Orphan nuclear receptor Nurr1 as a potential novel marker for progression in human pancreatic ductal adenocarcinoma

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Abstract. Nuclear receptor related-1 protein (Nurr1) is a novel orphan member of the nuclear receptor superfamily (the NR4A family) involved in tumorigenesis. The aim of the present study was to investigate the expression and possible function of Nurr1 in pancreatic ductal adenocarcinoma (PDAC). The expression pattern of Nurr1 protein was determined using immunohistochemical staining in 138 patients with PDAC. Elevated Nurr1 expression was more commonly observed in PDAC tissues and cell lines compared with healthy controls. Elevated expression was significantly associated with histological differentiation (P=0.041), lymph node metastasis (P=0.021), TNM classification of malignant tumors stage (P=0.031) and poor survival (P=0.001). Further experiments demonstrated that suppression of endogenous Nurr1 expression attenuated cell proliferation, migration and invasion, and induced apoptosis of PDAC cells. In conclusion, these results suggest that Nurr1 has an important role in the progression of PDAC and may be used as a novel marker for therapeutic targets.

Introduction

Pancreatic cancer is one of the most aggressive types of cancer and is the sixth leading cause of mortality in China (1). Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and has a poor patient prognosis. Despite recent refinement of systematic treatment, including surgery, chemotherapy, radiotherapy, and combination therapy, the 5-year survival rate remains <5% (2,3). The reasons for this high mortality rate include late diagnosis and a lack of effective treatment (4). Therefore, identification of potential novel biomarkers for early diagnosis, prognosis prediction, and novel therapeutic target is required.

Nuclear receptors (NRs) are transcription factors that participate in important biological processes ranging from the regulation of metabolism, to growth and development (5). To date, 48 NRs have been identified in the human genome. The defining features of NRs are two highly conserved regions, the DNA binding domain (DBD) and the ligand binding domain (LBD), which are able to function independently (6). Of the 48 NRs identified, 25 are referred to as orphan NRs, as their endogenous ligands are yet to be elucidated (7). There is a growing body of evidence to support the hypothesis that classical ligand-activated and orphan NRs are associated with the pathogenesis of PDAC (5). Nuclear receptor related-1 protein (Nurr1), which is also known as NR4A2 and RNR-1, is an orphan NR of the NR4A subfamily that has been associated with cell proliferation, differentiation and apoptosis in a cell type-specific manner (8). Orphan NRs are known to exhibit tissue-specific expression, and have context and tissue-specific roles (6). The oncogenic activities of Nurr1 have been detected in various types of cancer. Previous studies have demonstrated that Nurr1 may promote the progression of colorectal cancer, suppress apoptosis in cervical cancer cells, and is an independent prognostic marker for survival in patients with bladder cancer (9-11). Furthermore, it has been indicated that Nurr1 and Nur77 are markedly expressed in human PDAC cell lines (12). However, the significance of Nurr1 expression in human PDAC has not yet been reported, to our knowledge.

In the present study, the high expression levels of Nurr1 in human PDAC specimens and cell lines were further verified and the association between Nurr1 expression and clinicopathological characteristics was evaluated.

Materials and methods

Patients and tissue specimens. A tissue microarray of 138 matched pairs of primary PDAC samples and adjacent normal tissues was obtained from the Department of Pathology

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at the Affiliated Hospital of Nantong University (Nantong, China). The sample population consisted of 97 males and 41 females with PDAC who underwent surgery between 2004 and 2013 (age, 37-78 years). Principal clinical and pathological variables are summarized in Table I. Total cases of patients with PDAC were collected using protocols approved by the Ethics Committee of Affiliated Hospital of Nantong University and written informed consents were obtained from all patients.

Immunohistochemistry. A graded ethanol series was used to deparaffinize the tissue microarray, and endogenous peroxidase activity was subsequently blocked by soaking in 0.3% hydrogen peroxide for 30 min. Sections were heated to 120˚C in an autoclave for antigen retrieval in 10 mM citrate buffer (pH 6.0) for 20 min. Following rinsing in phosphate-buffered saline (PBS; pH 7.2), slides were exposed to 10% normal goat serum for 40 min to block any nonspecific reactions, followed by overnight incubation in a humidified chamber (temperature, 4˚C) with Nurr1 antibody (1:100; rabbit polyclonal; sc-991) and Ki-67 antibody (1:500; mouse monoclonal; sc-23900; both Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Negative control slides were included in all assays, using a non-specific immunoglobulin (monoclonal anti-rabbit; I0138; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) as a primary antibody. The peroxidase-antiperoxidase method (Dako, Glostrup, Netherlands) was used to process slides. Following washing with PBS, the peroxidase reaction was visualized by incubating the sections with diaminobenzidine solution. Hematoxylin was used for counterstaining following rinsing of the sections in water. Sections were subsequently dehydrated with graded alcohol and cover slipped.

Immunohistochemical analyses. Using fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany), two independent blinded pathologists examined all immunostained sections, at random. Five high-power fields were randomly chosen per slide and ≥500 cells were counted per view. Expression score was determined using staining intensity and immunoreactive cell percentage. Intensity of staining was evaluated subjectively on a scale of 0-3: 0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining. Extent of staining was recorded on a scale of 0-4: 0, <1%; 1, 1-25%; 2, 26-50%; 3, 51-75% and 4, >75%. These two scores were subsequently combined and each section was classified as low/no (0-4) or high Nurr1 expression (>4), according to the scores. Ki-67 expression was scored in a semi-quantitative fashion. A cut-off value of 50% or more positively stained in five high-power fields was used to identify Ki-67 staining, as follows: High-expression group (≥50%) and low-expression group (<50%).

Cell lines. Human PDAC cell lines (BxPC-3, CFPAC-1, PANC-1) and the human pancreatic duct epithelial cell line (HPDE6C-7) were all purchased from Chinese Academy of Sciences Cell Bank (Shanghai, China). PANC-1 and CFPAC-1 were cultured in Dulbecco's modified Eagle medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), whereas BxPC-3 and HPDE6C-7 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (all Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator containing 5% CO_2.

Immunofluorescence analysis. Cells were cultured on preferred glass coverslips (Thermo Fisher Scientific, Inc.) overnight. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Hatfield, PA, USA) for 30 min, permeabilized for 15 min with 0.1% Triton X-100/PBS, and subsequently incubated in a 1% bovine serum albumin (BSA)/PBS blocking solution for 2 h. To detect Nurr1, a rabbit polyclonal antibody was used (1:100) and incubated at 4˚C overnight. Incubation with the Alexa Fluor 568-conjugated goat anti-rabbit secondary antibody (1:1,000; A-11,011; Invitrogen; Thermo Fisher Scientific, Inc.), was performed for 2 h at room temperature. To visualize nuclei, cells were stained with 5 mg/ml 4',6-diamidino-2-phenylindole (Sigma-Aldrich; Merck Millipore). Images were captured using a Nikon confocal microscope (Nikon Corp., Tokyo, Japan).

Silencing of Nurr1. Two small interfering RNA (siRNA) duplexes targeting Nurr1 (siRNA#1 and #2) and one control scramble siRNA duplex were purchased from Shanghai GenePharma (Shanghai, China). Effective sequences were as follows: siRNA#1, 5'-GGCUUGUAAAUU UACCCAATT-3'; siRNA#2 5'-CCUCCAACUUGCAGA AUAUTT-3'; and nonspecific scramble siRNA, 5'-UUCUCC GAACGUGUCACGUTT-3', which was a random sequence that was not related to Nurr1 mRNA. One day prior to transfection, BxPC-3 cells were plated onto a 6- or 96-well plate (Corning Inc., Corning, NY, USA) at 50-60% confluence. Nurr1-targeting and control siRNA oligos were transfected into the cells 24 h later, using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from cells, and reverse transcription reactions were performed using random primers and a RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PCR was performed using SYBR Green Master Mix (Rox Klimatechnik GmbH, Weitefeld, Germany) on a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Primer sequences were designed as follows: Nurr1, forward 5'-GGCATGGTG AAGGAAGTTGT-3' and reverse 5'-CAGGGAAGTGAGGAG ATTGG-3'; and β-actin, forward 5'-CACCAACTGGGACGA CATG-3' and reverse 5'-GCACAGCCTGGATAGCAAC-3'. β-actin was used as a reference for Nurr1. The volumes of the reaction mixture were as follows: 10 μl SYBR Green I Mix; 2 μl cDNA; 0.2 μl forward primer; 0.2 μl reverse primer; and 7.6 μl RNase-free H_2O. PCR cycling conditions were as follows: 40 cycles of 50˚C for 2 min; 95˚C for 10 min; 58˚C for 3 sec; and 72˚C for 30 sec. Each sample was analyzed in triplicate. The 2-ΔΔCq method was used to determine the relative quantification of gene expression levels (13).

Western blotting. Cellular proteins were extracted using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and a Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA,

Table I. Correlation between Nurr1 expression and clinicopathologic features of patients with pancreatic ductal adenocarcinoma.

Characteristic	Total	Nurr1		χ^2 value	P-value
		Low n=72	High n=66		
Gender, n (%)				0.360	0.549
Male	97	49 (50.5)	48 (49.5)		
Female	41	23 (56.1)	18 (43.9)		
Age, years (%)				0.592	0.442
<60	82	45 (54.9)	37 (45.1)		
≥60	56	27 (48.2)	29 (51.8)		
Tumor location, n (%)				0.392	0.822
Head-neck	85	45 (52.9)	40 (47.1)		
Body-tail	41	20 (48.8)	21 (51.2)		
Total	12	7 (58.3)	5 (41.7)		
Tumor size, n (%)				5.769	0.056
<2 cm	31	20 (64.5)	11 (35.5)		
2-4 cm	67	28 (41.8)	39 (58.2)		
>4 cm	40	24 (60.0)	16 (40.0)		
Histological differentiation, n (%)				6.386	**0.041**
Well	36	18 (50.0)	18 (50.0)		
Moderate	81	48 (59.3)	33 (40.7)		
Poor	21	6 (28.6)	15 (71.4)		
Lymph node metastasis, n (%)				5.500	**0.021**
Yes	91	54 (59.3)	37 (40.7)		
No	47	18 (38.3)	29 (61.7)		
Nerve invasion, n (%)				0.790	0.374
Yes	39	18 (46.2)	21 (53.8)		
No	99	54 (54.5)	45 (45.5)		
TNM stage, n (%)				4.657	**0.031**
I-II	88	52 (59.1)	36 (40.9)		
III-IV	50	20 (40.0)	30 (60.0)		
Ki-67 expression, n (%)				21.876	**<0.001**
High	62	46 (74.2)	16 (25.8)		
Low	76	26 (34.2)	50 (65.8)		

Statistical analyses were carried out using Pearson χ^2 test. Bold text indicates a statistically significant (P<0.05) difference. Nurr1, nuclear receptor related-1 protein; TNM, TNM classification of malignant tumors.

USA) was subsequently used to determine the protein concentration. Equal amounts of protein (150 μg) were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride filter membranes (EMD Millipore, Billerica, MA, USA) and blocked for 2 h at room temperature with 5% non-fat milk in 20 mM tris-buffered saline and 0.05% Tween-20 (TBST) supplemented with 150 mM NaCl. The membrane was subsequently incubated overnight with rabbit anti-Nurr1 (1:200) and mouse anti-β-actin antibodies (1;1,000; sc-47778; both Santa Cruz Biotechnology, Inc.) at 4˚C, and washed three times with TBST buffer containing 0.1% Tween-20. Horseradish peroxidase-conjugated secondary human anti-mouse or anti-rabbit antibodies (1:5,000; Pierce Biotechnology, Inc., Rockford, IL, USA) were incubated at room temperature for 2 h and subsequently detected using an enhanced chemiluminescence detection system (Pierce Biotechnology, Inc.). Data were adjusted to β-actin as a loading control. ImageJ software (version 1.48; National Institutes of Health, Bethesda, MA, USA) was used to measure band intensity.

Cell proliferation assay. BxPC-3 cells transfected with Nurr1-siRNA or negative control-siRNA were seeded into 96-well cell culture cluster plates at a concentration of $2x10^4$ cells/well with 100 μl RPMI 1640 complete medium and cultured at 37˚C for 72 h. To measure proliferation, 10 μl cell counting kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well at different time points (0, 24, 48 and 72 h) for 100 min. A microplate reader (Bio-Rad Laboratories, Inc.) was used

to measure cell absorbance at 450 nm with a reference wavelength of 630 nm. Experiments were repeated at least three times. Detection of cell absorbance was performed every 24 h.

Plate colony formation assay. BxPC-3 cells transfected with Nurr1-siRNA or negative-control siRNA were seeded into 6-well culture plates at a concentration of ~1,000 cells/plate. Following two weeks of incubation, cell colonies were washed twice with PBS, fixed with 4% para-formaldehyde for 30 min and subsequently stained with crystal violet for 30 min. Individual clones >0.5 mm were counted.

Wound healing assay. BxPC-3 cells transfected with Nurr1-siRNA or negative-control siRNA were seeded into 6-well culture plates at a concentration of $5x10^5$ cells/well. Cells progressed to confluency for 24 h, and wounds were subsequently scratched in the monoculture using a 10-μl pipette tip. Three reference marks were made on the wells, and light-phase images were captured at 0 and 48 h post-wounding.

Cell invasion and migration assay. To evaluate the invasive and migratory potential of cells, 24-well cell cultures with 8-μm pores (Corning, Inc.) were used. For the invasion assay, $1x10^5$ BxPC-3 cells, transfected with negative-control or Nurr1 siRNA, suspended in 100 μl serum-free medium were seeded into an insert chamber pre-coated with Matrigel matrix (BD Biosciences, San Jose, CA, USA), and 0.5 ml 10% FBS medium was added to the matched lower chamber. Chambers were incubated for 48 h at 37˚C in an atmosphere containing 5% CO_2. Invasive cells on the lower surface of the membrane, which had invaded the Matrigel and migrated through the polycarbonate membrane, were fixed in 4% formaldehyde for 30 min and stained by crystal violet for 30 min. A similar procedure was utilized for the migration assay, with no Matrigel used and a 24-h permeating time for cells. Following each assay, five microscopic fields were randomly chosen to count the invasive and migrated cells.

Flow cytometry analysis of cell apoptosis. Transfected cells were harvested 48 h after transfection. To analyze apoptosis, cells were incubated for 15 min at room temperature with 5 μl recombinant fluorescein isothiocyanate-conjugated Annexin V and 5 μl propidium iodide (BD Biosciences) in darkness. Apoptotic cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

Statistical analysis. Statistical analysis was performed using the StatView version 5.0 software package. The chi-square test was used to analyze the association between Nurr1 and clinicopathological features of PDAC. For analysis of survival data, Kaplan-Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed using the Cox proportional hazards model and the 95% confidence interval was recorded for every marker. SPSS statistical software (version 21.0; IBM SPSS, Armonk, NY, USA) was used to conduct statistical analysis. Data are presented as means ± standard error of the mean, from at least three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Nurr1 expression is elevated in human PDAC cells and tissues. Nurr1 expression levels were measured in PDAC cell lines and tissues, to observe any changes in expression levels. The expression of Nurr1 mRNA and protein was significantly upregulated in three PDAC cell lines, when compared with the HPDE6C-7 pancreatic duct epithelial cell line ($P<0.05$; Fig. 1A and B). Immunofluorescence assays demonstrated that Nurr1 was markedly expressed in the cytoplasm of BxPC-3 and PANC-1 cells (Fig. 1C). To confirm the expression of Nurr1 in PDAC, the expression of Nurr1 protein was also analyzed by immunohistochemistry in 138 pairs of PDAC tissues. Nurr1 and Ki-67 expression was elevated in PDAC cells compared with the normal non-tumorous tissue (Fig. 2). Furthermore, linear regression analysis demonstrated that the expression of Nurr1 in PDAC tissues was significantly positively correlated with that of Ki-67 ($P<0.001$; Fig. 3). These findings indicated that Nurr1 may be associated with the malignant progression of PDAC.

Nurr1 expression is correlated with histological differentiation, lymph node metastasis, TNM stage and Ki-67 expression. A significant correlation was detected between Nurr1 expression and histological differentiation ($P=0.041$), lymph node metastasis ($P=0.021$), TNM stage ($P=0.031$) and Ki-67 expression ($P<0.001$; Table I). However, there was no significant correlation with other clinicopathological factors, including gender, age, tumor location or nerve invasion.

Nurr1 expression predicts the prognosis of patients with PDAC. Kaplan-Meier analysis and log-rank test were performed to further assess the prognostic status of Nurr1 expression in patients with PDAC. Survival curves indicated that elevated Nurr1 expression was significantly associated with poor overall survival ($P=0.001$; Fig. 4). Univariate analysis indicated that lymph node metastasis ($P=0.040$), TNM stage ($P=0.002$), Ki-67 expression ($P<0.001$) and Nurr1 expression ($P<0.001$) were significant prognostic predictors (Table II). Multivariate analysis indicated that Nurr1 expression ($P=0.040$) and Ki-67 expression ($P=0.002$) were significant independent prognostic indicators for patients with PDAC (Table II).

Depletion of Nurr1 restrains cell proliferation and induces apoptosis. Among the PDAC cell lines, BxPC-3 cells exhibited markedly increased mRNA expression levels of Nurr1 (Fig. 1A); therefore BxPC-3 cells were used to study the biological functions of endogenous Nurr1. An siRNA-oligo targeting Nurr1 was used to knockdown the endogenous expression of Nurr1 in BxPC-3 cells. Direct immunofluorescence observation and western blotting were used to determine the Nurr1 levels in siRNA-transfected cells. Immunofluorescence observation demonstrated that the majority of cancer cells expressed fluorescein at a relatively high level (Fig. 5A). A knockdown effect was observed using western blot analysis, and siRNA#1 and siRNA#2 demonstrated greater efficacy in silencing Nurr1 expression compared with the negative control (Fig. 5B).

Subsequently, the effect of decreased Nurr1 expression on pancreatic cancer cell proliferation was evaluated *in vitro* using a CCK-8 assay. Silencing of Nurr1 induced a significant

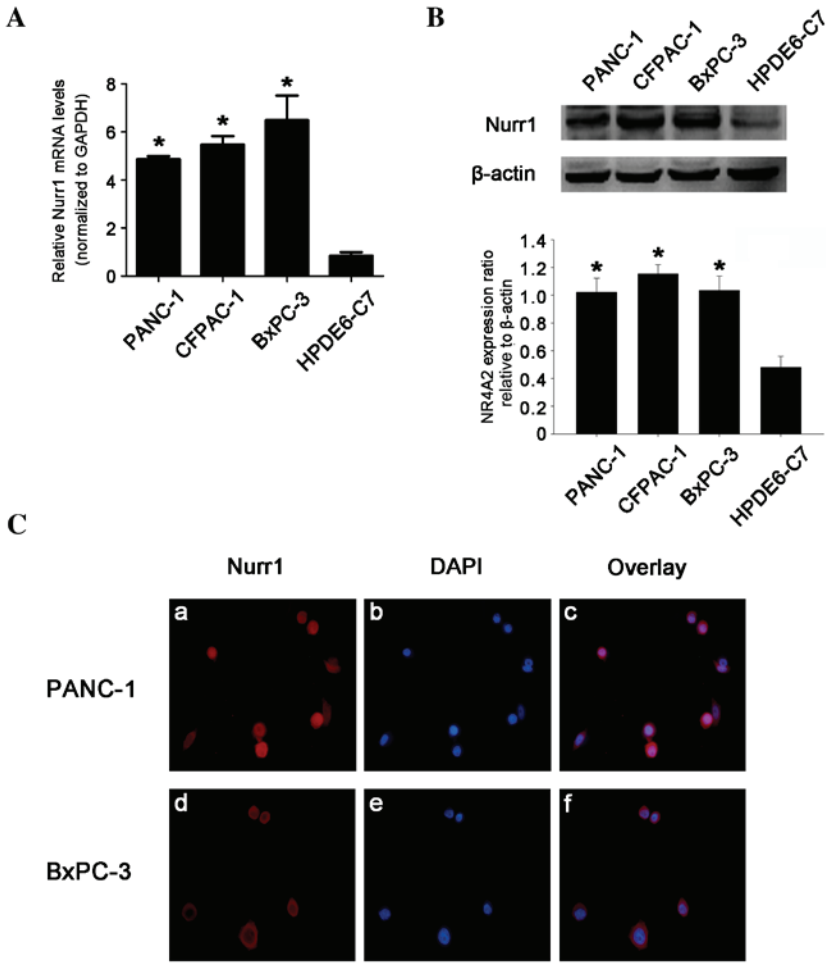

Figure 1. Nurr1 was upregulated in three PDAC cell lines: PANC-1, CFPAC-1 and BxPC-3. (A) Nurr1 mRNA levels were evaluated by reverse transcription-quantitative polymerase chain reaction in three PDAC cell lines. Normal human pancreatic duct epithelial cells, HPDE6-C7, were used as controls. (B) Nurr1 and β-actin protein in the different PDAC cell lines and normal pancreatic duct epithelial cells, analyzed by western blotting. (C) Immunofluorescence analysis of Nurr1 expression in PANC-1 and BxPC-3 cells. Nurr1 was dominantly expressed in the cytoplasm of the PDAC cells. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. control HPDE6-C7 cells. Nurr1, nuclear receptor related-1 protein; PDAC, pancreatic ductal adenocarcinoma; GADPH, glyceraldehyde 3-phosphate dehydrogenase; DAPI, 4',6-diamidino-2-phenylindole.

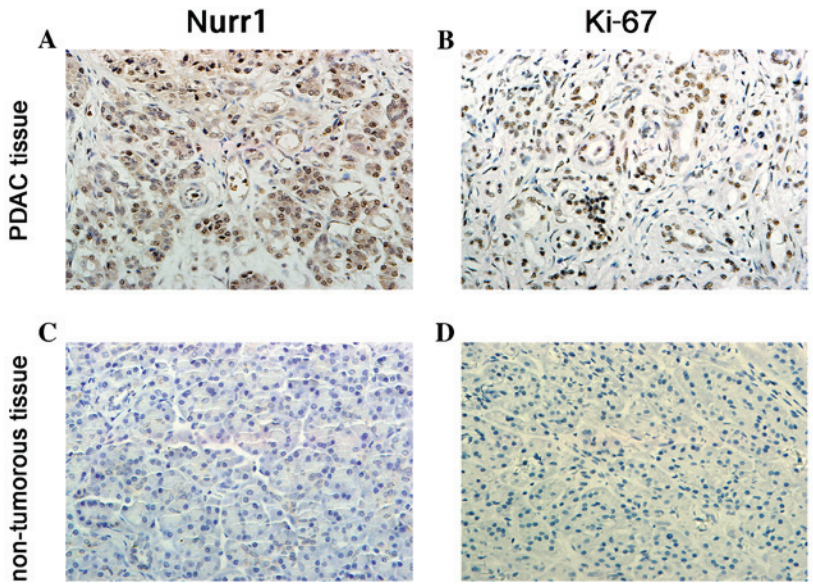

Figure 2. Nurr1 and Ki-67 expression and distribution was examined using immunohistochemistry in PDAC and paired normal tissues. (A and B) Nurr1 and Ki-67 expression was greater in PDAC cells (C and D) than in non-tumorous tissue. Nurr1, nuclear receptor related-1 protein; PDAC, pancreatic ductal adenocarcinoma.

Table II. Univariate and multivariate analyses showing the overall survival rate of patients with pancreatic ductal adenocarcinoma.

	Univariate cox regression			Multivariate cox regression		
Characteristic	HR	95% CI	P-value	HR	95% CI	P-value
Gender	0.917	0.627-1.342	0.657	-	-	-
Age	1.251	0.878-1.782	0.215	-	-	-
Tumor location	1.136	0.868-1.487	0.352	-	-	-
Histological	1.144	0.917-1.427	0.234	-	-	-
differentiation	1.181	0.881-1.584	0.265	-	-	-
Lymph node metastasis	1.465	1.018-2.108	**0.040**	1.003	0.670-1.501	0.987
Nerve invasion	1.057	0.731-1.529	0.768	-	-	-
TNM stage	1.763	1.226-2.536	**0.002**	1.397	0.932-2.095	0.105
Ki67	4.013	2.170-7.421	**<0.001**	1.925	1.285-2.883	**0.002**
Nurr1	1.959	1.368-2.807	**<0.001**	1.498	1.019-2.204	**0.040**

Statistical analyses were performed using Cox proportional hazards regression. Bold text indicates a statistically significant (P<0.05) difference. HR, hazard ration; CI, confidence intervals; TNM, TNM classification of malignant tumors; Nurr1, nuclear receptor related-1 protein.

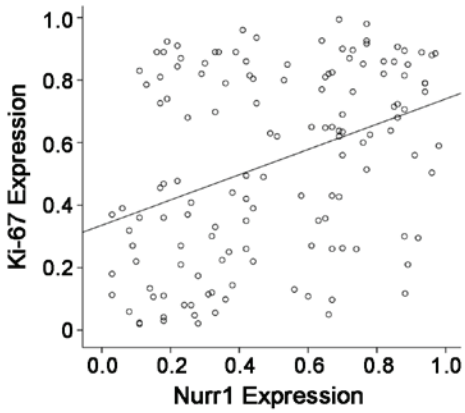

Figure 3. Association between Nurr1 and Ki-67 expression in 138 PDAC specimens. Scatter plots of Nurr1 vs. Ki-67 expression with a regression line showing a significant correlation (P<0.001) using the Spearman's correlation coefficient (r=0.34). Nurr1, nuclear receptor related-1 protein; PDAC, pancreatic ductal adenocarcinoma.

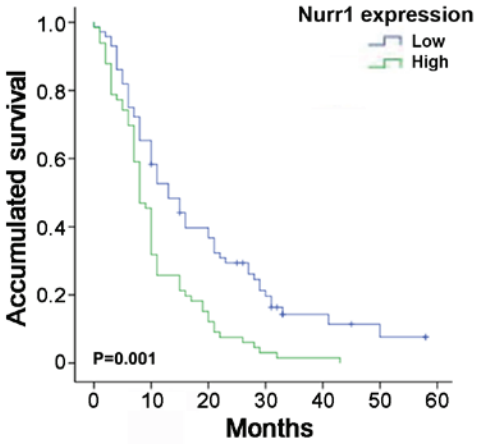

Figure 4. Kaplan-Meier survival curves for 138 patients with PDAC according to Nurr1 protein expression status (long-rank test). Nurr1 expression was scored according to immunohistochemical staining intensity, on a scale of 0-3, and immunoreactive cell percentage, on a scale of 0-4. These two scores were subsequently combined and expression was classified as low (0-4) or high Nurr1 expression (>4). Patients with low Nurr1 expression had a longer survival than those with higher expression (P=0.001; log-rank test). Nurr1, nuclear receptor related-1 protein; PDAC, pancreatic ductal adenocarcinoma.

decrease in cell viability in BxPC-3 cells (P<0.05; Fig. 5C). This finding was supported by analysis of the plate clone formation assay (Fig. 5D). Furthermore, analysis of cell apoptosis following Nurr1 knockdown revealed that the number of apoptotic Nurr1-siRNA cells significantly increased compared with control siRNA-transfected cells (P<0.05; Fig. 5E). These results suggest that Nurr1 may promote PDAC cell proliferation and apoptosis.

Knockdown of Nurr1 inhibits in vitro migration and invasion. A wound-healing assay and a Transwell migration assay were used to examine the effect of Nurr1 on PDAC migration. Transwell migration assay demonstrated that, following 24 h incubation, the number of migrated cells in the Nurr1-siRNA groups was significantly lower than that in the control siRNA cells (P<0.05; Fig. 6A). The wound-healing assay demonstrated that the migratory areas of Nurr1-siRNA cells were significantly smaller than control cells (P<0.05; Fig. 6B), indicating a decreased migratory ability following Nurr1 downregulation. Changes in cell invasiveness following 48-h incubation were evaluated using a chamber pre-coated with Matrigel. Compared with the control siRNA cells, Nurr1-siRNA cells demonstrated significantly decreased invasiveness (P<0.05; Fig. 6C). These results suggest that Nurr1 promoted the migration and invasion of PDAC cells.

Discussion

Pancreatic cancer is the eighth leading cause of cancer-associated mortality (14). The majority of patients (80-85%) are not diagnosed until the cancer has metastasized to other organs (1,2). The incidence and progression of pancreatic cancer is associated with various accumulations of genetic

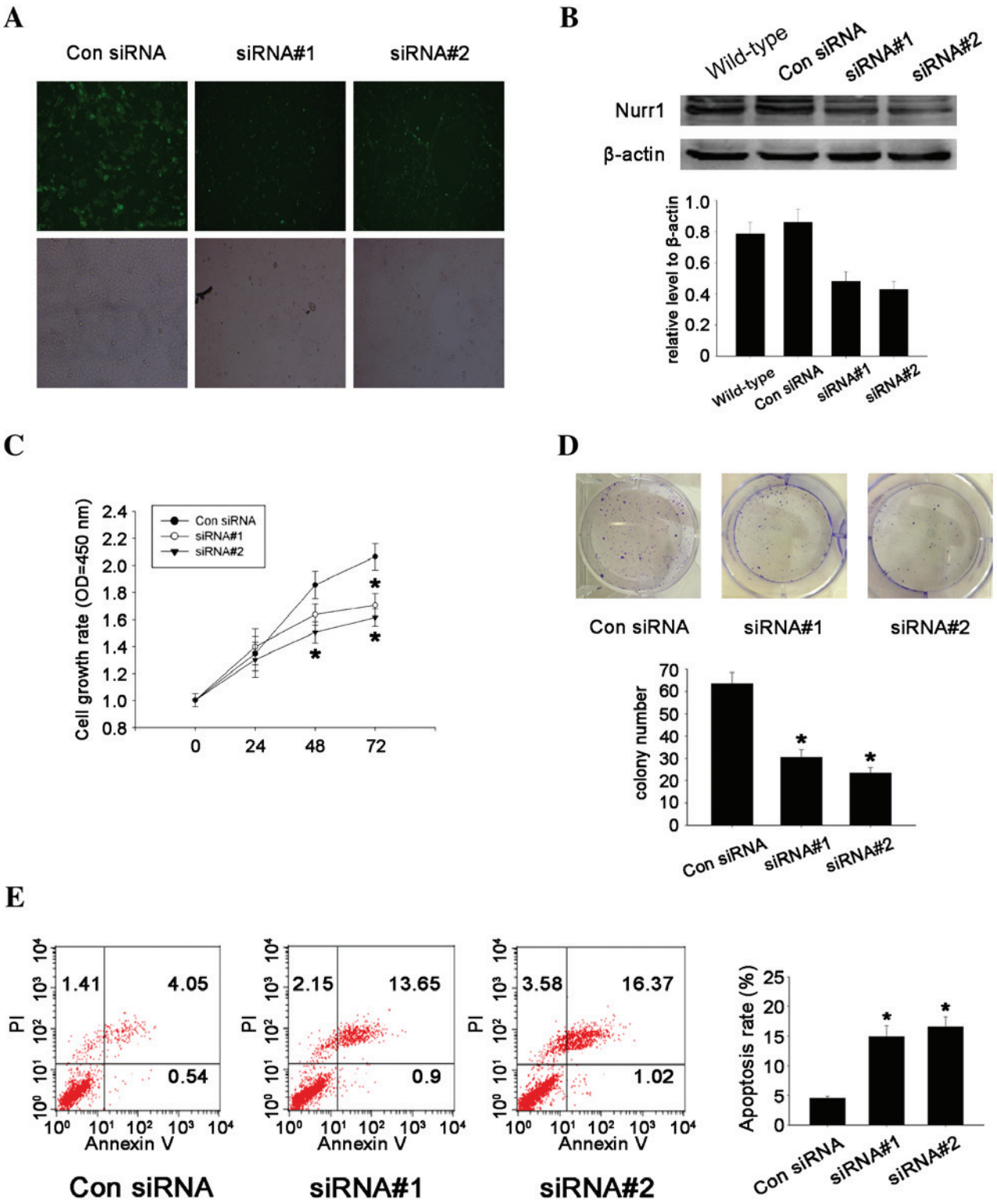

Figure 5. Depletion of Nurr1 reduced cell proliferation and induced apoptosis. (A) Fluorescence observation of siRNA transfection efficacy on the downregulation of Nurr1 in PDAC cells (BxPC-3; magnification, x100). (B) Nurr1 expression was detected using western blotting in BxPC-3 cells at 48 h following transfection with Nurr1-siRNA, whereas siRNA#1 and #2 exhibited greater downregulation. Relative level of Nurr1 expression was evaluated using densitometry. (C) At 48 h post-transfection, a cell counting kit-8 assay was performed to determine the proliferation of BxPC-3 cells. (D) Nurr1 knockdown suppressed plate colony formation. (E) At 48 h post-transfection, apoptotic BxPC-3 cells were detected via flow cytometry. Data are presented as the mean ± standard error of the mean, and are representative of three independent experiments. *P<0.05 vs. control. Nurr1, nuclear receptor related-1 protein; siRNA, short interfering RNA; PDAC, pancreatic ductal adenocarcinoma; Con, control; OD, optical density.

alterations (15). However, the molecular mechanisms of pancreatic cancer remain poorly understood. Nurr1 and NR4A1 have been associated with pancreatic cancer. Endogenous nuclear NR4A1 enhances pancreatic cancer cell proliferation and

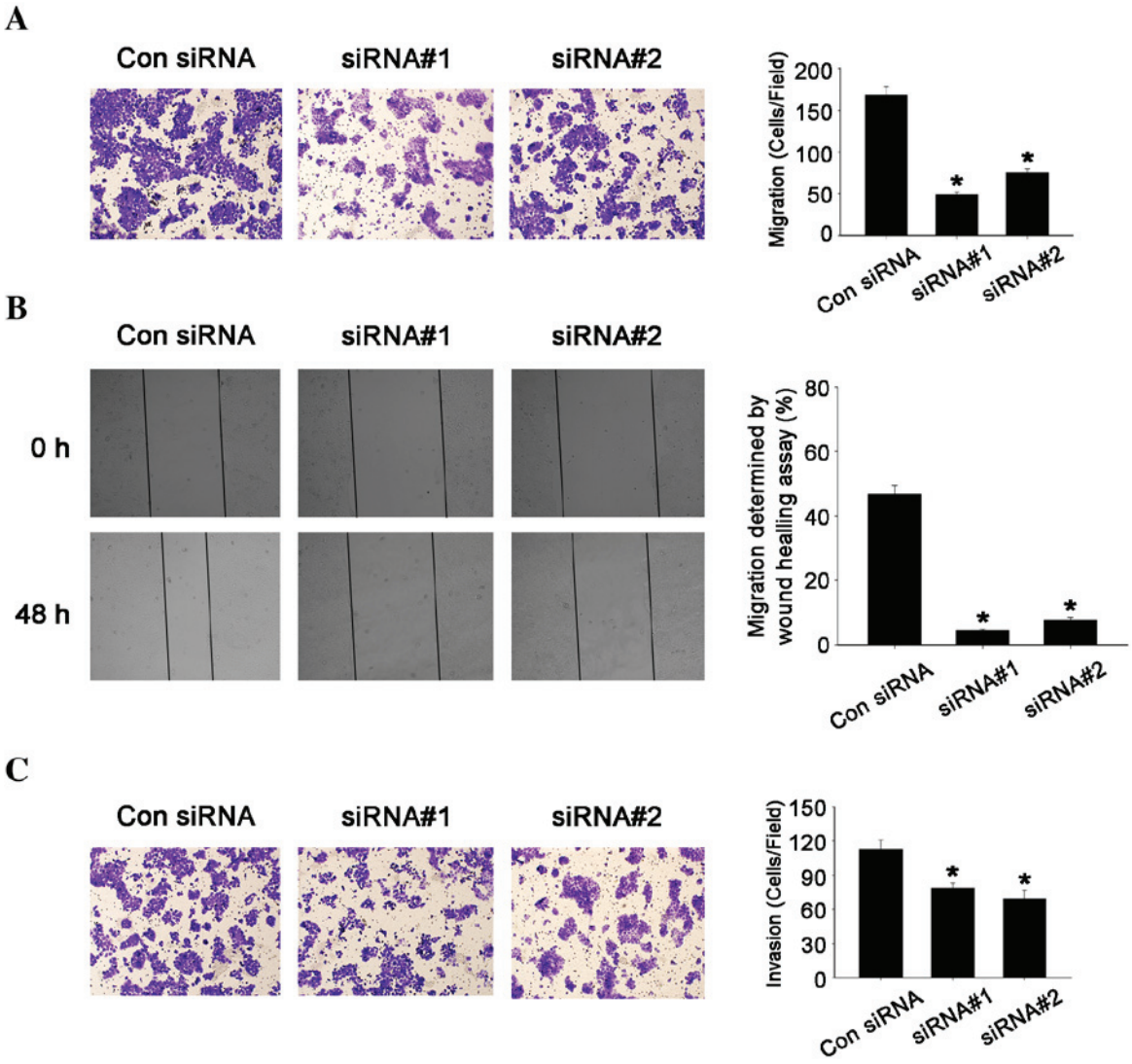

Figure 6. Knockdown of Nurr1 suppresses migration and invasion of BxPC-3 PDAC cells. (A) BxPC-3 cells were transfected with Con and Nurr1-siRNAs (#1 and #2), and cell migration was assessed using the Transwell migration assay. Results demonstrated that BxPC-3 cell migration was significantly impaired following Nurr1 knockdown. (B) The wound-healing assay demonstrated that the migratory areas of Nurr1-siRNA cells were significantly smaller than control cells. (C) BxPC-3 cell invasiveness was evaluated via Transwell invasion assays following transfection with Nurr1-siRNAs, and the results showed that cell invasion was significantly decreased compared with cells transfected with Con siRNA. Data are presented as the mean ± standard error of the mean, and are representative of three independent experiments. *P<0.05 vs. control. Nurr1, nuclear receptor related-1 protein; siRNA, short interfering RNA; PDAC, pancreatic ductal adenocarcinoma; Con, control.

survival, and exhibits pro-oncogenic activity (16); however, the clinical significance and biological functions of Nurr1 in pancreatic cancer remain unknown. In the present study, it was demonstrated that Nurr1 was more commonly expressed in PDAC tissues and cells compared with healthy pancreatic tissues and pancreatic ductal epithelial cells, and that elevated expression of Nurr1 is correlated with histological differentiation, lymph node metastasis and the TNM stage of patients with PDAC patients. Moreover, Kaplan-Meier analysis demonstrated that patients with high levels of Nurr1 expression exhibited significantly reduced survival times compared with those with low levels. Multivariate analysis further demonstrated that Nurr1 expression was an independent predictor of poor survival of patients with PDAC. These observations indicate that Nurr1 may function as an oncogenic factor and a predictor of survival in human PDAC.

Proliferation, invasion and migration are predominant characteristics of cancer development, and apoptosis has an important role in the regulation of oncogenesis. BxPC-3 cells exhibiting the higher expression level of endogenous Nurr1, and those with siRNA-induced knockdown of Nurr1, were used to investigate the biological functions of Nurr1 in PDAC. Suppressed Nurr1 expression decreased the viability of cancer cells and promoted cell apoptosis, which are indispensable for PDAC growth. These results suggest an oncogenic role of Nurr1 in human PDAC development.

Nurr1 is a member of the NR4A subfamily which includes two other members, Nur77, also known as NR4A1 or NGFI-B, and Nor-1, also known as NR4A3 (17). The NR4A subfamily are orphan NRs which act as transcription factors to positively or negatively regulate target gene expression (18). These orphan receptors, which exhibit pro-oncogenic or tumor

suppressor-like activity, have essential, time-specific roles in cancer (19). For example, Nur77 has tumor-promoting (colon cancer) and tumor-suppressive (acute myeloid leukemia) functions (20,21). Physiologically, Nurr1 is essential for dopaminergic neuronal survival and development (22). Therefore, consistent with Nur77, Nurr1 is associated with oncogenic activity in colorectal cancer (9) and breast cancer (23). Nurr1 is a potential novel marker for progression in human prostate cancer (24). High cytoplasmic expression of Nurr1 predicts poor survival in bladder cancer and nasopharyngeal carcinoma (11,25). Nurr1 is also a key target gene of thromboxane A2 receptors and is critical for the proliferation of lung carcinoma cells (26). Furthermore, Nurr1 expression and activity may be induced by several compounds, including 6 mercaptopurine and 1,1-bis(3'-indolyl)-1-(aromatic) methane (C-DIM) analogs (27,28). The ligand-binding domain of Nurr1 has reportedly been activated by 1,1-bis 3'-indolyl)-1-(p-chl orophenyl)-methane (DIM-C-pPhCl), resulting in decreased cell survival and induction of cell death pathways in bladder cancer (28). DIM-C-pPhBr and related compounds have also been found to activate nuclear Nurr1 in pancreatic cancer (12). Therefore, these results identify Nurr1 as a potential target for cancer therapy and Nurr1-active C-DIMs as a potential novel agent for the clinical treatment of cancer.

In conclusion, the present study demonstrated that Nurr1 was highly expressed in PDAC tissues and positively associated with Ki-67. High Nurr1 expression may be considered as an independent prognostic factor in patients with PDAC. Furthermore, Nurr1 enhanced cell viabilities and inhibited cell apoptosis, which ultimately promoted cell growth in PDAC. Nurr1 is a member of the orphan NR family, for which no natural ligands have yet been identified. Therefore, identification of specific chemicals able to inhibit the expression or transcriptional activity of Nurr1 would be beneficial for novel therapeutic strategies. Further study is required to delineate the detailed mechanism mediated by Nurr1.

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