ORIGINAL ARTICLE

Novel Long Non-coding RNA Markers for Prognostic Prediction of Patients with Bladder Cancer

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Objective To explore novel long non-coding RNA (lncRNA) molecular markers related to bladder cancer prognosis and to construct a prognostic prediction model for bladder cancer patients.

Methods LncRNA expression data of patients with bladder cancer were downloaded from TCGA database. Univariate Cox regression and likelihood-based survival analysis were used to discover prognosis related lncRNAs. Functional studies of prognosis related lncRNAs were conducted by co-expression analysis and pathway enrichment analysis. Multivariate Cox regression analysis was used to establish risk score model, and Receiver Operating Characteristic analysis was used to determine the optimal cut-off point of the model. The risk score model was validated through Kaplan Meier estimation method and log-rank test.

Results Seven prognosis related lncRNAs (OCIAD1-AS1, RP11-111J6.2, AC079354.3, RP11-553A21.3, RP11-598F7.3, CYP4F35P and RP11-113K21.4) which can predict survival of bladder cancer patient were discovered. Co-expression analysis and pathway analysis of these novel lncRNA signature and their target genes further revealed that these lncRNAs play important roles in the occurrence and development of bladder cancer. Additionally, a seven-lncRNA signature based risk score model for prognostic prediction of bladder cancer patients was established and validated. Notably, we identified the potential significance of two tumor-related antisense lncRNAs (OCIAD1-AS1 and RP11-553A21.3) in the prognosis of bladder cancer.

Conclusion Our results suggest that these lncRNA markers may serve as potential prognosis predictors for bladder cancer and deserve further functional verification studies.

BLADDER cancer is the most common malignancy of the urinary system and the ninth most common cancer in the world. Bladder cancer is a highly prevalent disease that mainly affects the elderly, killing an estimated 165 000 men and women each year.^[1] The high incidence, recurrence and mortality of bladder cancer prompted researchers to look for biomarkers that could be used for early diagnosis and improving prognosis.^[2]

It has been confirmed that a large part of the genome has been transcribed into RNAs, which is much higher than previously assumed,^[3] and a large part of the transcription products are composed of non-coding RNA (ncRNAs). Long non-coding RNAs (lncRNAs) are a kind of such ncRNAs containing more than 200 nucleotides lacking protein-coding abilities.^[4] Increasing evidences have shown that lncRNA can participate in gene regulation, post-transcriptional regulation and epigenetic regulation as a key participant of genomic regulatory network.^[5] Abnormal expression of lncRNAs is closely related to human cancer^[6] and certain lncRNAs can be used as markers for tumor diagnosis and prognosis.^[7-12] Also, many studies have shown that lncRNAs can influence development and progression of bladder cancer,^[13-15] and be the new potential prognostic molecular markers for bladder cancer.^[16, 17] Thus, this study aimed to explore novel lncRNA molecular markers related to bladder cancer prognosis and to construct a prognostic prediction model for bladder cancer.

MATERIALS AND METHODS

Data downloading and preprocessing

Datasets of lncRNAs expression profiling of bladder cancer patients and their clinical information of survival time were downloaded from the Cancer Genome Atlas (TCGA) database GDC (Genomic Data Commons) data portal (<https://portal.gdc.cancer.gov/>), and the final expression level of lncRNAs was defined as the $\log_2(\text{FPKM}+1)$ for the subsequent process. If an lncRNA is not expressed in more than half of the samples ($\text{FPKM}=0$), it was filtered out. Then the data were randomly divided into two groups: training set and test set. The training set was used to find the lncRNAs related to prognosis and to construct a prognostic prediction model. The test set was used to validate the model.

Identification of prognostic related lncRNAs

Survival package^[18] in R software was used to analyze

the relationship between the expression of lncRNAs and the prognosis of patients. When the P value of a lncRNA is less than or equal to 0.05, it is considered to have statistical significance. Because of the large number of lncRNAs with statistical significance, further screening of these lncRNAs was needed in order to have more practical application value. Likelihood-based survival analysis was performed using Rbsurv package^[19] in R software to select final prognostic related lncRNAs.

Co-expression and pathway enrichment analysis

Co-expression analysis was performed using the calculation of Pearson correlation coefficients (PCC) between the expression level of lncRNAs and protein coding genes (PCGs). The PCGs with PCC larger than 0.4, indicating positively correlated, or those with PCC smaller than -0.4, indicating negatively correlated, were defined as lncRNAs related PCGs and were selected. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and interaction network construction of lncRNAs related PCGs were performed using Enrichr.^[20, 21]

Establishment and validation of prognostic prediction model

Those prognostic-related lncRNAs were incorporated into the multivariate Cox regression analysis in the training set to estimate the Cox regression coefficients and establish a prognostic risk score formula. According to this formula, the prognostic risk score of each patient in the training set was calculated. The patients were divided into two groups: the high-risk group and the low-risk group, using intermediate risk score as the cut-off point. Using R survival ROC package,^[22] a Receiver Operating Characteristic (ROC) curve was constructed. By choosing the optimal cut-off point with the highest sensitivity and specificity, the samples were further divided into the low-risk group and the high-risk group again. The Kaplan-Meier estimation method was used for multivariate analysis and log-rank test was used for comparison. The prognostic risk score formula was further validated by fitting validation set and all data sets.

RESULTS

Summary of the datasets

Altogether, we obtained the clinic information of 341

samples and 14 371 lncRNA expression profiles from TCGA database. Among the acquired lncRNAs, we found 6737 were abundantly expressed in bladder cancer patients based on the cutoff we set. The 341 bladder cancer samples then were randomly assigned to a training series containing 171 cases or a test series containing 170 cases.

Identification of bladder cancer prognosis related lncRNAs

Through univariate Cox regression analysis in the training data, 671 lncRNAs ($P < 0.05$) with significant difference expression were screened out. Likelihood-based survival analysis was performed on the 671 differentially expressed lncRNAs and 7 core prognosis related

lncRNAs were obtained (**Table 1**). Three of them (CYP4F35P, RP11-111J6.2 and RP11-113K21.4) are long intergenic non-coding RNAs (lincRNAs) and four of them (OCIAD1-AS1, AC079354.3, RP11-553A21.3 and RP11-598F7.3) are antisense RNAs. Two antisense RNAs (AC079354.3 and RP11-598F7.3) overlap with known gene regions. RP11-553A21.3 locates in the promoter region of gene CD109 (**Figure 1A**), while OCIAD1-AS1 locates in the gene body of OCIAD1 (ovarian cancer immuno-reactive antigen domain containing 1) (**Figure 1B**).

Functional implication of the prognostic lncRNAs

We analyzed the co-expression of the seven prognostic lncRNAs and all expressed PCGs. Finally, we acquired

Table 1. Overall information of seven novel prognosis related lncRNAs

Ensembl ID	Gene symbol	Hazard ratio	P value	- logL	AIC
ENSG00000248256.1	OCIAD1-AS1	0.352498	0.000426	313.55	629.1
ENSG00000260331.1	RP11-111J6.2	0.124571	0.000367	309.8	623.61
ENSG00000222035.3	AC079354.3	132322.9	9.81E-06	304.4	614.79
ENSG00000231652.2	RP11-553A21.3 (AL590428.1)	28.95384	4.98E-05	300.78	609.56
ENSG00000256948.1	RP11-598F7.3	0.324872	0.012715	293.77	597.55
ENSG00000265787.1	CYP4F35P	0.473391	0.001861	291.21	594.41
ENSG00000255503.1	RP11-113K21.4	0.15136	0.000223	289.07	592.15

lncRNAs: long non-coding RNAs; AIC: Akaike Information Criterion.

Figure 1. Chromosome location and potential target genes of two antisense lncRNAs.

A. RP11-553A21.3 (also named AL590428.1), an antisense RNA is located in the promoter region of CD109. B. OCIAD1-AS1, an antisense RNA, is located in the gene body of OCIAD1.

923 PCGs in the positively correlated group and 219 PCGs in the negatively correlated group, respectively. We noted that lncRNA OCIAD1-AS1 had the most positively correlated PCGs (**Figure 2A**), while lncRNA RP11-553A21.3 had the most negatively correlated PCGs (**Figure 2B**), suggesting that the two lncRNAs play more important roles than other lncRNAs in the prognosis of bladder cancer.

Then, KEGG enrichment analysis was performed for the above screened positive and negative correlation PCGs to further study the potential biological functions of these lncRNAs. The results demonstrated that the top enriched KEGG pathways mostly associated with cancer cell cycle or cancer microenvironment, such as focal adhesion, extracellular matrix (ECM)-receptor

interaction, glycosaminoglycan biosynthesis and proteoglycans in cancer in the positively correlated group (**Figure 2C, 2E**), and tight junction, PPAR signaling pathway, bladder cancer pathway in the negatively correlated group (**Figure 2D, 2F**). Our results suggest that these seven lncRNA markers may play a role in bladder cancer by regulating the co-expression PCGs, thus participating in some known cancer-related pathways.

Risk score model of seven-lncRNA signature

In order to comprehensively study the relationship between the seven prognosis related lncRNAs and the prognosis of bladder cancer, we obtained the regression coefficients of each molecular marker based on multivariate Cox regression, and established a risk

Figure 2. Co-expressed protein coding genes (PCGs) for prognostic lncRNAs and their KEGG pathway enrichment network. Top 100 positively (A) or negatively correlated PCGs (B), and top ten KEGG pathways of positively (C) or negatively correlated PCGs (D) are presented. The significance of each term is represented by the length and color of the bar graph. Interaction networks of top ten KEGG pathways of positively (E) or negatively correlated PCGs (F) are shown.

scoring formula based on the seven lncRNA markers:
 $\text{Risk score} = -(0.6782 \times \text{OCIAD1-AS1}) - (0.8582 \times \text{RP11-111J6.2}) + (9.1474 \times \text{AC079354.3}) + (1.7133 \times \text{RP11-553A21.3}) - (1.1868 \times \text{RP11-598F7.3}) - (0.4080 \times \text{CYP4F35P}) - (0.9838 \times \text{RP11-113K21.4})$.

Then, we calculated the risk scores of the seven lncRNAs markers for each patient in the test dataset and ranked them according to the risk scores. Then, the patients were divided into the high-risk group (85 cases) and the low-risk group (86 cases). It can be seen from **Figure 3A** and **Figure 3B** that survival time of bladder cancer patients and their risk value distribution showed a certain pattern, that is, most of the deaths had higher risk scores, while those with longer survival time had lower risk scores. **Figure 3C** shows that the expression levels of RP11-553A21.3 and AC079354.3 were higher in the high-risk group, while the expression levels of the other five lncRNAs were lower in the high-risk group. Kaplan-Meier curve and log-rank test showed that the survival time of the low-risk group was longer than that of the high-risk group ($P < 0.001$) (**Figure 3D**).

Optimizing prognostic indicators through ROC analysis

We compared the sensitivity and specificity of risk scoring formulas by ROC analysis of seven prognostic factors based on lncRNAs. The result showed that the area under the curve (AUC) was as high as 0.84, and most samples can be well divided into two

groups: the high-risk group and the low-risk group. When the cut-off point was 1.202, the sensitivity and specificity both reached the highest, which can be used as the optimal cut-off point (**Figure 4A**). At this optimal threshold, bladder cancer patients can be further divided into the high-risk group which contained 70 cases and the low-risk group which contained 101 cases. Kaplan-Meier curve and logarithmic rank test further showed that there were significant differences in survival time between the high-risk group and the low-risk group ($P < 0.001$) (**Figure 4B**).

Validation of the prognostic model

We used the same model and risk formula, as well as the optimal cut-off point determined in the previous step, to evaluate the validity of the risk scoring model based on the seven lncRNA molecular markers in all data sets and test sets, respectively. Firstly, based on the best cut-off point, the patients in the data set were divided into high-risk 147 cases and low-risk 194 cases. From the Kaplan-Meier curve of the two groups, it was found that the survival time of high-risk patients was significantly shorter than that of low-risk patients ($P < 0.001$) (**Figure 4C**). Then, based on the best cut-off point, the test data were divided into high-risk group (77 cases) and low-risk group (93 cases), and a similar conclusion was reached that the survival time of high-risk patients was significantly shortened ($P = 0.004$) (**Figure**

Figure 3. LncRNA risk score analysis in the training data.

A. Distribution of risk scores; B. Distribution of survival time; C. Relative expression levels of the seven lncRNAs related to prognosis; D. The Kaplan-Meier curve used the median risk score as a cut-off point to divide patients into the high-risk group and low-risk group.

4D). Although the sample size of each group was unbalanced, the analysis results were consistent, which proves that our model can effectively predict the prognostic risk of bladder cancer.

DISCUSSION

In this study, we identified seven prognosis related lncRNAs that significantly differentially expressed among the bladder cancer patients. To the best of our knowledge, none of the seven lncRNAs has been reported in cancer or other diseases directly, but we can trace some potential function from their chromosome location and overlapped genes. Two of them, RP11-553A21.3 and AC079354.3, are adversely associated with the prognosis of bladder cancer. RP11-553A21.3

(also named AL590428.1) is an antisense RNA locating in the promoter region of gene CD109, which is a negative regulator of TGF- β signaling and a putative risk marker for cancer.^[23, 24] Antisense RNA has the function of regulation the expression level of its target genes.^[25] Thus, differed expression level of RP11-553A21.3 between the high risk group and low risk group maybe affects the expression level of CD109, and further contributes to the risk of bladder cancer patients. AC079354.3 is also an antisense RNA locating within gene KIAA2012, but the function of this gene remains elusive. The down-regulated expression of the other five lncRNAs was significantly associated with lower mortality and prolonged survival. CYP4F35P, RP11-111J6.2 (also named AC079148.1) and RP11-113K21.4 (also named AP001767.3) are all novel lin-

Figure 4. Determination of the optimal threshold and validation of the model.

A. The sensitivity and specificity of survival time were analyzed by Receiver Operating Characteristic. The red dot represents the risk score at the optimal cut-off point. B. In the Kaplan-Meier curve, the optimal cut-off point is used to estimate the patient's survival time in the training data. C. Kaplan-Meier curve is used to estimate the survival time of all patients. D. Kaplan-Meier curve is used to estimate patient's survival time in the test data.

crRNAs. RP11-598F7.3 (also named AC026369.3) is an antisense RNA of gene IQSEC3. OCIAD1-AS1 is an antisense RNA of gene OCIAD1. A previous study found that OCIAD1 was over-expressed in metastatic ovarian cancer tissues and the effect of OCIAD1 on cell adhesion may be related to its function in ovarian cancer.^[26] Thus, differed expression level of OCIAD1-AS1 between the high risk group and low risk group maybe affects the expression level of OCIAD1, and further contributes to the risk of bladder cancer patients. It should be noted that two antisense lncRNAs, P11-553A21.3 and OCIAD1-AS1, have the most co-expressed PCGs, further confirm their potential regulation role to the target PGCs. To our knowledge, this should be the first study to report the relationship between these two antisense lncRNAs and the prognosis of bladder cancer. These finding further emphasized the significance of lncRNAs in the cancer prognosis researches.

Recent studies have shown that lncRNA participates in different biological processes by regulating gene expression at the transcriptional and post-transcriptional levels. Therefore, it is feasible to study the biological function of lncRNAs by co-expression with PCGs.^[27, 28] In this study, we performed co-expression analysis and KEGG pathway enrichment analysis of PCGs co-expressed with the seven lncRNAs to predict the potential biological function of lncRNAs. We found that co-expressed PCGs were enriched in known cancer-related pathways, confirming the potential biological significance of these lncRNA markers in bladder cancer. In detail, the top enriched KEGG pathways in the positively and negatively correlated group mostly related to cancer cell cycle or cancer microenvironment. For example, focal adhesion is a type of adhesive contact between cell and ECM, it can anchor cell to the substratum and can mediate biochemical signaling which involved in tumor formation and progression.^[29] ECM, a complex mixture of structural and functional macromolecules, plays an important role in the morphogenesis of tissues and organs and the maintenance of the structure and function of cells and tissues.^[30] The pathways of ECM-receptor interaction, glycosaminoglycan biosynthesis and proteoglycans play roles as cancer ECM or microenvironment, and regulate several aspects of cancer biology, including tumorigenesis, tumor progression and metastasis.^[31-33] Tight junctions mediate attachment of the cells to one other, and expression of tight junction proteins may be up- or down-regulated and thus influence tumor spread.^[34-36]

PPAR signaling pathway is a well-researched pathway related to tumor and other diseases, and it as a potential target for cancer prevention and therapy has gained a strong interest in recent years.^[37-39] Additionally, bladder cancer pathway is also enriched in this study, providing direct evidence that these seven lncRNAs are related to prognosis of bladder cancer.

Since standard univariate Cox regression may generate hundreds of potentially significant biomarkers, a likelihood-based survival analysis was further used to discover a set of robust and presentative prognosis related biomarkers containing just several lncRNAs, which may be applicable for the construction of risk prediction model. We established a bladder cancer risk prediction model based on the seven prognosis related lncRNAs, and further determined the optimal cut-off point of the model by ROC analysis. By applying this model to training data and test data, we found that this model has good reproducibility. The prognosis of high-risk patients is poor, the survival time of low-risk patients is long, and the survival curves of high-risk and low-risk patients show significantly difference, which proves that our model is effective. However, further experiments are still needed to confirm the biological functions of these molecular markers.

In conclusion, we studied the potential value of lncRNAs in predicting the prognosis of bladder cancer. Seven novel lncRNAs related to the prognosis of bladder cancer were screened out, and a risk prediction model was constructed to evaluate the prognosis risk of bladder cancer patients. The co-expression analysis of the seven lncRNA markers, together with KEGG enrichment analysis and lncRNA localization analysis, further revealed the biological significance of lncRNAs in the prognosis of bladder cancer. Two antisense lncRNAs, P11-553A21.3 and OCIAD1-AS1, should be paid particular concern and are worth of further study.

Conflict of Interests Statement

The authors declare no competing interests.

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