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Original Article

PROM2 upregulation promotes cancer cell migration and confers a poor prognosis in lung squamous cell carcinoma

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Abstract: Lung squamous cell carcinoma (LUSC) remains a difficult-to-treat disease with a poor prognosis. While prominin-1 (*PROM1/CD-133*) is largely investigated in a variety of malignancies, the role of prominin-2 (*PROM2*), the other member of the prominin family, has not been studied in LUSC. Transcriptomic data derived from matched tumor and adjacent non-tumorous lung tissues of LUSC patients were employed to conduct an in-depth analysis of the genetic and epigenetic regulation of prominin genes within LUSC, utilizing bioinformatic approaches. Furthermore, cellular behavior experiments were executed to discern the biological functions of *PROM2*. It was observed that *PROM2*, in contrast to *PROM1*, exhibited significant upregulation and overexpression at both the mRNA and protein levels in LUSC, and this upregulation was correlated with shortened patient survival. Transcriptomic analysis unveiled DNA methylation as an epigenetic regulatory mechanism associated with *PROM2* expression. Notably, two transcription factors, *CBFB* and *NRIP1*, were identified as potential regulators of *PROM2* expression. Subsequent *in vitro* investigations demonstrated that knocking down *PROM2* led to the inhibition of cancer cell migration and the epithelial-to-mesenchymal transition (EMT). In summary, the pronounced upregulation of *PROM2* in LUSC patients was linked to an unfavorable prognosis, possibly attributable to its influence on cancer cell migration and EMT. These findings suggest that *PROM2* could serve as a promising diagnostic biomarker and therapeutic target in the management of LUSC. Consequently, further research into the mechanistic aspects and potential therapeutic interventions targeting *PROM2* is warranted in the clinical context.

Keywords: Lung squamous cell carcinoma, PROM2, epithelial-to-mesenchymal transition

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide, with 2.2 million new cases and 1.8 million deaths reported by the World Health Organization (WHO) in 2020, imposing a substantial global healthcare burden [1]. It is categorized into small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), further subdivided into adenocarcinoma (LUAD), squamous cell carcinoma (LUSC), and large cell carcinoma [2]. Patients are often asymptomatic until late stages, leading to delayed diagnoses, particularly in Taiwan, where 44% of NSCLC and 76% of SCLC patients are diagnosed with stage IV disease, precluding curative treatment [3]. LUSC, constituting 25%-30% of NSCLC cases, differs significantly from LUAD in histopathology, clinical features, and prognosis [4]. LUSC patients are typically older smokers [5]. While historically sharing treatment options with LUAD, the advent of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors led to divergent management. LUAD benefits from targeted therapy due to identified oncogenic alterations, whereas a lim-

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ited understanding of LUSC biology results in fewer treatment options and shorter survival [6]. Therefore, exploring efficient and tolerable LUSC treatments is imperative.

Prominins are cholesterol-binding, pentaspan transmembrane glycoproteins. The prominin family includes prominin-1 (PROM1) and prominin-2 (PROM2), which are widely conserved in the animal kingdom. However, Prominin-3 is now only found in non-mammals, such as zebrafish [7]. PROM1 (*CD133*) is the prototype of the prominin protein, mostly located at the apical plasma membrane in various embryonic, epithelial, and stem cells. Hence, it is widely used as a famous stem cell marker which plays a key role in cell differentiation, proliferation, and apoptosis in various cancers [8]. PROM2, a paralogue of PROM1, shares only 30% similarity with PROM1 in terms of amino acid sequences. Unlike PROM1, PROM2 is expressed exclusively in epithelial cells with a non-polarized, or basolateral predominant distribution [9].

The cellular and biochemical properties of PROM1 and PROM2 are thought to be comparable based on their structural similarities. However, The biological functions of PROM2 are less studied compared to PROM1, especially in cancer biology. In this article, we aimed to study the role of the prominin family, especially PROM2, in LUSC patients.

Materials and methods

Next-generation sequencing (NGS)

The transcriptome analysis was obtained from 3 pairs of lung tumors and adjacent non-tumor tissues of LUSC. The specimens were acquired from elective surgical resection of three confirmed LUSC patients with treatment intent. The study protocol was reviewed and approved by the Institutional Review Board of Kaohsiung Medical University Hospital (KMUH-IRB-20130054, May 24, 2013). The surgical tissues were freshly isolated without fixation and were immediately extracted for total RNA preparation. RNA library, including small RNAs, was constructed by an Illumina sample preparation kit, according to the protocol of the TruSeq RNA or Small RNA Sample Preparation Guide. The mRNA profiling was examined by NGS on the Illumina platform (Welgene Biotechnology Company, Taipei, Taiwan). The criteria used for picking up the differentially expressed genes (DEGs) after NGS analysis were fold change (the expression ratio in tumor compared to non-tumor region) more than 2 or less than 0.5 and a p-value < 0.05, as well as the fragments per kilobase per million (FPKM) > 0.3 in each sample.

Bioinformatics

The mRNA expression of *PROM1* and *PROM2* was derived from The Cancer Genome Atlas (TCGA) database on UALCAN website (accessed on December 1, 2021; ualcan.path.uab.edu). Using the interactive website, the expression levels of both *PROM1* and *PROM2* mRNAs were reclassified according to lymph node metastasis (N0-N3) and tumor stage (pathologic stages 1 to 4). The ratio of mRNA expression in lung cancer and normal specimens (cancer vs. normal) was derived from Oncomine database (accessed on December 1, 2021; http://www.oncomine.org), Compendia biosciences, Ann Arbor, MI, USA.

The protein expression of PROM1 and PROM2 was retrieved from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) database (accessed on January, 2021). The expression levels of both proteins were reclassified under the criteria of lymph node metastasis (N0-N3) and tumor stage (stages 1 to 4). The relationship between the prominins and overall survival time in LUSC was assessed by either the RNA-seq or the RNA gene chip cohort in the KM plotter (accessed on August 1, 2020; http://kmplot.com/analysis/). Patients were divided into 2 groups (high and low expression) based on the best cut-off in RNA-seq cohort and by the median value in the RNA gene chip cohort. The probability of survival between the two groups was computed. The hazard ratios (95% confidence intervals, CIs) were calculated using the Cox proportional model.

Immunohistiochemistry (IHC) staining for PROM2 protein

The in-house formalin-fixed paraffin-embedded (FFPE) LUSC tissues were collected. The FFPE tissue block was subsequently sectioned into 8 μm slices. De-waxing of these sections was carried out using xylene, followed by a rehydration process involving a descending ethanol

gradient. Antigen retrieval was performed via heat-mediated techniques within a pressure cooker, with a duration of 90 seconds. To inactivate endogenous peroxidase activity, sections were subjected to a 10-minute incubation with 3% hydrogen peroxide at room temperature. Furthermore, to mitigate non-specific antibody binding, sections were treated with 3% bovine serum albumin (MilliporeSigma) for a period of 20 minutes at room temperature. In this investigation, an assessment of PROM2 protein expression was carried out employing a rabbit polyclonal antibody specific to PROM2 (Catalog Number GTX47864, GeneTex Inc., Taiwan) at a dilution ratio of 1:200. The incubation was conducted at a temperature of 4°C overnight. Subsequently, immunoreactivity was probed using horseradish peroxidase-conjugated anti-rabbit secondary antibodies (dilution 1:1000; cat. no. ab6721, Abcam) for a duration of 20 minutes at room temperature. The ensuing steps included PBS washing and the application of 3,3' diaminobenzidine staining for 2 minutes at room temperature. Histological sections were subjected to examination through an ICC50 HD light microscope (Leica Microsystems, Inc.) equipped with a digital camera.

Investigations of DNA methylation and copy number

The extent of *PROM2* DNA methylation was compared according to the tissue source, the tumor stages, and the stages of lymph node metastasis on the UALCAN website (accessed on January 1, 2021; ualcan.path.uab.edu). The profiles of gene-specific DNA methylation data, copy number (gene level) and clinical information of patients with LUSC were extracted from the dataset TCGA Pan-Cancer (PANCAN) from the UCSC Xena website (https://xena.ucsc.edu/). Pearson's correlation between *PROM2* mRNA expression level and the copy number and/or DNA methylation was calculated using the metadata.

MicroRNAs and PROM2 correlation

Pearson's correlation between *PROM2* and all microRNAs in the TCGA LUSC dataset was calculated. The miRNAs were ranked by r value and validated by TargetScan (accessed on August 1, 2020; http://www.targetscan.org/vert_72/) from the top-ranked miRNAs. Excluding the miRNAs with context++ score percentile < 90, only miR-490-3p remained with a context++ score more than 70th percentile.

Predicting the transcriptional factors (TFs) responsible for regulating PROM2

The potential TFs of PROM2 were retrieved from the hTFtarget website (http://bioinfo.life.hust.edu.cn/hTFtarget#!/). hTFtarget website has compiled an extensive set of TF-target interactions by leveraging large-scale ChIP-Seq data involving human TFs. hTFtarget also developed a tailored analytical framework aimed at identifying robust TF-target interactions. Subsequently, the mRNA expression profiles of the identified TFs were extracted from our internal LUSC samples and the TCGA LUSC dataset. TFs that failed to demonstrate upregulated expression levels specifically within the tumor compartments in both cohorts were methodically eliminated from consideration, as they were presumably less likely to function as regulators of *PROM2*. In the final step, we assessed the survival implications associated with the remaining TFs using KM plotter (http://kmplot.com/analysis/) which was described in previous section "2.2. Bioinformatics". Only TFs demonstrating a detrimental effect on survival were retained as potential candidates for regulating *PROM2*.

Exploration of the associating pathways of PROM2

Gene Set Variation Analysis (GSVA) (http://bioinfo.life.hust.edu.cn/GSCA/#/expression) is a non-parametric and unsupervised computational method utilized to quantify the fluctuations in gene set enrichment across samples within an expression dataset. GSVA enables the evaluation of pathway enrichment specific to each sample. The resulting GSVA enrichment scores facilitate the functional enrichment analysis, survival assessment, sample clustering, analysis of copy number variations (CNV) in the context of pathways, and cross-tissue pathway investigations.

Gene Set Enrichment Analysis (GSEA) was also employed to explore the associating biological pathways of *PROM2*. The positively *PROM2*-correlated genes were extracted from the TCGA LUSC dataset on UALCAN website, using a criterion of the Pearson correlation

coefficient > 0.3. Then the extracted geneset (number = 230, gene list not shown here) was input into GSEA.

Cell lines and cell culture

Human LUSC cell lines H1703 and H520 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). LUSC cells were cultured in F-12K Medium (ATCC) with the supplement of 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific, Boston, MA, USA). H1703 and H520 cells were authenticated by the short tandem repeat analysis (Promega, Madison, WI, USA) and ascertained negative for mycoplasma contamination by MycoAlert™ mycoplasma detection kit (Lonza, Switzerland) every 3 months.

PROM2 knockdown LUSC cells

PROM2-knockdown cells were established using a shRNA expression system obtained from the National RNAi Core Facility (Taipei, Taiwan). The stable clone of *PROM2* shRNA-expressing cells was transfected with the shRNA plasmid. One day after transfection, the transfected cells were selected with 2 μg/ml puromycin until the presence of stable knockdown efficiency. The knockdown efficiency of *PROM2* shRNA plasmid was determined by qRT-PCR. In addition, the *PROM2*-knockdown LUSC cells were also subjected to the NGS as the method described previously. The transcriptomic data of 3 independent stable clones were sequenced which demonstrated the persistency of *PROM2* knockdown effect.

Transwell migration and wound healing assays

For the migration assays, cells were initially seeded into inserts containing polyester membranes with a pore size of 8 μm (EMD Millipore, Burlington, MA, USA). In the lower wells of the insert, a complete cell culture medium was added to serve as a chemo-attractant for 48 hours. The migratory cells were subsequently visualized through crystal violet staining. Alternatively, cells were cultured in a 12-well plate until they reached 100% confluence. The extent of cell movement was quantified by assessing the migration of cells into an acellular region created by the controlled application of a sterile tip. The quantitative analysis of transwell migration was conducted using a counting methodology.

Immunoblotting for EMT markers

Cellular total protein was extracted from LUSC cells with or without *PROM2* knockdown using RIPA lysis buffer (EMD Millipore, Billerica, MA, USA) supplemented with the protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). An equal amount of cellular protein was denatured by heating and then separated by SDS-PAGE. Proteins were transferred to PVDF membranes (EMD Millipore, Burlington, MA, USA) and probed with various primary antibodies for 4-16 h, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell-Signaling Technology, Danvers, MA, USA). Signals of specific proteins were detected using a chemiluminescence kit (EMD Millipore, Burlington, MA, USA). The primary antibodies used in the EMT experiment included N-cadherin (Catalog #610921, BD Biosciences, San Jose, CA, USA), E-cadherin (Catalog #610182, BD Biosciences, Franklin Lakes, NJ, USA), Vimentin (Catalog #550513, BD Biosciences, Franklin Lakes, NJ, USA), α-SMA (Catalog #A5228, Sigma-Aldrich, St. Louis, MO, USA), Slug (Catalog #9585, Cell-Signaling Technology, Danvers, MA, USA), and GAPDH (Catalog #MAB374, EMD Millipore, Burlington, MA, USA).

Statistical analysis

Results of the cell experiments are presented as mean ± standard deviation (SD). Differences between the two tested groups were compared using the Student's *t*-test with GraphPad Prism software (7.04 version, Graphpad Software, San Diego, CA, USA). Statistical significance was defined as p-value < 0.05.

Results

PROM2, but not PROM1, was upregulated in human LUSC

As a result of transcriptomic analysis, the DEG profile of in-house LUSC specimens was illustrated by the volcano plot which facilitates the visualization of significantly up- or down-regulated mRNAs. The prominin family, e.g. *PROM1* and *PROM2,* were specifically labeled. The

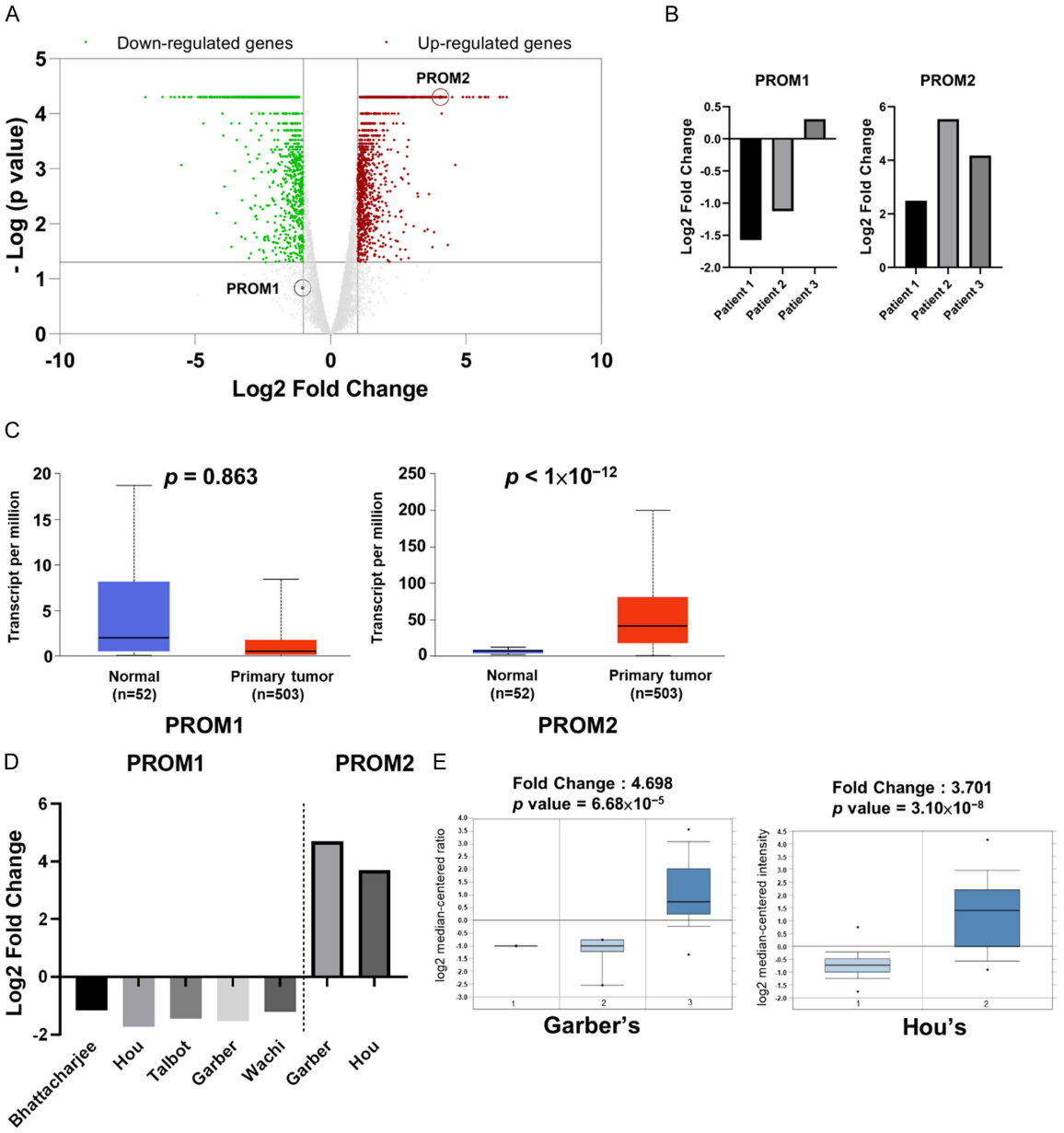


Figure 1. *PROM2,* but not *PROM1*, was up-regulated in lung squamous cell carcinoma (LUSC). A. The volcano plot summarizes the sequencing results of the in-house samples. Red dots represent the upregulated genes whereas green dots are representative of downregulated genes. B. Differential transcriptomic expression pattern of *PROM1* and *PROM2*, expressed as fold change, in our 3 pairs of LUSC samples. C. Differential expression patterns of *PROM1* and *PROM2* in The Cancer Genome Atlas (TCGA) LUSC cohort. D. *PROM1* and *PROM2* expression patterns in the Lung Cancer cohort in the Oncomine database. E. Higher *PROM2* expression in cancerous parts is reported in 2 independent LUSC datasets (Garber's and Hou's datasets).

expression of *PROM2*, but not *PROM1*, was significantly upregulated in tumor tissue, compared with adjacent non-tumorous tissue (**Figure 1A**). Transcriptomic analysis revealed remarkable upregulation of *PROM2* mRNA in all tumor tissues from the 3 LUSC patients. In contrast, the expression pattern of *PROM1* was not uniform in these samples (**Figure 1B**). To validate this finding, the public transcriptome data of LUSC patients, including 503 tumoral and 52 normal specimens, were extracted from TCGA by using the UALCAN website. The result demonstrated a significant upregulation (*p*-value < 1×10^{-12}) of *PROM2* but not *PROM1* in LUSC

tumor parts (**Figure 1C**). The Oncomine database was also searched for transcriptomic datasets of LUSC. Five datasets of the *PROM1* expression profiles and two of the *PROM2* were obtained respectively. Similarly, *PROM2* was significantly upregulated in both datasets available (**Figure 1D**, **1E**). As regards *PROM1*, the expression level in the tumor part seemed to be non-significantly down-regulated both in TCGA and Oncomine datasets (**Figure 1C**, **1D**).

Clinical significance of PROM2 upregulation in LUSC

The clinical significance of *PROM1* and *PROM2* was searched on UALCAN website using TCGA data. The mRNA expression level of *PROM1* and *PROM2* in different-stage tumors was demonstrated. The result showed a statistically significant upregulation of *PROM2* across different stages compared to that in normal tissue. However, this trend was not observed with *PROM1* (**Figure 2A**). In addition, *PROM2* in tumors with different lymph node metastatic stages showed higher expression than those in the normal part. Again, the significance was not found with *PROM1*, the other member of the prominin protein family (**Figure 2B**).

The survival association of a specific gene is crucial since it may indicate the oncogenic importance of the molecule. The survival significance of PROM1 and PROM2 in LUSC were searched on the KM plotter website respectively. The result showed the upregulation of PROM2 was associated with shorter overall survival in LUSC patients with a hazard ratio of 1.48 (CI 1.11-1.97, P = 0.008). Nonetheless, PROM1 did not demonstrate a survival association (**Figure 2C**).

Over-expression of PROM2 in protein level in LUSC

The tumor and normal parts of the resected samples were stained immunohistochemically by PROM2 antibody and showed obvious staining in LUSC tumor cells compared with the normal part (**Figure 3A**). Furthermore, we extracted the proteomic data from the CPTAC database to determine the protein expression extent of PROM2 in LUSC. The investigation, again, revealed a higher PROM2 protein expression in the LUSC tumor part compared with that in non-tumorous adjacent lung tissue (**Figure 3B**).

To investigate the clinical importance of the PROM2 protein, we also compared the PROM2 expression level in LUSC samples categorized by cancer stage and lymph node metastatic status. In samples of stages 1&2 or 3&4, the PROM2 was significantly expressed at a higher extent (**Figure 3C**). However, the expression difference between stages 1&2 and 3&4 was not significant. Similarly, the higher PROM2 expression was noted in N0 and N1&2 groups, compared with normal samples (**Figure 3D**).

Epigenetic regulation of PROM2 in LUSC

DNA methylation, CNV, and microRNA regulation are important epigenetic controls for specific gene expression. For methylation, data was extracted from the TCGA LUSC cohort. Methylation of *PROM2* was significantly suppressed in tumors. In advanced tumor stage or lymph node metastasis, the *PROM2* methylation also declined in tumors (**Figure 4A**). Besides, there was a strong negative correlation between the DNA methylation of *PROM2* and the expression of *PROM2* with r = -0.407 and *p*-value = 2.67×10^{-16} (**Figure 4B**). The copy number of *PROM2* did not exhibit a significant correlation with *PROM2* mRNA expression levels (r = 0.003, *p*-value = 0.955) within the TCGA LUSC cohort (**Figure 4C**).

For microRNA investigations, we searched miRNAs with a negative correlation with *PROM2* expression. After filtering the context score > 70, miR-490-3p was a potential regulating miRNA of *PROM2* (**Figure 4D**). miR-490 also suppressed in tumor part and high expression of miR-490 correlated with better clinical outcome (**Figure 4E**, **4F**). However, the context score percentile of miR-490 was only 73, reflecting that miR-490 might not be an optimal candidate miRNA regulating *PROM2* expression (**Figure 4G**).

Transcription regulation of PROM2 in LUSC

The predicted TFs of *PROM2* were retrieved from the website hTFtarget. Two potential TFs, CBFB and NRIP1, remained after the full analysis as described in section 2.6. *CBFB* and *NRIP1* were upregulated in mRNA level in our in-house LUSC samples (**Figure 5A**) as well as in TCGA LUSC cohort (**Figure 5B**). Upregulated *CBFB* and *NRIP1* were associated with poor survival (**Figure 5C**). Moreover, to demonstrate the transcriptional effect of *CBFB* and *NRIP1*

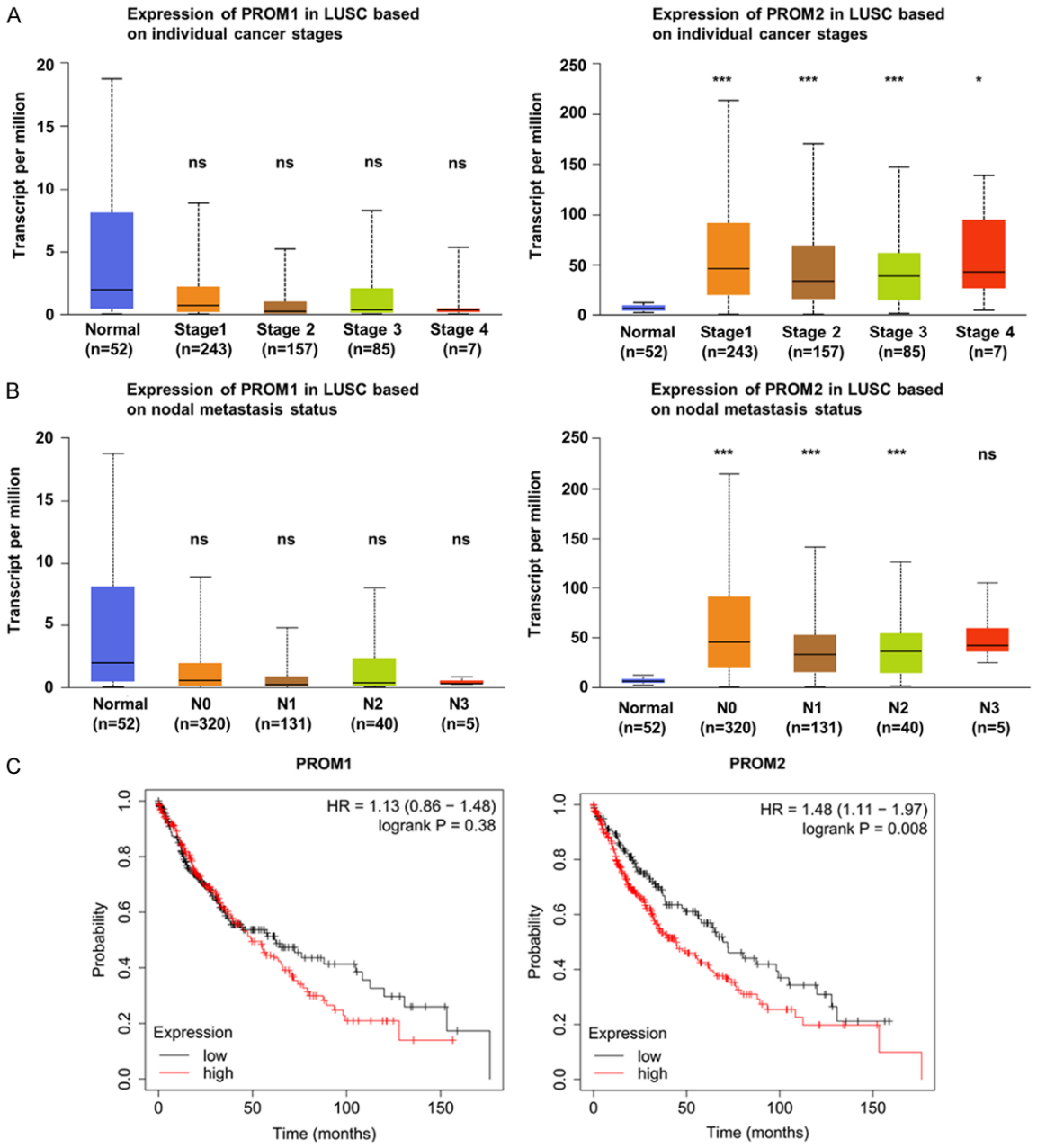


Figure 2. Up-regulated *PROM2* in LUSC was associated with extensive involvement and poor prognosis. A. The *PROM1* and *PROM2* expression patterns are illustrated by different cancer stages. B. The *PROM1* and *PROM2* expression patterns are illustrated by the different extent of lymph node involvement. C. The overall survival curves of LUSC patients are stratified by high/low expression of *PROM1* or *PROM2*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

on *PROM2*, we analyzed the combination effect of *PROM2* with *CBFB* or *NRIP1* on the survival. As a consequence, the adverse influence of *PROM2* on survival was solely evident in instances characterized by elevated expression levels of *CBFB* or *NRIP1* (**Figure 5D**). Conversely, this detrimental effect was not observed within the context of diminished *CBFB* or *NRIP1* expression (**Figure 5E**), implicating the regulatory roles of the two TFs on *PROM2*. Utilizing the TCGA LUSC cohort, we substantiated a positive correlation between *CBFB* or *NRIP1* and *PROM2* (**Figure 5F**). Furthermore, at the protein level, it was

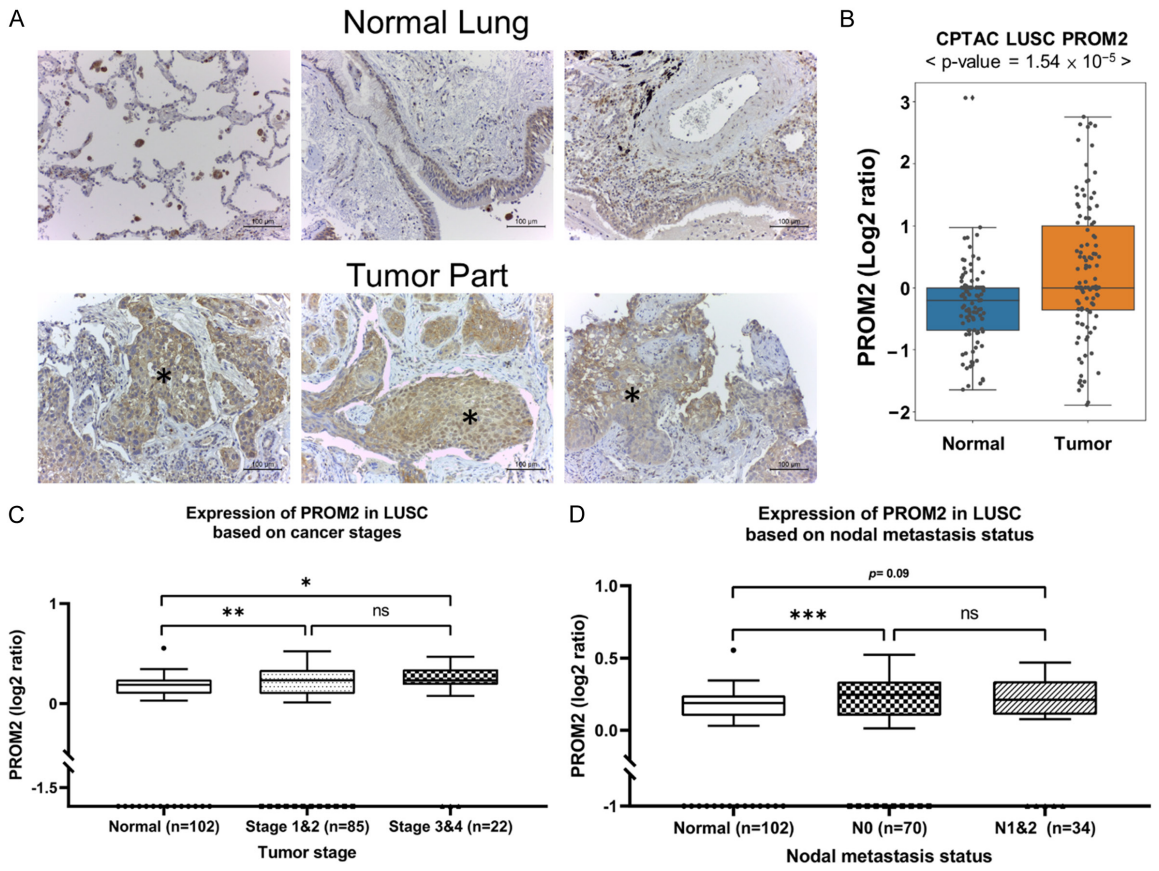
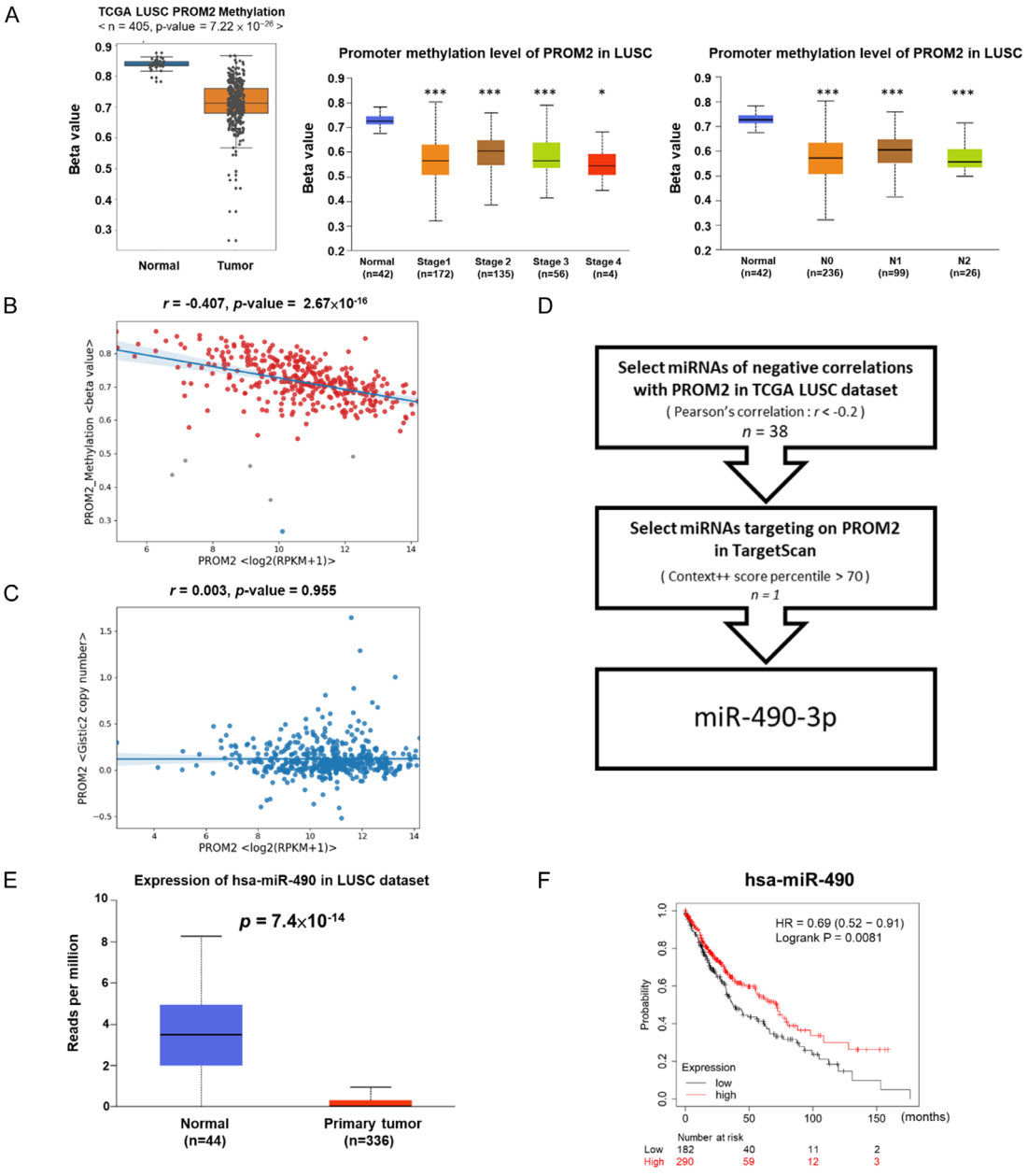


Figure 3. PROM2 over-expressed in protein level in LUSC. A. The immunohistochemistry (IHC) staining of PROM2 in normal and tumor parts of LUSC specimens respectively. The asterisk denotes the compartment of cancer cells. B. PROM2 protein expression patterns in LUSC dataset retrieved from the public Clinical Proteomic Tumor Analysis Consortium (CPTAC) database. C. PROM2 protein expression patterns in the CPTAC LUSC dataset stratified by different cancer stages. D. PROM2 protein expression patterns in the CPTAC LUSC dataset stratified by the different nodal metastasis status. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

observed that CBFB and NRIP1 exhibited pronounced expression within the tumor regions in the CPTAC LUSC cohort (**Figure 5G**). Notably, PROM2 protein expression also displayed a significant elevation in samples characterized by high CBFB or NRIP1 levels (**Figure 5H**).

EMT was the biological pathway promoted by the up-regulation of PROM2 in LUSC

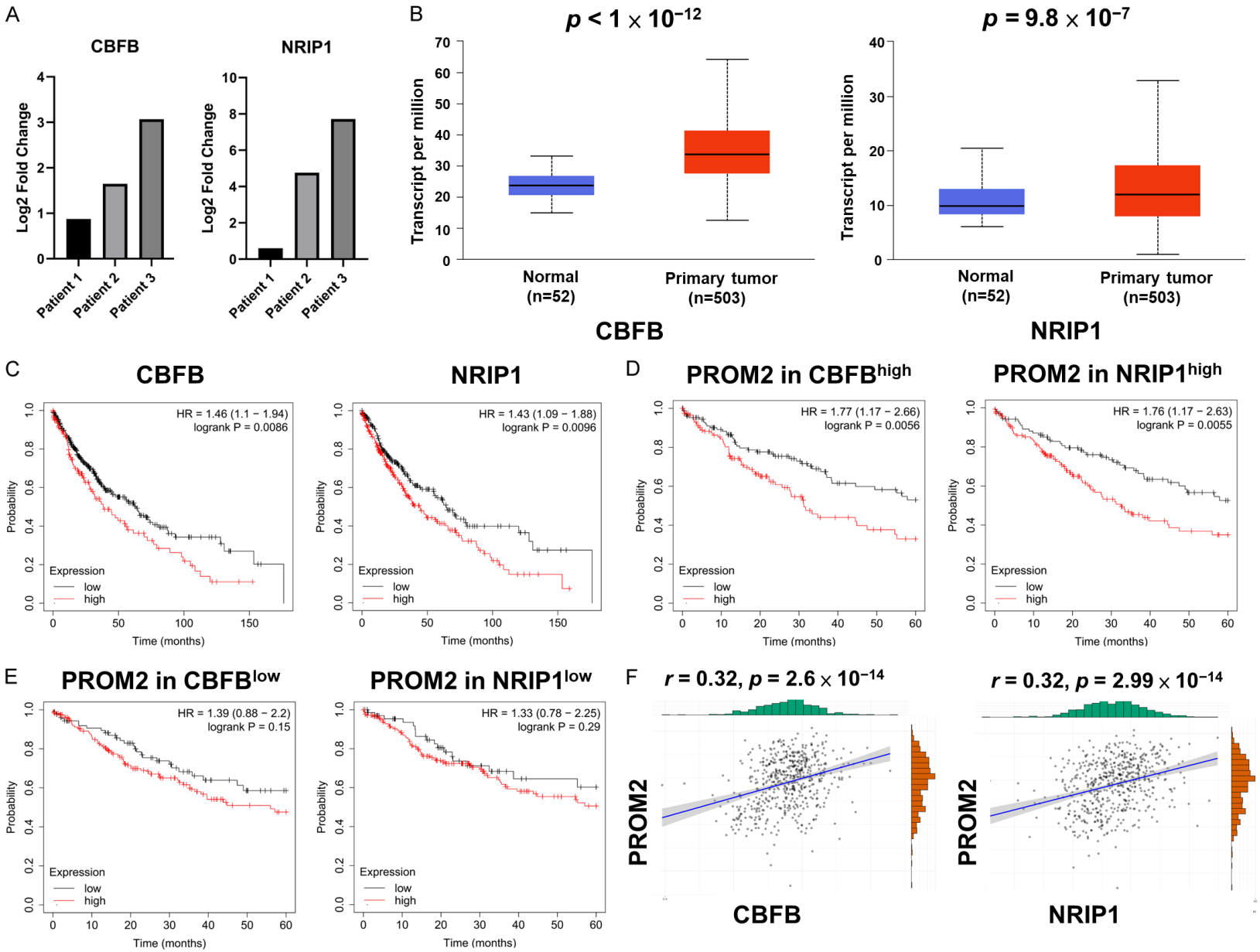
PROM2-knockdown H520 and H1703 cell lines underwent NGS for transcriptomic characterization. Subsequently, the downregulated genes within each cell line were systematically investigated, considering them statistically significant if they satisfied the criteria of a fold change less than 0.5 and a *p*-value below 0.05. The comparison of gene lists revealed 30 common downregulated genes attributed to shPROM2 knockdown in both cell lines (**Figure 6A**). According to the GSVA, the gene set associated with *PROM2* in the cell lines exhibited a statistically significant correlation with the epithelial-mesenchymal transition (EMT) pathway (**Figure 6B**). This association was further established as being predictive of unfavorable clinical outcomes (**Figure 6C**), including shortened overall survival (OS), progression-free survival (PFS), and disease-specific survival (DSS) in LUSC. In addition to the *in vitro* sequencing data, we sought validation by cross-referencing the findings with a publicly available human transcriptomic database. The results of GSEA reinforced the alignment with biological pathways, most notably the EMT. Furthermore, the *PROM2* co-expressive gene set demonstrated a significant link to shortened survival in the context of lung cancer (**Figure 6D**).



G

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type	Context ++ score	Context++ score percentile	Weighted context++ score	Conserved branch length	PCT
Position 1129-1135 of PROM2 3' UTR hsa-miR-490-3p	5' ...UCUGGGUCCUUAAAACCAGGUUU... 3' GUCGUACCUCAGGAGGUCCAAC	7mer -m8	-0.08	73	-0.08	0.509	<0.1

Figure 4. Epigenetic regulation of *PROM2* in LUSC. A. The pattern of promoter methylation of *PROM2* stratified by tissue compartment, cancer stages, and nodal involvement status, respectively, in the TCGA LUSC dataset. B. The correlation between methylation level and *PROM2* gene expression. C. The correlation between the level of copy number alteration and *PROM2* gene expression. D. The searching algorithms and filtering processes for the potential miRNAs targeting *PROM2* mRNA. E. The expression pattern of miR-490 in LUSC specimens retrieved from TCGA database. F. The survival significance of high- or low-expressions of miR-490 in LUSC patients. G. The profiles and predicting ability of miR-490 on the Targetscan website. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.


A
CBFB
NRIP1
Log2 Fold Change
Patient 1
Patient 2
Patient 3
B
p < 1 × 10−12
p = 9.8 × 10−7
Transcript per million
Normal (n=52)
Primary tumor (n=503)
CBFB
NRIP1
C
CBFB
NRIP1
HR = 1.46 (1.1 − 1.94)
logrank P = 0.0086
HR = 1.43 (1.09 − 1.88)
logrank P = 0.0096
Probability
Expression
low
high
Time (months)
D
PROM2 in CBFBhigh
PROM2 in NRIP1high
HR = 1.77 (1.17 − 2.66)
logrank P = 0.0056
HR = 1.76 (1.17 − 2.63)
logrank P = 0.0055
E
PROM2 in CBFBlow
PROM2 in NRIP1low
HR = 1.39 (0.88 − 2.2)
logrank P = 0.15
HR = 1.33 (0.78 − 2.25)
logrank P = 0.29
F
r = 0.32, p = 2.6 × 10−14
r = 0.32, p = 2.99 × 10−14
PROM2
CBFB
NRIP1

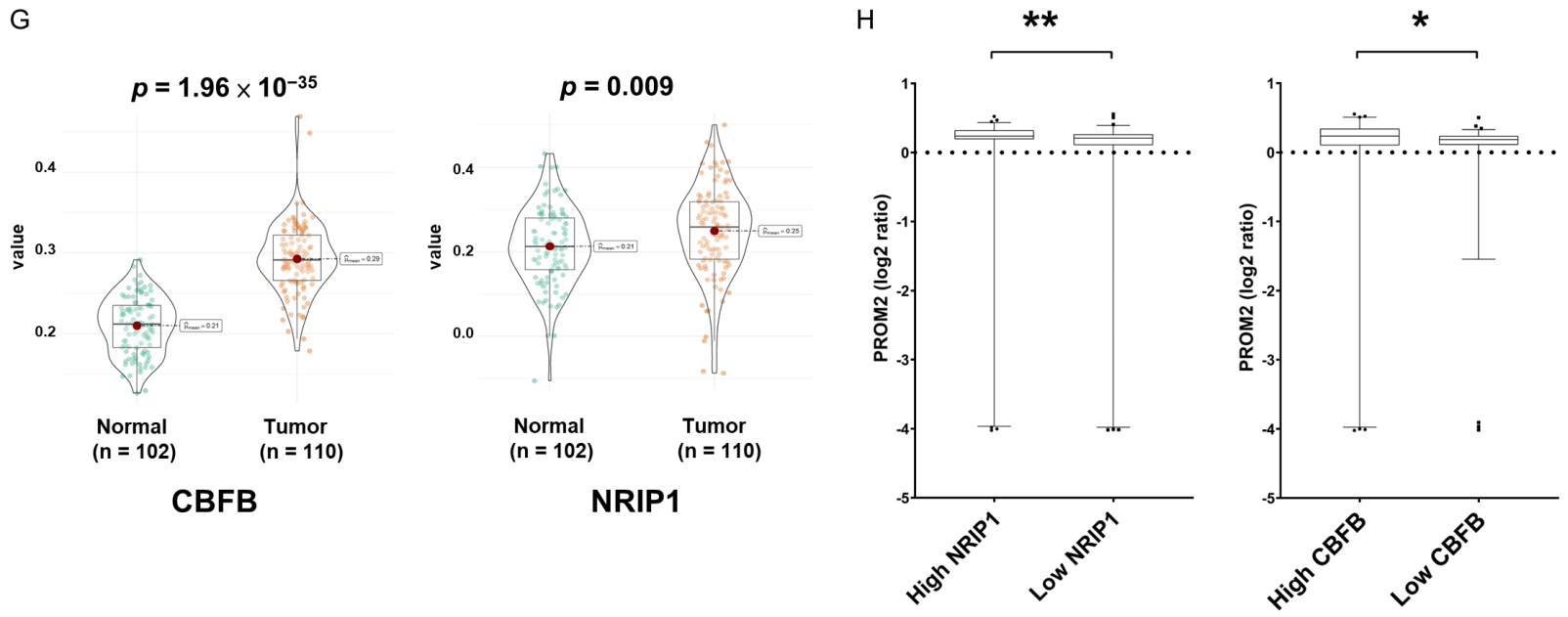


Figure 5. The transcriptional regulation for *PROM2* in LUSC. A. The expression pattern of the two potential transcription factors, *CBFB* and *NRIP1*, in the in-house LUSC specimens. B. The mRNA expression patterns of *CBFB* and *NRIP1* in the TCGA LUSC dataset. C. The survival significance of *CBFB and NRIP1* in TCGA LUSC cohort. D. The differential survival significance of *PROM2* in the context of high-CBFB or high-*NRIP1*-expressed LUSC. E. The differential survival significance of *PROM2* in the context of low-CBFB or low-*NRIP1*-expressed LUSC. F. The correlation between the *PROM2* and *CBFB/NRIP1* mRNA expression levels. G. The protein expression patterns of CBFB/NRIP1 in the CPTAC LUSC dataset. H. The association of PROM2 protein expression levels with CBFB/NRIP1 in the CPTAC LUSC dataset. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

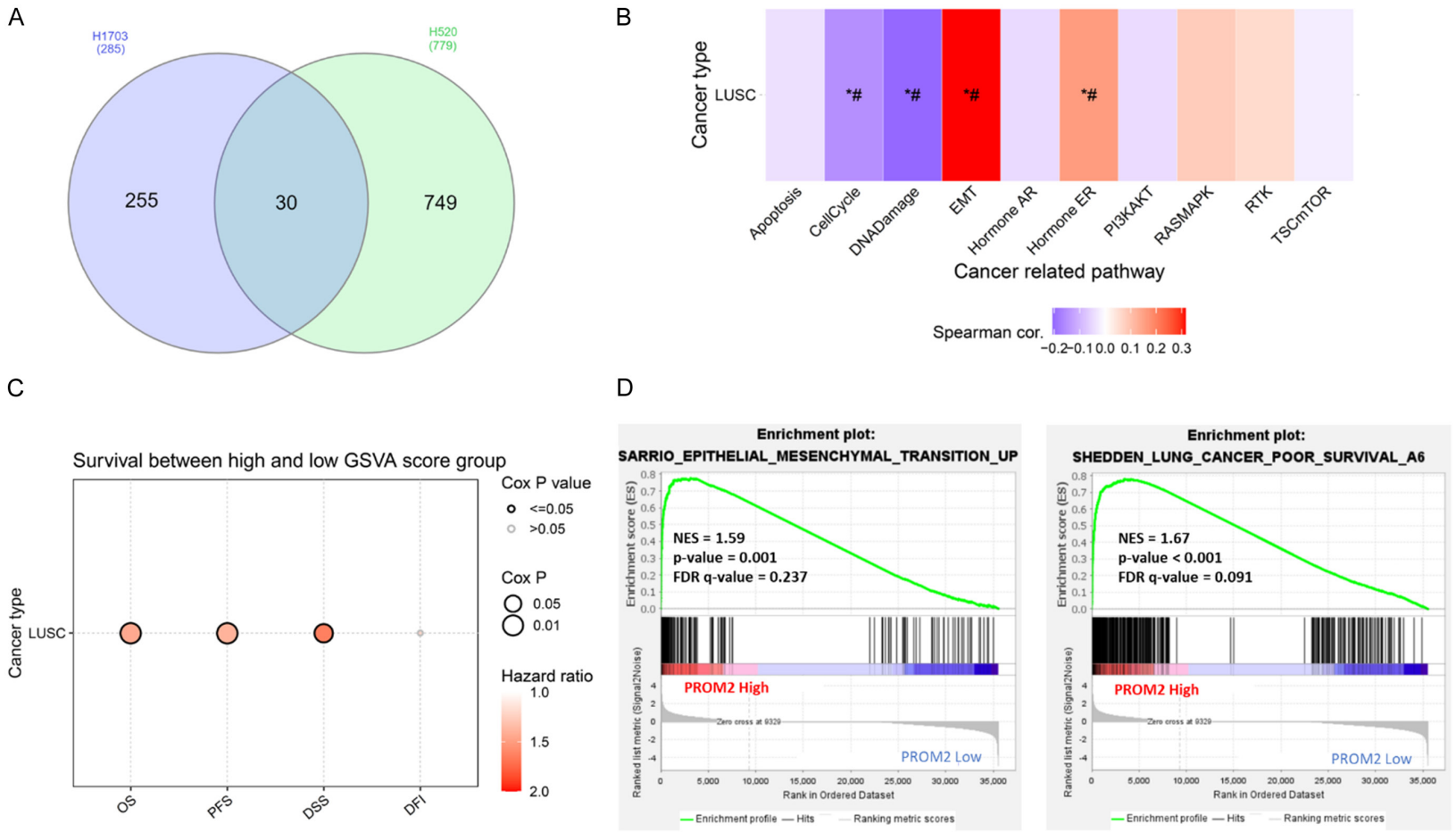


Figure 6. The functional pathways associated with *PROM2* in LUSC. A. Venn diagram shows the intersection of the significantly down-regulated genes after PROM2 knockdown in both cell lines. B. The biologic pathways of the common down-regulated genes after *PROM2* knockdown. C. The common down-regulated genes after *PROM2* knockdown are associated with poor outcomes. D. Enriched pathways associated with *PROM2*-correlating gene set, demonstrated by Gene Set Enrichment Analysis (GSEA). Abbreviations: DFI, disease-free interval; DSS, disease-specific survival; FDR, false discovery rate; GSVA, gene set variation analysis; NES, normalized enrichment score; OS, overall survival; PFS, progression-free survival.

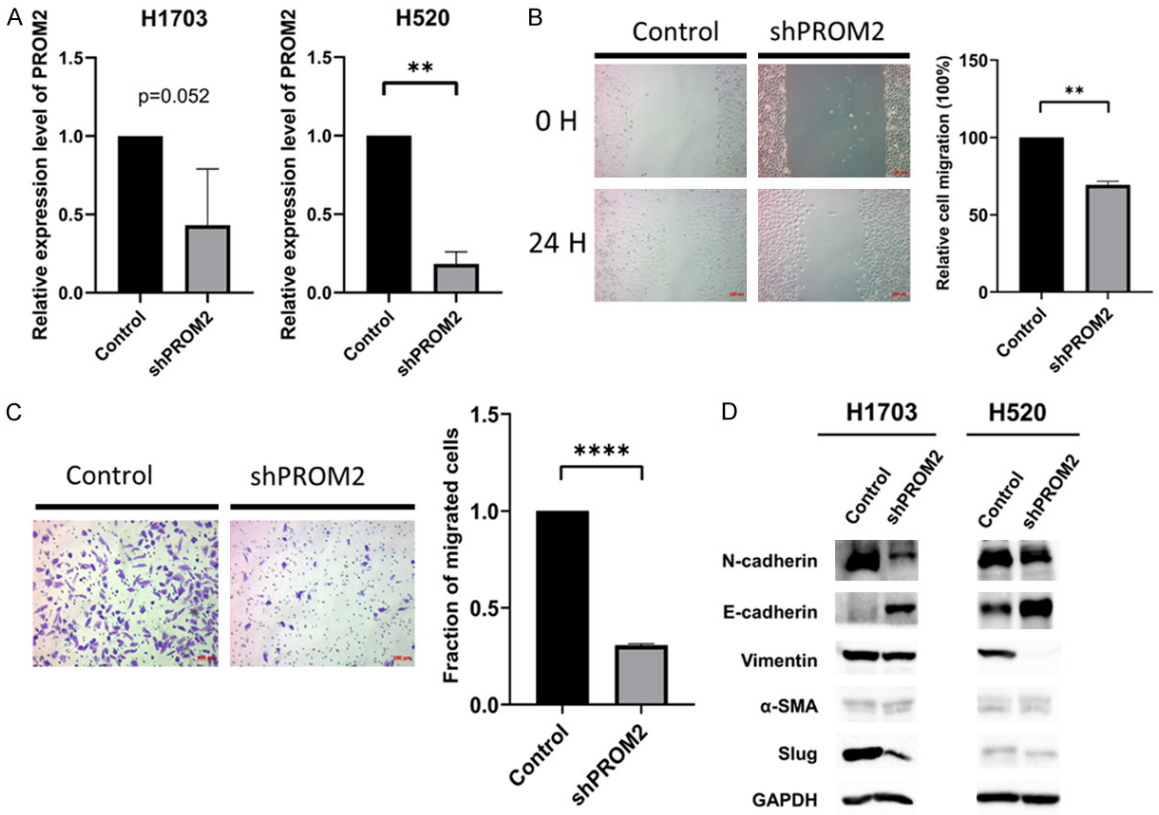


Figure 7. Higher *PROM2* promoted cancer cell migration and epithelial-mesenchymal transition (EMT) processes. A. The mRNA expression levels in *PROM2*-knockdown LUSC cell lines, H1703 and H520, respectively. B. The migration ability of LUSC cells with *PROM2* knockdown in the wound healing assay. C. The migration ability of LUSC cells with *PROM2* knockdown in the transwell assay. D. The Western Blot of EMT markers of LUSC cell lines before and after *PROM2* knockdown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

PROM2 facilitated cancer cell migration and promoted EMT

In order to explore the functional significance of *PROM2* in the context of LUSC, we utilized *PROM2*-knockdown cells (**Figure 7A**) for subsequent *in vitro* validation experiments. In the wound healing assay, the cell migration was disturbed by the inhibition of *PROM2* expression by shPROM2 (**Figure 7B**). Similarly, the fraction of cancer cell migration was reduced by the *PROM2* knockdown (**Figure 7C**). These results confirmed the facilitating ability of *PROM2* on the cancer cell migration of LUSC. Furthermore, we investigated the promoting effect of *PROM2* on the EMT process. As illustrated, the mesenchymal markers, such as N-cadherin, were reduced by inhibiting the *PROM2* expression both in H1703 and H520 cell lines. Conversely, the epithelial marker, E-cadherin, was elevated with the *PROM2* inhibition (**Figure 7D**). These results suggested the promoting role of *PROM2* on EMT in LUSC.

Discussion

The current study demonstrated that *PROM2*, but not *PROM1*, was upregulated in LUSC tumor tissues and showed a negative impact on LUSC patients' outcomes. LUSC patients with high *PROM2* expression had shorter overall survival. This implicates the potential diagnostic and therapeutic utilities of *PROM2* in LUSC.

PROM1 (CD133) has been studied extensively regarding its association with cancer formation and prognosis. Most importantly, *PROM1* is a well-known marker of cancer stem cells [10], which is associated with shorter survival, rapid tumor progression, and early tumor recurrence in various cancer types [11]. In a previous review, high expression of *PROM1* is linked to poorer survival in NSCLC [12]. Studies also showed a negative correlation between *PROM1* expression level with the prognosis focusing on LUAD [13, 14]. We searched the literature and there was no study aiming to investigate the

role of *PROM1* in LUSC. In our study, nonetheless, *PROM1* is not up-regulated in cancerous parts both in in-house samples and in the public dataset of LUSC. *PROM1* also showed no survival association in LUSC patients. This implicates the heterogenous tumor transcriptomics and their influences across different histologies in lung cancer.

Few studies investigate the role of *PROM2* in cancers. A review of public databases demonstrated that *PROM2* is up-regulated in ovarian and lung cancers, associated with poor survival [15]. *PROM2* is regarded as a signature gene of ferroptosis resistance in cancer [16], and targeting *PROM2* may reverse the resistance [17]. In LUAD, it has been reported that the *PROM2*-overexpressed A549 and PC-9 cells demonstrate enhanced cell viability, proliferation, migration, and invasion among *in vitro* experiments [18]. Moreover, *PROM2* overexpression links to the resistance of chemotherapeutic agents, while enhanced drug sensitivity to paclitaxel and cisplatin was observed in *PROM2*-knockdown endometrial and lung cancer cell lines [18, 19], respectively. On the contrary, Winnepenninckx et al. observed that higher expression of *PROM2* was linked to non-metastatic melanoma, suggesting an anti-metastatic role of *PROM2* [20]. The conflicting results may implicate the complex microenvironments across different tumors. Our study result is also suggestive of the pro-cancerous role of *PROM2* in LUSC. Through transcriptomic analysis, EMT is associated with the upregulation of *PROM2* and its correlated genes. In cell studies, *PROM2* knockdown was shown to suppress cell migration both in wound healing assay and transwell assay. Meanwhile, the decline of E-cadherin with *shPROM2* also confirmed the ability of *PROM2* knockdown to reverse the EMT process. To our knowledge, this is the first report that *PROM2* may involve the EMT process, implicating a potential role in cancer progression and metastasis.

In our study, we try to elucidate the epigenetic regulation of *PROM2*. Since the copy number did not correlate well with the *PROM2* expression, it is not thought of as a regulating factor. The microRNAs extracted from public databases are not good candidates due to low predicting scores. DNA methylation may be a possible regulating mechanism based on the strong negative correlation between methylation and mRNA expression level.

Transcription factors curated from public databases, such as *CBFB* and *NRIP1*, potentially control the expression of *PROM2* and both confer poor prognosis in LUSC, as *PROM2* does. The role of *CBFB* in lung cancer has never been studied in the literature. Previously, *CBFB* has demonstrated a suppressive role for breast cancer [21] while *NRIP1* was shown a promotive role for gastric cancer [22]. Our study first explored the involvement of *CBFB* and *NRIP1* in lung cancer. The survival influence of *PROM2* in LUSC is more significant only under the circumstances of high *CBFB* or *NRIP1*, indicating the strong interactive link between the two transcription factors and their transcriptional target, *PROM2*.

Conclusion

In short, the overexpression of *PROM2* is epigenetically modulated by hypo-methylation of its promoter and potentially regulated by transcription factors *CBFB* and *NRIP1.* Moreover, *PROM2* overexpression facilitates cancer cell migration and promotes EMT, resulting in a reverse correlation with the survival time of LUSC patients. Therefore, *PROM2* demonstrated potential diagnostic and therapeutic utilities in LUSC. Our study enlarges the body of knowledge regarding the biological role of *PROM2* in cancer and further molecular studies are needed to elucidate the operating mechanisms.

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Disclosure of conflict of interest

None.

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