NELFE promoted pancreatic cancer metastasis and the epithelial-to-mesenchymal transition by decreasing the stabilization of NDRG2 mRNA

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Abstract. Negative elongation factor E (NELFE) has been demonstrated to promote cancer progression as an RNA-binding protein (RBP). However, the expression patterns, biological role and molecular mechanism of NELFE in pancreatic cancer (PC) remain largely unknown. The expression levels of NELFE in 120 pairs of PC tissues and adjacent non-tumor clinical samples collected from patients with PC were examined via reverse transcription-quantitative (RT-q) PCR and immunohistochemistry. The mRNA expression levels of NELFE, N-Myc downstream-regulated gene 2 (NDRG2), c-Myc, survivin and cyclin D1 were detected via RT-qPCR. The protein expression levels of NELFE, NDRG2, total β-catenin, nuclear β-catenin, cytosolic β-catenin, E-cadherin, N-cadherin and Vimentin were measured by western blotting. NELFE and NDRG2 were then knocked-down by short hairpin (sh)RNA. PC cell proliferation was detected by MTT and colony formation assays. Invasion and migration were detected by transwell assays. The interaction between NELFE and NDRG2 was detected by luciferase reporter assays, mRNA decay assays and RNA immunoprecipitation. NELFE expression was increased in PC tissues compared with the paired non-cancerous tissues. NELFE expression was upregulated in PC cells when compared with normal pancreatic cells (HPDE6-C7). The present study revealed that knockdown of NELFE inhibited the proliferation, invasion and migration of PC cells. In addition, transfection of the sh-NELFE vector inhibited the epithelial-to-mesenchymal transition in PC cells by suppressing the expression and nuclear accumulation of β-catenin. Further mechanistic studies revealed that NELFE activates the Wnt/β-catenin signaling pathway by decreasing the stabilization of NDRG2 mRNA in PC. To the best of our knowledge, these results revealed the promotional function of NELFE on PC tumorigenesis and metastasis for the first time, helping to provide a promising strategy for the treatment of patients with PC.

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Introduction

Pancreatic cancer (PC) is the third leading cause of cancer-associated mortality worldwide (1), with a total of 1,619 cases succumbing to PC reported by China's Disease Surveillance Point System (DSPS) between 1991 and 2000 (2). Due to the high rate of metastasis and lack of effective treatment for patients with metastatic cancer, the overall 5-year survival rate of PC is <10% (3). Thus, it is critical to further elucidate the molecular mechanisms underlying PC metastasis.

The epithelial-to-mesenchymal transition (EMT) is at least in part responsible for the metastatic progression of multiple types of cancer, and this process results in cancer cells losing epithelial features and acquiring mesenchymal features (4,5). Among the various signaling pathways that contribute to the development of PC, the Wnt/β-catenin signaling pathway plays a prominent role in the EMT and metastasis in PC (6). The expression and subcellular localization of β-catenin is closely associated with PC tumor formation and development. The activation of Wnt signaling occurs upon nuclear accumulation of β-catenin (7), which activates downstream target genes. Therefore, investigation of the pivotal upstream regulator of the Wnt/β-catenin pathway is definitely warranted for a comprehensive understanding of the molecular mechanism underlying the metastasis and EMT of PC.

RNA-binding proteins (RBPs), which bind directly to the RNA of target genes, contribute to tumor biology and progression by regulating RNA at multiple levels, for example,

through RNA stabilization, translation, localization and degradation (8-10). Although nearly 2,000 RBPs have been identified (11), the molecular mechanisms by which RBPs modulate human cancer progression remain largely unclear. Negative elongation factor E (NELFE) is one of the subunits of NELF, which is a multi-subunit that cooperates with DRB sensitivity-inducing factor (DSIF) and results in the inhibition of Pol II elongation (12). Recent research revealed that NELFE could also serve as an RBP oncogene in hepatocellular carcinoma through the regulation of MYC signaling (13). However, the expression patterns and the potential physiological functions of NELFE in other types of human cancer, including PC, remain largely unknown.

The present study investigated the role of NELFE in PC cell proliferation, migration and invasion and assess the correlation between NELFE expression in clinical PC tissues and prognosis. The aim of the present study was to determine whether NELFE is a potential therapeutic target and an effective predictive biomarker for patients with PC.

Materials and methods

Patients and tissue samples. The Ethics Committee of Clinical Research of Xi'an Jiaotong University (Xi'an, China) approved the present study, and written informed consent was obtained from each patient prior to the study start. The 120 pairs of PC tissues and adjacent non-tumor clinical samples were collected from patients with PC who underwent surgical resection at The Second Affiliated Hospital of Xi'an Jiaotong University between May 2013 and May 2015. The adjacent tissues were collected ≥5 cm away from the edge of PC cancerous tissue. Inclusion criteria were as follows: All patients were determined to have PC via pathological diagnosis and had not received chemotherapy, radiotherapy or immunotherapy prior to resection; and the exclusion criteria were as follows: Patients aged <20 and >70 years. Table I presents the clinicopathological data of the patients. Every pair of tissues observed in the present study contained both cancerous and distant non-cancerous tissues, and were frozen in liquid nitrogen immediately after surgery, and then stored at -80˚C.

Immunohistochemistry. The immunohistochemistry assay was performed as previously described (14). The anti-NELFE antibody used in the immunohistochemistry assay was purchased from Beijing Biosynthesis Biotechnology (1:100; cat no. bs-19198R). The biotinylated goat anti-rabbit antibody (1:200; cat. no. A0279; Beyotime Institute of Biotechnology) was used as the secondary antibody. The diaminobenzidine tetrachloride (cat. no. P0203; Beyotime Institute of Biotechnology) was used for staining at 37˚C for 10 min.

Cell culture and transfection. The normal pancreatic cell line, HPDE6-C7, was purchased from CELLBIO Cell Center, and the PC cell lines, including PaCa-2, PANC-1, SW1990, AsPC-1 and BxPC-3, were purchased from the American Type Culture Collection. All cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich, Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.). The specific short hairpin (sh)-RNA vector (sh-NELFE and sh-NDRG2) to knockdown NELFE or NDRG2 expression in PC and their control vectors were purchased from Genepharma. PC cells were cultured in 6-well plates until they reached 40-50% confluence, and the 50 nM vectors were transfected into PC cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In order to establish stable clones, PC cells were expanded by trypsinizing after 48 h, diluted and then seeded in a 96-well plates with DMEM containing 10% FBS and 500 μg/ml G418 (Gibco; Thermo Fisher Scientific, Inc.). The PC cells were inspected daily under the fluorescence microscope to ensure the colonies were derived from a single cell within a well. After 20-25 days selection, the PC cells were trypsinized and re-seeded into 24-well plates, and then into 6-well plates in DMEM containing 10% FBS with 500 μg/ml G418. Finally, the NELFE or NDRG2 expression levels in the PC cells collected from G418 positive colony were assessed by reverse transcription-quantitative (RT-q) PCR and western blotting.

RT-qPCR. RT was performed on 0.1 μg total RNA, which had been extracted from PC cell lines or tissue by TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to complementary DNA using a RT-PCR kit (ABI; Thermo Fisher Scientific, Inc.) 45 min at 50˚C. qPCR reactions were performed in triplicate using a SYBR Premix Ex Taq Real-Time PCR kit (Takara Bio, Inc.) according to the manufacturer's protocol. The housekeeping genes GAPDH, β-actin and Hydroxymethyl bilane synthase (HMBS) were selected to serve as the candidate internal controls in the present study, as they were steadily expressed at a similar level in the examined PC cell lines. All the three internal controls were expressed stably, and the RT-qPCR results calculated using the three internal controls were similar. GAPDH is taken as the internal control in the figures listed in the paper. The NELFE and NDRG2 mRNA Expression levels were examined using a relative quantification approach ($2^{-\Delta\Delta Cq}$ method) compared with the level of GAPDH (15), which is expressed at a similar level in the examined PC cell lines. The RT-qPCR thermocycling conditions were as follows: Initial denaturation (90˚C, 5 min), 40 cycles of denaturation (90˚C, 10 sec), annealing (75˚C, 6 sec) and elongation (75˚C, 30 sec), final elongation (75˚C, 10 min) and a final hold (4˚C). The primers used in the present study were as follows: NELFE: Forward, 5'-GCATATCCATATGCAGGAATGCCTGGAGAAGTTCC-3', reverse, 5'-GCGGATCCTTATTCGGCCAGTCGGTAGATTAGC-3'; NDRG2: Forward, 5'-CACTCCAGTGACAGCACCTCT-3'; reverse, 5'-GGCTCCAACACCAACTCCAATT-3'; and GAPDH: Forward, 5'-AATGGACAACTGGTCGTGGAC-3'; reverse, 5'-CCCTCCAGGGGATCTGTTTG-3'.

Western blotting. The protein was collected from PC cell lines via lysing by RIPA Buffer (Thermo Fisher Scientific, Inc.), and the protein concentrations were determined by BCA assays (Cell Signaling Technology, Inc.). Each of the samples were quantitatively released by the RIPA Buffer at the same concentration.

Total proteins (25 μg per lane) were separated via SDS-PAGE (10% gel). The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore) and blocked in 5% skimed milk PBS with 0.1%

Table I. Association between NELFE expressions and clinicopathological features in pancreatic cancer.

Variable	Total no. of patients n=120	NELFE expression, n		P-value
		High	Low	
Age, n (%)				0.259
<60 years	48 (40.0)	36	12	
≥60 years	72 (60.0)	47	25	
Sex, n (%)				0.646
Female	45 (37.5)	30	15	
Male	75 (62.5)	53	22	
Chronic pancreatitis, n (%)				0.546
Yes	57 (47.5)	44	13	
No	63 (52.5)	39	24	
Tumor size, n (%)				0.04
<2 cm	42 (35.0)	34	8	
≥2 cm	78 (65.0)	49	29	
Differentiation, n (%)				0.136
Well-moderate	69 (57.5)	44	25	
Poor	51 (42.5)	39	12	
Lymph-node metastasis, n (%)				0.001
Yes	69 (57.5)	39	30	
No	51 (42.5)	44	7	
TNM stage, n (%)				0.003
I-II	63 (52.5)	51	12	
III-IV	57 (47.5)	32	25	

P<0.05 was considered to indicate a statistically significant result. NELFE, negative elongation factor R; TNM, Tumor-Node-Metastasis.

Triton X-100 at 37˚C for 1 h. The membranes were then incubated with the primary antibodies at 4˚C overnight and the secondary antibodies at room temperature for 1 h. Finally, the results were visualized using an ECL blotting analysis system (GE Healthcare Biosciences). The primary antibodies used in the present study were: Anti-NELFE (catalog no. ab170104; 1:1,000), anti-GAPDH (catalog no. ab8245; 1:5,000), anti-E-cadherin (catalog no. ab1416; 1:1,000), anti-N-cadherin (catalog no. ab202030, 1:1,000), anti-Vimentin (catalog no. ab193555; 1:2,000), anti-β-catenin (catalog no. ab32572; 1:1,000), anti-NDRG2 (catalog no. ab174850; 1:2,000), anti-α-tubulin (catalog no. ab210797; 1:2,000) and anti-LaminB1 (catalog no. ab252351; 1:2,000) (all from Abcam). The goat anti-rabbit secondary antibody (1:10,000; cat. no. bs-40295G-IRDye8) was purchased from Beijing Bioss Biotechnology. Finally, the membranes were tested using a bio-imaging system (DNR Bio-Imaging Systems). ImageJ software (version 1.6.0; National Institutes of Health) was used to quantify the intensity of protein bands and normalized by GAPDH.

MTT assays. The PC cells were seeded into the 96-well plates at a density of 500-700 cells per well. The cells in each well were cultured in 100 μl DMEM medium with 10% FBS. At the time points 0, 24, 48, 72 and 96 h, MTT solution (10 μl, 5 mg/ml) were added into the culture medium. After incubation for 4 h at room temperature, the medium containing MTT solution was removed, and dimethyl sulfoxide (DMSO; volume, 200 μl; concentration, 1 mg/ml) was added into each well to dissolve the purple formezan. Finally, the plate reader (Bio-Rad Laboratories) was used to observe the absorbance at the 492 nm wavelength.

Colony-forming assays. A total of 100 PC cells per well were seeded into a 6-well cell culture cluster containing 2 ml DMEM supplemented with 10% FBS in each well. Following culture in a humidified atmosphere for 10 days at 37˚C, the colonies were fixed with methanol, and then stained in 0.1% crystal violet solution at 37˚C for 30 min. The cells were then washed with PBS 2-3 times and then dried overnight. The colonies forming units (consisting of ≥50 cells) were assessed under an inverted fluorescence microscope (Nikon, magnification, x40).

Cell migration and invasion assays. The 8.0 μm pore Transwell chambers (EMD Millipore) inserted in 24-well plates were used for the migration and invasion assays. Migration assays: The PC cells ($2x10^4$) in 200 μl serum-free media were applied to the upper chamber, while normal culture media (DMEM supplemented with 10% FBS, 700 μl) was added to the bottom compartment. After incubation in cell incubator for 36 h

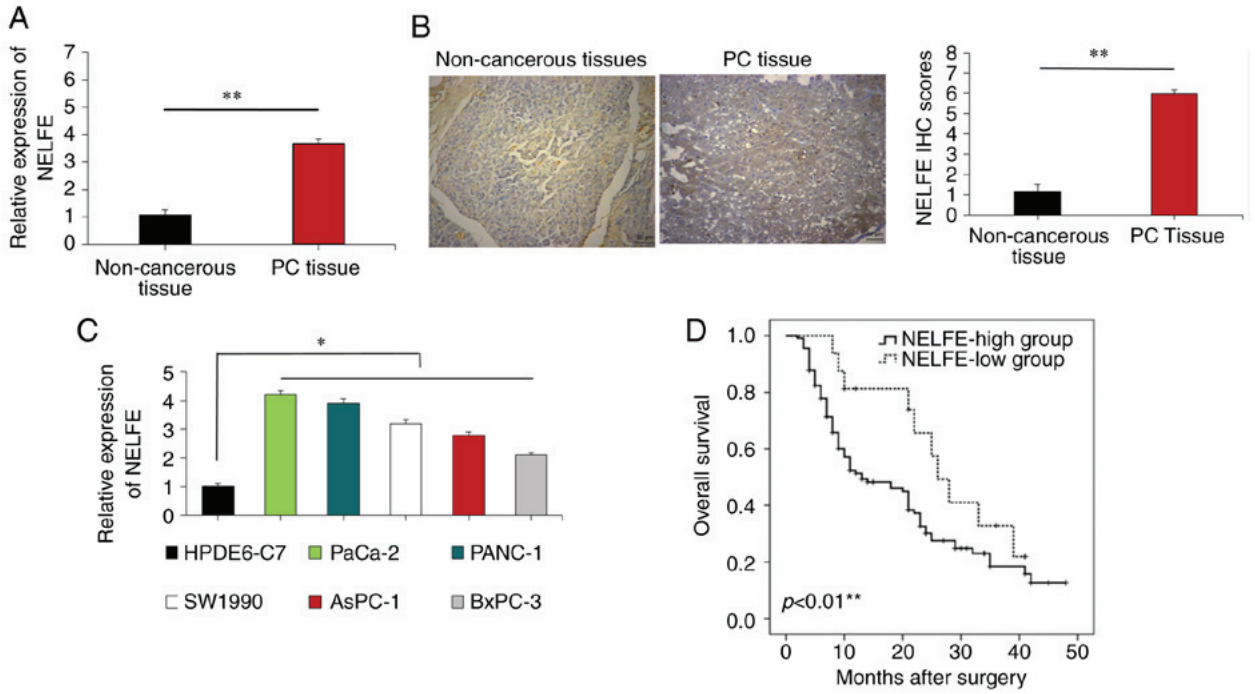

Figure 1. Increased NELFE expression in PC tissues and cells. (A) The expression of NELFE in samples was analyzed by reverse transcription-quantitative PCR. (B) Analysis of the expression of NELFE in samples using an immunohistochemistry assay. The positive expression of NELFE was brown and yellow. The nuclei of cells were blue. (C) NELFE expression levels in various human PC cell lines and HPDE6-C7 cells. (D) Comparison of Overall survival between PC patients with high and low NELFE expression levels (n=120). The 120 pair of PC tissues and adjacent non-tumor clinical samples were removed from patients with PCs. *P<0.05 vs. HPDE6-C7; **P<0.01 vs. non-cancerous tissues or NELFE-low group. NELFE, negative elongation factor E; PC, pancreatic cancer.

at 37˚C, the invaded cells moved into the lower side of the membranes were fixed with 4% paraformaldehyde at 37˚C for 1 h and then stained with 0.1% crystal violet solution for 30 min at 37˚C. Invasion assay: The procedures was same as those aforementioned in the migration assay except that the membranes in the upper chamber were pre-coated with 15 μg Matrigel (Becton Dickinson Bioscience). Finally, the migrated cells were examined in 10 randomly selected fields of view under a fluorescence microscope (magnification, x200).

Luciferase reporter assays. The PC cells (1x10^5/well) in a 24-well plate were co-transfected with *Renilla* luciferase (phRL-TK), sh-NELFE vector or sh-NELFE negative control (NC) empty vector, and pGL3-NDRG2-3'UTR reporter vectors (or pGL3-TCF promoter) (GenePharma Co., Ltd.) using Lipofectamine® 3000 (1 μl/well; Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 24 h, the luciferase activities were measured using a Dual-Luciferase Reporter Assay System kit (Promega Corporation) according to the manufacturer's protocol. The relative Firefly luciferase activity was measured by normalizing to Renilla luciferase activity.

mRNA decay assay. The PC cells were seeded into 6-well plates supplemented with actinomycin D (Sigma Aldrich; Merck KGaA) at a concentration of 0.8 μg/ml. RT-qPCR assay was performed as aforementioned in order to detect NDRG2 RNA in PC cells at scheduled time points following transfection with sh-NELFE or the control vectors, including 2, 4, 6, 8, 10 and 12 h.

RNA immunoprecipitation (RIP assay). The RIP assay was performed according to the instructions of the RIP RNA-binding protein Immunoprecipitation kit (EMD Millipore) following the manufacturer's protocol. The PC cell lysates were added with RIP Lysis Buffer and NELFE antibody, normal mouse IgG (negative control) (1:200; cat. no. PP6421-K; EMD Millipore) and agarose beads and incubated overnight at 4˚C. A High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used to reverse transcribe the immunoprecipitated RNAs. Finally, RT-qPCR was performed as aforementioned in order to examine the targets transcripts.

Statistical analysis. Statistical analyses were performed using SPSS statistical software (version 19.0; IBM Corp.) and the data are expressed as the mean ± standard error. Student's t-test was used to evaluate the difference between two groups. The Kaplan-Meier method and log-rank test was used to plot the survival curves. Person correlation was performed in order to assess the correlation between NELFE and NDRG2 mRNA expression in PC tissues. P<0.05 was considered to indicate a statistically significant result. All experiments were repeated triplicate.

Results

NELFE is increased in PC tissues and cell lines. In order to determine the expression pattern of NELFE in clinical PC samples, the present study investigated NELFE expression in 120 pairs of PC tissues and adjacent non-tumor clinical samples removed from patients with PC. RT-qPCR assays suggested that NELFE mRNA expression was primarily higher in the PC tissues than in the paired non-cancerous

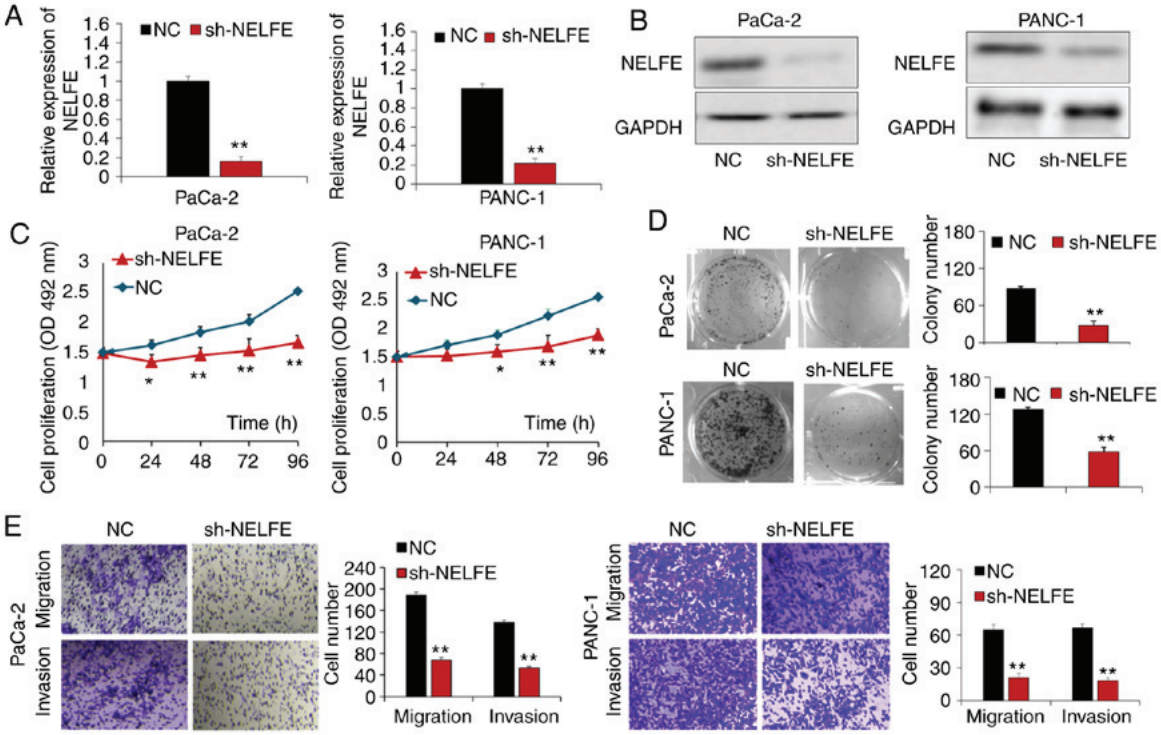

Figure 2. NELFE promoted the malignant biological behavior of PC cells. (A) The mRNA expression of NELFE was decreased following transfection of the NELFE-shRNA vector in PaCa-2 and PANC-1 cells. (B) The protein expression of NELFE was decreased following transfection of the NELFE-shRNA vector in PaCa-2 and PANC-1 cells. (C) MTT assay demonstrated that decreased NELFE suppressed PC cell growth. (D) The decreased NELFE inhibited clone formation of PC cells. (E) Transwell assay revealed that knockdown of NELFE inhibited PC cell invasion and migration. The invaded cells were quantified by counting the cells in 10 randomly selected fields (x200 magnification). All experiments were repeated in triplicate. *P<0.05, **P<0.01. NELFE, negative elongation factor E; PC, pancreatic cancer; shRNA, short hairpin RNA; NC, negative control.

tissues (P<0.01; Fig. 1A). Immunohistochemistry assays also revealed that NELFE expression was largely upregulated in PC tissues compared with that in non-cancerous tissues from patients with PC (P<0.01; Fig. 1B). The present study also analyzed the clinical significance of NELFE in patients with PC. A high NELFE expression level was significantly associated with lymph node metastasis (P=0.001), large tumor size (P=0.04) and advanced Tumor-Node-Metastasis (TNM) stage PC (16) (P=0.003; Table I). The present study further measured the expression levels of NELFE in PC cells using RT-qPCR, and the results revealed that the expression level of NELFE was higher in PC cell lines than in normal pancreatic cells (HPDE6-C7) (P<0.05; Fig. 1C). Among the PC cell lines, the expression of NELFE was highest in the PANC-1 and PaCa-2 cell lines. In addition, the Kaplan-Meier analysis revealed that compared with those with higher levels of NELFE, patients with lower expression levels of NELFE had a shorter survival time (P<0.01; Fig. 1D).

NELFE promotes PC cell proliferation, invasion and migration. The present study transfected PaCa-2 and PANC-1 cells with a sh-NELFE vector or control vector. RT-qPCR and western blot assays demonstrated that compared with transfection of the control vector, transfection of the sh-NELFE vector markedly decreased the mRNA and protein expression levels of NELFE in PC cells (P<0.01; Fig. 2A and B). The MTT assay suggested that the proliferation ability of both PaCa-2 and PANC-1 cells was significantly inhibited following knocking-down NELFE expression (P<0.05; Fig. 2C). In addition, the colony formation results demonstrated that downregulated NELFE expression inhibited the PC cell colony formation ability (P<0.01; Fig. 2D). The present study also performed transwell assays in order to assess the function of NELFE in the migration and invasion ability of PC cells. As presented in Fig. 2E, decreased NELFE significantly suppressed the migration and invasion ability of PC cells (P<0.01). These results further suggested that NELFE serves as an oncogene in PC cells.

NELFE promotes EMT and Wnt/β-catenin signaling in PC cells. EMT is well known as the foundation and key mechanism of the invasion and metastasis of PC cells. Thus, the present study performed a western blot assay to measure the expression levels of epithelial markers (E-cadherin) and mesenchymal markers (N-cadherin and Vimentin) in PC cells transfected with control vector or sh-NELFE vector, in order to investigate whether NELFE promoted PC cell migration and invasion via regulating the EMT. As presented in Fig. 3A, decreased NELFE levels inhibited the expression of mesenchymal markers and enhanced the expression of epithelial markers, which indicated that NELFE promoted PC cell EMT. β-catenin is recognized as a key effector of Wnt/β-catenin signaling, which is widely implicated in EMT in a number of different types of cancer, including PC (17). The results from the present study also revealed that decreased NELFE resulted in the downregulation of β-catenin expression in PC cells. A decrease in β-catenin breakdown and its subsequent accumulation in the cytoplasm will facilitate its nuclear translocation.

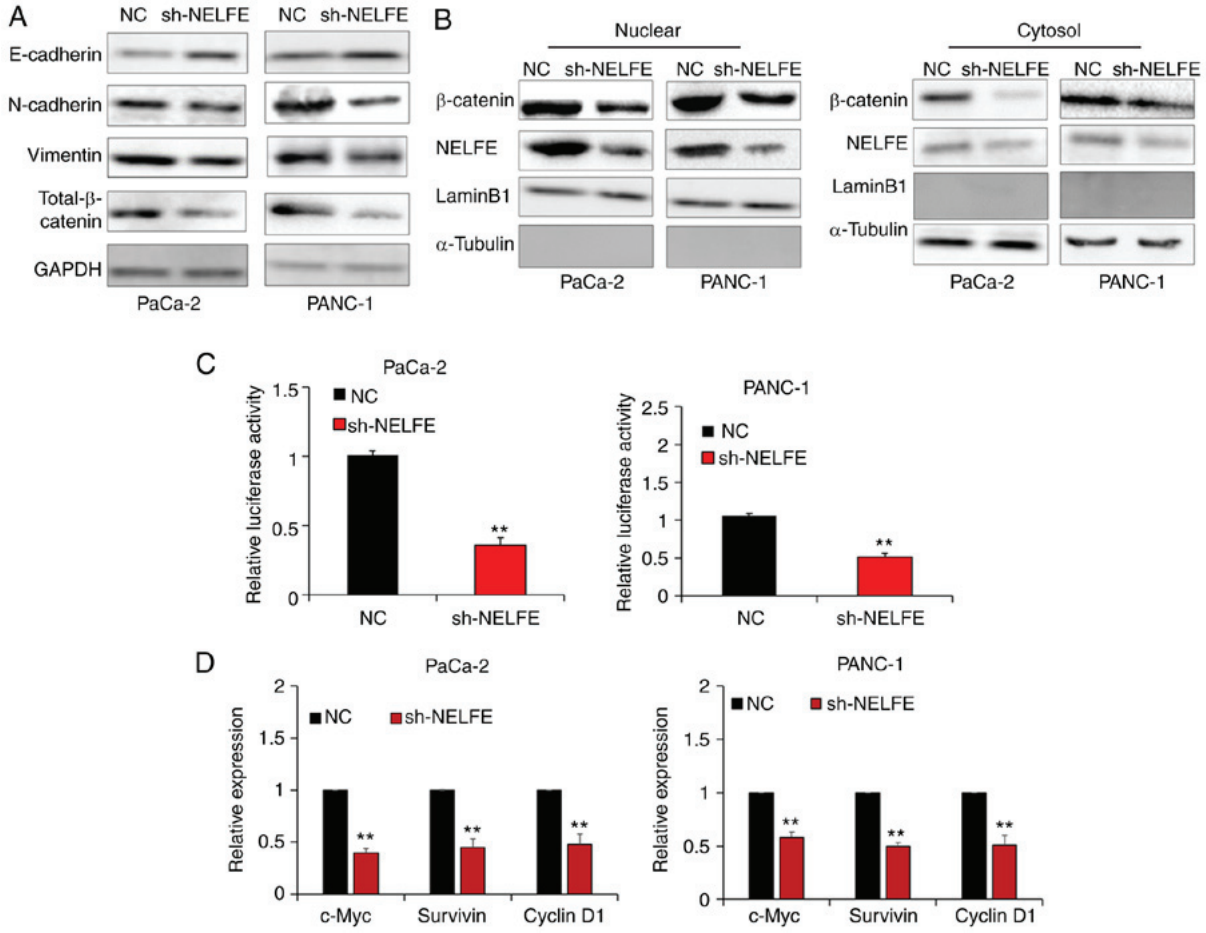

Figure 3. NELFE promoted EMT and Wnt/β-catenin signaling way in PC. (A) Western blot analysis of EMT-associated protein and β-catenin expression after the downregulation of NELFE in PC cells. GAPDH was used as an internal control. (B) Downregulation of NELFE in PC cells reduced the nuclear accumulation of β-catenin. (C) The dual luciferase reporter assay showed that decreased NELFE inhibited the transactivation of the TCF reporter in PC cells. (D) Reverse transcription-quantitative PCR assay revealed that the expression of the Wnt/β-catenin downstream genes (c-Myc, Survivin and cyclinD1) was decreased due to the knockdown of NFLFE in PC cells. All experiments were repeated three times. *P<0.05, **P<0.01 vs NC group. NELFE, negative elongation factor E; EMT, epithelial-to-mesenchymal transition; PC, pancreatic cancer; shRNA, short hairpin RNA; NC, negative control.

Then, β-catenin can bind to TCF transcription factors in the nucleus and successfully transcriptionally activate a series of genes associated with the EMT (18). Thus, the present study performed subcellular fractionation assays in order to evaluate whether NELFE activated the Wnt/β-catenin signaling pathway in PC cells. The results revealed that downregulation of NELFE in PC cells markedly decreased the nuclear accumulation of β-catenin (Fig. 3B). In addition, a dual luciferase reporter assay demonstrated that decreased NELFE significantly inhibited the transactivation of the TCF reporter in PC cells (Fig. 3C). Furthermore, the present study measured the expression levels of Wnt/β-catenin target genes, such as c-Myc, survivin and cyclin D1, via RT-qPCR. The results revealed that Wnt/β-catenin downstream gene expression was significantly downregulated following NELFE knockdown in PC cells (Fig. 3D). Overall, these results suggested that NELFE promotes EMT via activating the Wnt/β-catenin signaling pathway in PC.

NELFE activates the Wnt/β-catenin signaling pathway by inhibiting NDRG2 expression in PC. NDRG2, a tumor suppressor belonging to the NDRG family, has been reported to be decreased in human cancer tissues, including PC (19). Accumulating studies have revealed that NDRG2 is involved in the regulation of EMT via inhibiting β-catenin/c-Myc signaling and then inducing E-cadherin degradation in human cancers (20-22). Notably, the present study aimed to determine the potential molecular mechanisms underlying the regulation of the Wnt/β-catenin signaling pathway by NELFE, and revealed that NDRG2 expression was strongly increased following NELFE knockdown (Fig. 4A). In addition, the present study performed a RT-qPCR to assess whether there is a correlation between the mRNA expression levels of these two genes in clinical samples of PC tissues. The pearson correlation analysis of RT-qPCR results revealed that NELFE mRNA levels were negatively correlated with NDRG2 mRNA levels (R=-0.776, P<0.01; Fig. 4B). Thus, it was speculated that NELFE may activate the Wnt/β-catenin signaling pathway by inhibiting the expression of NDRG2. The present study transfected the sh-NDRG2 vector or control vector into PC cells with NELFE knockdown. As presented in Fig. 4C, knockdown of NDRG2 in PC cells with decreased NELFE increased the expression levels of total β-catenin (including nuclear β-catenin and cytosol β-catenin) and mesenchymal markers (N-cadherin and Vimentin), and decreased the expression level of an epithelial marker (E-cadherin) (Fig. 4C). In addition, knockdown

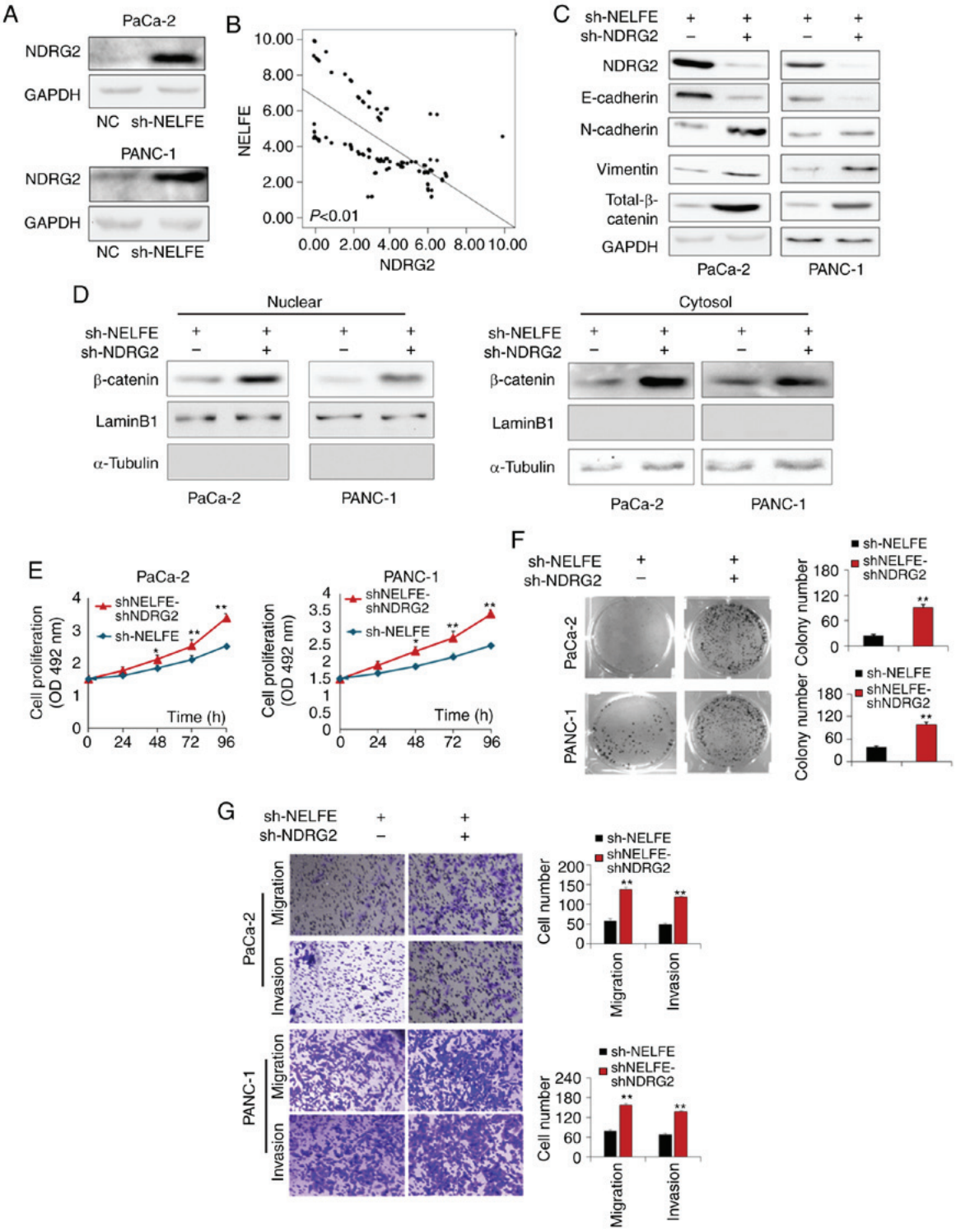

Figure 4. NDRG2 is an essential downstream effect of NELFE in PC. (A) NDRG2 expression was measured by western blotting in PC cells with NELFE knockdown. (B) The correlation between NELFE and NDRG2 mRNA expression in 120 samples from patients with PC was analyzed via person correlation analysis (R=-0.776). (C) Western blot analysis of associated protein and β-catenin expression following the downregulation of NDRG2 in PC cells with decreased NELFE. (D) Decreased NDRG2 enhanced the nuclear accumulation of β-catenin in PC cells with decreased NELFE. (E) MTT assay showed downregulation of NDRG2 rescued the function of sh-NELFE on PC cells proliferation. (F) The downregulation of NDRG2 rescued the function of decreased NELFE on PC cells colony formation ability. (G) Knockdown of NDRG2 rescued the inhibition function of decreased NELFE on PC cells migration and invasion. The PC cells with NELFE-NDRG2 double knockdown cells were used in the panels of C-G. All experiments were repeated in triplicate. *P<0.05, **P<0.01 vs. NC group. NDRG2, N-Myc downstream-regulated gene 2; NELFE, negative elongation factor E; EMT, epithelial-to-mesenchymal transition; PC, pancreatic cancer; shRNA, short hairpin RNA; NC, negative control.

of NDRG2 promoted the nuclear accumulation of β-catenin in PC cells, even though the expression of NELFE was downregulated (Fig. 4D). Furthermore, sh-NDRG2-mediated downregulation of NDRG2 significantly promoted the proliferation, invasion and migration of sh-NELFE-transfected PC cells (Fig. 4E-G). Taken together, these results suggested that

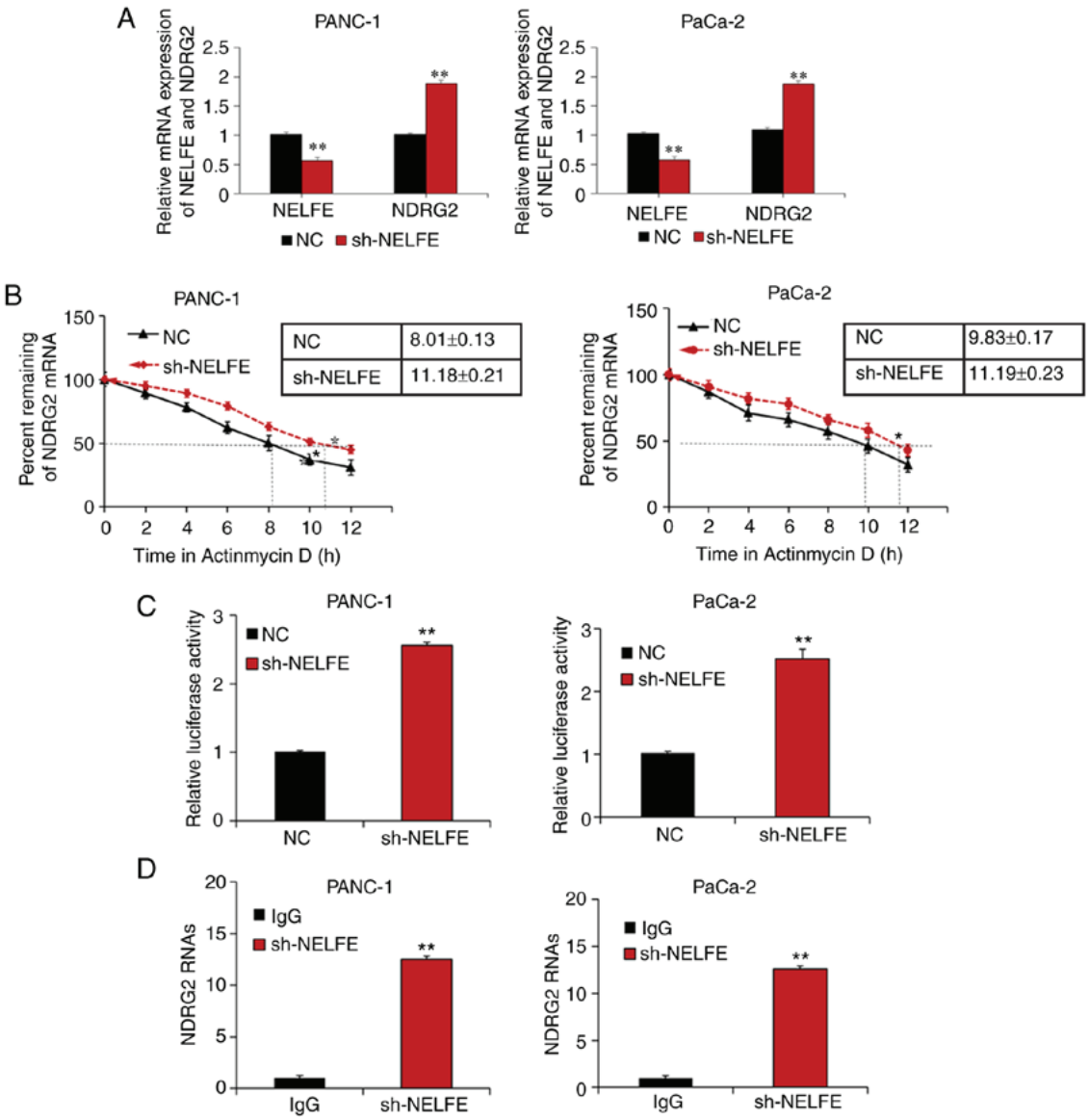

Figure 5. NELFE decreased NDRG2 by interacting with its mRNA. (A) Reverse transcription-quantitative PCR results revealed that the mRNA levels of NDRG2 were increased following the knockdown of NELFE. (B) The half-life NDRG2 mRNA increased after NELFE knockdown in PC cells. (C) The decreased NELFE enhanced the luciferase activity of NDRG2 3'untranslated region. (D) RNA immunoprecipitation assays demonstration NELFE directly binding to NDRG2 mRNA. All experiments were repeated three times. *P<0.05, **P<0.01 vs. NC group. NEFLE, negative elongation factor E; NDRG2, N-Myc elongation factor E; PC, pancreatic cancer.

NELFE activated the Wnt/β-catenin signaling pathway and promoted PC tumor progression in a manner at least partly dependent on NDRG2 expression downregulation.

NELFE decreases NDRG2 expression by directly interacting with its mRNA. The RT-qPCR results further demonstrated that the mRNA expression level of NDRG2 was significantly increased following NELFE knockdown (Fig. 5A), suggesting that NELFE regulated NDRG2 at the transcriptional level. The effect of NELFE on the stability of NDRG2 mRNA was also measured, and the results revealed that the half-life of NDRG2 mRNA was prolonged after NELFE was decreased in PC cells (Fig. 5B). A luciferase reporter assay demonstrated that decreased NELFE enhanced the luciferase activity of the NDRG2 3' UTR (Fig. 5C). In order to further investigate whether NELFE binds directly to NDRG2 mRNA, the present study performed RIP assays, and the results revealed that NDRG2 mRNA was more enriched in sh-NELFE transfected PC cells than the control (IgG) (Fig. 5D). Thus, the results from the present study demonstrated that NELFE inhibited NDRG2 expression by promoting decay of its transcript.

Discussion

To the best of our knowledge, the present study is the first to demonstrate that NELFE acts as an RBP to promote EMT and metastasis in PC by activating the Wnt/β-catenin signaling pathway via post-transcriptional inhibition of NDRG2 expression. The data from the present study contributes to the current understanding of the molecular mechanism by which NELFE overexpression promotes tumorgenesis and progression in PC.

NELFE is an important part of NELF, which is well known as the pivotal regulatory factor of the Pol II pausing complex (12,23,24). NELF contains four multi-functional sub-units, NELFA, NELFB, NELFC and NELFE, which are all involved in the regulation of MYC signaling (25-27). In addition, the four sub-units have been identified to be involved in a variety of different types of human cancer (23,28,29). Midorikawa *et al* (26) reported that the overexpression of NELFE contributed to the tumorigenesis of hepatocellular carcinoma (HCC). A recent study demonstrated that NELFE is upregulated in HCC tissues, and the overexpression of NELFE promoted the progression of HCC via directly binding to and enhancing MYC (13). Further research on the role of NELF will be helpful for understanding the potential molecular mechanism of PC, and may provide a novel treatment strategy. To the best of our knowledge, the present study demonstrated for the first time that NELFE was increased in PC tissues and cell lines. These data are consistent with other recent results suggesting that NELFE functions as an oncogene in human cancers (13). The present study further confirmed that the upregulation of NELFE was associated with poor outcomes in patients with PC. In addition, the *in vitro* results demonstrated that NELFE downregulation weakened the proliferation, invasion and migration capacities of PC cells, suggesting that NELFE enhanced the malignant biological behavior of PC cells. Considering these results have indicated the lower expression of NELFE in adjacent non-cancerous tissues from PC patients and normal cells, respectively, compared with that in the PC tissue and PC cell lines, it was speculated that the abnormally increased expression of NELFE is associated with the development and progression of PC. These data strongly suggest that NELFE may work as a potential therapeutic target for patients with PC.

PC is characterized as one of the most aggressive types of human tumor, which causes its high probability of cancer-associated mortality. The poor outcome is in part associated with the high rate of metastasis for patients with PC. Thus, it is important to further understand the potential molecular mechanisms underlying the role of NELFE in promoting PC metastasis. EMT is recognized as a response to the metastasis of human cancer (30), and in this process, epithelial cells lose differentiation characteristics, such as cell adhesion and cell polarity, and are transformed into mesenchymal cells and gain the ability to invade and migrate (31). The present study used a western blot assay to demonstrate that knockdown of NELFE significantly increased E-cadherin expression and decreased N-cadherin and vimentin expression in PC cells. These data suggest that NELFE promotes EMT in PC cells. Previous research has revealed that a series of signaling factors participate in the regulation of EMT processes (32), including Wnt/β-catenin, TGF-β, Notch and HIF-1α (33-35). Among these signaling pathways, Wnt/β-catenin is well known to serve as the key mediator of EMT (36). The western blot assay results revealed that decreased NELFE inhibited the expression and nuclear accumulation of β-catenin, suggesting that NELFE promoted EMT via activating the Wnt/β-catenin signaling pathway in PC.

Although increasing research has been focused on the involvement of RBPs in a range of different types of human cancer in previous years (37-39), more studies on the details of the biological effect and underlying mechanisms of RBPs are still required. To the best of our knowledge, the present study provided the first evidence that NELFE acts as an RBP to promote PC tumorigenesis and metastasis via the post-transcriptional regulation of NDRG2 expression. NELFE has been verified to contain an RNA recognition motif domain (40), which preferentially binds directly to RNAs. At first, studies focused on only NELFE revealed that it binds to certain special RNAs (41,42). Until recently, the role of NELFE in the regulation of human cancer progression through binding to certain target genes began to attract researchers' interest (13,43). The present study identified NDRG2 as a target gene of NELFE in PC. The use of the luciferase, mRNA decay and RIP assays demonstrated that NELFE inhibits NDRG2 expression by binding directly to its 3'UTR. NDRG2 is involved in the regulation of cancer cell differentiation and proliferation as a tumor suppressor (19). Accumulating studies revealed that NDRG2 expression was significantly lower in various cancer tissues, including liver cancer, pancreatic cancer and glioblastoma, compared with normal tissues, and decreased NDRG2 expression was closely associated with a shorter overall survival in patients with tumors (22,44). NDRG2 has been revealed to regulate the EMT of human cancer in a series of reports. Chen *et al* (45) reported that NDRG2 suppressed human cancer upon the metabolic reprogramming. Kim *et al* (46) demonstrated that NDRG2 repressed breast cancer EMT via STAT3/Snail signaling. It has also been confirmed that NDRG2 could inhibit the prostate cancer cell invasion and migration through regulating EMT-associated genes (47) and suppress EMT of esophageal cancer cells via regulating the AKT/XIAP signaling pathway (48). Notably, other studies have confirmed that NDRG2 plays a pivotal role in the modulation of EMT by inhibiting β-catenin expression (49,50). Thus, the results of the present study are rational. The present study also demonstrated that NDRG2 could contribute to rescuing the function of NELFE in PC cell invasion and migration, further suggesting that NELFE promoted PC metastasis and EMT by activating the Wnt/β-catenin signaling pathway via inhibiting NDRG2 expression. These results may help to open novel avenues for treatment strategies for PC.

In summary, to the best of our knowledge, the present study revealed the overexpression of NELFE in PC for the first time. The overexpression of NELFE promoted malignant phenotypes in PC cells, including proliferation, migration and invasion. In addition, the results demonstrated that NELFE promoted the EMT by enhancing the expression and nuclear accumulation of β-catenin. The present study further revealed that NELFE inhibited NDRG2 expression via binding with its 3'UTR. The present study also revealed the significant role of NDRG2 in mediating the function of NELFE in PC cells. The data also demonstrated for the first time the significance of the NELFE/Wnt/β-catenin/NDRG2 axis in PC and may offer a novel therapeutic strategy for PC.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

LH collected the clinical samples and performed most of the experiments. YZ performed the experiments and the statistical analysis. CH conceived and designed the study. SZ assisted with the design of the study and drafting of the manuscript. All authors have read and approved the final version of this published manuscript.

Ethics approval and consent for publication

The present study was approved by the Ethics Committee of Clinical Research of Xi'an Jiaotong University (Xi'an, China) and was performed in accordance with the 1964 Declaration of Helsinki. Written informed consent was obtained from all patients prior to the study start.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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