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Original Article

LMNTD2-AS1 regulates immune cell infiltration and promotes prostate cancer progression by targeting FUS to regulate NRF2 signal pathway

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Abstract: Numerous studies have demonstrated that long non-coding RNAs (lncRNAs) play crucial roles in tumor progression. This study aimed to identify lncRNAs associated with overall survival (OS) and progression-free interval (PFI) in prostate cancer (PCa) patients and to elucidate the driving mechanisms and functions of these lncRNAs. We utilized the TCGA database to screen for lncRNAs linked with OS and PFI. KM survival analysis, ROC curve analysis, and Cox survival analysis were employed to assess the prognostic significance of lncRNAs in PCa patients. We conducted a loss-of-function assay to explore the role of lncRNAs in PCa. Correlation analysis was performed to study the relationship between lncRNAs and immune cell infiltration. Lasso regression analysis was performed to screen proteins which might interact with lncRNAs, while rescue experiments verified the integrity of the signaling pathway. LMNTD2-AS1 was found to be the only lncRNA in PCa patients associated with both OS and PFI with significantly elevated levels in PCa. Elevated LMNTD2-AS1 expression was significantly linked to advanced stage, grade, primary treatment outcomes, residual tumors, and Gleason scores in PCa patients. Moreover, multivariate Cox regression analysis revealed that high LMNTD2-AS1 expression independently predicted PFI in PCa patients. The AUC of LMNTD2-AS1 for predicting 3-year OS and 5-year OS in PCa patients was 0.877 and 0.807, respectively, while for 3-year PFI and 5-year PFI it was 0.751 and 0.727, respectively. Overexpression of LMNTD2-AS1 correlated with infiltration of neutrophils, macrophages, pDC, NK CD56bright cells, and other immune cells. Furthermore, FUS and NRF2 are both potential binding proteins and related signaling pathways downstream of LMNTD2-AS1. Functional experiments demonstrated that LMNTD2-AS1 knockdown significantly inhibited migration, invasion, and proliferation of PCa cells while overexpression of FUS was found to rescue the functional inhibition caused by LMNTD2-AS1 knockdown. LMNTD2-AS1 functions as an oncogene in PCa, influencing patient prognosis and the immune microenvironment; it may regulate immune cell infiltration and promote PCa progression by interacting with the NRF2 signaling pathway via FUS binding.

Keywords: Prostate cancer, LMNTD2-AS1, FUS, immune cell infiltration

Introduction

Prostate cancer (PCa) is the most common malignancy among men worldwide, with an estimated 288,300 new cases and 34,700 deaths annually in the United States in 2020 [1]. Interactions between acquired somatic gene alterations, inherent germline susceptibility, macro-environmental and micro-environmental factors play important roles in the oncogenesis of PCa [2]. Early-stage PCa typically accumulates large-scale genomic structural rearrangements and copy number alterations [3]. Gene fusions, particularly between TMPRSS2 and ERG, represent the most frequent chromosomal aberrations observed in PCa, instigating carcinogenesis in over 50% of patients [4]. The intrinsic germline predisposition is evidenced by the fact that PCa-initiating cells can be tubular or basal prostate epithelial cells.

Furthermore, chronic inflammation or infections induced by urologic microbes promote prostate carcinogenesis via oxidative stress, leading to the production of reactive oxygen species that cause DNA damage, subsequently selecting for mutant cells [5, 6]. While surgery was once the primary treatment for PCa [7], the past decade has witnessed a paradigm shift in PCa treatment strategies, with targeted therapies notably enhancing patients' quality of life and survival rates [8].

Long non-coding RNAs (lncRNAs) are distinct transcriptional units that produce non-coding RNAs exceeding 200 nucleotides in length. These molecules play integral roles in gene expression and various physiological and pathological processes [9-11]. Cellular localization of lncRNAs determines their diverse biological functions, including nuclear translocation, recruitment of chromatin-modifying complexes, RNA stability and selective splicing, microRNA (miRNA) sponge action, and gene targeting [12, 13]. Recent investigations have illuminated the intricate and precise regulatory functions of lncRNAs in cell proliferation, differentiation, invasion, metastasis, and even metabolic reprogramming of cancer cells, exhibiting remarkable tissue specificity [14-16]. For instance, lncRNA-SLERCC was shown to be significantly down-regulated in tumour tissue and correlated with clinical staging of renal cell carcinoma. This can be attributed to SLERCC's ability to bind directly to UPF1, thereby exerting tumor-suppressive effects through the Wnt/β-catenin signaling pathway and inhibiting RCC progression and metastasis [9]. Furthermore, a growing body of evidence underscores the involvement of lncRNAs in immune system regulation, showing cell-type-specific patterns within immune cells [17]. A prior study indicated a significant association of lncRNA LMNTD2-AS1 with OS and PFI in PC patients. However, its functional implications remain to be fully understood, and no research has comprehensively explored the predictive roles of LMNTD2-AS1 in PCa progression [18].

In this study, we began by retrieving lncRNA expression profiles and clinical data of PCa patients from the TCGA database and identified LMNTD2-AS1 as a key lncRNA closely associated with overall survival (OS) and progression-free interval (PFI). We found that LMNTD2-AS1 expression was significantly increased in PC tissues and correlated with poor prognosis in PC patients. Subsequently, we analyzed the relationship between LMNTD2-AS1 and PCa immune cell infiltration. Finally, we screened for possible lncRNA binding proteins by Lasso regression analysis and elucidated the role of LMNTD2-AS1 in PCa and possible signaling pathways by loss-of-function experiments and rescue assays.

Materials and methods

Screening for LMNTD2-AS1 associated with OS and PFI in PCa patients

RNA-seq data, including transcripts per million (TPM) and fragments per kilobase million (FPKM) for PCa patients, were obtained from The Cancer Genome Atlas (TCGA) database. The genome annotation file was downloaded to identify the Ensembl ID of lncRNAs, which were subsequently filtered in the gene type column of the expression matrix. Clinical information, sample data, and gene files of the patients were downloaded from the "Clinical, Metadata and cart" sections, respectively. All data were integrated using R software. The TCGA-PCa database contains clinical information for 553 patients, including 52 normal patients and 500 patients with complete clinical information. The lncRNA LMNTD2-AS1 was identified for its association with OS and PFI in PCa patients using screening criteria of $P < 0.05$ and a hazard ratio (HR) ≥ 4. Furthermore, the mean expression levels of LMNTD2-AS1 in normal and cancerous tissues were compared across pan-cancers.

Screening for LMNTD2-AS1-interacting proteins

Potential proteins interacting with LMNTD2-AS1 were identified using the StarBase database (http://starbase.sysu.edu.cn/) and matched with prognostic data from TCGA-PCa patients. Lasso analysis was employed to identify key proteins associated with OS and PFI in PCa patients. Patients were classified into high and low-risk groups based on risk score, and Kaplan-Meier survival curves were used to illustrate OS and PFI for patients in different risk groups.

Relationship between LMNTD2-AS1 and FUS expression and clinicopathological characteristics of PCa patients

Clinicopathological characteristics data of PCa patients were downloaded from the TCGA data-

base. The expression levels of LMNTD2-AS1 and FUS were compared among patients with different clinicopathological characteristics, including pathological T-stage, pathological N-stage, clinical T-stage, residual tumor, primary therapy outcomes, age, PSA, and Gleason score.

Relationship of LMNTD2-AS1 and FUS with OS and PFI in PCa patients

Prognostic data of PCa patients were downloaded from the TCGA database, and patients were divided into two groups according to LMNTD2-AS1 and FUS expression levels. The effects of high and low LMNTD2-AS1 and FUS on OS and PFI were analyzed using Kaplan-Meier (KM) survival curves. The statistical results were expressed as hazard ratios (HR) and 95% confidence intervals (CI), and the effects of LMNTD2-AS1 and FUS on PFI were compared in different subgroups.

Prognostic value of LMNTD2-AS1 and FUS

The predictive performance of LMNTD2-AS1 and FUS on 3-year and 5-year OS and PFI in PCa patients was assessed using receiver operating characteristics (ROC). The diagnostic value was presented as the area under the curve (AUC). Additionally, temporal ROC curves for OS and PFI at 1-5 years were constructed.

Relationship between LMNTD2-AS1 and the immune microenvironment

The correlation between immune cell scores and LMNTD2-AS1 in PCa tissues was analyzed using the ssGSEA algorithm. The relationship between each immune cell and LMNTD2-AS1 was presented as correlation coefficients and *p*-values. Furthermore, we investigated the differences in immune cell infiltration in the high and low LMNTD2-AS1 expression groups.

Cell lines and cultures

The human prostate cancer cell line LNCap was procured from the Shanghai Cell Bank, Chinese Academy of Sciences. LNCap cells were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and maintained in a 5% CO_2 incubator at 37°C.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using Trizol reagent (TaKaRa, China) following the manufacturer's instructions. cDNA was synthesized by reverse transcription using cDNA kits (Vazyme Biotech, Nanjing), and CT values were detected using SYBR Green PCR kits (Vazyme Biotech, Nanjing). The relative expression of FUS and NRF2 was calculated using the $2^{-\Delta\Delta CT}$ method, with GAPDH serving as an internal standard.

Primers for FUS, NRF2 and GAPDH are listed below. FUS-fw: 5' ATGGCCTCAAACGATTATACCCA 3'; FUS-rev: 5' GTAACTCTGCTGTCCGTAGGG 3; NRF2-fw: 5' ACACGGTCCACAGCTCATC 3'; NRF2-rev: 5' TGTCAATCAAATCCATGTCCTG 3; GAPDH-fw: 5' AACGGATTTGGTCGTATTG 3'; GAPDH-rev: 5' GGAAGAGATGGTGATGATT 3'.

Western blotting

Total protein from the cells was extracted using RIPA. 20 μg of protein extract was loaded onto an SDS-PAGE gel and then transferred to a PVDF membrane. The samples were blocked with 5% bovine serum albumin (BSA) for 1 hour at room temperature. Subsequently, samples were incubated overnight at 4°C with primary antibodies: anti-FUS (ab124923, Abcam), anti-NRF2 (ab62352, Abcam). After washing with TBST, samples were incubated with HRP-labelled secondary antibodies for 1 hour at room temperature. After washing, the blots were visualized with ECL (Epizyme, China) reagents [19].

EdU assay

Pretreated cells were cultured in 24-well plates and treated with 10 μM EdU (Yeasen, China) for 2 hours at 37°C and 5% CO_2. Cells were assayed using EdU after fixation and pro-permeabilisation operations, and finally DAN restaining was performed and EdU fluorescence was observed using a microscope (Leica, Germany).

Clone formation assay

In a 6-well plate (Corning, USA), 500 treated cells were seeded in each well. The medium was discarded when the cells had grown to visible colonies, washed in PBS, fixed in anhydrous

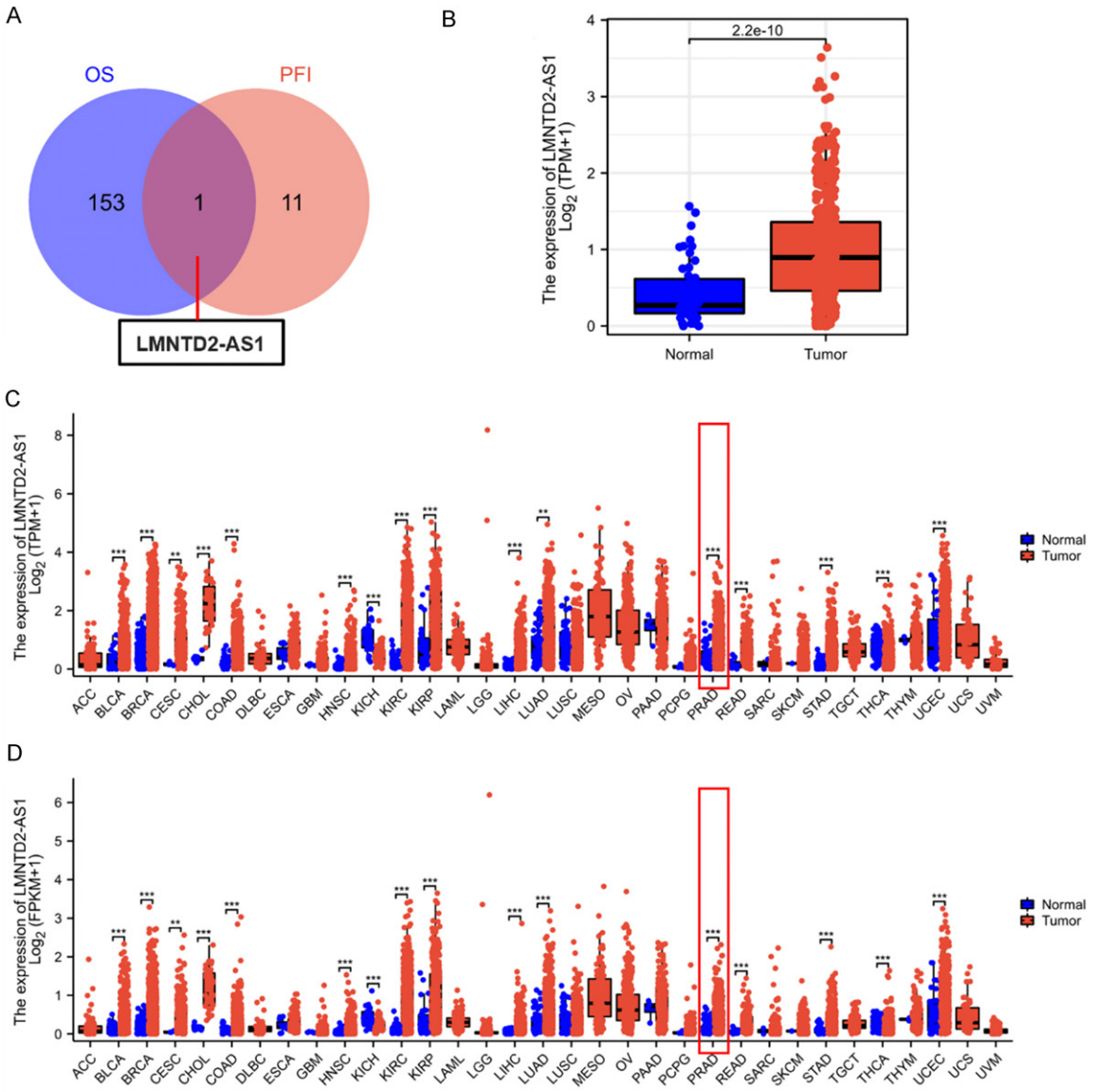

Figure 1. LMNTD2-AS1 is a highly expressed lncRNA associated with OS and PFI in PCa patients. A. Venn diagram screening for OS and PFI related lncRNAs in PCa patients. B. LMNTD2-AS1 expression in TCGA-PCa database. C. LMNTD2-AS1 expression in pan-cancerous tissues in TCGA-TPM type data. D. LMNTD2-AS1 expression in pan-cancerous tissues in TCGA-FPKM type data. **P < 0.01, ***P < 0.001.

ethanol, then stained with 0.1% crystal violet (Vicmed, China) and imaged.

Wound healing assay

Treated cells were seeded in 6-well plates (Corning, USA). When cells reached 80% confluence, the cell layer was scratched with a 200 μl pipette tip. Debris was washed with PBS buffer. Medium containing 2% FBS was added to each well. Images were taken at 0 h, 24 h, and 48 h post-wounding using an Olympus microscope (Tokyo, Japan).

Transwell assays

Migration and invasion capabilities of cells were assessed using Transwell chambers (Corning, USA). For invasion assays, the upper chamber was coated with 100 μl Matrigel (BD Biosciences, USA). Specifically, transfected cells (5 × 10^4) were seeded in the upper cham-

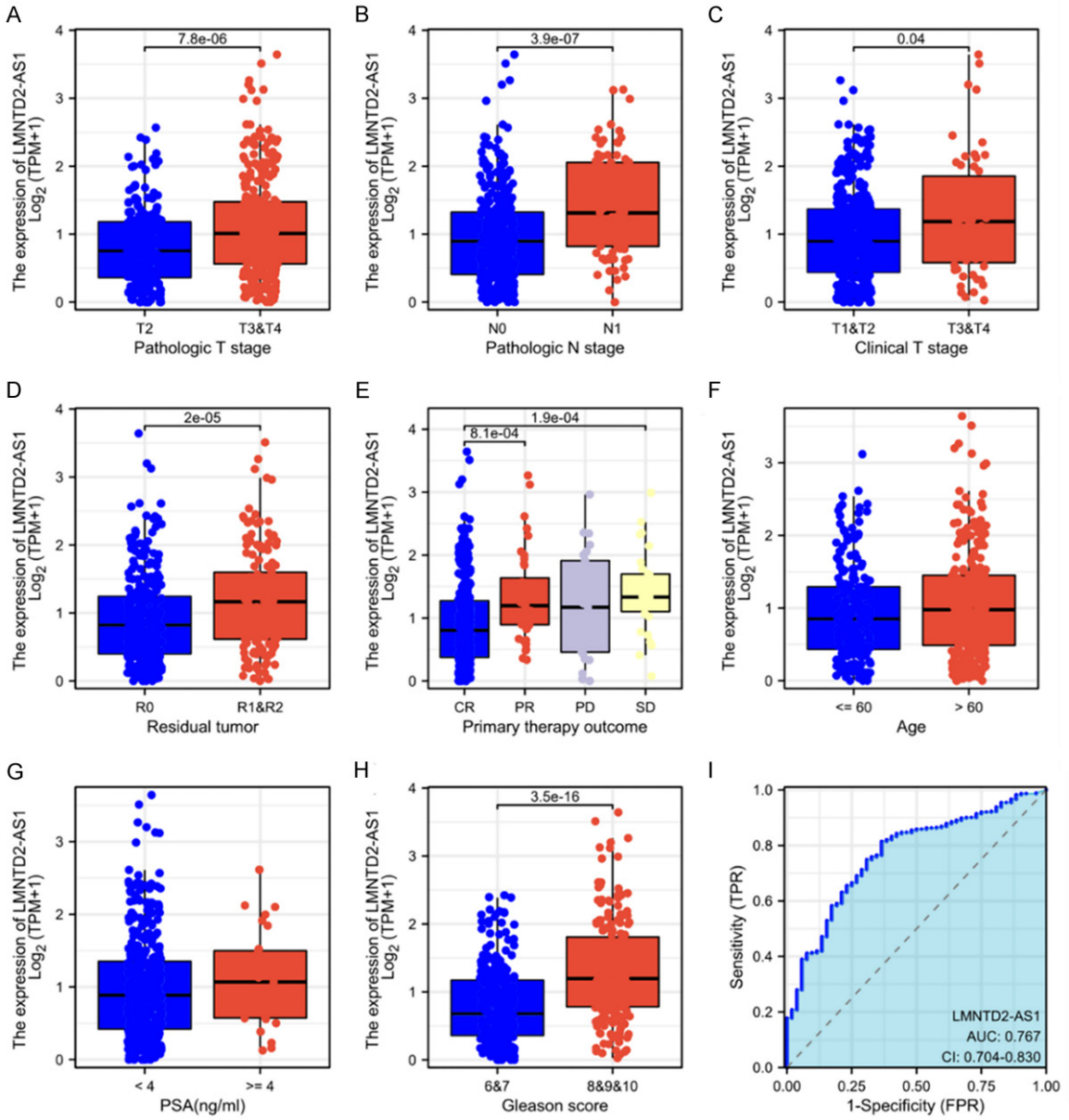

Figure 2. LMNTD2-AS1 expression in clinicopathological features of PCa patients. A. Pathological T stage. B. Pathological N stage. C. Clinical T stage. D. Residual tumor. E. Primary therapy outcomes. F. Age. G. PSA. H. Gleason score. I. LMNTD2-AS1 predicts AUC curves in tumor and normal tissues.

ber, and medium with 10% FBS was added to the lower chamber. After 12-24 h, invading and migrating cells were fixed, stained with 0.1% crystal violet (Vicmed, China), imaged, and counted using an inverted microscope (Leica, Germany).

Statistical analysis

Univariate and multivariate Cox regression analyses were employed to pinpoint independent predictors of OS and PFI in PCa patients. Kaplan-Meier survival curves were utilized to evaluate OS and PFI in PCa patients. All statistical evaluations were conducted using R software (version 4.0.2), SPSS (version 25.0), and GraphPad Prism (version 8.3). A *p*-value less than 0.05 was deemed statistically significant.

Results

LMNTD2-AS1 is overexpressed and correlates with OS and PFI in PCa patients

To explore potential functional lncRNAs associated with prostate cancer prognosis, we screened 154 lncRNAs associated with OS and 12 lncRNAs associated with PFI in PCa

Table 1. The clinical information of PCa patients in TCGA depend on LMNTD2-AS1 expression

Characteristics	Low expression of LMNTD2-AS1	High expression of LMNTD2-AS1	*P* value
N	250	251	
Pathologic T stage, n (%)			< 0.001
T2	115 (23.3%)	74 (15%)	
T3&T4	131 (26.5%)	174 (35.2%)	
Pathologic N stage, n (%)			0.001
N0	174 (40.7%)	174 (40.7%)	
N1	24 (5.6%)	56 (13.1%)	
Clinical T stage, n (%)			0.036
T1&T2	175 (43%)	177 (43.5%)	
T3&T4	19 (4.7%)	36 (8.8%)	
Residual tumor, n (%)			< 0.001
R0	177 (37.7%)	139 (29.6%)	
R1&R2	59 (12.6%)	95 (20.2%)	
Primary therapy outcome, n (%)			< 0.001
CR	190 (43.2%)	151 (34.3%)	
PR	11 (2.5%)	29 (6.6%)	
PD	11 (2.5%)	18 (4.1%)	
SD	6 (1.4%)	24 (5.5%)	
Age, n (%)			0.081
≤ 60	122 (24.4%)	103 (20.6%)	
> 60	128 (25.5%)	148 (29.5%)	
PSA (ng/ml), n (%)			0.768
< 4	213 (48%)	204 (45.9%)	
≥ 4	13 (2.9%)	14 (3.2%)	
Gleason score, n (%)			< 0.001
6&7	185 (36.9%)	109 (21.8%)	
8&9&10	65 (13%)	142 (28.3%)	

Abbreviations: PCa, Prostate cancer; TCGA, The Cancer Genome Atlas; CR, Complete response; PR, Partial response; PD, Progressive disease; SD, Stable disease; PSA, Prostate specific antigen.

patients from the TCGA database. LMNTD2-AS1 emerged as the only lncRNA associated with both OS and PFI (**Figure 1A**). Further analysis revealed elevated LMNTD2-AS1 expression in PCa tumor tissues compared to normal tissues (**Figure 1B**). Using TPM and FPKM data from TCGA, we found significant overexpression of LMNTD2-AS1 in BLCA, BRCA, CESC, CHOL, COAD, HNSC, KIRC, KIRP, LIHC, LUAD, PRAD, READ, STAD, THCA, and UCEC tumour tissues, with a down-regulation in KICH tumor tissues (**Figure 1C**, **1D**).

High expression of LMNTD2-AS1 correlates with advanced clinical and pathological grade of PCa patients

We investigated the relationship between LMNTD2-AS1 expression levels and the clinical and pathological grade of PCa patients. High expression of LMNTD2-AS1 was associated with advanced pathological T stage (P < 0.001, **Figure 2A**), pathological N stage (P < 0.001, **Figure 2B**), clinical T stage (P=0.036, **Figure 2C**), residual tumor (P < 0.001, **Figure 2D**), primary therapy outcomes (**Figure 2E**) and Gleason score (P < 0.001, **Figure 2H**), but not to age (P=0.081, **Figure 2F**) and PSA (P=0.768, **Figure 2G**). Chi-square analysis further confirmed that high LMNTD2-AS1 was associated with pathological T stage, pathological N stage, clinical T stage, residual tumor, primary therapy outcomes, and Gleason score (All P < 0.05, **Table 1**). Furthermore, ROC analysis indicated that LMNTD2-AS1 effectively distinguished tumor from normal tissue (AUC=0.767) (**Figure 2I**).

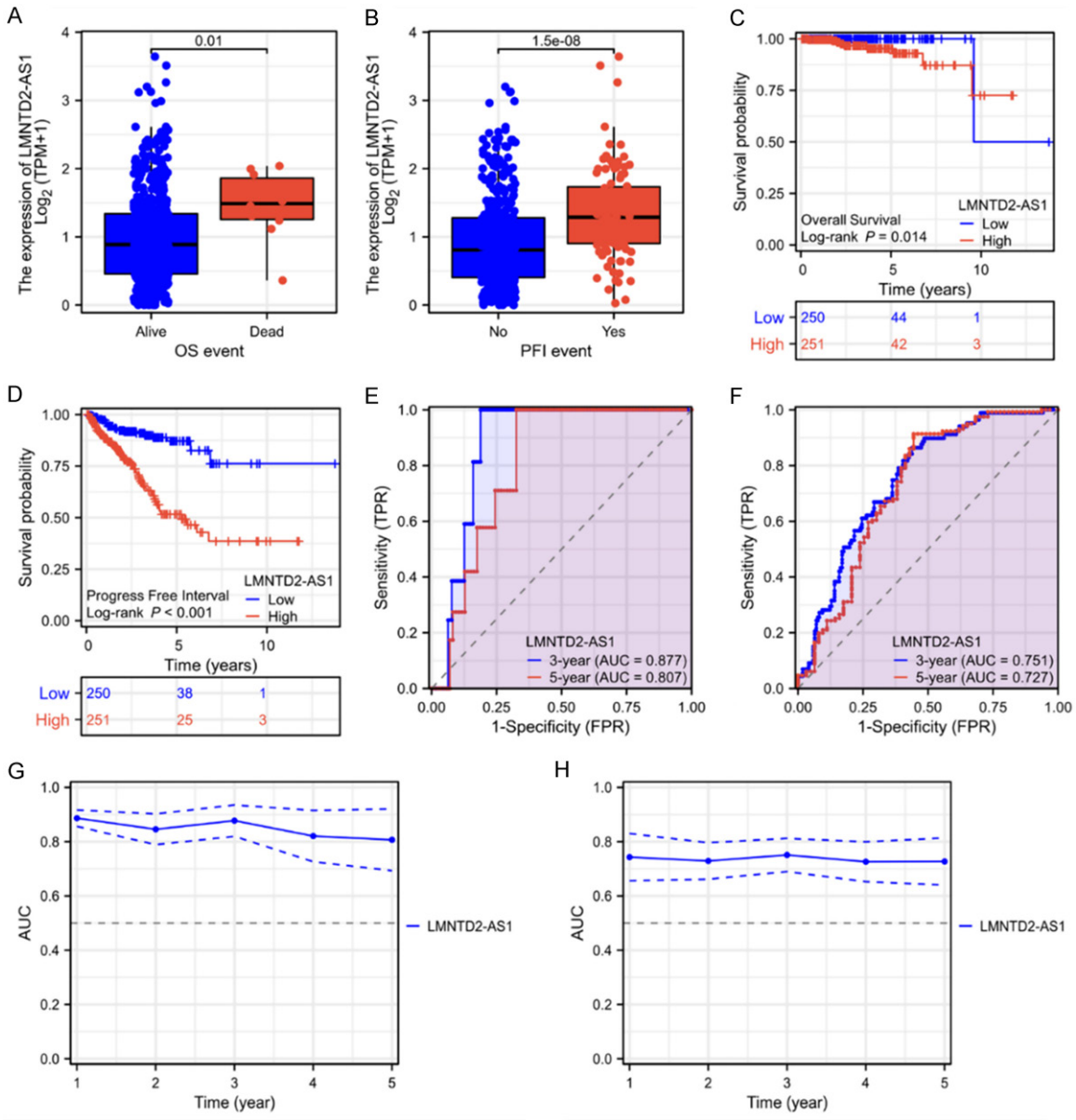

Figure 3. High expression of LMNTD2-AS1 was associated with poor prognosis. (A, B) Expression of LMNTD2-AS1 in OS (A) or PFI (B) positive and negative. (C, D) OS (C) or PFI (D) survival curve of LMNTD2-AS1 high and low groups. (E, F) AUC curves of LMNTD2-AS1 predicting 3- and 5-year OS (E) or PFI (F). (G, H) AUC curves of LMNTD2-AS1 predicting 1-5-year OS (G) or PFI (H).

High expression of LMNTD2-AS1 was associated with poor prognosis

Given the association of the lncRNA LMNTD2-AS1 with OS and PFI in PCa patients, we further examined its relationship with OS and PFI. Our findings revealed that LMNTD2-AS1 expression was notably higher in deceased patients (**Figure 3A**) and in the PFI-positive cohort (**Figure 3B**). Kaplan-Meier survival curves indicated that patients with lower LMNTD2-AS1 levels had significantly improved OS (high group vs low group, HR=8.755 (1.098-69.803), **Figure 3C**) and PFI (high group vs low group, HR=3.894 (2.429-6.243), **Figure 3D**) compared to those with higher LMNTD2-AS1 levels. Additionally, elevated LMNTD2-AS1 expression was linked to a worse PFI across various subgroups (Figure S1). Multivariate Cox regression analysis identified LMNTD2-AS1 as an independent risk factor for PFI (**Tables 2**, **3**). ROC curves further demonstrated that the AUC of the LMNTD2-

Table 2. The OS related factors in PCa patients

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Pathologic T stage	494		0.134		
T2	189	Reference			
T3&T4	305	3.277 (0.608-17.655)	0.167		
Pathologic N stage	428		0.126		
N0	348	Reference			
N1	80	3.470 (0.767-15.695)	0.106		
Clinical T stage	407		**0.005**		
T1&T2	352	Reference		Reference	
T3&T4	55	9.299 (2.079-41.582)	**0.004**	20.507 (2.199-191.268)	**0.008**
Residual tumor	470		0.157		
R0	316	Reference			
R1&R2	154	2.587 (0.693-9.654)	0.157		
Primary therapy outcome	440		**0.013**		
CR	341	Reference		Reference	
PR	40	5.411 (0.481-60.836)	0.171	1.401 (0.065-30.185)	0.830
PD	29	15.798 (2.855-87.411)	**0.002**	7.370 (0.578-94.023)	0.124
SD	30	4.177 (0.376-46.396)	0.245	3.157 (0.163-61.310)	0.448
Age	501		0.479		
≤ 60	225	Reference			
> 60	276	1.578 (0.441-5.650)	0.484		
PSA (ng/ml)	444		**0.007**		
< 4	417	Reference		Reference	
≥ 4	27	10.528 (2.481-44.681)	**0.001**	6.542 (0.705-60.730)	0.098
Gleason score	501		**0.007**		
6&7	294	Reference		Reference	
8&9&10	207	6.654 (1.371-32.298)	**0.019**	1.981 (0.126-31.168)	0.627
LMNTD2-AS1	501		**0.008**		
Low	250	Reference		Reference	
High	251	8.755 (1.098-69.803)	**0.041**	2.669 (0.261-27.274)	0.408

Abbreviations: OS, Overall survival; PCa, Prostate cancer; CR, Complete response; PR, Partial response; PD, Progressive disease; SD, Stable disease; PSA, Prostate specific antigen. Note: P < 0.05 is labeled in bold. Proportional risk hypothesis testing using the survival package and Cox regression analysis. Variable screening strategy: samples meeting P < 0.05 in the univariate go into the multivariate Cox to build a model.

AS1 high group for predicting 3-year and 5-year OS was 0.877 and 0.807 (**Figure 3E**), respectively, and the AUC for 3-year and 5-year PFI was 0.751 and 0.727 (**Figure 3F**), respectively. Temporal ROC curves also highlighted the accuracy of LMNTD2-AS1 in predicting 1-5-year OS (**Figure 3G**) and PFI (**Figure 3H**).

Knockdown of LMNTD2-AS1 inhibits the proliferation, migration and invasion of PCa cells

The above studies identified LMNTD2-AS1 as a potential oncogene upregulated in PCa tumor tissues. To understand its functional role in PCa, we conducted wound healing assays, which revealed that LMNTD2-AS1 si#1 and LMNTD2-AS1 si#2 knockdown significantly inhibited LNCap cell migration (**Figure 4A**). Transwell assays further demonstrated that LMNTD2-AS1 si#1 reduced the migratory and invasive ability of LNCap (**Figure 4B**). We then selected LMNTD2-AS1 si#1 as the target siRNA. Furthermore, EdU and clone formation assays revealed that knockdown of LMNTD2-AS1 significantly inhibited the proliferative capacity of the cells (**Figure 4C**, **4D**).

LMNTD2-AS1 was significantly associated with the PCa immune microenvironment

Numerous studies have underscored the pivotal role of the immune microenvironment in PCa

Table 3. The PFI related factors in PCa patients

Characteristics	Total (N)	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
Pathologic T stage	494		**< 0.001**		
T2	189	Reference		Reference	
T3&T4	305	3.745 (2.118-6.623)	**< 0.001**	1.162 (0.524-2.574)	0.712
Pathologic N stage	428		**0.013**		
N0	348	Reference		Reference	
N1	80	1.901 (1.175-3.078)	**0.009**	0.925 (0.504-1.695)	0.800
Clinical T stage	407		**< 0.001**		
T1&T2	352	Reference		Reference	
T3&T4	55	3.799 (2.357-6.123)	**< 0.001**	1.969 (1.083-3.581)	**0.026**
Residual tumor	470		**< 0.001**		
R0	316	Reference		Reference	
R1&R2	154	2.353 (1.559-3.552)	**< 0.001**	0.627 (0.336-1.172)	0.144
Primary therapy outcome	440		**< 0.001**		
CR	341	Reference		Reference	
PR	40	9.441 (5.441-16.380)	**< 0.001**	7.355 (3.304-16.372)	**< 0.001**
PD	29	5.778 (3.203-10.424)	**< 0.001**	4.863 (2.191-10.793)	**< 0.001**
SD	30	4.797 (2.631-8.746)	**< 0.001**	3.650 (1.442-9.238)	**0.006**
Age	501		0.202		
≤ 60	225	Reference			
> 60	276	1.304 (0.865-1.967)	0.205		
PSA (ng/ml)	444		**< 0.001**		
< 4	417	Reference		Reference	
≥ 4	27	4.223 (2.108-8.459)	**< 0.001**	1.955 (0.821-4.655)	0.130
Gleason score	501		**< 0.001**		
6&7	294	Reference		Reference	
8&9&10	207	4.665 (2.951-7.375)	**< 0.001**	2.423 (1.243-4.723)	**0.009**
LMNTD2-AS1	501		**< 0.001**		
Low	250	Reference		Reference	
High	251	3.894 (2.429-6.243)	**< 0.001**	2.617 (1.355-5.057)	**0.004**

Abbreviations: PFI, Progress free interval; PCa, Prostate cancer; CR, Complete response; PR, Partial response; PD, Progressive disease; SD, Stable disease; PSA, Prostate specific antigen. Note: P < 0.05 is labeled in bold. Proportional risk hypothesis testing using the survival package and Cox regression analysis. Variable screening strategy: samples meeting P < 0.05 in the univariate go into the multivariate Cox to build a model.

progression. We evaluated the association between LMNTD2-AS1 expression and various immune cells, conducting a comprehensive correlation analysis (**Figure 5A**). The results showed that LMNTD2-AS1 was positively correlated with the levels of pDC (P < 0.001, **Figure 5B**), NK CD56bright cells (P < 0.001, **Figure 5C**), and CD8 T cells (P < 0.001, **Figure 5D**); and negatively correlated with neutrophils (P < 0.001, **Figure 5E**), B cells (P=0.004, **Figure 5F**), mast cells (P < 0.001, **Figure 5G**), DCs (P < 0.001, **Figure 5H**), iDC (P < 0.001, **Figure 5I**), macrophages (P < 0.001, **Figure 5J**), Th1 cells (P < 0.001, **Figure 5K**), Th2 cells (P < 0.001, **Figure 5L**), Th17 cells (P < 0.001, **Figure 5M**), Tgd (P < 0.001, **Figure 5N**), TFH (P < 0.001, **Figure 5O**), NK cells (P=0.011, **Figure 5P**) and eosinophils (P < 0.001, **Figure 5Q**). In addition, B cells, pDCs, DCs, iDCs, Eosinophils, macrophages, mast cells, neutrophils, CD8 T cells, Th1 cells, Th17 cells, Th2 cells, Tgd, TFH and CD56bright cells showed significant differences between LMNTD2-AS1 high and low expression groups (Figure S2).

FUS is a possible binding protein for LMNTD2-AS1

We continue to explore the possible mechanism of action of LMNTD2-AS1 in PCa. Here, we focus on the RNA binding protein (RBP)

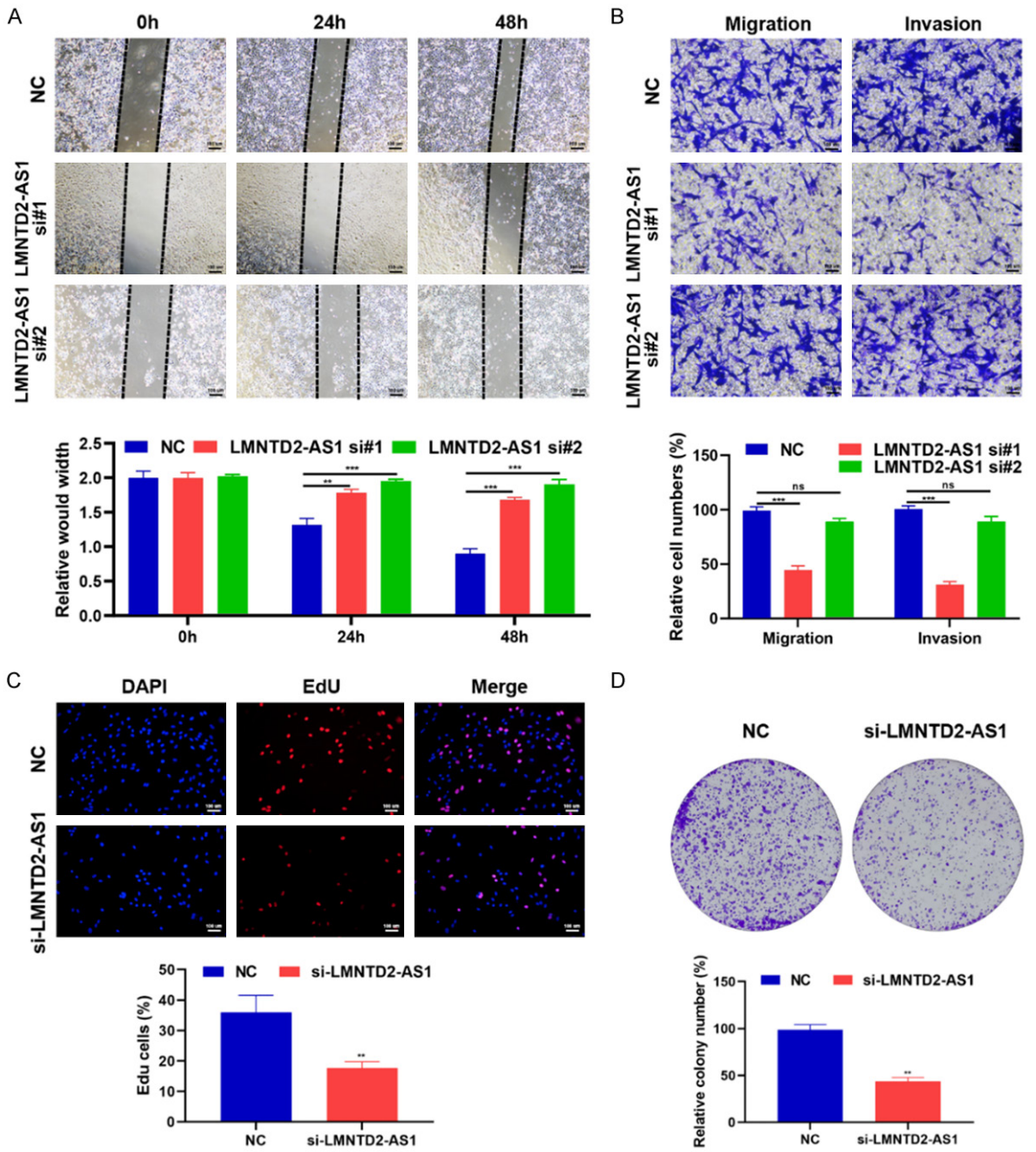

Figure 4. Knockdown of LMNTD2-AS1 inhibits the proliferation, migration and invasion of PCa cells. Wound healing assay (A), transwell assay (B), EdU proliferation assay (C) and clone formation assay (D) of LNCap cells after LMNTD2-AS1 knockdown. **P < 0.01, ***P < 0.001.

mode of action of lncRNAs. Using the starbase database, we predicted 47 proteins that could potentially bind to LMNTD2-AS1. We screened 2 key binding proteins associated with PCa OS (**Figure 6A-C**) and 3 key binding proteins associated with PCa PFI (**Figure 6D-F**) through Lasso regression analysis. Co-expression heat maps showed that high expression of LMNTD2-AS1 was positively correlated with FUS and RANGAP1 and negatively correlated with SMNDC1 and CSTF2T (**Figure 6G**). Venn diagram showed that FUS was the only binding protein with crossover (**Figure 6H**), and LMNTD2-AS1 was positively correlated with FUS expression in PCa (R=0.565, P < 0.001) (**Figure 6I**). In addition, we investigated the expression of FUS in PCa and the relationship with OS and PFI.

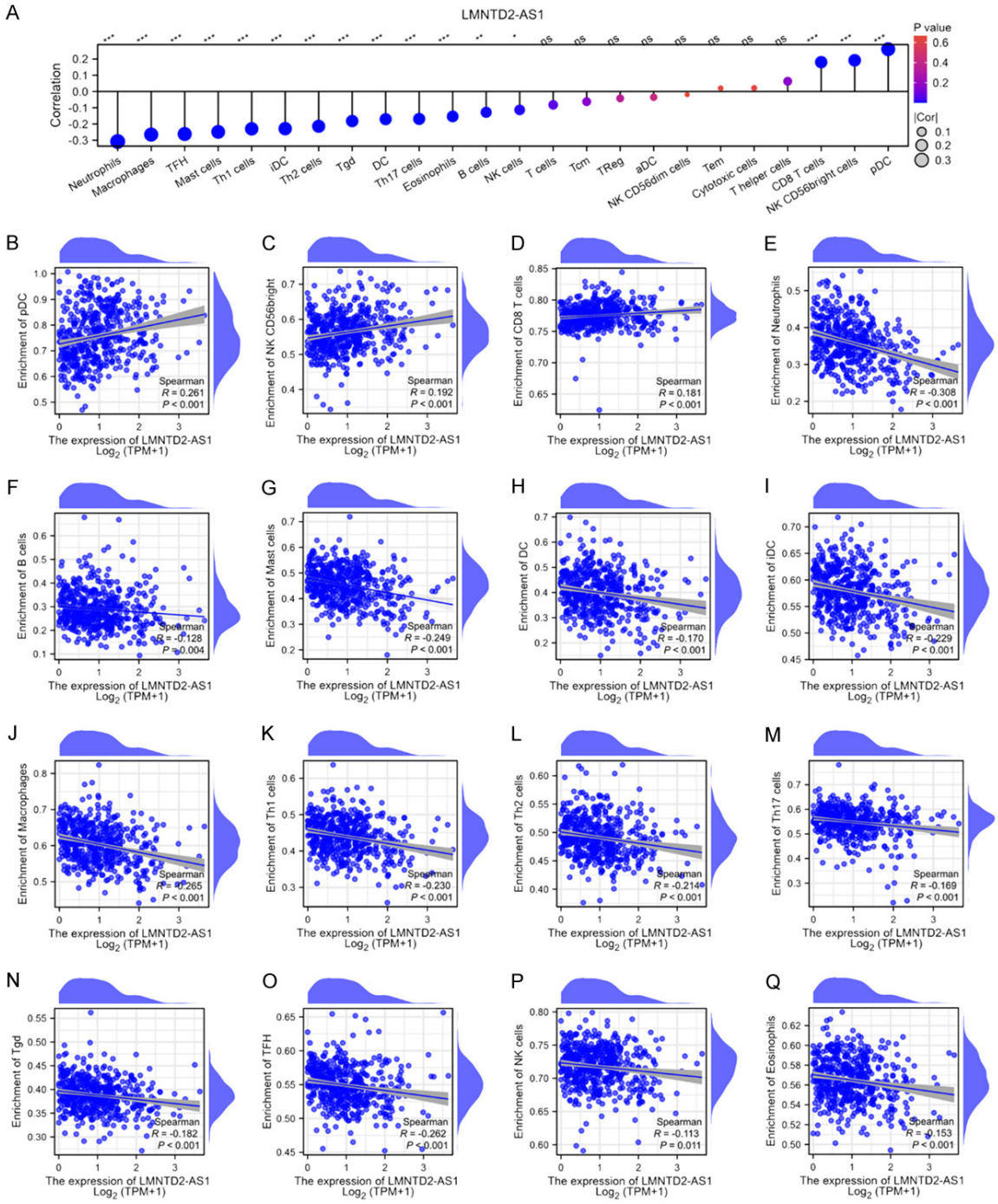

Figure 5. LMNTD2-AS1 was significantly associated with the PCa immune microenvironment. A. Relationship of LMNTD2-AS1 with various immune cells. B. Mast cells. C. Macrophages. D. Eosinophils. E. Neutrophils. F. B cells. G. pDC. H. DC. I. iDC. J. CD8 T cells. K. Th1 cells. L. Th2 cells. M. Th17 cells. N. Tgd. O. TFH. P. NK cells. Q. NC CD-56bright cells. *P < 0.05, **P < 0.01, ***P < 0.001.

FUS expression was upregulated in PCa and high FUS expression was associated with advanced grade and poorer prognosis in PCa patients

We examined FUS expression in PCa and found it to be significantly upregulated in PCa tumor tissues (P < 0.001, **Figure 7A**). Elevated FUS expression was associated with advanced pathological T-stage (P < 0.001, **Figure 7B**), pathological N-stage (P < 0.001, **Figure 7C**), clinical T-stage (P < 0.001, **Figure 7D**), primary treatment outcome (**Figure 7E**), residual tumor (P=0.030, **Figure 7F**), and Gleason score (P <

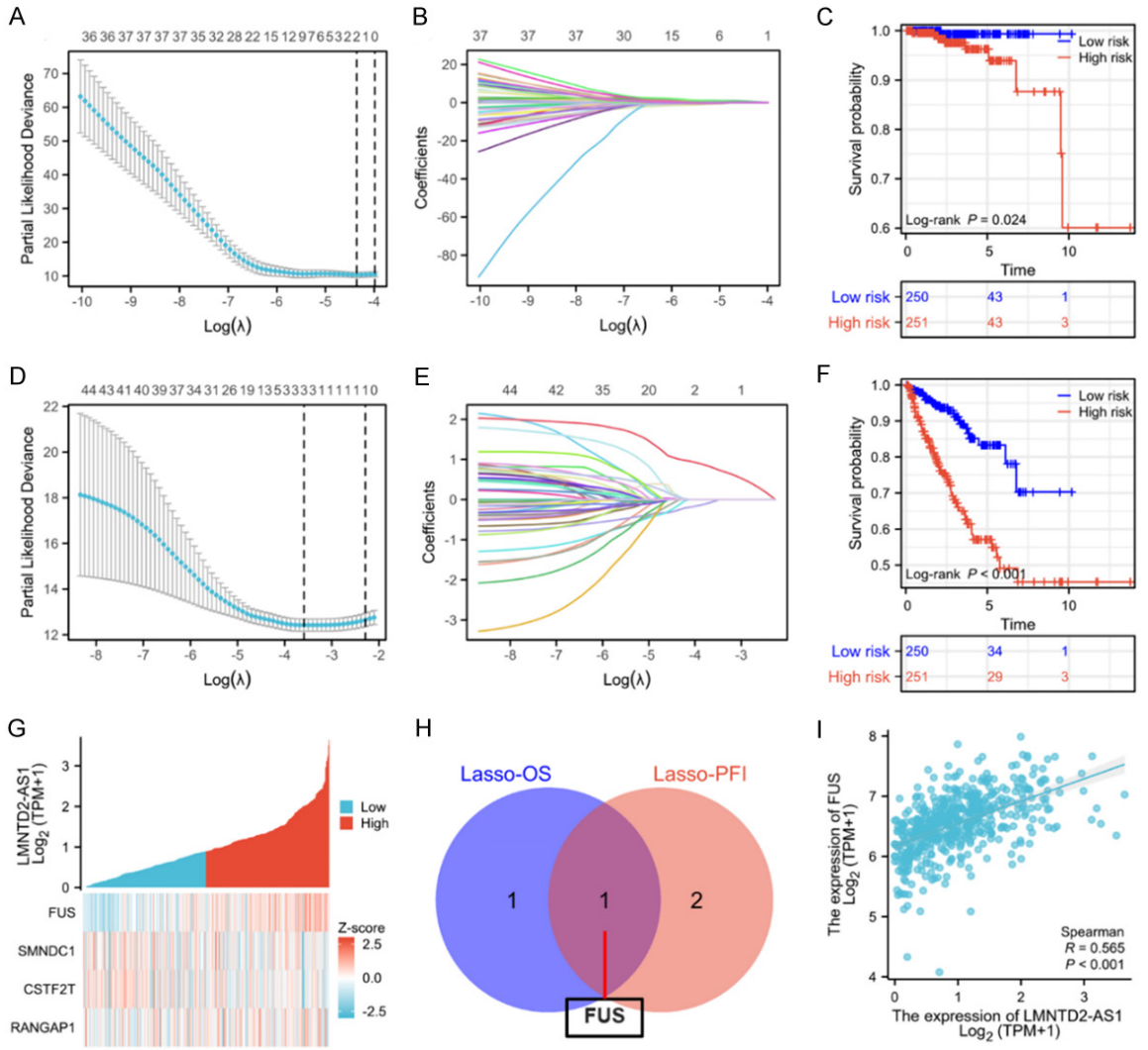

Figure 6. FUS is a possible binding protein for LMNTD2-AS1. A, B. OS Lasso regression analysis of LMNTD2-AS1 related proteins. C. OS survival curves for the high and low risk groups. D, E. PFI Lasso regression analysis of LMNTD2-AS1 related proteins. F. PFI survival curves in the high and low risk groups. G. Co-expression heat map of LMNTD2-AS1 and related proteins. H. Venn diagram of OS and PFI-related proteins. I. Scatter diagram of LMNTD2-AS1 versus FUS.

0.001, **Figure 7H**), but not to PSA (P=0.934, **Figure 7G**). In addition, the ROC curve showed that FUS also efficiently differentiated tumour and normal tissue (AUC=0.752) (**Figure 7I**). In addition, we investigated the relationship between FUS and PCa survival. The results showed that high expression of FUS was associated with poor OS and PFI in PCa patients, and that FUS was also a good predictor of 1-5-year OS and PFI in PCa patients (Figure S3).

FUS overexpression rescues the functional inhibition caused by LMNTD2-AS1 knockdown

Recent studies suggest that enhanced m6A methylation can augment FUS expression, subsequently inhibiting the NRF2/HO-1 signaling pathway, suppressing ferroptosis, and promoting prostate cancer progression [20]. We then evaluated the effect of FUS on LMNTD2-AS1 in LNCap cells. qRT-PCR and WB experiments demonstrated that knockdown of LMNTD2-AS1 significantly inhibited FUS and promoted NRF2 expression (**Figure 8A**), while overexpression of FUS partially rescued FUS expression and reduced NRF2 driven by LMNTD2-AS1 knockdown (**Figure 8B**, **8C**). In addition, wound healing assays and Transwell assays also showed that FUS overexpression could rescue the functional inhibition caused by LMNTD2-AS1 knockdown (**Figure 8D**, **8E**).

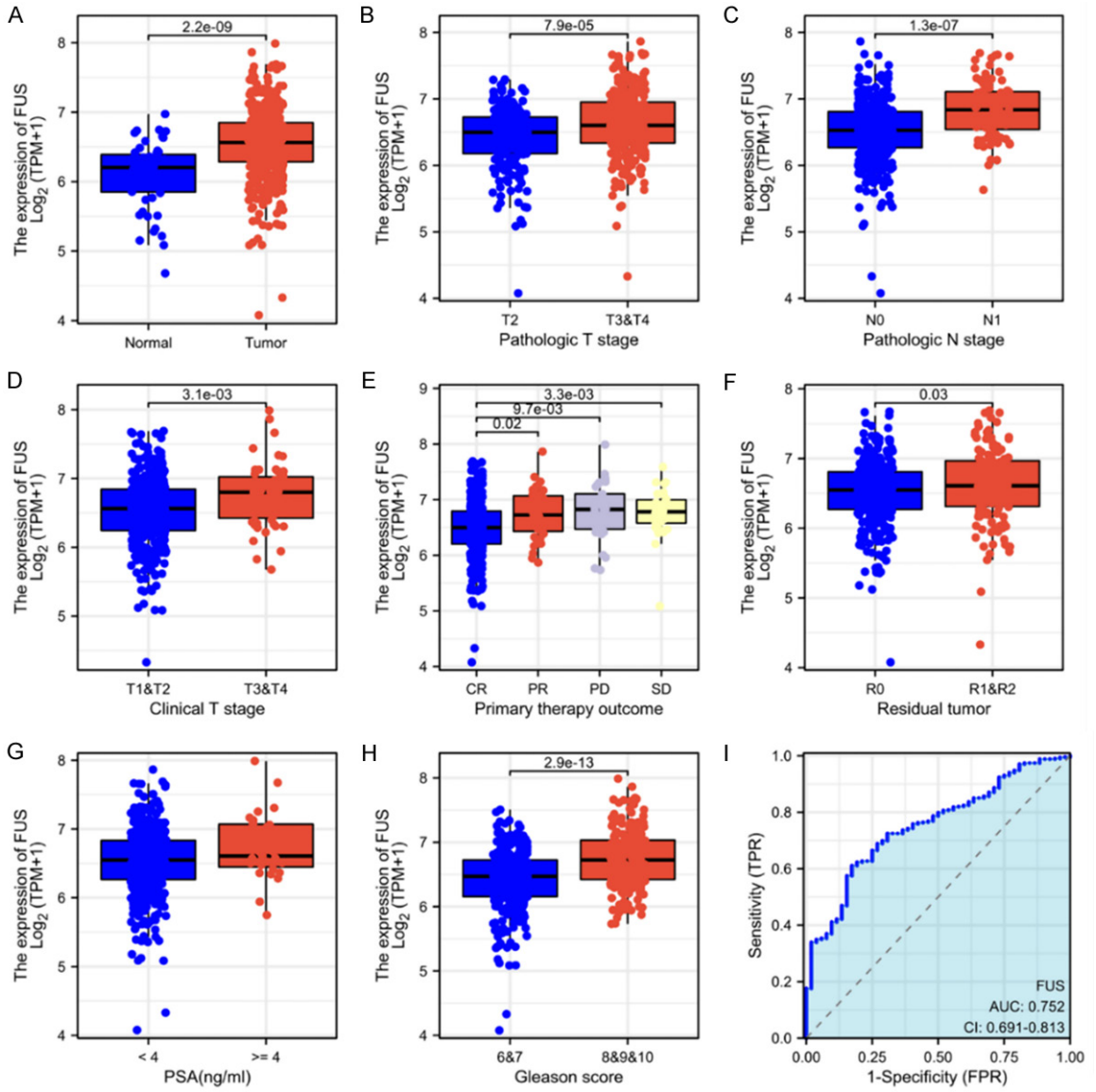

Figure 7. FUS expression in clinicopathological features of PCa patients. A. FUS expression in TCGA-PCa database. B. Pathological T stage. C. Pathological N stage. D. Clinical T stage. E. Primary therapy outcomes. F. Residual tumor. G. PSA. H. Gleason score. I. FUS predicts AUC curves in tumor and normal tissues.

Discussion

In our analysis of the TCGA-PCa database, LMNTD2-AS1 emerged as the sole lncRNA associated with both OS and PFI in PCa patients. Elevated LMNTD2-AS1 expression correlated with advanced grading and a less favorable prognosis. Functional assays revealed that knockdown of LMNTD2-AS1 expression significantly reduced the proliferation, migration and invasive capacity of LNCap cells. Furthermore, we analyzed the relationship between LMNTD2-AS1 and the PCa immune microenvironment and found that LMNTD2-AS1 was associated with multiple immune cell infiltrations. Moreover, rescue experiments demonstrated that LMNTD2-AS1 may promote PCa cell progression through the NRF2 signaling pathway by regulating the RNA-binding protein FUS.

LncRNAs, defined as transcripts exceeding 200 nucleotides without protein-coding potential, have been described as “barren areas in the desert” [21]. Recent studies have shown that most lncRNAs play a critical role in the biologi-

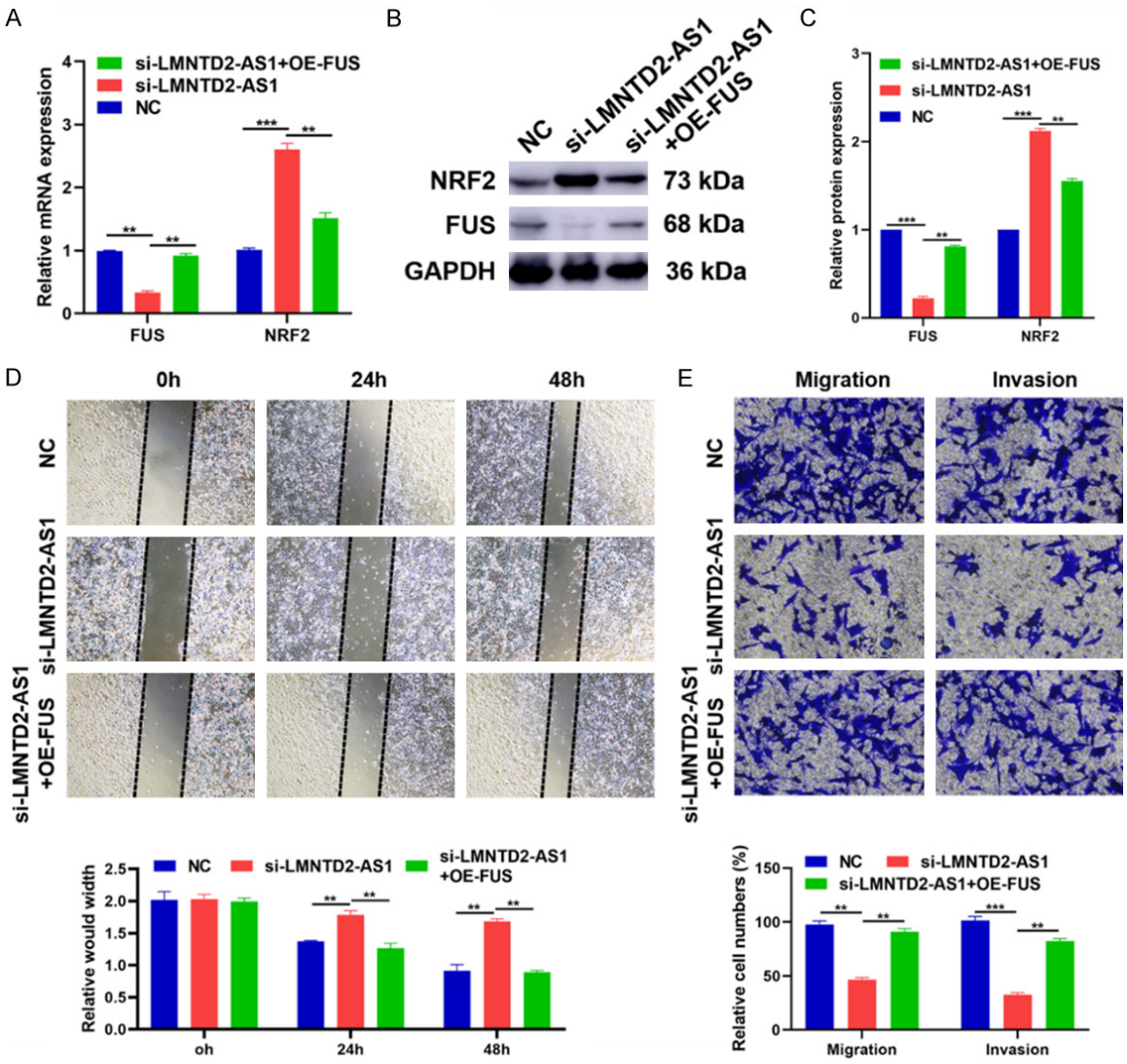

Figure 8. FUS overexpression rescues the functional inhibition caused by LMNTD2-AS1 knockdown. A. Changes in mRNA levels of FUS and NRF2 after LMNTD2-AS1 knockdown and overexpression of FUS. B, C. Changes in protein levels of FUS and NRF2 after LMNTD2-AS1 knockdown and overexpression of FUS. D. Wound healing assay in LNCap cells after LMNTD2-AS1 knockdown and overexpression of FUS. E. Transwell assay of LNCap cells after AS1 knockdown and overexpression of FUS. *P < 0.05, **P < 0.01, ***P < 0.001.

cal functions of cells and diseases [22]. Aberrant lncRNA expression has been shown to disrupt downstream effector molecules pivotal in tumor development [23]. Moreover, recent findings highlight the potential of lncRNAs as biomarkers for diverse malignancies [24]. Given these insights, it becomes imperative to identify novel lncRNAs implicated in PCa and elucidate their mechanistic roles. Our findings underscore the potential of LMNTD2-AS1 as a biomarker for PCa, given its high accuracy (AUC=0.767) in differentiating between PCa tumors and normal tissues.

The recombinant protein FUS, located on chromosome 16, is an RNA binding protein originally identified as a fusion oncogene in human liposarcoma, containing 15 exons that encode a protein of 526 amino acids [25]. Studies have demonstrated that aberrant expression of FUS is closely associated with human cancer [26]. Functional experiments have demonstrated that deletion or overexpression of FUS affects cell proliferation and migration in several tumour cell lines [27, 28]. Numerous studies have found that FUS can play an important role in disease progression as an lncRNA binding pro-

tein. Chen et al. [29] found that LINC00659 can positively regulate SLC10A1 expression in hepatocellular carcinoma (HCC) by recruiting FUS, thereby inhibiting HCC progression. Similarly, Liu et al. [30] showed that lncRNA HOTAIR could modulate STAT3 expression by interacting with FUS, influencing myocardial cell dynamics to ameliorate myocardial ischemia/reperfusion. Our research demonstrates that FUS overexpression can rescue functional suppression induced by LMNTD2-AS1 knockdown, suggesting a potential oncogenic role of FUS in PCa through its interaction with LMNTD2-AS1.

With advancements in tumour research, tumour immunity has received more widespread attention. Research in recent decades has shown that the immune system is linked with all stages of tumour progression [31]. Non-coding RNAs, a popular research topic in recent years, have been shown to be involved in the regulation of systemic immune responses; lncRNAs, a non-coding RNA, play a very important role in different stages of cancer immunity, including immune cell infiltration, tumour antigen release, antigen presentation, immune cell activation and migration [32, 33]. As our understanding deepens, individual or combined immune-related lncRNAs have been recognized for their unique value in cancer research, serving as novel biomarkers for early diagnosis and prognostic indicators across a spectrum of cancers, including those of the liver, lung, and stomach [34-36].

Conclusion

In summary, our study confirms that LMNTD2-AS1 is a highly expressed oncogene in PCa, and that high expression of LMNTD2-AS1 correlates with poor prognosis and the immune microenvironment characteristics of PCa patients. LMNTD2-AS1 may regulate the NRF2 signaling pathway by binding to FUS, which regulates immune cell infiltration and promotes the progression of PCa. Through these results, LMNTD2-AS1 is expected to be a target for PCa patient immunotherapy.

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Disclosure of conflict of interest

None.

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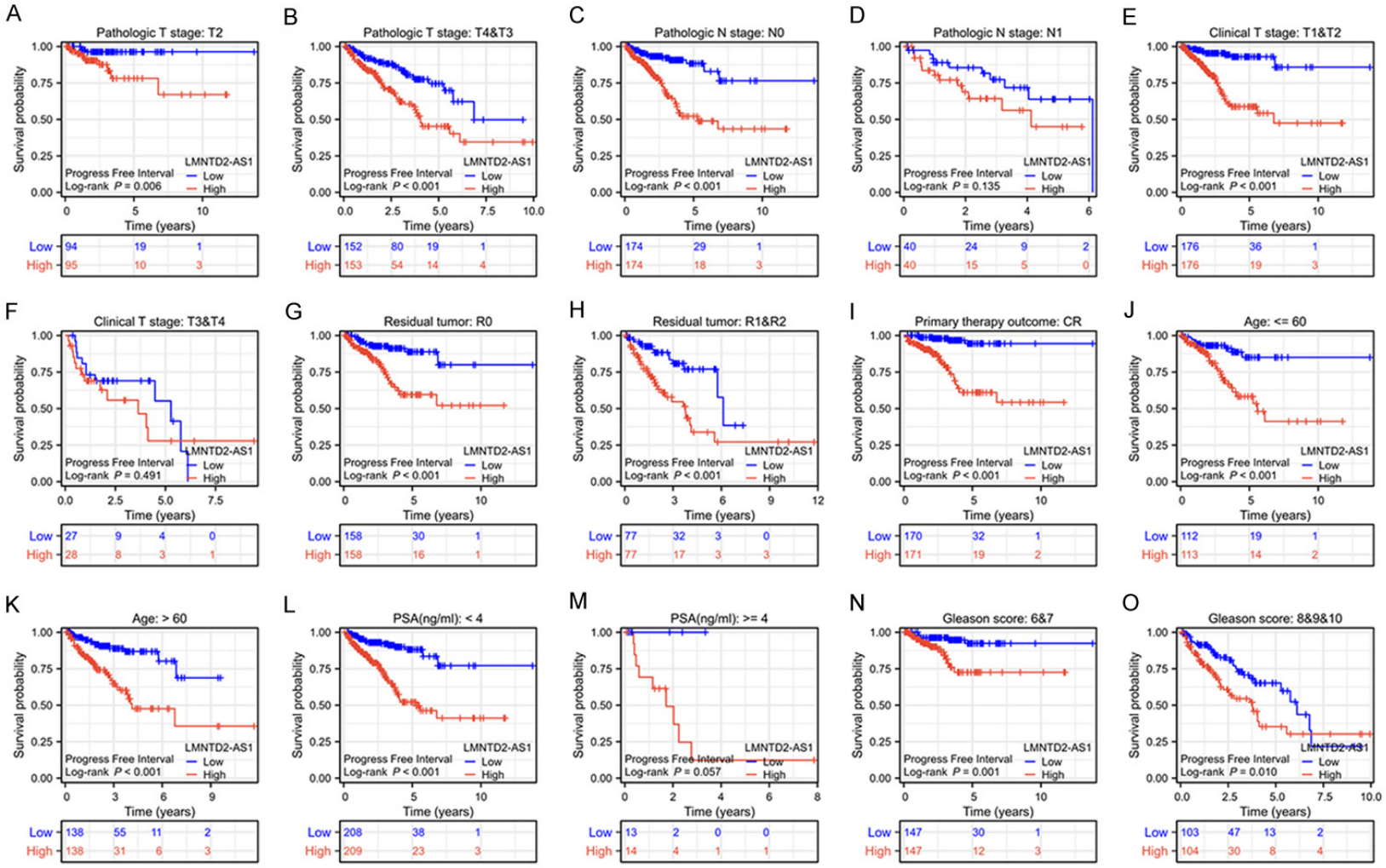

Figure S1. PFI survival curves of different LMNTD2-AS1 in each subgroup. A. Pathological T2 stage. B. Pathological T3&4 stage. C. Pathological N0 stage. D. Pathological N1 stage. E. Clinical T1&2 stage. F. Clinical T3&4 stage. G. Residual R0. H. Residual R1&2. I. Primary CR therapy outcomes. J. Age ≤ 60 years. K. Age > 60 years. L. PSA < 4 ng.ml. M. PSA ≥ 4 ng.ml. N. Gleason score 6&7. O. Gleason score 8&9&10.

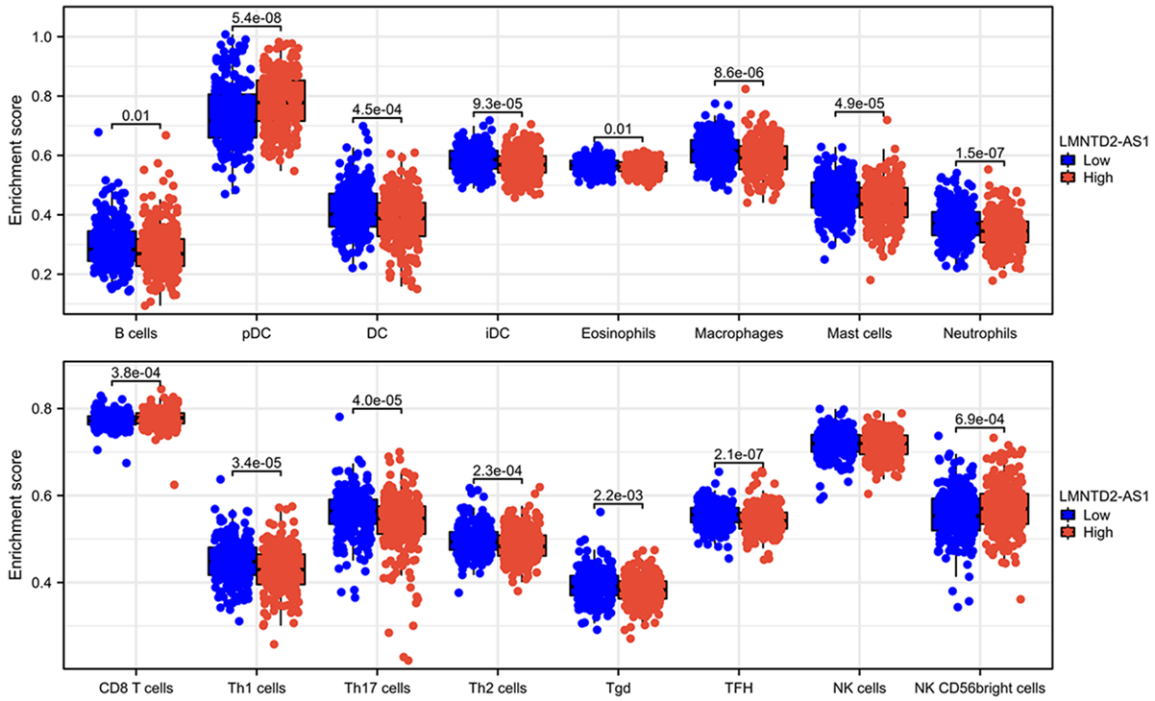

Figure S2. Relationship of LMNTD2-AS1 with various immune cells.

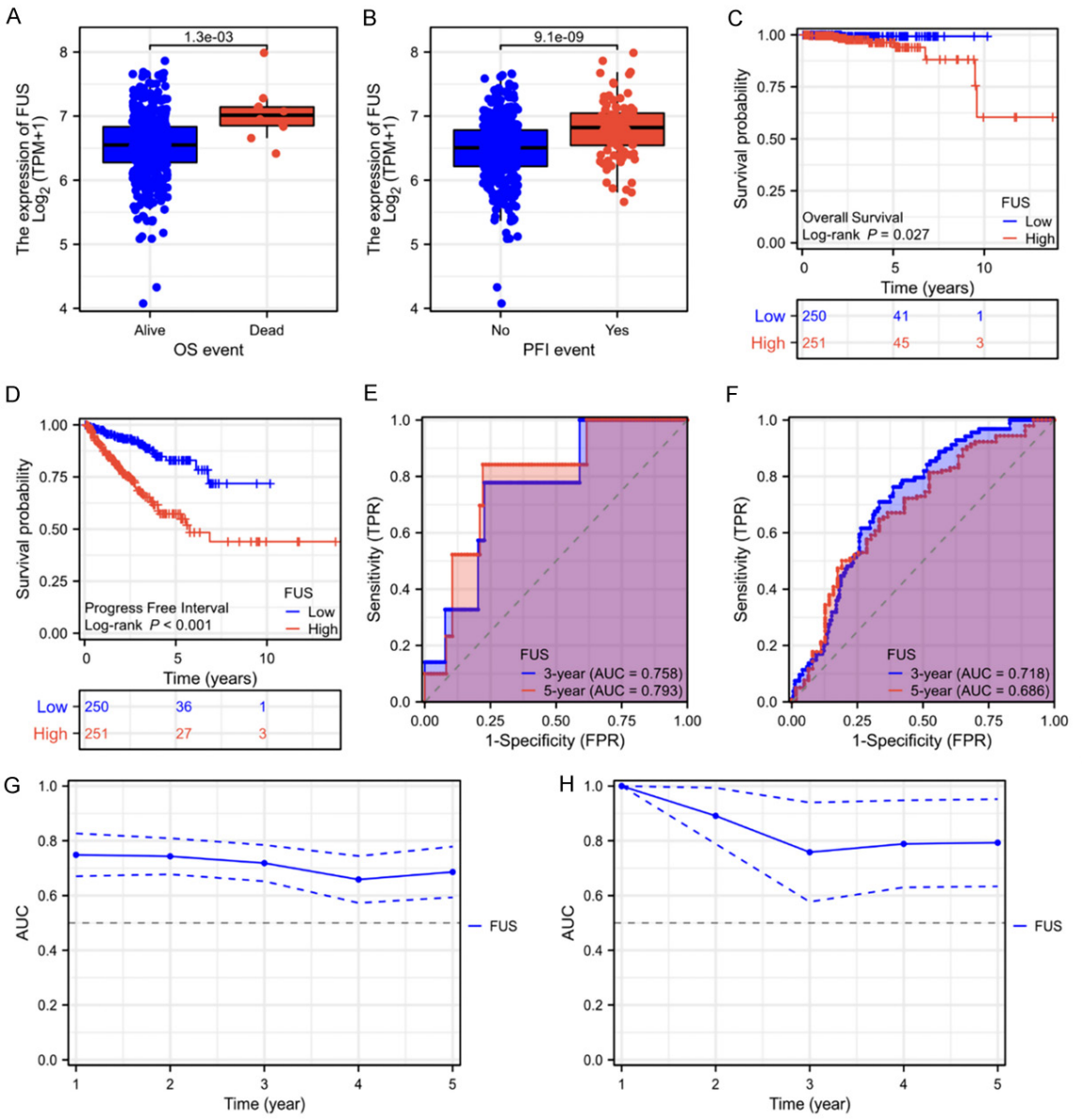

Figure S3. High expression of FUS was associated with poor prognosis. (A, B) Expression of FUS in OS (A) or PFI (B) positive and negative. (C, D) OS (C) or PFI (D) survival curve of FUS high and low groups. (E, F) AUC curves of FUS predicting 3- and 5-year OS (E) or PFI (F). (G, H) AUC curves of FUS predicting 1-5-year OS (G) or PFI (H).