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ORIGINAL RESEARCH

Extracellular Matrix–Related Six-lncRNA Signature as a Novel Prognostic Biomarker for Bladder Cancer

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Introduction: Bladder cancer (BC) is the fourth-commones cancer and the sixth-leading cause of cancer-related death among men. However, a lack of reliable biomarkers remains a problem forprognosis and treatment of BC. lncRNAs have been shown to play important roles in various cancers, and have emerged as promising biomarkers for cancer prognosis and treatment.

Methods: In this study, using univariate and multivariate Cox regression analysis, we examined the differential expression profiles of 1,651 lncRNAs in the TCGA BLCA cohort and created a prognostic gene signature composed of six lncRNAs (for *SNHG12*, *MAFG-DT*, *ASMTL-AS1*, *LINC02321*, *LINC01322*, and *LINC00922*), designed the SMALLL signature.

Results: The SMALLL signature displayed significant prognostic power for overall survival for BC patients in multiple cohorts. Gene Ontology analysis showed that genes coexpressed with the SMALLL signature were associated with the extracellular matrix network, and immune cell–infiltration analysis showed that activated naïve B cells, regulatory T cells, M0 macrophages, eosinophils, resting memory CD4 T cells and resting NK cells were significantly different in high- and low-risk groups. We also confirmed differential expression of the lncRNAs of the SMALLL signature in BC tissue and paracancer normal tissue by qRT-PCR analysis. Cell-invasion and -migration experiments showed that *MAFG-AS1*, *ASMTL-AS1*, *LINC02321*, and *LINC00922* significantly affected cell invasion and migration.

Conclusion: Our study revealed that the lncRNA signature is an important predictive factor of prognosis and provides a promising biomarker for BC.

Keywords: long noncoding RNA, bladder cancer, extracellular matrix, immune-cell infiltration, prognosis, signature

Introduction

Bladder cancer (BC) is the fourth-commonest cancer and the sixth-leading cause of cancer-related death among men.[1] With regard to histological characteristics, nearly 90% of BCs are urothelial carcinoma, of which approximately 75%–85% are classified as non–muscle invasive BC (NMIBC) and the rest MIBC.[2] At present, treatment options for BC include surgery,[3] radiotherapy, chemotherapy,[4] immunotherapy,[5] and recently developed immunocheckpoint inhibitors.[6] In general, the American Joint Committee on Cancer TNM-staging system is used to guide prognosis and management options for BC patients. However, although patients with NMIBC have favorable survival after initial treatment, 5-year recurrence and 5-year progression remain as high as 70% and 30%, respectively.[7,8] As such, there

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is an urgent need for development of effective prognostic biomarkers for earlier detection and precise recurrence prediction of BC.

lncRNAs are RNA transcripts that are longer than 200 bases and lack translational potential.[9] Although the functions of lncRNAs have not been fully elucidated, studies have shown that lncRNAs play an important role in dose-compensation effects, epigenetic regulation, cell proliferation and differentiation, chromatin modification, immunity, and other biological processes.[10,11] Mounting evidence indicates that the deregulation of lncRNA is implicated in the tumorigenesis and progression of various types of cancers, including BC.[12,13] For example, *UCA1* lncRNA is upregulated in BC.[14] *UCA1* overexpression promotes cell-cycle progression, carcinogenesis, and cancer invasion of BC cells through enhancing ERK1/2, MAPK, and PI3K–Akt pathways.[15] *H19* is another well characterized lncRNA that is highly expressed in BC and many other cancers.[16,17]

Lack of an effective prediction approach is one of the main causes of poor prognosis of BC patients. Emerging evidence has suggested that lncRNAs may serve as novel biomarkers for accurate prognosis of cancer patients.[18] Various studies have shown that lncRNA expression is significantly associated with survival in patients with ovarian cancer,[19] papillary thyroid carcinoma,[20] pancreatic cancer,[21] non–small cell lung cancer,[22] and esophageal cancer.[23] Recently, a set of immune-related lncRNAs was found to be significantly correlated with progression of diverse cancer types, including hepatocellular carcinoma,[24] breast cancer,[25] non–small cell lung cancer,[26] anaplastic gliomas,[27] glioblastoma multiforme,[28] and diffuse large B–cell lymphoma.[29] In addition, multiple lncRNA-based gene signatures have been developed to predict recurrence in hepatocellular carcinoma,[30] breast cancer,[31] and colorectal cancer,[32] as well as BC.[33–35] These findings indicate better efficacy of lncRNA signatures in predicting prognosis than individual lncRNAs.

Changes in the composition, physical properties, and spatial conformation of the extracellular matrix (ECM) play an important role in the process of tumor proliferation and invasion. Witkowski et al found that there is extensive immunomicroenvironment remodeling in B-cell acute lymphoblastic leukemia.[36] Varn et al found that there are complex interactions between different immune-cell types in the tumor microenvironment and proved that these interactions have a significant impact on patient survival.[37] Through the pathway enrichment analysis of genes related to SMALL (*SNHG12*, *MAFG-DT*, *ASMTL-AS1*, *LINC02321*, *LINC01322*, and *LINC00922*) characteristics, we found that these genes significantly enriched pathways related to the ECM, including ECM structural constituents, cell adhesion–molecule binding, actin binding, and glycosaminoglycan binding.

In this study, we developed a novel lncRNA signature for survival prediction of BC patients, with increased patient samples and improved methods. We conducted a comprehensive analysis of lncRNA-expression profiles in a cohort of 372 BC patients from the Cancer Genome Atlas (TCGA) database, and identified and validated a six-lncRNA signature to predict overall survival (OS) of patients with BC. In addition, we verified the accuracy of the signature for survival prognosis in two external data sets (GSE31684 and GSE32894), and compared the signature we established with other existing BC lncRNA prognostic signatures.

Methods

BC Data-Set Retrieval and Differential Gene-Expression Analysis

To screen lncRNAs that can be used as effective prognostic markers for BC patients, gene-expression profiles and clinical data of the TCGA Urothelial Bladder Carcinoma (BLCA) cohort were downloaded from the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov). The TCGA BLCA cohort comprises 429 cases, and detailed information is available on the portal. Gene-expression profiles and survival data of GSE31684 and GSE32894 were downloaded from the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo).

After transformation of Ensembl IDs to gene symbols, mRNA and lncRNA profiles were extracted for differential analysis using the R program. Samples were sorted into a tumor group and a normal group. Then the EdgeR program was used to normalize the data and determine differentially expressed genes. Briefly, the estimateCommonDisp and estimateTagwiseDisp functions were used to calculate ordinary dispersion and dispersion within the gene range, respectively, then the exactTest function was used to perform Fisher's exact test. Finally, the topTags function was used to extract differentially expressed genes. In order to analyze correlations between expression characteristics of lncRNA and OS and recurrence of BC patients, 372 BC cases with complete clinical information were included in this study. These were randomly divided into a training group and a validation group. Detailed clinical features of all groups were listed in Table 1.

Table 1 Clinical Features of BC Patients in Training and Validation Groups

	Training (188)	Validation (184)	*P*-value	SMD
Time, mean (SD)	795.09 (881.34)	758.40 (768.58)	0.669	0.044
Status, n (%)				
Alive	113 (60.1)	106 (57.6)	0.674	0.051
Dead	75 (39.9)	78 (42.4)		
Age (years), n (%)				
<70	97 (51.6)	98 (53.3)	0.757	0.033
≥70	91 (48.4)	86 (46.7)		
Sex, n (%)				
Female	45 (23.9)	50 (27.2)	0.479	0.074
Male	143 (76.1)	134 (72.8)		
Grade, n (%)				
High	178 (94.7)	176 (95.7)	0.81	0.045
Low	10 (5.3)	8 (4.3)		
Disease stage, n (%)				
I	2 (1.1)	0	0.124	0.248
II	48 (25.5)	58 (31.5)		
III	76 (40.4)	58 (31.5)		
IV	62 (33.0)	68 (37.0)		
T, n (%)				
T1	3 (1.6)	1 (0.5)	0.636	0.166
T2	53 (28.2)	59 (32.1)		
T3	101 (53.7)	88 (47.8)		
T4	26 (13.8)	30 (16.3)		
TX	5 (2.7)	6 (3.3)		
M, n (%)				
M0	85 (45.2)	90 (48.9)	0.69	0.089
M1	4 (2.1)	5 (2.7)		
MX	99 (52.7)	89 (48.4)		
N, n (%)				
N0	114 (60.6)	101 (54.9)	0.007	0.4
N1	24 (12.8)	19 (10.3)		
N2	28 (14.9)	46 (25.0)		
N3	8 (4.3)	0		
NX	14 (7.4)	18 (9.8)		
Race, n (%)				
Caucasian	153 (81.4)	145 (78.8)	0.604	0.065
Others	35 (18.6)	39 (21.2)		
Recurrence, n (%)				
No	109 (58.0)	116 (63.0)	0.372	0.104
Yes	79 (42.0)	68 (37.0)		

Abbreviation: BC, bladder cancer.

Risk-Score Calculation

Univariate Cox proportional-hazard regression analysis was carried out for the differentially expressed lncRNAs to screen the candidates that significantly correlated with OS of BC patients. Next, correlation coefficients of these candidate lncRNAs were generated by multivariate Cox regression analysis. Expression of the selected lncRNAs was weighted linearly according to their coefficients, and a risk-score formula was obtained:

$$\text{risk score} = \sum_{i=}^{n}(Exp(lncRNAi) * Coe(lncRNAi)$$

where i is the identifier of selected lncRNAs, n the number of lncRNA genes in prognosis, *Exp* the expression value of lncRNA, and *Coe* the estimated regression coefficient of lncRNA on multivariate Cox proportional-hazard regression analysis. This risk-scoring model fully considers the function of each prognostic lncRNA gene. Each patient received a risk score: a linear combination of significant levels of lncRNA expression weighted by their respective Cox regression coefficients.

Pathway-Enrichment Analysis and Possible Mechanism Exploration

All coexpressed genes were selected for pathway enrichment analysis. The R package ClusterProfiler[38] was used for enrichment. The threshold of significance of false positive ratefor biological processes and pathway of significant enrichment was set at 0.05. GSEA version 4.0.2[39,40] was used for biological processes and pathway-enrichment analysis. The threshold of significance was set as 0.05, and normalized enrichment score (NES) was set at |NES|>1. We introduced coexpressed genes with correlation coefficients >0.6 into STRING (http://string-db.org) to establish a PPI network. Next, we imported the PPI network into Cytoscape and used the CytoHubba plug-in to find the hub genes.

Table 2 Nucleotide sequence of primers for lncRNA qPCR

lncRNA	Sequence
ASMTL-AS1	Forward: 5′-TGACAGTCTCTGCACCAAGG-3′
ASMTL-AS1	Reverse: 5′-CTCAGTCCTTCTCCGCACTC-3′
LINC01322	Forward: 5′-AGCAAACCACAGGAAACCAC-3′
LINC01322	Reverse: 5′-GCAATCTGTTTTGGCTCCCA-3′
SNHG12	Forward: 5′-AAGGACGGGTTTTAGGCATT-3′
SNHG12	Reverse: 5′-CAGTCTTGATGGGACCGTTT-3′
LINC00922	Forward: 5′-CACTCACGAAGCACACACAA-3′
LINC00922	Reverse: 5′-TCTGGCAGGGGTACATTCTG-3′
MAFG-AS1	Forward: 5′-TCCCGTGTCTGGACTTTCTC-3′
MAFG-AS1	Reverse: 5′-GGGAGGTGAGGAGATCTTCG-3′
LINC02321	Forward: 5′-ACCCTTCTGACCACCAAGTG-3′
LINC02321	Reverse: 5′-CAAGCCAAGCCTTGAAAAAG-3′

Immune Cell–Infiltration Analysis

As an important part of the tumor microenvironment, immune cells play an important role in tumor growth and metastasis.[36] Therefore, we performed an in silico deconvolution of 22 immune-cell types through the CIBERSORT algorithm (https://cibersort.stanford.edu).[41] After deleting samples with no statistical significance, 304 samples remained, and these were split into high-risk (n=153) and low-risk (n=151) cohorts by the risk model. A nonparametric Wilcox test was performed to assess whether the infiltration of immune cells differed between the high-risk and low-risk groups. A violin plot was selected to show results, in which blue represented the low-risk cohort and red the high-risk cohort.

lncRNA-Expression Analysis in Human BC Tissue

All the BC tissue samples (n=20) were obtained by surgery with the written consent of patients who underwent surgery at the First Affiliated Hospital of Kunming Medical University. All samples were pathologically confirmed as urothelial carcinoma by two pathologists independently. Ethics consent was approved by the First Affiliated Hospital of Kunming Medical University's Committees for Ethical Review of Research Involving Human Subjects. Total RNA was extracted with the Eastep Super Total RNA-extraction kit, followed by cDNA synthesis using the GoScript reverse-transcription system (Promega). Expression of lncRNAs was determined by qPCR using Go-taq qPCR Master Mix (Promega). Primers (Table 2) designed using Primer3 (http://primer3.ut.ee), and DNA oligos were synthesized by Xinfan Biotechnology, Shanghai, China.

shRNA Interference

DMEM, FBS, penicillin–streptomycin, trypsin–EDTA and PBS were purchased from Biological Industries (Kibbutz Beit Haemek, Israel). SVHUC1, RT4, T24 and UMUC3 cell lines were purchased from Kunming Institute of Zoology (Chinese Academy of Sciences, Kunming, China). Cell cultures were routinely grown at 37°C with 5% CO_2 using DMEM + 10% FBS + 1% penicillin–streptomycin solution. Plasmids of shRNA targeting *MAFG-AS1* and negative

shRNA were designed and synthesized by Shanghai Jikai Gene Technology (GenePharma). The shRNA sequence was CAGGGCAATTCCAACCAAGAA. Transfections were performed according to the protocol provided by the manufacturer (Lipo3000, Thermo Fisher Scientific). Cell-invasion and -migration assays were performed using the Cultrex (Trevigen, MD, USA) BME.

Statistical Analysis

To discover potential factors affecting the prognosis of BC patients, univariate Cox proportional-hazard regression was applied to determine associations between lncRNA expression and OS in the training group. If corrected $P<0.05$, corresponding lncRNAs were statistically significant and considered as candidate prognostic lncRNAs of BC. Multivariate Cox proportional-hazard regression was carried out among the pool of candidate prognostic lncRNAs, and those with corresponding $P<0.005$ were identified as optimal prognostic lncRNAs impacting the survival of BC patients.

An individual risk score for each patient was built for predicting prognosis by including expression of each optimal prognostic lncRNA, weighted by their estimated regression coefficients on multivariate Cox regression model:

$$\text{risk score } = \sum_{i=}^{n}(Exp(lncRNAi) * Coe(lncRNAi)$$

The risk-score model was a measure of prognostic risk for each BC patient. Based on the median risk score of the training group as the cutoff, patients with BC were sorted into two groups: high risk and low risk. A high risk score indicated poor survival for BC patients.

In each group, we validated the reliability and validity of the risk-score formula. Kaplan–Meier analysis was utilized to compare the survival rate of the groups. The survival difference between the low-risk and high-risk groups was assessed by the log-rank test. Time-dependent ROC-curve analysis for OS was used to display the performance of the lncRNA risk model. Univariate and multivariate analyses with Cox proportional-hazard regression for OS were performed on individual clinical risk factors with and without the six-lncRNA signature in each cohort. HRs and 95% CIs were estimated.

In addition, multivariate Cox proportional-hazard analysis and stratified data analysis were used to test whether the risk score was independent of other clinical features, with age, sex, race, pathological grade, TNM stage, and recurrence status as covariates. $P<0.05$ was considered statistically significant.

All the analyses were implemented in SPSS version 24.0 or R version 3.6.1 with the following packages:"Limma", "EdgeR", "Pheatmap", "Caret", "Survival", "Magrittr", "Survminer", "SurvivalROC", "RMS", "PreprocessCore", "Cibersort", "Vioplot", "org.Hs.eg.db", "ClusterProfiler", "ggpubr", and "ggplot2". All the hypotheses were two-sided, and $P < <0.05$ was considered statistically significant.

Results

The TCGA BLCA cohort (n=372) was randomly divided into a training group (n=188) and a validation group (n=184). There were no significant differences in survival time, survival state, age, sex, race, pathological grade, disease stage, T stage, M stage, or recurrence between the two groups, but for N stage, $P<0.05$ (Table 1). To determine the correlation between of lncRNA expression and the OS rate of BC patients, we carried out a variation analysis of mRNA and lncRNA expression, respectively, in the training group (Figure 1A, B). Next, we analyzed the differentially expressed lncRNAs in the training group by univariate Cox regression analysis. Interestingly, we found thatexpression levels of several lncRNAs were closely related to OS of the patients ($P<0.05$). Then, multivariate Cox proportional-hazard regression analysis resulted in six top candidates for a gene signature, designated the SMALLL signature, based on the first letter of the component genes: *SNHG12*, *MAFG-AS1*, *ASMTL-AS1*, *LINC02321*, *LINC01322*, and *LINC00922*. Among the six lncRNAs, positive coefficients indicated that expression of *MAFG-AS1*, *LINC02321*, *LINC01322*, and *LINC00922* correlated with longer OS, whereas expression of *SNHG12* and *ASMTL-AS1* inversely correlated with survival of patients (Table 3).

To assess whether the combination of the identified lncRNAs improved power in predicting OS of BC patients, we established a risk-scoring formula for OS prediction according to expression of the six lncRNAs: risk score= (−0.301151077 × expression value *SNHG12*) + (0.316595657 × expression value *MAFG-AS1*) + (0.172517499 × expression value *LINC02321*) + (0.179091241 × expression value *ASMTL-AS1*) + (0.158178271 × expression value *LINC01322*) + (0.124204122 × expression value *LINC00922*). Then the risk scores of the SMALLL signature were calculated for each patient in the training group. Next, we ranked the patients according to their risk scores and divided them into high-risk (n=94) and low-risk (n=94) groups, with the median risk score of the training group as the cutoff value (Figure 1C). As expected, OS of BC patients with higher risk scores (39.36%) was shorter than that of BC patients with lower risk scores (77.02%, Figure 1E). In addition, expression of high-

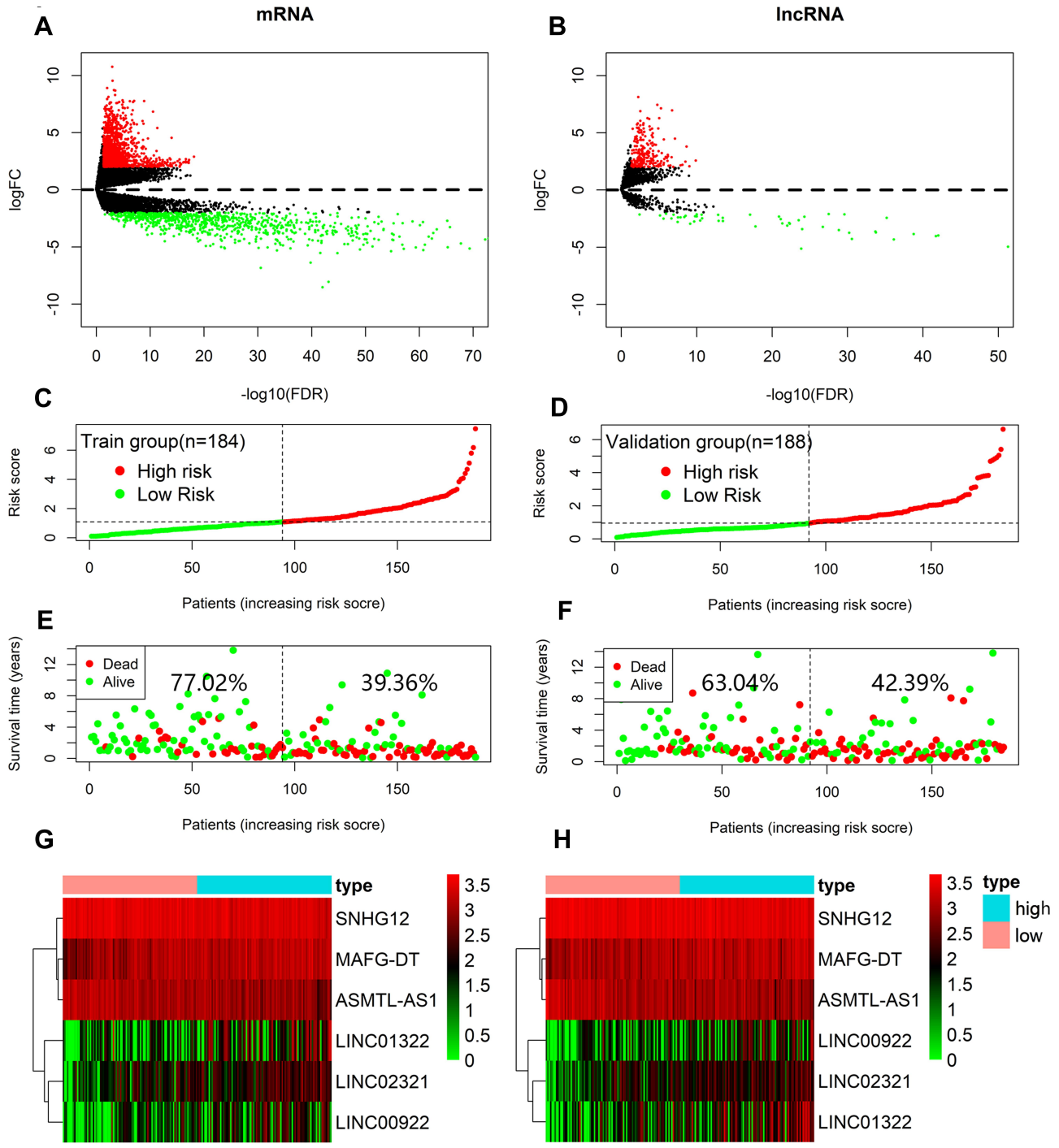

Figure 1 Construction of the six-lncRNA risk model of BC. Volcano graph of mRNA differential analysis results. (**B**) Volcano graph of lncRNA differential analysis results. (**C, D**) lncRNA signature risk–score distribution in the training group (**C**) and validation group (**D**). (**E, F**) BC patients' survival status in the training group (**E**) and validation group (**F**). (**G, H**) Heatmap of lncRNA-expression profiles in the training group (**G**) and validation group (**H**). Rows represent lncRNAs, and columns represent patients. Red, high expression; blue, low expression.

risk lncRNAs (*MAFG-AS1*, *LINC02321*, *LINC01322*, and *LINC00922*) was higher in the high-risk group, while there was no significant difference in expression of low-risk lncRNAs (*SNHG12* and *ASMTL-AS1*) between the high-risk and low-risk groups (Figure 1G). To validate the prognostic performance of the SMALLL signature, we evaluated its prognostic power in the validation group derived from the TCGA BLCA cohort. Using the risk-score formula, patients in the

Table 3 Six lncRNAs Significantly Associated With OS of BC Patients in the Training Group

	Ensembl	Coefficient[a]	Exp(coef)	SE(Coef)	Z	Pr(>|Z|)[b]
SNHG12	ENSG00000197989	−0.3012	0.7400	0.1725	−1.7456	0.0809
MAFG-AS1	ENSG00000265688	0.3166	1.3724	0.1050	3.0156	0.0026
LINC02321	ENSG00000258884	0.1725	1.1883	0.0782	2.2072	0.0273
ASMTL-AS1	ENSG00000236017	−0.1791	0.8360	0.1042	−1.7192	0.0856
LINC01322	ENSG00000244128	0.1582	1.1714	0.0461	3.4279	0.0006
LINC00922	ENSG00000261742	0.1242	1.1322	0.0614	2.0245	0.0429

Notes: [a]Derived from univariate Cox proportional-hazard regression analysis in the 188 training-group patients. [b]Obtained from permutation test repeated 10,000 times.
Abbreviations: BC, bladder cancer; OS, overall survival.

validation group were divided into high-risk (n=92) and low-risk (n=92, Figure 1D) groups. OS of BC patients and expression patterns of the six lncRNAs were similar to the training group (Figure 1F and H).

SMALLL Signature Significantly Predicted OS of BC Patients

Kaplan–Meier analysis showed that OS of the high-risk group was significantly lower than the low-risk group (HR 2.89, 95% CI 0.24–0.58; P=4.626^{-66}, Figure 2A) in the training group (n=188). The OS rate of the high-risk group was 59.1% in 24 months, 38.9% in 48 months, 31.9% in 72 months, 25.8% in 96 months, and 85.6%, 69.1%, 60.6% and 57.9% in the low-risk group, respectively. A similar effect was also observed in the validation group (Figure 2B). To further test the utility of this signature, we examined its prognostic performance in the survival of BC patients in two additional publicly available BC cohorts: the GSE31684[42] and GSE32894[43] data sets. The results showed that the survival rate in the high-risk group was significantly lower than the low-risk group, with P=3.677^{-4} (GSE31684, Figure 2C) and 3.552^{-3} (GSE32894, Figure 2D), respectively. Three distinct lncRNA gene signatures have been reported to have significant prognostic performance on survival of BC patients.[34,44,45] The composition of our signature is different from these published signatures. In addition, ROC analysis showed that the SMALLL signature performed better than the three lncRNA signatures (Figure 2E).

Survival Prediction by SMALLL Signature Was Independent of Classic Clinical Factors

We conducted multivariate Cox proportional-hazard regression analysis and multi-ROC analysis to assess whether the SMALLL signature predicted OS of BC independently of classic clinical and pathological factors. Selected covariates included patient age, sex, race, pathological grade, TNM stage, and recurrence status (Tables 4 and 5). Our results showed that the prognostic risk score for the SMALLL signature (HR 1.797, 95% CI 1.350–-2.393; P=0.001) was independent of these clinical features in the training group (Figure 3A). Consistently, similar results were obtained in the validation group and all samples: validation group — high-risk group vs low-risk group (HR 1.002, 95% CI 0.717–1.401; P=0.05); all samples — high-risk group vs low-risk group (HR 1.662, 95% CI 1.293–2.137; P<0.001, Figure 3B and C). We noticed that in the validation group, the statistical significance of the multivariate analysis was marginal (Figure 3B, right panel). The variation might have been due to limited sample size, as the statistical significance improved markedly when all samples were included (Figure 3C, right panel). To compare sensitivity and specificity between various clinical parameters and the SMALLL signature in OS prediction for BC patients, we carried out ROC analysis. As shown in Figure 3D, the predictive capability of the SMALLL signature (AUC 0.753) had greater predictive power than the classic clinical parameters (AUC 0.514–0.671).

Functional Enrichment Analysis and Possible Mechanism Exploration

To determine potential biological roles involved in the SMALLL signature, we performed a functional enrichment analysis for coexpression of genes. Gene Ontology (GO) analysis showed that these genes were significantly enriched in cancer-related networks, including ECM structural constituents, cell adhesion–molecule binding, focal adhesion, and regulation of actin cytoskeleton (Figure 4A). Additionally, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that these genes were significantly enriched in focal adhesion, regulation of actin cytoskeleton, the Fanconi anemia pathway, ECM-receptor

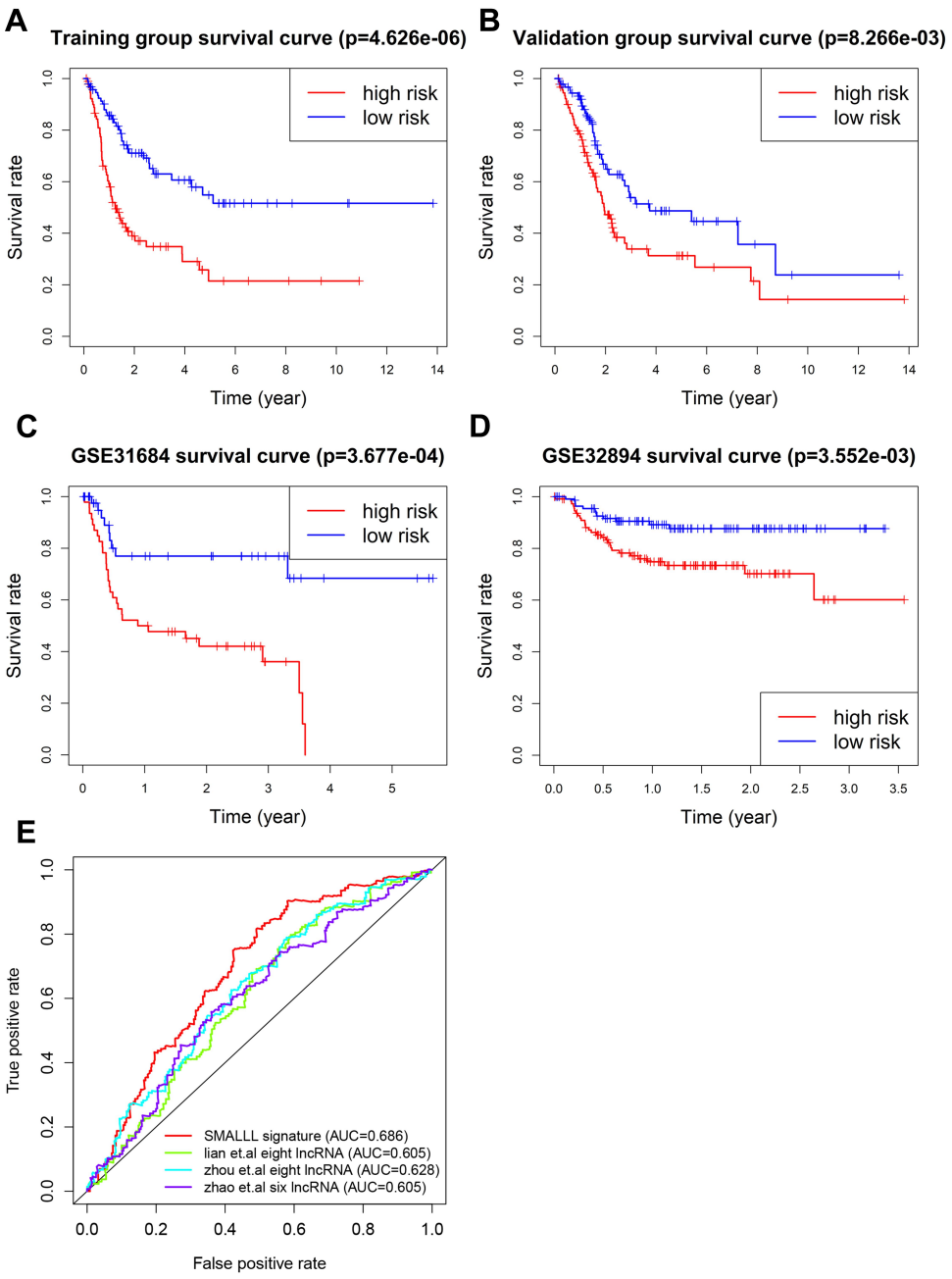

Figure 2 Effectiveness of the risk model. (**A–D**) Survival analysis of different risk cohorts in the training group (**A**), validation group (**B**), GSE31684 (**C**), and GSE32894 (**D**). (**E**) Multi-ROC curve of different clinical characteristics (age, sex, stage, pathological T stage, pathological M stage, pathological N stage, race, recurrence status), and risk score.

interaction, and DNA replication (Figure 4B). GSEA results showed that the transcriptional profile of the high-risk group was positively correlated with the pathways of adherens-junction organization (P<0.05, |NES|>1), cell-matrix adhesion (P<0.05, |NES|>1), cell substrate–junction assembly (P<0.05, |NES|>1), glycoprotein-metabosm processes (P<0.05, |NES|>1), and response to inorganic substances (P<0.05, |NES|>1; Figure 4C–G). Then, we found hub genes through the PPI network of co-express ed genes. The top-nine hub genes — *COL3A1*, *COL1A2*, *COL1A1*, *COL5A1*, *COL6A1*, *COL5A2*, *COL6A2*, *COL6A3*, and *COL12A1* — all belong to the gene family encoding collagen (Figure 4H). These results indicated that the lncRNAs of the SMALLL signature might be functionally implicated in the regulation of ECM networks.

Table 4 Univariate and Multivariate Cox Regression Analysis on Associations between SMALLL Signature and OS of BC Patients in Training Group

	Total	High risk score	Low risk score	Univariate		Multivariate	
		Cases, n (MST)	Cases, n (MST)	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Overall	188	94 (15.7)	94 (62.9)	1.9 (1.48–2.43)	<0.001	1.8 (1.35–2.39)	<0.001
Age, years							
<70	98	42 (19.4)	56 (55.4)	1.01 (0.98–1.05)	0.52	1 (0.95–1.04)	0.84
≥70	90	52 (13.3)	38 (–)				
Sex							
Female	45	28 (8.8)	17 (–)				
Male	143	66 (16.8)	77 (60.3)	0.37 (0.17–0.81)	0.01	0.76 (0.29–1.98)	0.58
Grade							
High	179	93 (14.6)	86 (58.2)	—	—	—	—
Disease stage							
II	53	16 (55.2)		2.1 (1.27–3.47)	<0.05	0.76 (0.31–1.84)	0.54
III	65	37 (13.4)	28 (47.1)				
IV	68	40 (11.7)	28 (26.8)				
T							
T2	56	30 (12.7)	26 (–)	2.05 (1.24–3.39)	<0.05	1.66 (0.91–3.02)	0.10
T3	88	43 (16.8)	45 (–)				
T4	36	18 (14.6)	18 (55.4)				
M							
M0	88	41 (12.6)	47 (65.4)	2.53 (0.59–10.85)	0.20	1.11 (0.22–5.73)	0.90
MX	96	51 (16.4)	45 (33.2)				
N							
N0	108	53 (16.4)	55 (–)	1.9 (1.27–2.85)	<0.05	1.52 (0.74–3.13)	0.26
N1	27	13 (16.8)	14 (–)				
N2	31	20 (14.7)	11 (32.4)				
NX	16	8 (12.5)	8 (41)				
Race							
Caucasia	153	78 (11.9)	75 (–)	0.67 (0.3–1.46)	0.31	0.44 (0.18–1.03)	0.06
Others	35	16 (16.4)	19 (–)				
Recurrence							
No	112	51 (53.9)	61 (–)	2.88 (1.35–6.13)	<0.05	2.09 (0.88–4.96)	0.09
Yes	76	43 (14.7)	33 (30.3)				

Abbreviations: BC, bladder cancer; MST, median survival time; OS, overall survival.–

Immune Cell–Infiltration Analysis

By analyzing the abundance of immune-cell infiltration for different clinical characteristics and different risk groups, we found that the abundance had a greater correlation with tumor grade, T staging, and M staging and less correlation with age, sex, recurrence status, tumor location, and N staging (Figure 5A). Immune cell–infiltration abundance in the low-risk group was significantly higher than the high-risk group (Figure 5B). After analyzing each immune cell individually, it was found that naïve B cells, regulatory T cells, M0 macrophages, and eosinophils infiltrated more abundantly in the high-risk group, while resting memory CD4 T cells and resting NK cells infiltrated more abundantly in the low-risk group (Figure 5C).

Expression of lncRNAs in SMALLL Signature

To further validate our findings, we measured the expression of the six lncRNAs in cancer and paracancerous

Table 5 Univariate and Multivariate Cox Regression Analysis on Associations between SMALLL Signature and OS of BC Patients in Validation Group

	Total	High risk score	Low risk score	Univariate		Multivariate	
		Cases, n (MST)	Cases,n (MST)	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Overall	184	92 (26.4)	92 (43.4)	1.07 (0.83–1.38)	0.04	1 (0.72–1.4)	0.05
Age, years							
<70	96	45 (65.2)	51 (91.2)	1.05 (1.01–1.1)	0.03	1.05 (1–1.1)	0.04
≥70	88	47 (19.1)	41 (22.3)				
Sex							
Female	50	24 (26.9)	26 (43.4)	0.74 (0.33–1.66)	0.47	0.62 (0.26–1.45)	0.27
Male	134	66 (26.4)	68 (44.1)				
Grade							
High	175	91 (26.1)	84 (40.3)	1.57 (0.93–2.67)	0.09	1 (0.2–5.11)	1
Low	9	1 (–)	8 (–)				
Disease stage							
II	53	19 (26.4)	34 (91.2)				
III	69	39 (34.7)	30 (95.3)				
IV	62	28 (23.1)	34 (22.3)				
T							
T2	56	20 (–)	36 (85.2)	1.38 (0.74–2.57)	0.31	1.42 (0.39–5.13)	0.59
T3	101	57 (10.3)	44 (5.5)				
T4	27	15 (19.7)	12 (19.3)				
M							
M0	87	36 (25)	51 (63.2)	1.89 (0.44–8.08)	0.39	0.93 (0.13–6.78)	0.95
M1	5	4 (–)	1 (–)				
MX	92	52 (26.2)	40 (24.5)				
N							
N0	108	53 (95.3)	55 (91.2)	1.38 (0.93–2.05)	0.11	1.24 (0.42–3.67)	0.70
N1	16	9 (33.4)	7 (37.5)				
N2	44	24 (21.4)	20 (19.9)				
NX	16	6 (8.5)	10 (21.9)				
Race							
Caucasian	145	66 (26.9)	79 (43.5)	1.47 (0.55–3.94)	0.44	1.15 (0.34–3.91)	0.82
Others	39	13 (25.7)	26 (63.6)				
Recurrence							
No	113	51 (44.1)	62 (95.3)	1.49 (0.68–3.23)	0.32	1.2 (0.43–3.35)	0.73
Yes	71	41 (19.8)	30 (37.5)				

Abbreviations: BC, bladder cancer; MST, median survival time; OS, overall survival.

tissue in 20 patients with BC by qPCR. Our data showed that expression of high-risk lncRNAs (*MAFG-AS1*, *LINC02321*, *LINC01322*, and *LINC00922*) was significantly higher in BC tissue than paracancerous bladder tissue. Consistently, expression of low-risk lncRNAs (*SNHG12* and *ASMTL-AS1*) was higher in paracancerous BC tissue (Figure 6A).

Next, we measured expression of the six lncRNAs in four BC cell lines (RT4, T24, UMUC3, and SVHUC1) by qPCR. The results showed that *ASMTL-AS1*, *LINC00922*, and LINC01322 were highly expressed in RT4 cells and *LINC02321*, *MAFG-AS1*, and *SNHG12* highly expressed in UMUC3 cells (Figure 6B). Therefore, RT4 and UMUC3 cells were used for RNA-interference experiments.

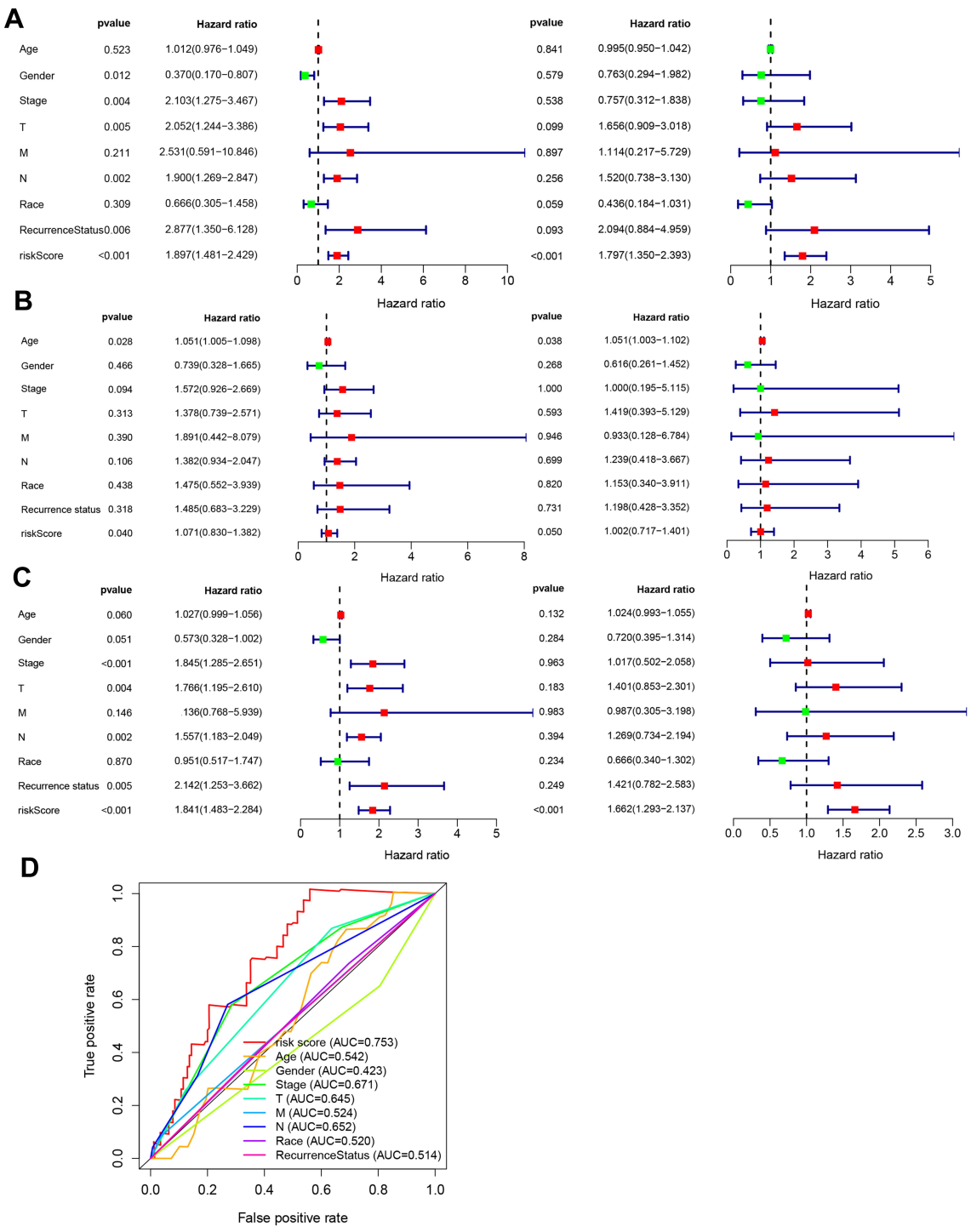

Figure 3 Clinical independence prognostic analysis. (**A**) Univariate and multivariate Cox clinical independence prognostic analysis in training group. (**B**) Univariate and multivariate Cox clinical independence prognostic analysis in validation group. (**C**) Univariate and multivariate Cox clinical independence prognostic analysis in all samples. (**D**) Comparison of prognostic performance between the SMALL signature and two published eight-lncRNA signatures using ROC-curve analysis.

Standard growth curves were examined for UMUC3 and RT4 cells (Figure 6C and D). As GO and KEGG analyses indicated connection of lncRNAs in the SMALLL signature in the ECM network, often implicated in cancer metastasis, and the difference in *SNHG12* between cancer and adjacent cancer was not significant. Therefore, we assessed the effect of *MAFG-AS1*, *ASMTL-AS1*, *LINC01322*, *LINC02321*, and

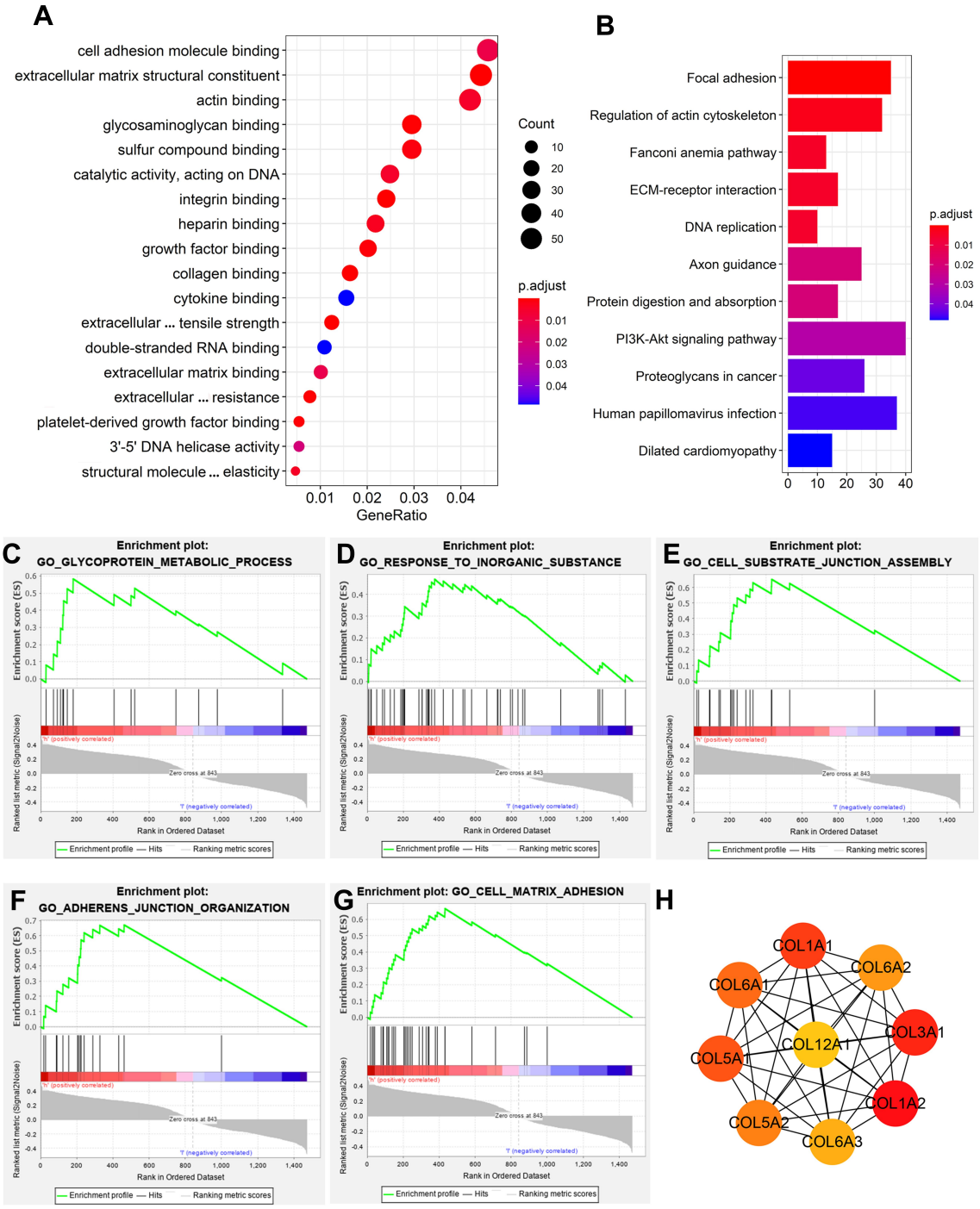

Figure 4 Pathway-enrichment analysis and possible mechanism exploration. (**A**) Gene Ontology (GO) enrichment analysis of coexpressed mRNAs. (**B**) Kyoto Encyclopedia of Genes and Genomes enrichment analysis of coexpressed mRNAs. (**C–G**) Gene set–enrichment analysis of coexpressed mRNAs: adherens-junction organization (**C**), cell-matrix adhesion (**D**), cell substrate–junction assembly (**E**), glycoprotein-metabolism processes (**F**), response to inorganic substances (**G**). (**H**) Top nine hub genes found in the PPI network of coexpressed genes by the Cytoscape plugin CytoHubba.

LINC00922 depletion on the invasion and migration of BC cells. To this end, knockdown of *MAFG-AS1* and *LINC02321* in UMUC3 cells was achieved by transient transfection of shRNA-expressing plasmids. Knockdown of *ASMTL-AS1*, *LINC00922*, and *LINC01322* in RT4 cells was achieved in the same way. Two days after transfection, cell migration and invasion were determined by transwell based in vitro assays, using calcein AM for cell quantification. As shown in Figure 6E–I, knockdown of *MAFG-AS1*,

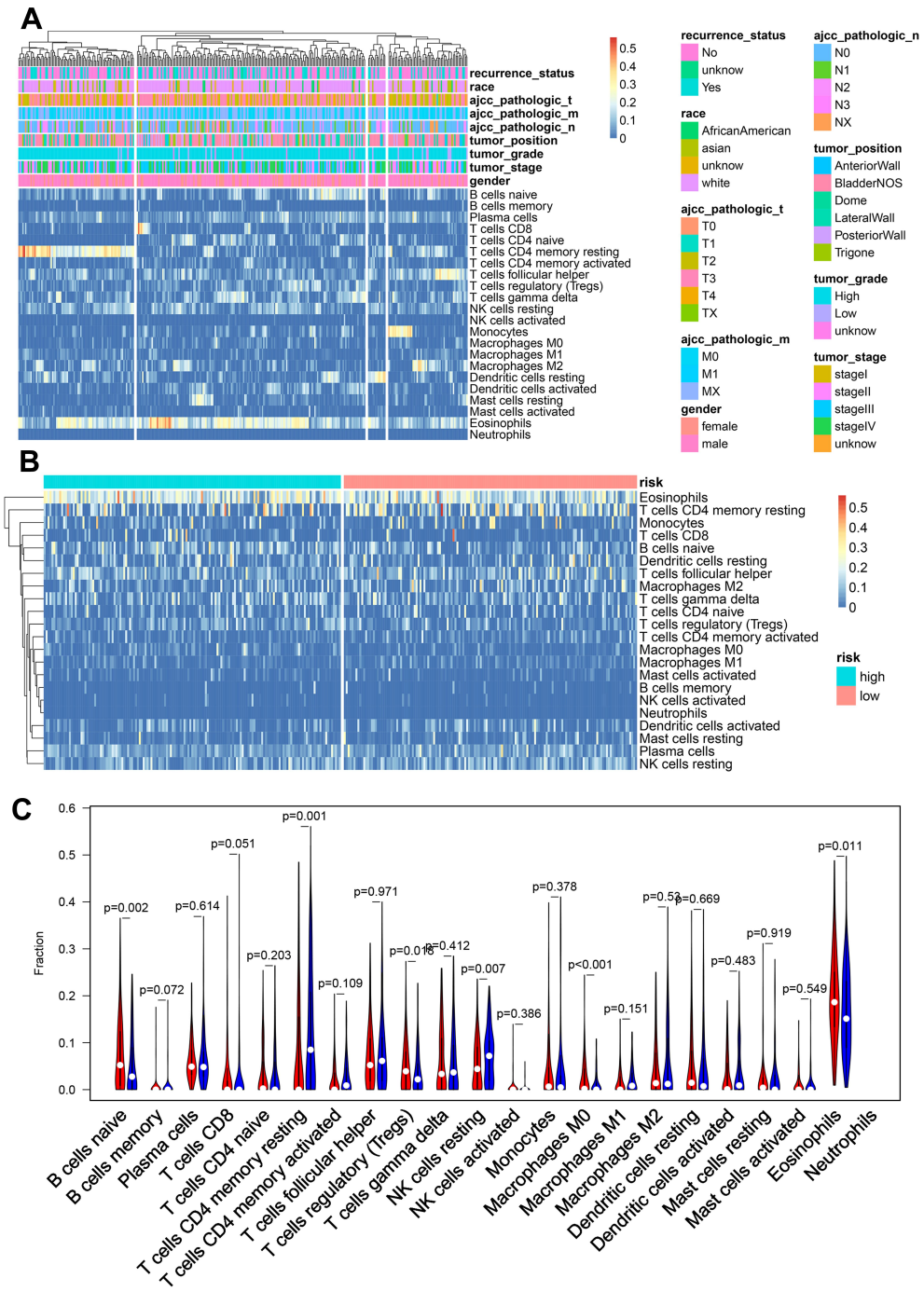

Figure 5 Immune cell–infiltration analysis. (**A**) Heat map of immune cell–infiltration abundance, grouped by clinical features. (**B**) Heat map of immune cell–infiltration abundance, grouped by risk scores based on SMALL signatures. (**C**) Violin chart of abundance of each type of immune-cell infiltration in different risk groups. Red represents the high-risk group and blue the low-risk group.

LINC02321, and *LINC00922* significantly inhibited the migration and invasion of UMUC3 and RT4 cells, and knockdown of *ASMTL-AS1* significantly promoted the migration and invasion of RT4 cells. The knockdown of *LINC01322* had no significant effect on the migration and invasion of RT4 cells. These results suggested that *MAFG-AS1*, *LINC02321*, and *LINC00922* overexpression may promote invasiveness of BC cells and low

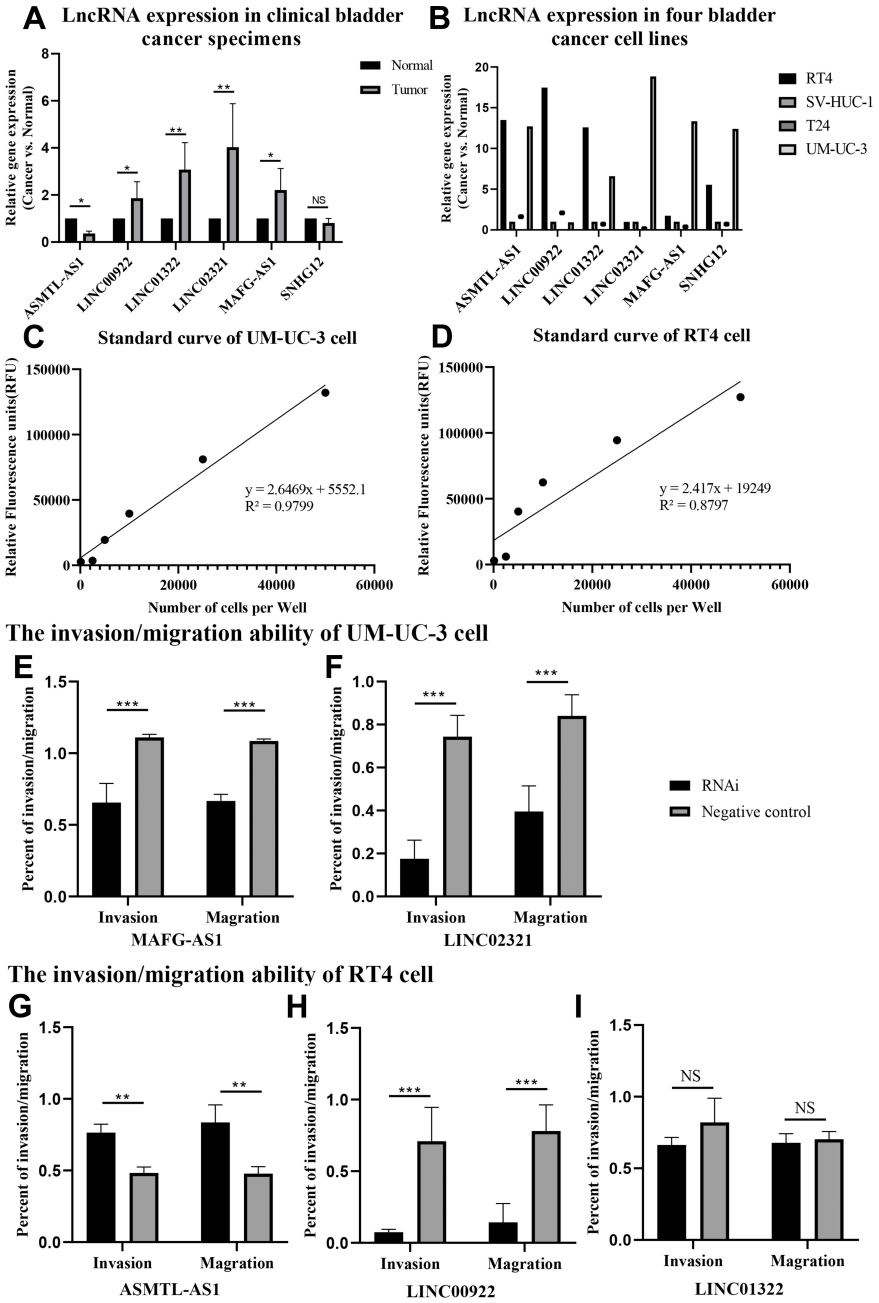

Figure 6 Expression of six lncRNAs in clinical BC specimens. (**A**) Real-time fluorescent qPCR results of six lncRNAs in 20 BC tissue types and their matched adjacent non–tumor tissue samples. Average gene expression in adjacent nontumor tissue was set at 1. Error bars represent SEM. NS, not significant; **P*<0.05; ***P*<0.01. (**B**) Real-time fluorescent qPCR results of six lncRNAs in four bladder cancer cell lines. Average gene expression in the SVHUC1 was set at 1. (**C, D**) Standard curve for cell-invasion and -migration assays. UMUC3 and RT4 cells were harvested, diluted, and incubated for fluorescence. The trend line and line equation are included on the graph. (**E, F**) UMUC3 cells were transfected with *MAFG-AS1* shRNA and *LINC02321* shRNA, followed by invasion and migration assays. Error bars represent SEM. ****P*<0.001. (**G–I**) RT4 cells were transfected with *ASMTL-AS1* shRNA, *LINC00922* shRNA, and *LINC01322* shRNA, followed by invasion and migration assays. Error bars represent SEM. NS, not significant; ***P*<0.01; ****P*<0.001.

expression of *LINC01322* has a similar effect. However, the mechanism of how those lncRNAs contribute to BC-cell invasion remains unknown, and further investigations are needed to elucidate it.

Discussion

Traditionally, treatment and prognosis of BC patients are based on pathological grading and imaging results. However, the risk of recurrence and cancer-related

death remains high, even after radical cystectomy, post-operative radiotherapy, and chemotherapy,[46,47] especially in MIBC patients. As such, traditional TNM staging and imaging assessment are unable to predict the invasiveness of BC and determine prognoses for BC patients properly. Therefore, it is very important to find new and effective prognostic biomarkers for BC patients.

Accumulating evidence has indicated that lncRNAs are involved in many physiological processes, and deregulated lncRNAs may contribute to the occurrence, development, and metastasis of cancer.[48,49] In addition, lncRNAs have shown great potential as novel molecular biomarkers in early diagnosis, treatment, and prognosis in many types of cancer.[50–52] In recent years, many tissue-specific lncRNAs have been found in normal tissue by transcriptional profiling, and lncRNA dysregulation has been found in a variety of human cancers.[13,53,54] Studies have shown that expression of some lncRNAs can predict the risk of cancer progression in patients with different types of cancer, such as breast cancer,[31] colorectal cancer,[32] and ovarian cancer.[19] However, due to the complexity of the lncRNA-transcription landscape, a single lncRNA may not accurately predict the prognosis of cancer patients. Although attempts to use lncRNAs in prognoses - for BC patients have been described,[55,56] further exploration and improvement is required in this field. By comprehensive mining of lncRNA-expression profiles in the TCGA cohort, we established a novel six-lncRNA signature, the SMALLL signature, that was significantly related to the prognosis of BC patients.

To the best of our knowledge, little is known about the implications of the six lncRNAs in the SMALLL signature in human cancers, except for *SNHG12*. *SNHG12* is a small nucleolar host gene that has been described in several types of cancer including breast,[57] gastric,[58] esophagus,[59] liver,[60] and prostate cancer.[61] Upregulation of *SNHG12* has shown proliferative and antiapoptotic effects in some cancer-cell types, and altered expression of *SNHG12* correlates with prognosis and survival of cancer patients.[62] Additionally, *SNHG12* is also differentially expressed in normal and malignant bladder tissue.[63] For the other five lncRNAs, *ASMTL-AS1* and *LINC00922* have only recently been described in papillary thyroid cancer[64] and lung cancer.[65] *ASMTL-AS1* downregulation in papillary thyroid cancer is positively linked to advanced clinical stage and unfavorable outcomes. It has been proposed that *ASMTL-AS1* is a tumor suppressor via the miR93-3p–miR660–FOXO1 axis.[64] *LINC00922* upregulation is associated with poor prognosis in lung cancer and favors cancer-cell proliferation and invasion.[65] However, their biological role in BC remains elusive.

Through pathway-enrichment analysis of the lncRNAs in the SMALL signature, we found that these lncRNAs may be involved in regulation of the ECM. Through PPI-network analysis of coexpressed genes, we found some hub genes, of which the first nine hub (*COL3A1*, *COL1A2*, *COL1A1*, *COL5A1*, *COL6A1*, *COL5A2*, *COL6A2*, *COL6A3*, and *COL12A1*) are members of the gene family involved in collagen coding. This further verified the hypothesis that these lncRNAs may be involved in regulation of the ECM.[66] Next, we confirmed through in vitro experiments that knockdown of these lncRNAs significantly affects the invasion and migration of BC cells.

Development and morbidity of BC are associated with various risk factors, such as aging, which correlates with accumulation of gene mutations.[67,68] Aging also causes alterations in sex hormones and their receptors, which are associated with the impact of sex differences on the morbidity and development of BC.[69] As a result, we propose that this might be the reason that the prognostic value of the SMALLL signature was more efficient in women.

The present study has some innate limitations inevitably. First of all, this study was retrospective because it was based on the TCGA data set and not validated in prospective clinical trials. Secondly, the mechanism behind lncRNAs in our classifiers is still completely unclear. More importantly, the TCGA cohort did not contain integrated information on some important features, such as pharmacological and surgical interventions. Therefore, further research on specific lncRNAs is required. Despite these shortcomings, the results show that our lncRNA-based classifier can be used as a reliable predictor of BC survival and recurrence. In summary, we identified a novel lncRNA signature that is significantly associated with OS in BC patients. Further analysis of the signature indicates that the lncRNAs were enriched in the extracted matrix-structu e pathway. Our findings provide insight into an lncRNA signature that might be a prognostic biomarker for BC.

Ethics and Consent

All patients in the study signed informed-consent forms before surgery at the First Affiliated Hospital of Kunming Medical University. Our research was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Kunming Medical University and complied with the ethical principles of the 1964 Declaration of Helsinki and later versions.

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Author Contributions

All authors made a significant contribution to the work reported, whether in conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas, took part in drafting, revising, or critically reviewing the article, gave final approval to the version to be published, have agreed on the journal to which the article has been submitted, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest for this work, and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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