The analysis of a ceRNA network and the correlation between lncRNA, miRNA, and mRNA in bladder cancer

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#These authors contributed equally to this study.

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Background: To explore the correlation between the lncRNA-miRNA-mRNA and ceRNA network through the differential expression analysis of lncRNAs, miRNAs and mRNAs in bladder cancer based on The Cancer Genome Atlas (TCGA) database combined with Gene Ontology (GO) and Kyoto Encyclopedia of Genes Genomes (KEGG) enrichment analysis.

Methods: Firstly, the expression profile data and corresponding clinical data of RNAs in bladder cancer were searched and downloaded from TCGA database, and aberrantly expressed long non-coding RNA (lncRNA), microRNA (miRNA), and messenger RNA (mRNA) were screened and found by using TCGA database. The relationship between lncRNA-miRNA-mRNA was established by comparing these lncRNAs, miRNAs, and mRNAs, while the ceRNA network was constructed. Combined with the analysis of the GO annotation and KEGG pathway, the effects of lncRNA-miRNA-mRNA interaction on the development of bladder cancer were explored.

Results: A total of 1,742 differentially expressed lncRNA, 511 differentially expressed miRNAs, and 4,373 differentially expressed mRNAs were identified, and 328 lncRNAs, 73 miRNAs, and 677 mRNAs were screened by survival analysis. With the lncRNA-miRNA-mRNA correlation analysis, a ceRNA network consisting of 45 lncRNAs, 14 miRNAs, and 29 mRNAs was successfully constructed. The GO annotation and functional enrichment of target gene mRNAs in the network are mainly concentrated in the signal pathways and include fatty acid biosynthesis, gap junction, insulin signaling pathway, and the MAPK signaling pathway biological processes such as positive regulation of cellular process and system development.

Conclusions: We successfully identified the target gene correlating lncRNA, miRNA, and mRNA, and constructed a ceRNA network. Our findings can provide a potential target for the study of the occurrence, development, diagnosis, treatment, and prognosis of bladder cancer.

Keywords: Bladder cancer; long non-coding RNA (lncRNA); microRNA (miRNA); messenger RNA (mRNA); The Cancer Genome Atlas (TCGA)

Introduction

Bladder cancer is a malignant tumor originating from the bladder uroepithelium. It is one of the most common malignant tumors in the urinary system and seriously endangers human health. The incidence of bladder cancer in the world ranks 11th among all malignant tumors, among which the incidence rate in males is 9.0/100,000, ranking 7th among male malignant tumors. In China, the incidence
of bladder cancer in men has exceeded that of prostate
cancer, ranking first among malignant tumors in the male
urinary system (1) (Standard for Diagnosis and Treatment

In recent years, great progress has been made in the
study of bladder cancer, but its mechanisms are still unclear.
Studies have shown that gene mutation and abnormal
regulation of gene expression play an important role in
the process of tumorigenesis and progression, and also play
an important role in the occurrence and development of
bladder cancer (2,3). MicroRNA (miRNA), for example,
has been widely shown to be involved in the development
of bladder cancer (4). Compared with miRNAs, there are
few studies on newly discovered long non-coding RNA
(lncRNA). lncRNA is a non-coding RNA transcript with
a length of more than 200 bp, which does not encode
proteins. LncRNA was once thought to have no biological
functions and to be a “noise” in transcription. However,
recent studies have shown that lncRNA plays an important
role in epigenetic regulation, transcriptional regulation,
and post-transcriptional regulation by acting as a signaling
molecule, bait molecule, scaffold molecule, or guide
molecule in the form of RNA, and participates in nearly all
physiological and pathological processes of organisms. The
abnormal expression of lncRNAs is closely related to the
occurrence, development, and metastasis of tumors (5,6).
The effect of abnormal expression of lncRNA in bladder
cancer tissues on the proliferation, invasion, and migration
of bladder cancer cells, and its guiding significance for
clinical diagnosis and prognosis have been reported (7,8).
miRNA is a non-coding single-stranded RNA encoded by
endogenous genes. Its length is 22 bp. It can regulate the
expression of mRNA through the microRNA response
element (MRE) on the mRNA (9). Each miRNA can
regulate the transcription and translation of multiple RNA,
and each MRE can interact with multiple miRNAs (10,11).

Salmena et al. (12) proposed the endogenous competitive
non-coding ceRNA hypothesis, indicating that RNA
transcripts can communicate with each other through
microRNA response elements (MRES), thus affecting the
expression level of various RNAs, which plays a crucial
role in tumorigenesis. Although progress has been made
in the study of bladder cancer, the detection of specific
lncRNAs associated with bladder cancer, survival analysis,
and other clinically significant lncRNAs have not been
reported. Therefore, we attempted to extract sample data
from the Cancer Genome Atlas (TCGA) database, conduct
differential expression, GO, KEGG, and survival analysis.

The aim of our study was to analyze the molecular functions
of the integrated lncRNA itself and the related signaling
pathways, and to find the related regulatory molecules. We
hope our findings can provide a reliable theoretical basis for
exploring the molecular mechanism of bladder cancer and
finding molecular targets for clinical diagnosis or treatment.

Methods

Data acquisition of bladder cancer and its adjacent samples

All the data in this study were collected from the TCGA
database until January 28, 2016, and included 408 cases of
expression of mRNA, 407 cases of expression of miRNAs,
and 412 cases of clinical samples, along with 19 matched
samples of cancer tissues and adjacent normal tissues. After
eliminating the duplicate data and ultra-low expression data,
only part of the data information and incomplete clinical
information were recorded in the follow-up research.

Screening of lncRNAs, miRNAs, and mRNAs differentially
expressed and significantly surviving in bladder cancer
tissues

The data of RNA expression profiles were collected, and the
differentially expressed lncRNAs, miRNAs, and RNAs were
analyzed by R language edgeR package. The differentially
expressed lncRNAs, miRNAs and mRNAs whose absolute
value of fold change (FC) was more than 2, and whose false
discovery rate (FDR) was less than 0.05, were screened. At
the same time, the volcano maps were drawn by ggplot2
package, and the differentially expressed lncRNAs,
miRNAs, and mRNA were obtained. Survival analysis was
then conducted by using R language Survival package, and
lncRNAs, miRNAs, and mRNAs with significant differences
in expression and survival analysis were screened out.

Correlation analysis between lncRNA and miRNA-RNA,
and construction of a ceRNA network in bladder cancer

The expression data of differentially expressed and surviving
significant RNA, lncRNA, and miRNAs were correlated
with corresponding expression data (i.e., differentially
expressed and surviving significant RNA corresponding
to lncRNA and miRNA), and the correlation coefficient
between them was calculated. The absolute value of the
correlation coefficient equal to or more than 0.5 represented
a significant correlation.
The interacting miRNAs were matched according to the miR-code database (http://www.mircode.org/) using the differentially expressed and surviving significant lncRNA and miRNAs from the above analysis (13). Target Scan (http://www.targetscan.org/vert_72/), miRDB (http://mirdb.org/), and miRTarBase (http://miRTarBase.mbc.nctu.edu.tw/php/index.php) databases were used to predict the target genes of miRNAs (14-16), and the target genes present in all three databases were screened. Finally, a subset of sections was obtained from the differentially expressed and surviving target genes screened, and the target genes differentially expressed and significantly surviving from earlier analysis. Based on the above-obtained lncRNA, miRNAs, and RNA, a ceRNA network between lncRNA and miRNAs was constructed using Cytoscape 3.7.1, and Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes Genomes (KEGG) enrichment analysis of the target genes in the network were carried out.

Enrichment analysis and survival analysis

The differentially expressed and surviving significant mRNAs, lncRNAs, significantly correlated mRNAs and miRNAs, along with significantly correlated mRNAs were analyzed by GO functional annotation and KEGG pathway enrichment, respectively. Combined with the survival data of patients with bladder cancer in TCGA, the K-M curve method was used to analyze the survival of patients with bladder cancer using the R language Survival package. The relationship between lncRNAs, miRNAs, and mRNAs and the prognosis of bladder cancer was analyzed. P<0.05 was considered to have statistical significance.

Results

Differentially expressed and significantly surviving lncRNAs in bladder cancer

lncRNA was extracted from the expression matrix file downloaded from the TCGA database, and 1,742 differentially expressed lncRNAs were screened according to the criteria of |log FC| (>2), FDR <0.05 using R language edgeR package. As shown in the volcano map (Figure 1), there were 1,292 up-regulated lncRNAs and 450 down-regulated lncRNAs. Survival analysis of differentially expressed lncRNAs showed that 328 differentially expressed and significantly analyzed lncRNAs were screened. As shown in the Wayne diagram (Figure 2), 260 of them were up-regulated, and 68 were down-regulated.

Differentially expressed and significantly surviving miRNAs in bladder cancer

To study the direct relationship between lncRNA and miRNAs, the differentially expressed miRNAs of bladder cancer in the TCGA database were analyzed. In total, 511 differentially expressed miRNAs were identified according to the screening criteria of |log FC| (>2) and FDR <0.05, of which 406 were differentially up-regulated, and 105 were...
differentially down-regulated \((\text{Figure 3})\). By performing K-M survival analysis on differentially expressed miRNAs, a total of 73 differentially expressed and surviving miRNAs were obtained \((\text{Figure 4})\), of which 41 were up-regulated and 32 were down-regulated.

\textit{mRNA differentially expressed and significantly survived in bladder cancer}

Similarly, according to the above method, the mRNA expression profiles of bladder cancer tissues and adjacent tissues in the TCGA database were compared, and differential mRNAs with \(|\log \text{FC}| \geq 2\) and FDR <0.05 were screened. The results showed that there were 4,373 differentially expressed mRNAs in bladder cancer tumor tissues compared with normal tissues \((\text{Figure 5})\), of which 2,254 were up-regulated mRNA and 2,119 were down-regulated mRNA. The differentially expressed mRNA was subjected to survival analysis, and 677 mRNAs with significant differences and survival were screened \((\text{Figure 6})\), of which 246 were up-regulated and 431 were down-regulated.
**Construction of ceRNA network of lncRNA-miRNA-mRNA in bladder cancer**

To further understand the role of differentially expressed lncRNAs in bladder cancer, we performed a correlation analysis between lncRNAs, miRNAs and mRNAs with variability of expression and significant survival. With 328 differentially expressed and significantly surviving lncRNAs and 73 miRNAs, the lncRNA-miRNA-mRNA ceRNA network was constructed. The results showed that 45 lncRNAs were involved in the regulation of 14 miRNAs (Table 1). After a subset of sections was obtained from 14 miRNA-regulated target genes and differentially expressed and surviving mRNAs, 29 target genes regulated by miRNAs were identified. Thus, 45 lncRNAs, 14 miRNAs, and 29 mRNAs constitute a direct regulatory relationship of lncRNA-miRNA-mRNA in bladder cancer (Figure 7).

**GO and KEGG enrichment analysis of mRNA in the lncRNA-miRNA-mRNA ceRNA network of bladder cancer**

To further investigate the effect of mRNA in the relationship of lncRNA, miRN, and mRNA in bladder cancer on the cause and development of bladder cancer, GO and KEGG enrichment analysis was performed on lncRNA (differentially expressed and significantly surviving)-correlated mRNAs, miRNA (differentially expressed and significantly surviving)-correlated mRNAs, and differentially expressed and significantly surviving mRNAs. The results showed that some target genes were enriched in the signaling pathways, including cell adhesion molecules (CAMs), MAPK signaling pathway, and PI3K Akt signaling pathway. MAPK signaling pathway has been associated with promoting cell proliferation (17,18), while PI3K-Akt signaling pathway has been associated with tumor invasion and metastasis (19). Other pathways have been associated with cancer, adhesion, and abnormality in gene transcription (Figures 8-10).

Similarly, we performed GO and KEGG enrichment analysis on mRNAs of the lncRNA-miRNA-mRNA ceRNA network, and the GO annotation results are shown in Figure 11 (only the top 10 are visible). The results of KEGG enrichment analysis showed that 29 mRNAs were involved in cancer-related signaling pathways such as fatty acid biosynthesis, gap junction, insulin signaling pathway, prostate cancer, regulation of actin cytoskeleton, and MAPK signaling pathway (Table 2).

**Discussion**

Bladder cancer is the most common malignant tumor in the urinary system in China, and its incidence and mortality are the first in urinary tract tumors. Advances in medical technology have made great progress in the treatment of bladder cancer. However, the recurrence rate of bladder cancer is high, and the 5-year survival rate is low. Therefore, patients with bladder cancer need to endure long-term detection and treatment. The difficulties of this disease may be due to an insufficient understanding of the potential mechanism and the lack of effective biomarkers. Earlier studies have shown that improper gene regulation may play an important role in bladder cancer (20) and has enormous potential as a biomarker. This article studies the mechanism of action in bladder cancer to better understand and identify biomarkers of bladder cancer. With the development of high-throughput sequencing technology, more and more studies have shown that lncRNA plays a crucial role in the development of cancer (2,3), and that the expression of lncRNA is closely related to the progress of different cancers. Studies have shown that lncRNA TSLNC8 can interact with TKT and STAT3, thereby regulating the phosphorylation levels of STAT3-Tyr705 and STAT3-Ser727 and the transcriptional activity of STAT3, leading to the inactivation of the IL-6/STAT3 signaling pathway and suppressing the development of tumors (21). At present, the research focus of related lncRNA in urinary tract tumors at home and abroad is focused on prostate cancer. The related lncRNAs include prostate cancer gene expression marker 1 (PCGEM1), prostate-specific antigen 3 (DD3/PCA3), prostate non-coding RNA1 (PRNCR1), and others. In bladder tumors, there are more studies on urothelial carcinoma-associated gene 1 (UCA1) and imprinted gene H19, and more lncRNA studies are still in their infancy. In this study, a series of lncRNAs were screened using public data of RNA expression profiles from TCGA database. Target gene enrichment analysis revealed correlations with pathways such as the cGMP-PKG signaling pathway.

As an area of intense research, miRNA has been studied in relation to various tumors, including bladder cancer. It has been proven that miR-141, miR-200c, miR-30b, miR-370, miR-1236, and miRNAs like miR-144, miR-223, and miR-let-7, etc. (22-25) play a key role in the proliferation, invasion, metastasis, apoptosis, and prognosis evaluation of bladder cancer. Yonemori et al. (26) found that by down-regulating miR-139-5p or miR-139-3p to enhance the migration and invasion of bladder cancer cells, and directly
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Table 1 (continued)
Table 1 (continued)

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IncRNA, long non-coding RNA; miRNA, microRNA.

Figure 7 ceRNA regulatory network of lncRNA, miRNA, and mRNA in bladder cancer. Note: the red represents miRNA, the blue represents lncRNA, and the yellow represents mRNA. IncRNA, long non-coding RNA; miRNA, microRNA; mRNA, messenger RNA.
Figure 8 GO and KEGG enrichment analysis results of lncRNA-related mRNA. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes Genomes; lncRNA, long non-coding RNA; mRNA, messenger RNA.
Figure 9 GO and KEGG enrichment analysis of miRNA-related mRNA. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes Genomes; lncRNA, long non-coding RNA; mRNA, messenger RNA.
Figure 10 GO and KEGG enrichment analysis of differentially expressed and significant mRNAs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes Genomes; mRNA, messenger RNA.
Figure 11 GO enrichment analysis on mRNAs of the lncRNA-miRNA-mRNA ceRNA network (top 10). GO, Gene Ontology; lncRNA, long non-coding RNA; miRNA, microRNA; mRNA, messenger RNA.

Table 2 Results of the KEGG enrichment analysis of 29 mRNAs in a bladder cancer ceRNA network

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KEGG, Kyoto Encyclopedia of Genes Genomes.
target MMP11, which can be used as a prognostic marker for bladder cancer patients. In this study, bioinformatics methods were used to identify several miRNAs with differential expression and significant survival, such as MAP3K8 and PDGFRA, using the public resources of bladder cancer in TCGA database. Further enrichment analysis showed that these miRNA-related mRNAs were enriched in signaling pathways such as the MAPK signaling pathway, and this finding can provide a certain data foundation for further research.

The proposed ceRNA hypothesis provides an important guiding direction for the pathogenesis of tumors and also offers a new theoretical basis for the diagnosis and treatment of tumors. The interaction of RNA molecules like lncRNA, miRNA, and mRNA has important biological significance. Studies have shown that lncRNA, miRNA, and mRNA are in a state of equilibrium in the ceRNA network. Once this balanced microenvironment state is broken, cancer disease ensues (27). Studies have confirmed that lncRNA-regulated ceRNA networks have important regulatory effects in cancers such as gastric cancer, liver cancer, and prostate cancer (28-31). In this study, a ceRNA network consisting of 45 lncRNAs, 14 miRNAs, and 29 mRNAs was constructed by RNA omics data from TCGA database, which provided a basis for further experimental verification.

The inadequacy of this study is that all the data consist of changes in RNA levels provided by TCGA, with no data from the proteome, and thus we were unable to analyze mRNA at the protein level in ceRNA networks. In the next study, we will conduct quantitative analysis and experiments such as immunohistochemistry or Western blotting to further explore and verify the results of this study.

**Conclusions**

In summary, we performed a differential analysis of lncRNA, miRNA, and mRNA data in the TCGA database, and combined them with survival analysis to screen out those lncRNAs, miRNAs, and mRNAs that were differentially expressed and significantly proliferated. The correlation between lncRNA, miRNA, and mRNA was also analyzed in pairs, and a ceRNA network was constructed to provide a potential target for the diagnosis, treatment, and prognosis evaluation of bladder cancer.

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