A risk model based on lncRNA-miRNA-mRNA gene signature for predicting prognosis of patients with bladder cancer

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Abstract.
OBJECTIVES: We aimed to analyze lncRNAs, miRNAs, and mRNA expression profiles of bladder cancer (BC) patients, thereby establishing a gene signature-based risk model for predicting prognosis of patients with BC.
METHODS: We downloaded the expression data of lncRNAs, miRNAs and mRNA from The Cancer Genome Atlas (TCGA) as training cohort including 19 healthy control samples and 401 BC samples. The differentially expressed RNAs (DERs) were screened using limma package, and the competing endogenous RNAs (ceRNA) regulatory network was constructed and visualized by the cytoscape. Candidate DERs were screened to construct the risk score model and nomogram for predicting the overall survival (OS) time and prognosis of BC patients. The prognostic value was verified using a validation cohort in GSE13507.
RESULTS: Based on 13 selected. lncRNAs, miRNAs and mRNA screened using L1–penalized algorithm, BC patients were classified into two groups: high-risk group (including 201 patients) and low risk group (including 200 patients). The high-risk group's OS time (hazard ratio [HR], 2.160; 95% CI, 1.586 to 2.942; $P = 5.678$e-07) was poorer than that of low-risk groups' (HR, 1.675; 95% CI, 1.037 to 2.713; $P = 3.393$ e-02) in the training cohort. The area under curve (AUC) for training and validation datasets were 0.852. Younger patients (age $\leqslant 60$ years) had an improved OS than the patients with advanced age (age > 60 years) (HR 1.033, 95% CI 1.017 to 1.049; $p = 2.544$E-05). We built a predictive model based on the TCGA cohort by using nomograms, including clinicopathological factors such as age, recurrence rate, and prognostic score.
CONCLUSIONS: The risk model based on 13 DERs patterns could well predict the prognosis for patients with BC.

Keywords: Competing endogenous RNA, prognosis, nomogram, bladder cancer

1. Introduction

Bladder cancer (BC) is the most prevalent carcinoma of urinary system in China and even the whole world, and the ninth killer in all kinds of cancers in the world [1,2]. Although current treatment of BC has improved the 5-year overall survival (OS), poor prognosis is also existing due to the absence of typical symptoms. And it is found that most cases of BC patients had advanced and distal metastasis [3,4,5]. Therefore, it is of great need to develop and improve the diagnosis and treatment methods of BC.

Recently, molecular biomarkers including lncRNA, miRNAs and mRNA can be used for detection, diagnosis, recurrence prediction and post-treatment monitoring of BC [6,7,8,9]. Moreover, it has been found that the lncRNAs could interact with miRNAs as competing endogenous RNA (ceRNA) to regulate BC. For example, LncRNA GAS6-AS2 has been identified as a new prognostic marker of BC could promote BC proliferation through miR-298-CDK9 axis [10]; LncRNA LINC00641 was a potential factor to predict prognosis

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and nhibit BC developing through miR-197-3p-KLF10-PTEN-PI3K-AKT aixs [11]. lncRNA TINCR can reduce the OS rate of BC patients by inhibiting miR-7 and regulating mTOR [12]. Above all, based on the above researches, we found that the RNAs could be a potential biomarkers for BC and also were associated with BC through the ceRNA mechanism [13,14,15].

Thus, this study aimed to construct a RNA-based risk model to predict the prognosis of BC. We firstly conducted a ceRNA regulatory network analysis to screen the RNAs which could be used as the biomarkers for BC, and subsequently, we integrated genomic and clinical features to build a nomogram model based on prognostic RNAs to predict prognosis of patients with BC.

2. Methods

2.1. RNAs expression data

The mRNA, lncRNA and miRNA profile was obtained from The Cancer Genome Atlas (TCGA) (https://gdc-portal.nci.nih.gov/), which included 401 BC samples and 19 healthy control samples with survival prognosis information. Additionally, GSE13507 dataset containing 256 samples were obtained from Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/), in which 165 samples contained the survival prognosis information.

2.2. Differentially expressed RNAs screening

We used limma package in R language [16] (Version 3.34.7) to identify the differentially expressed RNAs (DERs) including mRNA (DEmRNA), lncRNAs (DElncRNAs), and microRNAs (DEmiRNAs) in TCGA dataset. The criterion was set to FDR < 0.05 and the $|\log2 FC| > 0.5$. Pheatmap package in R was used for bi-directional hierarchical clustering (version 1.0.8) [17].

2.3. Competing endogenous RNAs regulatrory network construction

The relationship between the DEmiRNAs and DElncRNAs was predicted using DIANA-LncBasev2 [18, 19], and the correlation of DEmiRNAs and mRNA using starBase (Version 2.0) [20]. Subsequently, we retained the negative regulation among DEmiRNAs, DElncRNAs and mRNA. The ceRNA regulatory network including the DEmiRNAs, DElncRNAs and mRNA was

constructed and visualized by the cytoscape (Version 3.6.1) [21]. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment were performed to analyze the function of mRNA involved in the ceRNA regulatory network with the cutoff of p value < 0.05 [22,23].

2.4. Prognosis related RNAs screening

We used univariate and multivariate Cox regression analysis in the survival package (Version 2.41–1) [24] to identify DERs related to prognosis with the threshold of log-rank $p < 0.05$. What's more, penalized package (Version 0.9–50) [25] was applied to screen the optimized DERs with Cox-PH model [26].

2.5. Prognostic model establishment

In order to obtain the RNAs' performance in predicting prognosis and OS time for BC patients, we established risk score using coefficients weighted by the penalty Cox model, and calculated the risk score according to the follow formula

$$Risk\ score = \sum \beta_{RNAs} \times Exp_{RNAs}$$

β_{RNAs}, the coefficients; Exp_{RNAs}, the expression level of RNAs.

The median value of risk score in training dataset was considered the cut-off point as classifying cohort to high- and low-risk group. Subsequently, we analyzed the OS with Kaplan-Meier plots and used the receiver operating characteristic (ROC) analysis to evaluate their performance using the value of area under curve (AUC) [27,28]. The same analysis was performed in the validation cohorts of GSE13507. We performed univariate and multivariate Cox regression analysis to analyze the independent significance of different factors with the threshold of p value < 0.05. Covariates included age (older than 60 vs younger than 60), and tumor occurrence (no vs yes).

Nomograms were constructed using the rms package (https://cran.r-project.org/web/packages/rms/index.html) in R3.6.1. The multivariable Cox regression model's coefficients were used to generate nomograms. Calibration curve was graphically evaluated by plotting the observed rate versus the probability predicted by the nomogram, and the consistency index (C-index) [29, 30,31] was calculated.
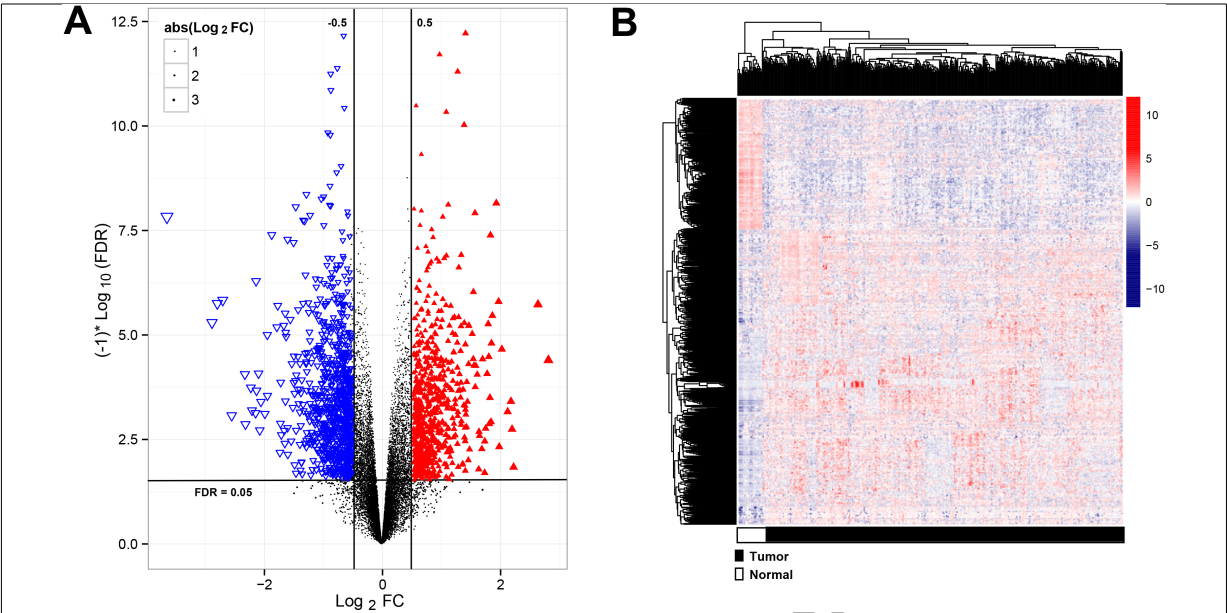
Fig. 1. Analysis of significantly differentially expressed RNAs (DERs). (A) the volcano plot showed the significantly DERs, red points were up-regulated RNAs, blue points were down-regulated RNAs; (B) The two-way clustering of DERs, $n = 401$.

3. Results

3.1. DERs analysis

A total of 15860 mRNA, 1116 lncRNAs and 458 miRNAs were obtained from TCGA dataset. Subsequently, 635 mRNA, 49 lncRNAs and 326 miRNAs were differentially expressed in the training cohorts, which were visualized using Volcano plots (Fig. 1A). The heatmap revealed that RNAs expression patterns between BC and healthy control were distinguishable (Fig. 1B).

3.2. Construction of ceRNAs regulatory network

According to the DIANA-LncBasev2, 130 regulatory relationships between DEmiRNAs and DElncRNAs were identified. Subsequently, a total of 148 regulatory relationships between DEmiRNAs and mRNAs were obtained using the starBase Version 2.0, which included 31 DElncRNAs, 30 DEmiRNAs and 51 mRNA (Fig. 2). The mRNA in ceRNA network were enriched in 3 KEGG pathways including axon guidance, cell adhesion molecules, stem cell pluripotency regulating signaling pathways, and 18 GO terms, such as cell morphogenesis involved in differentiation, axonogenesis, and sensory organ development (Table 1). Taken together, we successfully constructed a ceRNA network, the DERs of which might involve in biological function in BC.

3.3. Candidate DERs selection

A total of 31 DERs (20 mRNA, 5 DElncRNAs and 6 DEmiRNAs) were significantly related to the prognosis. Subsequently, 16 DERs were selected using the multivariate cox regression analysis. At last, 13 optimized DERs including 3 lncRNAs (LINC00161, ZFHX4-AS1 and DSCR9), 2 miRNAs (hsa-miR-431-5p and hsa-miR-590-3p) and 8 mRNAs (GPM6A, ISL1, SH3GL3, PRIMA1, KCNQ5, PCDH9, NTNG1 and POU4F1) were identified (Table 2). Collectively, the 13 optimized DERs were correlated with the prognosis of BC patients, which was selected for the construction of risk score model.

3.4. Construction of predictive signature

Following are the risk scores in the training cohort:
Risk score = $(-0.30118 \times$ LINC00161 expression level) $+ (0.40446 \times$ ZFHX4-AS1 expression level) $+ (-0.52441 \times$ DSCR9 expression level) $+ (0.11872 \times$ hsa-miR-431-5p expression level) $+ (-0.21469 \times$ hsa-miR-590-3p expression level) $+ (-0.19766 \times$ GPM6A expression level) $+ (0.15216 \times$ ISL1 expression level) $+ (0.04723 \times$ SH3GL3 expression level) $+ (0.12835 \times$ PRIMA1 expression level) $+ (0.11512 \times$ KCNQ5 expression level) $+ (0.10289 \times$ PCDH9 expression level) $- (0.38562 \times$ NTNG1 expression level) $+ (-0.32468 \times$ POU4F1 expression level).

Table 1
KEGG and GO enrichment for the genes in the ceRNA regulatory network

Category	GO	Description	P value	Enrichment	Gene count
Biology Process	GO:0000904	Cell morphogenesis involved in differentiation	7.94E-06	6.6	9
	GO:0007409	Axonogenesis	2.51E-06	9.1	8
	GO:0007423	Sensory organ development	1.00E-05	7.6	8
	GO:0007507	Heart development	1.00E-06	8.4	9
	GO:0007517	Muscle organ development	1.58E-10	15	11
	GO:0007610	Behavior	1.26E-05	7.2	8
	GO:0014706	Striated muscle tissue development	3.16E-08	13	9
	GO:0032990	Cell part morphogenesis	5.01E-08	8.6	11
	GO:0048667	Cell morphogenesis involved in neuron differentiation	1.26E-06	8.2	9
	GO:0048812	Neuron projection morphogenesis	2.51E-08	9.1	11
	GO:0048858	Cell projection morphogenesis	3.16E-08	8.8	11
	GO:0048880	Sensory system development	5.01E-07	11	8
	GO:0060537	Muscle tissue development	3.16E-09	13	10
	GO:0061061	Muscle structure development	3.16E-08	8.8	11
	GO:0061564	Axon development	5.01E-06	8.3	8
	GO:0070848	Response to growth factor	6.31E-05	5.9	8
	GO:0071363	Cellular response to growth factor stimulus	3.98E-05	6.1	8
	GO:0120039	Plasma membrane bounded cell projection morphogenesis	3.16E-08	8.9	11
KEGG Pathway	Hsa04360	Axon guidance	5.01E-03	8.4	3
	Hsa04514	Cell adhesion molecules	2.51E-03	11	3
	Hsa04550	Signaling pathways regulating pluripotency of stem cells	2.51E-03	11	3

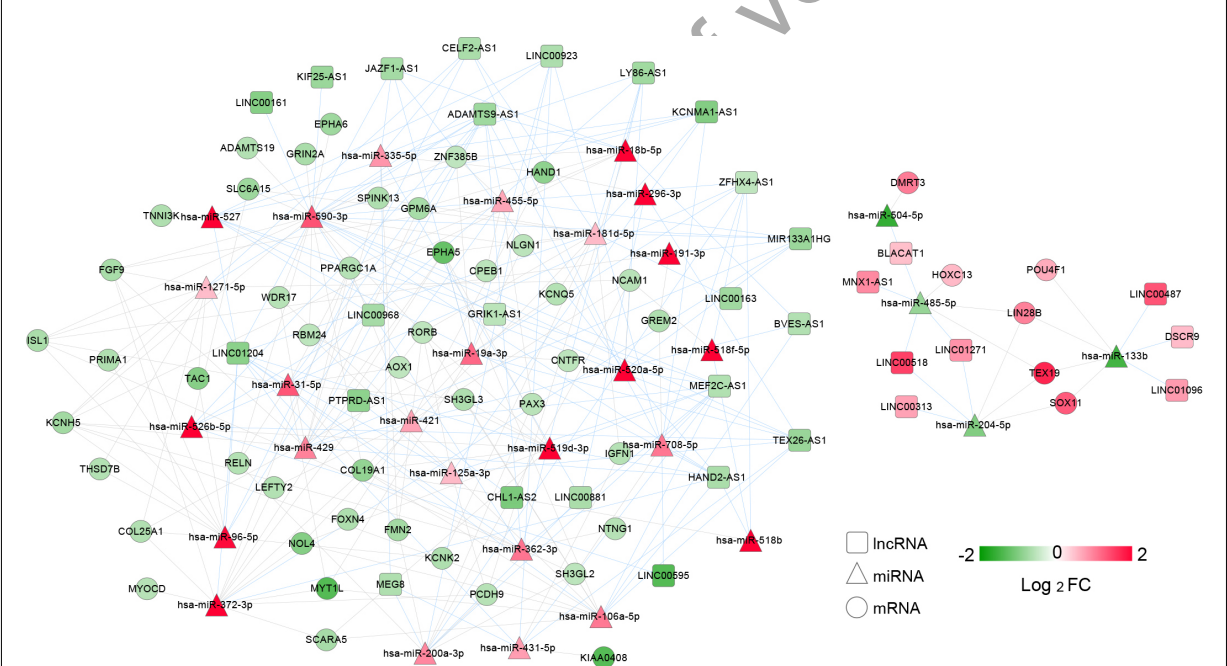Note: KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology.

Fig. 2. ceRNA regulatory network. Differentially expressed lncRNAs (DElncRNAs) were showed by the represented squares; the triangles showed differentially expressed microRNAs (DEmiRNAs) while differentially expressed mRNA (mRNA) were shown by the circles; the color key represented the log2FC. The red connecting lines represented the DElncRNA-DEmiRNA, while the gray connecting lines represented the DEmiRNA-DEmRNA regulatory connection.

With the cut-off point of median risk score in the training cohort, we found that 201 patients in high-risk (50.1%) group had a lower OS (hazard ratio (HR), 2.160; 95% CI cores, 1.586 to 2.942; $P = 5.678e-07$; Fig. 3A) than 200 patients in low-risk (49.9%) group. As for the validation cohort, 83 (50.3%) patients in high-risk group were associated with a poorer OS than 82 (49.7%) patients in low-risk group (HR, 1.675; 95%

Table 2
The optimized RNAs information

Symbol	Type	Multi-variate Cox regression analysis			Coef
		HR	95%CI	_P_ value	
LINC00161	lncRNA	0.964	0.935–0.993	1.540E-02	−0.30118
ZFHX4-AS1		1.039	1.001–1.079	4.990E-02	0.40446
DSCR9		0.923	0.867–0.983	1.300E-02	−0.52441
hsa-miR-431-5p	miRNA	1.133	1002–1.285	4.533E-02	0.11872
hsa-miR-590-3p		0.787	0.644–0.963	1.990E-02	−0.21469
GPM6A	mRNA	0.952	0.903–0.986	4.678E-02	−0.19766
ISL1		1.019	1.009–1.056	3.121E-02	0.15216
SH3GL3		1.011	1.006–1.045	4.537E-02	0.04723
PRIMA1		1.015	1.007–1.062	4.524E-02	0.12835
KCNQ5		1.013	1.007–1.066	4.656E-02	0.11512
PCDH9		1.011	1.005–1.090	4.797E-02	0.10289
NTNG1		1.049	1.003–1.114	1.226E-02	0.38562
POU4F1		0.965	0.936–0.996	2.520E-02	−0.32468

Note: HR, hazard ratio; CI, confidence interval; Coef, cox regression coefficient.

Table 3
Clinical factor statistics and cox regression analysis

Clinical characteristics	TCGA ($N =$401)	Uni-cox regression		Multi-cox regression	
		HR [95%CI]	Pvalue	HR [95%CI]	Pvalue
Age(years,mean $\pm$ sd)	68.01 $\pm$ 10.59	1.033 [1.017–1.049]	**2.544E-05**	1.265 [1.187–1.680]	**1.913E-02**
Gender(Male/Female)	296/105	0.869 [0.627–1.203]	3.963E-01	–	–
Pathologic_M(M0/M1/-)	194/11/196	3.366 [1.607–7.049]	6.283E-04	1.381 [0.245–7.782]	7.144E-01
Pathologic_N(N0/N1/N2/N3/-)	232/45/75/7/42	1.562 [1.326–1.840]	4.327E-08	1.198 [0.544–2.636]	6.539E-01
Pathologic_T(T1/T2/T3/T4/-)	4/117/190/58/32	1.742 [1.390–2.182]	1.205E-06	1.237 [0.546–2.800]	6.101E-01
Pathologic_stage(I/II/III/IV/-)	2/128/137/132/2	1.708 [1.410–2.068]	1.787E-08	1.074 [0.245–2.256]	6.015E-01
Neoplasm histologic grade(High/Low/-)	378/20/3	2.888 [0.715–11.68]	1.367E-01	–	–
Tumor metastasis(Yes/No/-)	68/33/300	1.002 [0.588–1.709]	9.934E-01	–	–
Radiotherapy(Yes/No/-)	20/355/26	1.065 [0.523–2.169]	8.624E-01	–	–
Lymphovascular invasion(Yes/No/-)	145/129/127	2.225 [1.528–3.240]	1.877E-05	1.949 [0.806–4.711]	1.387E-01
Recurrence (Yes/No)	85/248/68	1.873 [1.295–2.709]	7.075E-04	1.643 [1.252–3.591]	2.130E-02
PS status (High/Low)	200/201	2.160 [1.586–2.942]	5.678E-07	3.141 [1.449–6.810]	3.750E-03
Death (Yes/No)	176/225	–	–	–	–
Overall survival time(months,mean $\pm$ sd)	27.03 $\pm$ 27.90	–	–	–	–

Note: TCGA, The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval.

CI cores, 1.037 to 2.713; $P = 3.393$ e-02; Fig. 3B). The AUC for training data set was 0.852 while the validation was 0.740. Above all, these results implied that the prognostic model was with high sensitivity and specificity.

3.5. Construction of nomogram for predicting the OS of BC patients

To screen the clinical factors significantly related to prognosis, we analyzed the significance of clinical characteristics between BC and the controls using univariate and multivariate analyses. As shown in Table 3, we found that age, tumor recurrence and PS model status were the significant clinical factors related to prognosis. Further analyses of KM curves indicated that OS of patients aged younger than 60 years higher than the patients older than 60 years (HR 1.033, 95% CI 1.017

to 1.049; $p = 2.544$E-05). The OS of patients without recurrence better than the patients with recurrence (HR: 1.873, 95% CI 1.295 to 2.079; $p = 7.075$e-04). For the relapsed patients, low risk score meant an improved OS while the high risk one represented an poor OS (HR: 2.528, 95% CI 1.344 to 4.753; $p = 2.919$e-03). So did for the patients without recurrence (HR: 1.999, 95% CI 1.245 to 3.209; $p = 4.125$e-3) (Fig. 4).

A nomogram was built based on clinicopathological factors in the TCGA cohort to predict the prognosis of samples (Fig. 5). The predictors included age, recurrence and prognostic score. The calibration plots have good predictive ability for 3- and 5-year OS with the c index was 0.683 and 0.674, respectively.

The combination of all the features including age, tumor recurrence, 3 lncRNAs, 2 miRNAs, 8 mRNA could best discriminate the BC to healthy controls with the AUC of 0.901 for TCGA and 0.758 for GSE13507
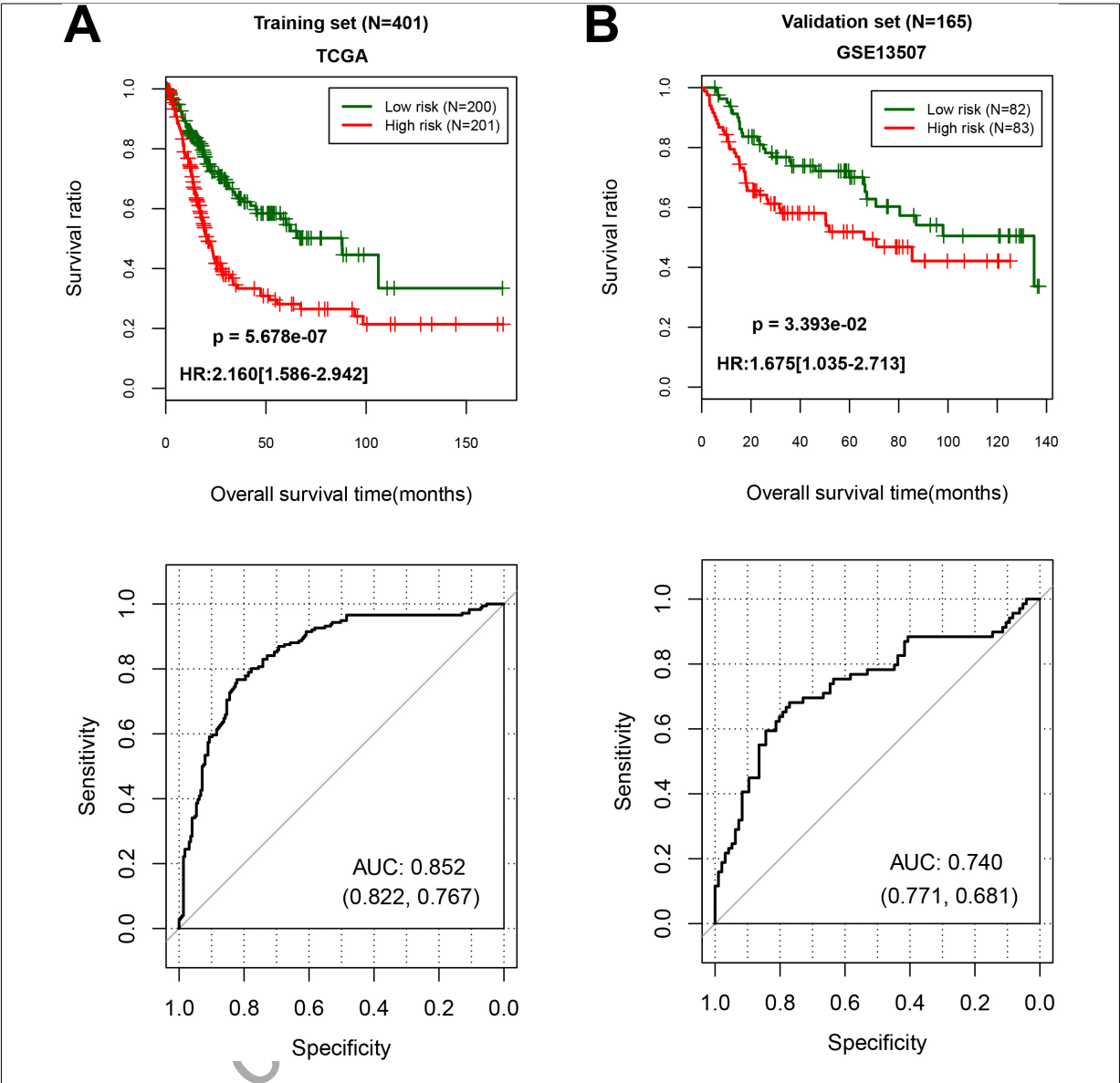
Fig. 3. The performance of 13 differentially expressed RNAs expression signature in predicting overall survival. (A) In training cohort, low risk group including 200 samples, and high risk group including 201 patients; (B) In validation cohort, low risk group including 82 samples, and high risk group including 83 patients.

(Fig. 6, Table 4). All these results suggested that the combined model based on the 13-DERs signature and clinical factors could accurately predict the prognosis and OS for BC patients.

4. Discussion

The present study identified novel prognostic RNAs (including 3 lncRNAs, 2 miRNAs , and 8 mRNA) in BC and constructed a risk model for predict the prog-

nosis of patients with BC. We validated the tool and found that it could improve the ability to predict prognosis in BC patients compared with clinical risk factors. Additionally, a nomogram comprising prognostic score and clinicopathological data including age and tumor recurrence was obtained to predict the OS. Our results indicated that the 13 DERs signature could be an indicator that categorized patients into a high-risk and a low-risk group, and the OS of the training and validation dataset differed significantly.

Table 4
ROC curve parameter information table for each type of model

| Type	AUC		C-index		*P* Value	
	TCGA	GSE13507	TCGA	GSE13507	TCGA	GSE13507
Age	0.595	0.564	0.606	0.549	2.087E-06	9.309E-11
Tumor recurrence	0.609	0.543	0.543	0.548	4.923E-02	2.989E-01
Clinical model	0.635	0.606	0.639	0.561	6.598E-09	1.643E-14
3 lncRNAs	0.678	0.661	0.611	0.552	2.242E-06	1.535E-01
2 miRNAs	0.648	0.665	0.573	0.558	3.728E-03	4.276E-02
8 mRNAs	0.785	0.738	0.608	0.605	3.073E-06	4.693E-03
Multi-RNAs prognostic score model	0.852	0.740	0.651	0.628	2.55E-11	6.74E-04
Combine model	0.901	0.758	0.694	0.651	6.26E-14	< 0.001

Note: TCGA, The Cancer Genome Atlas.
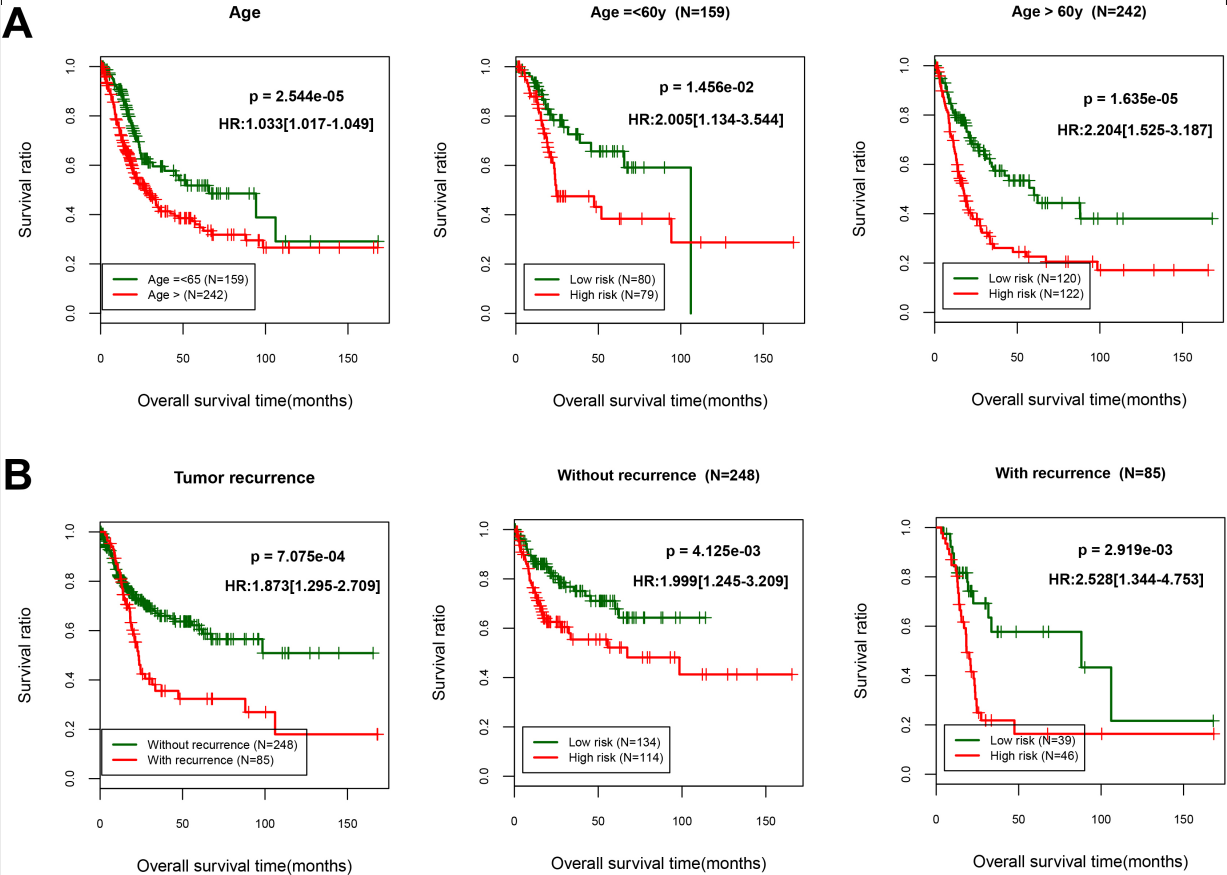
Fig. 4. Overall survival was evaluated using Kaplan-Meier curves in line with age and recurrence-tiered low-risk and high-risk scores. (A) Age, age $\geqslant 60$ years, $n = 159$, age > 60 years, $n = 242$; (B) Tumor recurrence, without recurrence, $n = 248$, with recurrence, $n = 85$.

We constructed a ceRNA-related 13-gene signature to explore the correlation between BC and lncRNA-associated ceRNA network based on the TCGA cohort using Lasso-penalized Cox regression algorithm. Previous studies have confirmed that the risk score model based on the novel signature could predict the prognosis and survival time of patients with BC [32,33,34,35]. These studies explored the model based on one type such as lncRNA or mRNA, which is inconsistent with our study. In our study, we constructed a ceRNA-related 13-gene signature including lncRNAs, miRNAs and mRNA that have high accuracy of prediction for prognosis of BC patients. Compared to unique type RNA biomarker, the biomarkers screened in this study is a mix of different type of RNAs including lncRNAs, miRNAs and mRNA [36,37,38]. The combination of the
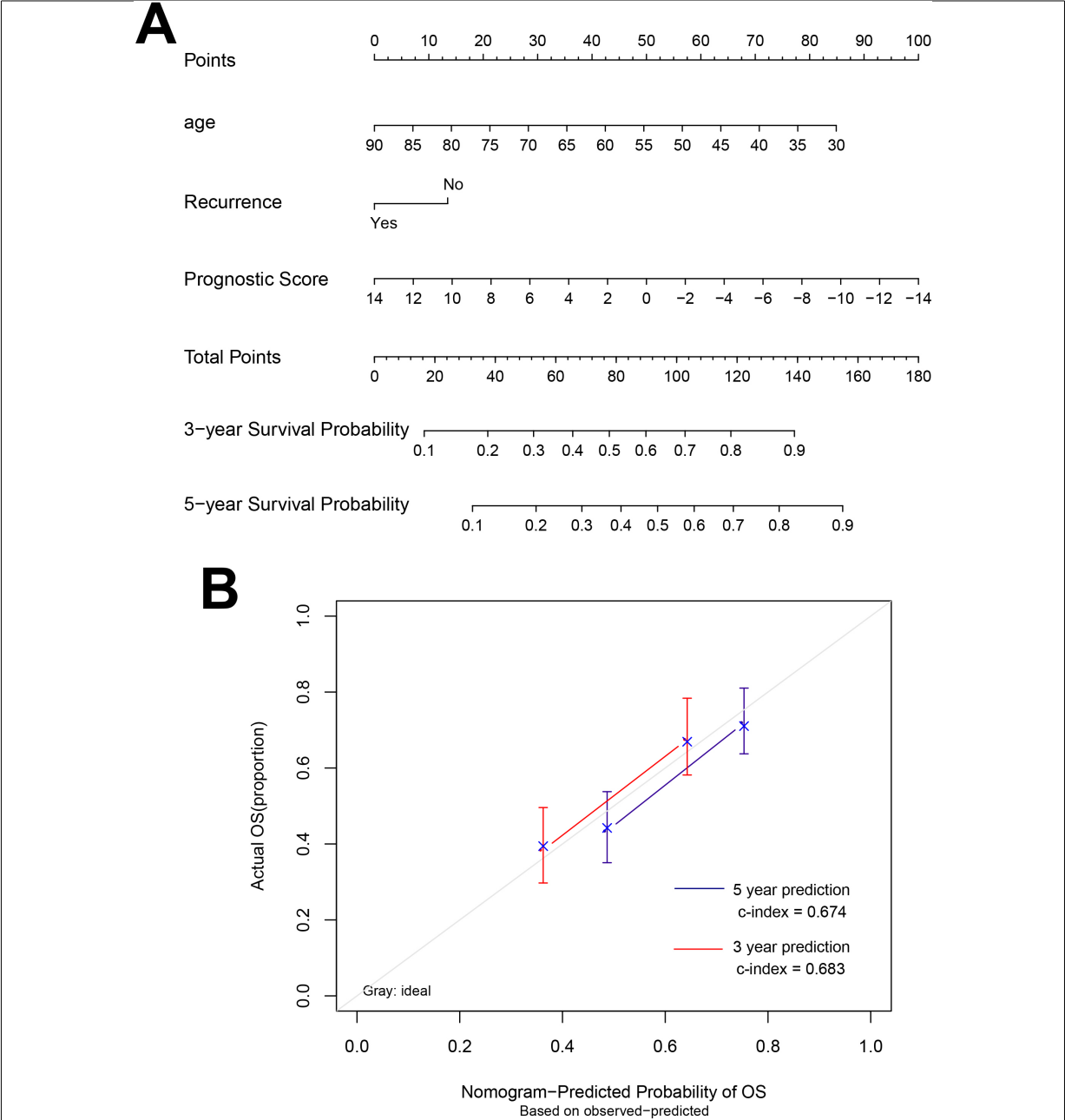
Fig. 5. Nomograms that predict the 3- and 5-year overall survival.

three type RNAs has the best AUC of 0.852 compared with the unique type of RNA.

Moreover, increasing evidence has implied that some of them might be involved in crucial biological functions in the progression of cancer. From the results of KEGG pathways, we found that NTNG1 (one mRNA in the 13-RNA signature) was involved in cell adhesion molecules pathway. Cell adhesion molecules are complex protein and carbohydrate molecules of many different types found on the surfaces of all cells. They are important in many aspects of cell biology including development, differentiation, and motility. These processes are frequently disturbed in cancer and recent work has demonstrated that disturbances in cell adhesion molecule expression are also common in malignancy [39]. Furthermore, NTNG1 has been reported
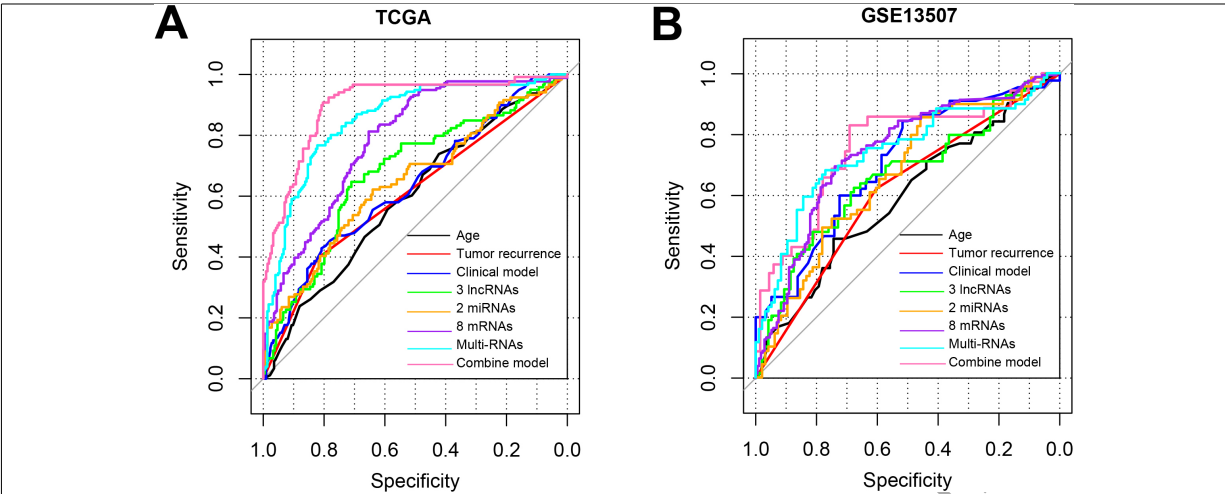
Fig. 6. The ROC curve of training set TCGA (A) and validation set GSE13507 (B) which based on the results of each type of model.

to be potential tumor diagnostic markers in multiple cancers including BC, Breast invasive carcinoma, and Colon adenocarcinoma [40]. In addition, ISL1 involved in Signaling pathways regulating pluripotency of stem cells. For classic bladder exstrophy, ISL1 has been reported as a significant susceptibility gene, and it regulates the development of urinary tract [41,42,43]. Its abnormal methylation could use to predict BC patients outcome [41,44,45,46]. The two mRNA were the important biomarkers to predict prognosis, which might be regarded for targeted therapy for patients with BC. The other DERs need much experiments to explore their function in BC. This study provided a basic therapies for application of the DERs in targeted therapy.

Notably, the signature was validated in another dataset related to BC (GSE13507). Interestingly, patients with BC in the low-risk group have a favorable survival advantage through KM curves. Indeed, the each AUC values of ROC plots in 1, 3 and 5 years were nearly high in the training dataset and validation dataset. These results suggested that the model had excellent accuracy for predicting the prognosis of patient with BC. Furthermore, we also found that risk score in model was related to age and recurrence. Previous studies have indicated that age and recurrence were significantly correlated with the prognosis of BC patients [47,48]. The two factors have potential prediction ability for patients with BC. Besides, we also found that a nomogram was built based on tumor recurrence and age to predict the OS which could provide simple, accurate prognosis predictions for BC.

The study also has limitations. First, in order to be used widely, an operation-friendly methods such as qPCR should be performed to further validate the model. Second, additional biological experiments should be performed to validate the mechanism of the biomarkers especially the non-coding RNAs. Third, the sample of the validation dataset used in this study is tissue which is not non-invasive. So the data from the blood or the urine should be included to validate the model in the future.

5. Conclusion

In summary, this study suggested that lncRNA-related ceRNA signature is highly correlated with BC. Moreover, we constructed the prognosis model based on 13-RNAs signature that is a potential prognostic tool for predicting the prognosis of BC patients. Additionally, the 13 lncRNA-related RNAs might be considered as the reliable therapeutic biomarkers for predicting the prognosis of BC patients.

Data availability statement

The datasets generated and/or analysed during the current study are available in the NCBI repository, https://www.ncbi.nlm.nih.gov/geo/. Accession number: GSE13507.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethics of approval statement

Not Applicable.

Patient consent statement

Not Applicable.

Permission to reproduce material from other sources

Not Applicable.

Clinical trial registration

Not Applicable.

Author contributions

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