enriched in GO terms, including collagen fibril organization, extracellular matrix (ECM) organization and pathways, such as focal adhesion (P<0.05). A PPI network including 197 genes and 510 interactions, was constructed. The top 21 genes in the network module were enriched in GO terms, including collagen fibril organization and pathways, such as ECM receptor interaction (P<0.05). Finally, 4 sub-pathways of cysteine and methionine metabolism, including paths 00270_4, 00270_1, 00270_2 and 00270_5, were obtained (P<0.01) and identified to be enriched through DNA (cytosine-5)-methyltransferase (*DNMT*)3A, *DNMT3B*, methionine adenosyltransferase 2α (*MAT2A*) and spermine synthase (*SMS*). The identified microRNAs, particularly hsa-miR-29a and its 4 associated target genes *DNMT3A*, *DNMT3B*, *MAT2A* and *SMS*, may participate in the prognostic risk mechanism of BUC.

## Introduction

Bladder urothelial carcinoma (BUC), a malignancy of the genitourinary system, is one of the most common types of bladder cancer (1). At present, the risk factors of BUC primarily comprise smoking and contact with aromatic amine chemicals (1). BUC may be divided into two categories: Non-muscle- and muscle-invasive BUC (2). Transurethral resection and radical cystectomy are the current treatment strategies for non-muscle- and muscle-invasive BUC, respectively (3). Although numerous methods have been suggested, an effective treatment remains elusive due to high recurrence rates. A more thorough understanding of the underlying molecular mechanism of prognostic risk may be beneficial for the development of therapeutic interventions, and therefore the prognosis of patients with BUC.

MicroRNAs are a group of non-coding small RNAs, comprising ~21 nucleotides, which regulate the expression of target genes through binding to 3'-untranslated regions (UTRs) (4). Previous studies have demonstrated the association between microRNAs and risk factors in the prognosis of BUC (5), including miR-141 expression, which was revealed to be significantly downregulated in invasive bladder cancer (6). miR-141 regulates kelch-like ECH-associated protein 1 and controls the oxidative stress response that is associated with the prognosis of BUC (7). In addition, miR-205 targets PH domain leucine-rich repeat-containing protein phosphatase 2 and phosphatase and tensin homolog (*PTEN*), further influencing protein kinase B signaling (8). Cathomas *et al* (9) demonstrated that the expression of *PTEN* was associated with the development of chemotherapy- and castration-resistant cancer, as well as patient prognosis. Additionally, members of the epidermal growth factor (EGF) family have been suggested as potential prognostic markers in BUC (10); at the same time, resistance of EGF receptor is reversed by miR-200 in BUC (11). Therefore, miR-200 serves an important role in the prognostic risk of BUC and is an independent marker associated with an increased risk of non-muscle-invasive bladder cancer recurrence (12).

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An improved understanding of microRNA-associated risk factors may clarify the prognostic molecular mechanism of BUC. In the present study, microRNA expression profile data and clinical data were downloaded, survival curves were created to estimate risk factors and target genes regulated by microRNA were analyzed. In addition, regulation networks were constructed and functional analysis of target genes was performed. Finally, a protein-protein interaction (PPI) network of target genes regulated by microRNA was analyzed and a sub-pathway analysis was performed.

## Materials and methods

*Data sources.* Clinical case data and expression profile data of microRNAs were downloaded from the Cancer Genome Atlas (TCGA; cancergenome.nih.gov) database on the BCGSC_IlluminaHiSeq_miRNASeq platform (Canada's Michael Smith Genome Sciences Centre, Vancouver, BC, Canada). The TCGA microRNA expression data were obtained from 529 patients with BUC (download cut-off date, August 11, 2014). Reads per kilobase of exon per million mapped reads (RPKM) was used to quantify the expression value of patient microRNA (13) using the following formula: RPKM = total microRNA reads/[total mapped reads (million) x microRNA sequence length (kb)]. Additionally, clinical case data comprised 411 patients with urothelial bladder carcinoma (download cut-off date, August 11, 2014). A total of 408 cases that exhibited microRNA expression profile data were selected for analysis.

*Survival analysis.* The mean expression value of each microRNA in the 408 cases was calculated as the critical value. All cases were divided into two groups: MicroRNA expression greater than the critical value, and microRNA expression equal to or less than the critical value of microRNA expression. A Kaplan-Meier estimator survival curve was created for microRNA in the two groups and a log-rank test was applied to analyze the significance. MicroRNAs exhibiting a significantly different survival curve were screened as candidates for prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference.

*Identification of risk-related miRNAs.* Cox's proportional hazards regression model was used to estimate the risk factors for collected clinical data and microRNA that demonstrated a significant effect on the survival curves. KMsurv (14) and survival (15) packages in R language were applied for the plotting of survival curves and Cox's proportional hazards regression model. Cox's proportional hazards regression model was created according to the backward selection method; variables were first introduced and subsequently the free variables with no significant differences were eliminated [hazard ratio (HR), 0.99997; P=0.0449].

*Analysis of key target genes regulated by microRNA.* MicroRNA target genes were predicted from relevant databases, including two validation databases, miRNecords (16) and miRWalk (17). To be applicable for the present study, the predicted regulatory association must have existed in at least three of the following databases: miRanda (18),

mirTarget2 (19), PicTar (20), PITA (21) and TargetScan (22). Genes that complied with the two aforementioned requirements were screened. A regulatory network was created and visualized using Cytoscape (23), based on the predicted target genes. Cytoscape is an open source software platform for visualizing complex networks and integrating these with any data type.

*Functional analysis of target genes.* The Database for Annotation, Visualization and Integrated Discovery, which provides analytical tools for extracting biological relevance from collections of genes (24), was used for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of target genes in the microRNA-regulated network. $P<0.05$ was used as the threshold criterion.

*PPI network analysis of microRNA target genes.* The PPI network of target genes was constructed using the Search Tool for Retrieval of Interacting Genes database, which provided integrated knowledge of the known and predicted associations for protein networks (25). PPI pairs with a combined score >0.4 were screened and visualized using Cytoscape.

*Sub-pathway analysis of target genes.* The K-clique method was used to divide metabolic pathways into sub-pathways, based on structural information, and to identify risk pathways using hypergeometric test (26). ISubpathway Miner limma (27) in R was applied for investigation of the processes of K-clique recognized risk sub-pathways. Sub-pathways with $P<0.05$ were considered to be risk sub-pathways. The associations between pathways and disease with target gene involvement were investigated.

## Results

*Survival analysis.* A total of 16 survival curves that significantly affected microRNA were obtained. Among them, the survival curves, including those for hsa-miR-3622a, hsa-miR-1292 and hsa-miR-3138 with significantly longer survival times and has-miR-29a with shorter survival time, were obtained on the condition that expression of microRNA was higher than the mean critical value. Another 12 survival curves exhibited significant longer survival time on the condition that the expression of microRNA was lower than mean value.

*Cox's proportional regression analysis.* Prognostic hazard ratios of microRNA were obtained using Cox's proportional regression analysis of the aforementioned 16 microRNA expression values. hsa-miR-29a was identified as a risk microRNA associated with the prognosis of UBC.

*Risk-related microRNA regulation network.* A regulation network of hsa-miR-29a was constructed by collecting and arranging database data of microRNA regulated target genes; a total 417 target genes were contained in the network (Fig. 1).

*Functional enrichment analysis of target genes.* Based on the results of enrichment analysis, the target genes of hsa-miR-29a were primarily enriched in GO terms, including collagen fibril
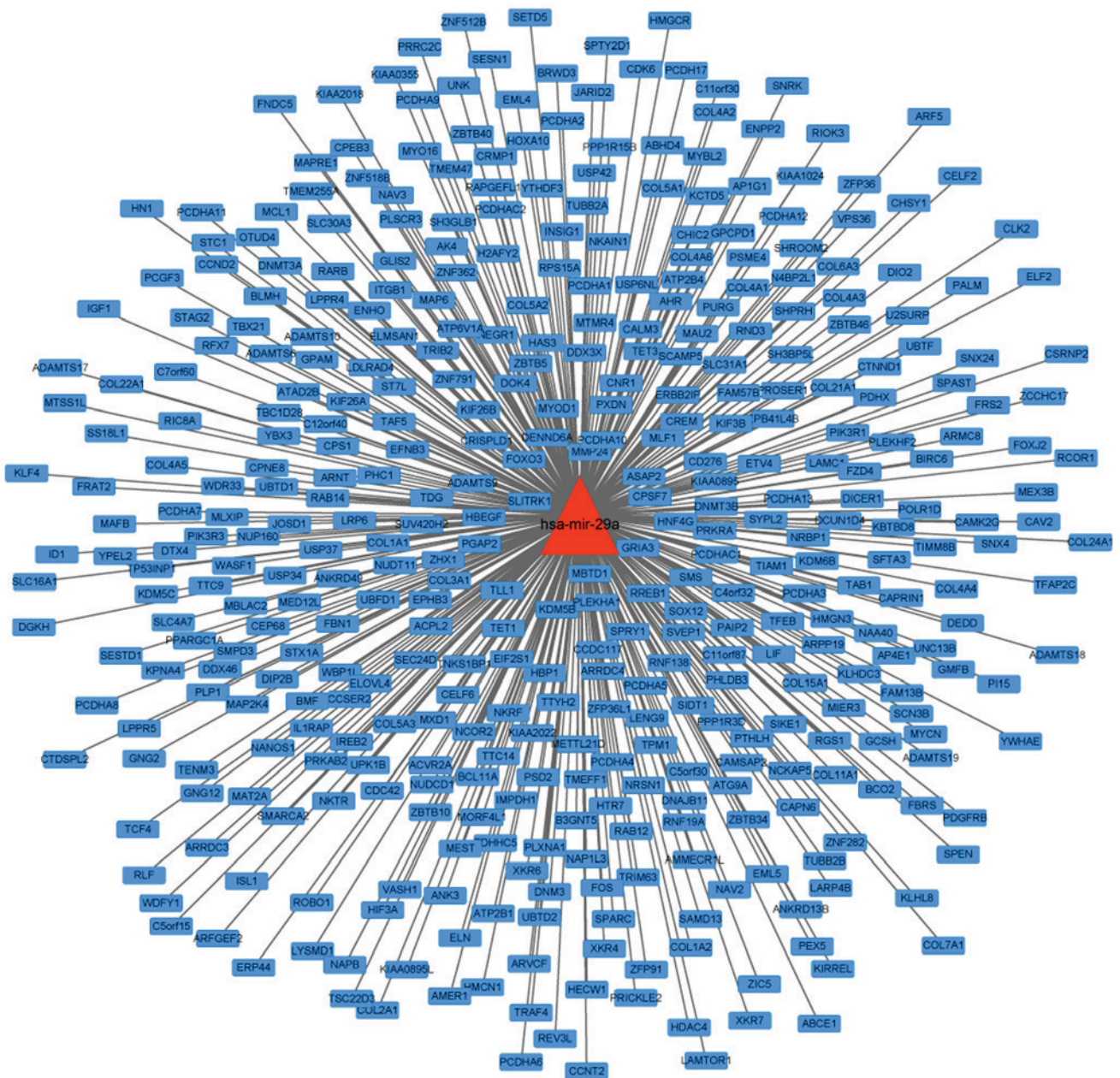
Figure 1. Regulatory network constructed using the risk microRNA, miR-29a, and its regulated target genes. The red triangle represents microRNA, and the blue rectangles represent target genes. miR, microRNA.

organization (P=2.64x10$^{-6}$), extracellular matrix (ECM) organization (P=2.02x10$^{-5}$), homophilic cell adhesion (P=3.66x10$^{-5}$) and extracellular structure organization (P=7.45x10$^{-5}$). These target genes were also enriched in pathways that included focal adhesion (P=5.06x10$^{-10}$), ECM-receptor interaction (P=1.16x10$^{-9}$) and small cell lung cancer (P=7.71x10$^{-6}$), and pathways in cancer (P=1.11x10$^{-3}$) (Table I).

*PPI network analysis of target genes.* A PPI network with 197 genes and 510 edges was constructed (Fig. 2). In this network, collagen type 1 α chain 2 (*COL1A2*)2, *COL1A1* and *COL3A1* were the top three nodes, the degrees of which were 25, 24 and 24, respectively. In addition, the top 5 pairs with the greatest combined score were phosphatidylinositol 3-kinase regulatory subunit 1-platelet-derived growth factor receptor β (0.999), *COL5A2-COL5A1* (0.992), *L5A2-COL11A1* (0.999),

*L5A1-COL5A3* (0.999) and *COL4A6-COL4A5* (0.999). Values in brackets are the combined score value.

Furthermore, a network module with 21 genes was screened from the PPI network (Fig. 3). The enrichment results of this module are presented in Table II. The genes in this module were primarily enriched in functions that included collagen fibril organization (P=2.97x10$^{-15}$), ECM organization (P=3.01x10$^{-15}$), cell adhesion (P=1.15x10$^{-13}$) and biological adhesion (P=1.17x10$^{-13}$).

*Risk sub-pathway analysis.* A total of 4 sub-pathways of cysteine and methionine metabolism were obtained, including paths 00270_4 (P=4.11x10$^{-4}$), 00270_1 (P=6.16x10$^{-4}$), 00270_2 (P=5.40x10$^{-3}$) and 00270_5 (P=6.26x10$^{-3}$). Paths 00270_4 and 00270_1 were enriched by DNA (cytosine-5)-methyltransferase 3α (*DNMT3A*), DNMT3β (*DNMT3B*), methionine

Table I. Top 5 GO terms and pathways enrichment of hsa-miR-29a target genes.

| Category | Term | Count | P-value |
| --- | --- | --- | --- |
| GOTERM_BP_FAT | GO:0030199~collagen fibril organization | 8 | $2.64 \times 10^{-6}$ |
| GOTERM_BP_FAT | GO:0030198~ECM organization | 12 | $2.02 \times 10^{-5}$ |
| GOTERM_BP_FAT | GO:0007156~homophilic cell adhesion | 13 | $3.66 \times 10^{-5}$ |
| GOTERM_BP_FAT | GO:0043062~extracellular structure organization | 14 | $7.45 \times 10^{-5}$ |
| GOTERM_BP_FAT | GO:0022610~biological adhesion | 33 | $9.53 \times 10^{-5}$ |
| GOTERM_CC_FAT | GO:0005581~collagen | 18 | $4.91 \times 10^{-20}$ |
| GOTERM_CC_FAT | GO:0044420~ECM part | 24 | $1.84 \times 10^{-16}$ |
| GOTERM_CC_FAT | GO:0005578~proteinaceous ECM | 34 | $2.30 \times 10^{-14}$ |
| GOTERM_CC_FAT | GO:0031012~ECM | 35 | $3.42 \times 10^{-14}$ |
| GOTERM_CC_FAT | GO:0005604~basement membrane | 15 | $6.33 \times 10^{-10}$ |
| GOTERM_MF_FAT | GO:0005201~ECM structural constituent | 19 | $9.83 \times 10^{-13}$ |
| GOTERM_MF_FAT | GO:0048407~PDGF binding | 7 | $6.57 \times 10^{-8}$ |
| GOTERM_MF_FAT | GO:0005198~structural molecule activity | 30 | $4.42 \times 10^{-4}$ |
| GOTERM_MF_FAT | GO:0003677~DNA binding | 76 | $1.83 \times 10^{-3}$ |
| GOTERM_MF_FAT | GO:0019838~growth factor binding | 9 | $3.22 \times 10^{-3}$ |
| KEGG_PATHWAY | hsa04510: Focal adhesion | 22 | $5.06 \times 10^{-10}$ |
| KEGG_PATHWAY | hsa04512: ECM-receptor interaction | 15 | $1.16 \times 10^{-9}$ |
| KEGG_PATHWAY | hsa05222: Small cell lung cancer | 11 | $7.71 \times 10^{-6}$ |
| KEGG_PATHWAY | hsa05200: Pathways in cancer | 17 | $1.11 \times 10^{-3}$ |
| KEGG_PATHWAY | hsa05214: Glioma | 7 | $1.85 \times 10^{-3}$ |
| REACTOME_PATHWAY | REACT_16888: Signaling by PDGF | 14 | $2.20 \times 10^{-10}$ |
| REACTOME_PATHWAY | REACT_18266: Axon guidance | 12 | $1.99 \times 10^{-9}$ |
| REACTOME_PATHWAY | REACT_13552: Integrin cell surface interactions | 11 | $4.20 \times 10^{-6}$ |
| REACTOME_PATHWAY | REACT_604: Hemostasis | 10 | $4.97 \times 10^{-2}$ |

ECM, extracellular matrix; PDGF, platelet-derived growth factor.

Table II. Top 5 GO terms and pathway enrichment of hsa-miR-29a target genes in network module 1.

| Category | Term | Count | P-value |
| --- | --- | --- | --- |
| GOTERM_BP_FAT | GO:0030199~collagen fibril organization | 8 | $2.97 \times 10^{-15}$ |
| GOTERM_BP_FAT | GO:0030198~ECM organization | 10 | $3.01 \times 10^{-15}$ |
| GOTERM_BP_FAT | GO:0007155~cell adhesion | 14 | $1.15 \times 10^{-13}$ |
| GOTERM_BP_FAT | GO:0022610~biological adhesion | 14 | $1.17 \times 10^{-13}$ |
| GOTERM_BP_FAT | GO:0043062~extracellular structure organization | 10 | $1.90 \times 10^{-13}$ |
| GOTERM_CC_FAT | GO:0005581~collagen | 18 | $2.85 \times 10^{-43}$ |
| GOTERM_CC_FAT | GO:0044420~ECM part | 18 | $7.38 \times 10^{-33}$ |
| GOTERM_CC_FAT | GO:0005578~proteinaceous ECM | 20 | $4.29 \times 10^{-30}$ |
| GOTERM_CC_FAT | GO:0031012~ECM | 20 | $1.86 \times 10^{-29}$ |
| GOTERM_CC_FAT | GO:0044421~extracellular region part | 20 | $6.82 \times 10^{-21}$ |
| GOTERM_MF_FAT | GO:0005201~ECM structural constituent | 16 | $8.63 \times 10^{-30}$ |
| GOTERM_MF_FAT | GO:0005198~structural molecule activity | 18 | $4.09 \times 10^{-20}$ |
| GOTERM_MF_FAT | GO:0048407~PDGF binding | 6 | $2.32 \times 10^{-12}$ |
| GOTERM_MF_FAT | GO:0019838~growth factor binding | 6 | $4.43 \times 10^{-7}$ |
| GOTERM_MF_FAT | GO:0005178~integrin binding | 4 | $9.62 \times 10^{-5}$ |
| KEGG_PATHWAY | hsa04512: ECM-receptor interaction | 14 | $2.60 \times 10^{-24}$ |
| KEGG_PATHWAY | hsa04510: Focal adhesion | 14 | $3.93 \times 10^{-19}$ |
| KEGG_PATHWAY | hsa05222: Small cell lung cancer | 5 | $4.42 \times 10^{-5}$ |
| KEGG_PATHWAY | hsa05200: Pathways in cancer | 5 | $7.63 \times 10^{-3}$ |
| REACTOME_PATHWAY | REACT_18266: Axon guidance | 12 | $2.01 \times 10^{-20}$ |
| REACTOME_PATHWAY | REACT_16888: Signaling by PDGF | 12 | $5.11 \times 10^{-19}$ |
| REACTOME_PATHWAY | REACT_13552: Integrin cell surface interactions | 9 | $3.37 \times 10^{-11}$ |

ECM, extracellular matrix; PDGF, platelet-derived growth factor.

Table III. Analyzed results of risk pathways.

| Pathway ID | Pathway name | P-value | Gene |
|---|---|---|---|
| path:00270_4 | Cysteine and methionine metabolism | $4.11 \times 10^{-4}$ | *DNMT3A*, *DNMT3B*, *MAT2A*, *SMS* |
| path:00270_1 | Cysteine and methionine metabolism | $6.16 \times 10^{-4}$ | *DNMT3A*, *DNMT3B*, *MAT2A*, *SMS* |
| path:00270_2 | Cysteine and methionine metabolism | $5.40 \times 10^{-3}$ | *DNMT3A*, *DNMT3B*, *MAT2A* |
| path:00270_5 | Cysteine and methionine metabolism | $6.26 \times 10^{-3}$ | *DNMT3A*, *DNMT3B*, *MAT2A* |

DNMT, DNA(cytosine-5)-methyltransferase; MAT, methionine adenosyltransferase; SMS, spermine synthase.
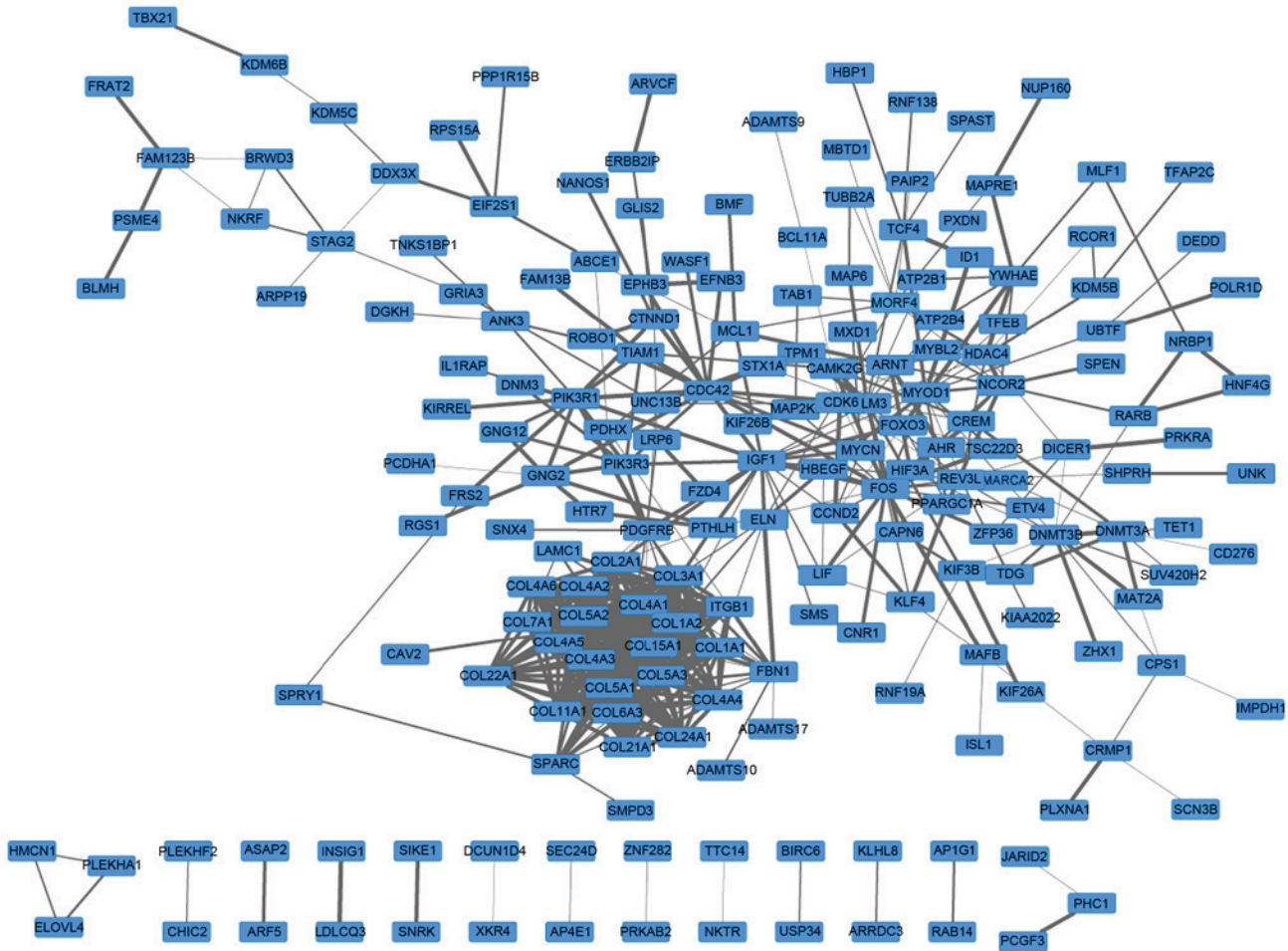


Figure 2. PPI network constructed using the target genes of miR-29a. Blue rectangles represent target genes of miR-29a and edges represent interactions between target genes. The thickness of each edge is in proportion to the combined score.

adenosyltransferase 2α (*MAT2A*) and spermine synthase (*SMS*), whereas paths 00270_2 and 00270_5 were enriched by *DNMT3A, DNMT3B and MAT2A* (Table III).

**Discussion**

BUC is a malignancy of the genitourinary system that is difficult to effectively treat due to high recurrence rates (28). In the present study, hsa-miR-29a was screened as a prognostic risk-related microRNA of BUC. In addition, 21 genes in the network module were enriched in GO terms, including collagen fibril organization and ECM organization, and were enriched in pathways, including ECM-receptor interaction and

focal adhesion. Finally, 4 pathways, including path00270_4, path00270_1, path00270_2 and path00270_5, were obtained and enriched by 4 target genes, *DNMT3A DNMT3B*, *MAT2A* and *SMS*.

hsa-miR-29a was the only microRNA that significantly affected the prognosis of BUC. hsa-miR-29a is a microRNA member of the miR-29 family, the dysregulation of which has been demonstrated to affect *DNMT3A* expression in the HL1 cell line (29). Notably, in the *DNMT3A* mutation samples, DNA methylation patterns were altered (30). In other types of cancer, including lung cancer, the miR-29 family reversed biological processes of aberrant DNA methylation and was associated with a poor prognosis in cancer (31). In addition,
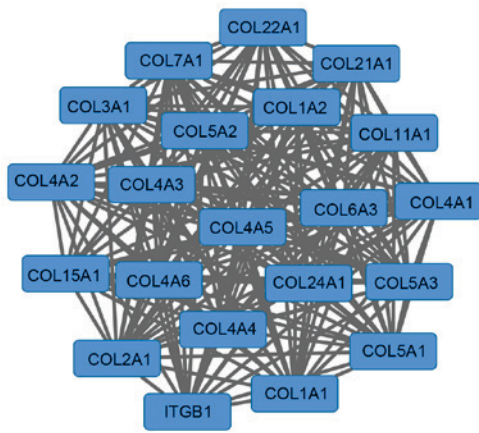
Figure 3. Network module 1 extracted from the PPI network. Blue rectangles represent target genes and edges represent interactions between target genes.

downregulated miR-29a may promote transforming growth factor β induction and further promote the fibrotic response by interacting with genes, including fibrillin, elastin and collagens (32). Similar to *DNMT3A, DNMT3B* also exhibits complementarities with the miR-29 family at 3'-UTRs (31). The synthesis of S-adenosyl-(L)-methionine (adoMet), the primary methyl group donor in humans, is the primary step in the process of methionine metabolism (33). Through Adohcy, the transfer of activated methyl groups is naturally catalyzed from AdoMet to C5 atom by *DNMT3A* and *DNMT3B* (34). Consistent with previous studies, results in the present study revealed that *DNMT3A* and *DNMT3B* were regulated by miR-29a, and enriched in the cysteine and methionine metabolism pathway, affecting the prognosis of *BUC*.

Furthermore, miR-29a has also been demonstrated to regulate *MAT2A* and *SMS*. *MAT2A* is a mammalian gene that encodes MAT (35). AdoMet is an intermediate metabolite that also functions as an intracellular control switch, which regulates essential functions (36). Furthermore, *MAT2A* serves a role in the methionine cycle pathway, which is an important metabolic pathway (37). Although the molecular mechanisms of *SMS* associated with BUC prognostic risk have not been reported, the results of the present study suggest that they may serve important roles in BUC prognostic risk through their involvement in the cysteine and methionine metabolism pathway.

In addition to the aforementioned pathways, miR-29a was also enriched in ECM organization and biological adhesion. Ioachim *et al* (38) demonstrated that thrombospondin type 1 serves an important role in the prognosis of cancer, being enriched in ECM organization pathways. β1-integrin has been demonstrated to downregulate expression of miR-29a, whilst increased expression of β1-integrin in BUC cells induces tissue invasion (39). Cell invasion is the primary factor associated with poor prognosis (40). Through these pathways, miR-29a may exhibit an important prognostic risk.

Although several key genes and pathways associated with BUC were identified using comprehensive bioinformatic methods, no experiment was conducted to verify the results and this therefore presents a clear limitation to the present study. Further experimental studies of diverse samples are thus required to validate the results of the present study.

In conclusion, the identified microRNAs, particularly hsa-miR-29a, may serve important roles in the prognostic risk mechanism of BUC through the regulation of 4 target genes, including *DNMT3A, DNMT3B, MAT2A* and *SMS*, and through involvement in cysteine and methionine metabolism pathways. However, further study is required to support the potential association between microRNAs, target genes and prognostic risk factors.

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