ΔNp63 promotes UM-UC-3 cell invasiveness and migration through claudin-1 *in vitro*

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Abstract. The p63 gene, a member of the p53 gene family, has two different promoter usage-generating proteins that contain or lack (ΔN) an NH2-terminus. Although p53 and p63 have high sequence and structural similarities, the molecules differ in function and expression profiles. p63 is critical for the development of epithelial organs or tissues, including the epidermis and other squamous epithelia, as well as the salivary, lachrymal, mammary and prostate glands and the urothelium. In addition, p63 is essential for the proliferative potential of stem cells in the epidermis. In contrast to p53, the role of ΔNp63 in tumors remains unclear and complex. Our previous study demonstrated that ΔNp63 is overexpressed in human bladder carcinoma tissues. The mechanism by which ΔNp63 promotes tumor cell development, including adhesion, proliferation and polarity, is unknown. Data demonstrate that ΔNp63 induces the invasiveness of cancer cells through specific downstream genes and the mechanism is associated with cell junctions. Claudin-1 is an important p63 target gene for normal skin development. Claudin-1, as a connexin, functions in a similar manner to other connexins to affect important events during cancer cell development. In the present study, ΔNp63 gene expression in bladder tumor tissues was found to be significantly higher than that in normal tissue, indicating that ΔNp63 is localized to the nucleus. In addition, ΔNp63 silencing decreased invasion and metastasis in UM-UC-3 cells and reduced claudin-1 expression.

Introduction

The p63 gene is a member of the p53 gene family and has two different promoter usage-generating proteins that contain (TA) or lack (ΔN) an NH2-terminus. The p53 and p63 molecules differ in function and expression profiles. p63 is critical for the development of various epithelial organs or tissues and is also essential for the proliferative potential of stem cells in the epidermis (1-4). ΔNp63 functions as a dominant negative regulator of the TA isoforms of p63 and p53, which have been revealed to inhibit apoptosis and promote stem cell proliferation *in vitro* (5,6). In contrast to p53, the role of ΔNp63 in tumors remains unclear and complex (7,8). Previous studies have demonstrated that ΔNp63 is overexpressed in carcinomas of squamous epithelial origin (9-11) and may play a role in promoting tumorigenesis (12,13). Our previous study demonstrated that ΔNp63 is overexpressed in human bladder carcinoma.

Cell-cell and cell-extracellular matrix interaction is crucial for tumor transformation and tumor invasion (14,15), in which the tight junction is an important constituent. Absence of tight junctions or defects in these complexes has been associated with the development of the neoplastic phenotype in epithelial cells (16-18). The disruption of tight junctions leads to cohesion loss, invasiveness and the lack of differentiation, thereby promoting tumorigenesis (19). Claudin-1 is a tight junction protein expressed in epithelial and endothelial cells (20).

The mechanism by which ΔNp63 promotes tumor cell development, including adhesion, proliferation and polarity, is unknown. Previous studies have reported that ΔNp63 induces cancer cell invasion (21,22). Specific downstream genes of ΔNp63 have been described (21-25) in association with cell junctions. Claudin-1 is an important p63 target gene required for normal skin development (26). As a connexin, it acts in a similar manner to these proteins to affect events important for cancer cell development.

In the present study, ΔNp63 expression in human bladder carcinoma UM-UC-3 cells was reduced *in vitro*. These results indicate that ΔNp63 is located in the nucleus. In addition, ΔNp63 silencing decreases invasion and metastasis of UM-UC-3 cells and reduces claudin-1 expression. These results indicate that claudin-1, as a ΔNp63 target gene, is associated with cell invasion and migration in UM-UC-3 bladder cancer cells.

Materials and methods

Cell culture and transfection. The human bladder carcinoma cell line, UM-UC-3, was purchased from the Institute of Cell Research (Chinese Academy of Sciences, Shanghai, China).

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The study was approved by the ethics committee of North Sichuan Medical College, Nan Chong, P.R. China. Cells were cultured in RPMI-1640 medium (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS; Sijixin Inc., Beijing, China) and 1% penicillin-streptomycin (Invitrogen, Shanghai, China). All cells were cultured at 37˚C with 5% CO_2. The expression plasmid that encodes ΔNp63 was kindly provided by Dr He Yunfeng (The First Affiliated Hospital, Chongqing Medical University, Chongqing, China)and has a structure consisting of two 19 bp stem-targeting ΔNp63 mRNA, a 9 bp loop and a short poly$(A)_6$ sequence. The sequences of two oligonucleotides were as follows: forward, 5'-GATCCGTGCCCAGACTCAATTTAGTTTCAAGACGA CTAAATTGAGTCTGGGCATTTTTTGTCTTCAAGACG ACTAAATTGAGTCTGGGCATTTTTTGTCGACA-3' and reverse, 5'-AGCTTGTCGACAAAAAATGCCCAGACT CAATTTAGTCGTCTTGAAACTAAATTGAGTCTGGGC ACG-3'. The sequences of the negative control shRNA were as follows: forward, 5'-GATCCGACTTCATAAGGCGCA TGCTTCAAGACGGCATGCGCCTTATGAAGTCTTTTTT GTCGACA-3' and reverse, 5'-AGCTTGTCGACAAAAAAG ACTTCATAAGGCGCATGCCGTCTTGAAGCATGCGCC TTATAAGTCG-3'. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell invasion and migration assay. Cell invasiveness was determined using a Transwell chamber (6.5 mm in diameter with polyvinylpyrrolidone-free polycarbonate filter of 8-μm pore size; Corning Inc., Corning, NY, USA) precoated with 30 μg Matrigel (BD Biosciences, San Jose, CA, USA). Approximately 100 μl of cells (10^5) transfected with siRNA or control plasmid were added to the upper compartment of the Transwell chamber. Then, 600 μl of 10% FBS medium was added to the lower chamber. Following 24 h incubation at 37˚C, the non-invading cells in the upper surface of the filter were removed using a cotton swab. The cells that penetrated into the lower surface of the filter were stained with trypan blue. Finally, the invading cells were counted under a microscope using a 10X objective in four random fields. Cells were plated in six-well plates for the migration assay. A wound was created on the monolayer cells when the cells reached full confluence by scraping a gap using a micropipette tip. The plate was then washed with serum-free RPMI-1640 medium to clean the dissociated cells. Cells were then incubated with serum-free RPMI-1640 medium at 37˚C in 5% CO_2. Cells that migrated into the unit length area were counted five times for each group at 0, 12 and 24 h following scraping.

Cell heterogeneity adhesion assay. Cells (~1x10^5/ml) were added into a 96-well plate covered with collagen IV and incubated at 37˚C in 5% CO_2 for 90 min. The plate was washed with phosphate-buffered saline (PBS) to clean the dissociated cells. Approximately 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) was then added to the culture medium. Following incubation for 10 min at room temperature, the culture medium was removed and then 200 μl dimethylsulfoxide was added into each well. Absorbance (A value) was measured at 570 nm. Each sample was assayed four times. The cell adhesion rate was compared with the ratio of adherent cells and the total A value of the cells.

Confocal microscopy. Cells were seeded on polylysine (10 μg/ml)-coated glass chamber slides at a density of 2,000 cells/chamber and washed, fixed in ice-cold 4% paraformaldehyde for 15 min and permeabilized in 100 mM phosphate buffer containing 0.2% Triton X-100 (Sigma-Aldrich) for 4 min. Cells were then incubated with 5% bovine serum albumin (BSA) and immunolabeled with anti-ΔNp63 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-claudin-1 antibodies (1:500; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Normal goat IgG was used instead of anti-p63 in specific experiments to serve as the negative control. Following incubation with the primary antibodies, the cells were washed and incubated for 1 h with fluorescein isothiocyanate-conjugated anti-ΔNp63 antibodies (1:500; Santa Cruz Biotechnology, Inc.) and Cy3-conjugated anti-claudin-1 antibodies (1:500; Santa Cruz Biotechnology, Inc.) for 1 h. Additional washes were performed and the cells were mounted using fluorescent mounting medium (Applygen Technologies, Inc., Beijing, China). Cells were viewed under a Leica SP2 upright microscope and the images were captured in LCS Light (Leica).

Reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated using an RNeasy mini kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen). Real-time PCR was conducted using an iCycler (Bio-Rad) with an iQ SYBR-Green Supermix (Bio-Rad), according to the manufacturer's instructions. The primer sequences designated from the coding region of the human gene cDNA were as follows: ΔNp63, 5'-CAGCCCATTGACTTGAACTTTG-3' (sense) and 5'-TGTTATAGGGACTGGTGGACGA-3' (antisense); claudin-1, 5'-GAGGATGGCTGTCATTGGG-3' (sense) and 5'-CTTGGTGTTGGGTAAGAGGTTG-3' (antisense). The internal controls were as follows: 5'-TGACGTGGA CATCCGCAAAG-3' (sense) and 5'-CTGGAAGGTGGACAG CGAGG-3' (antisense). The PCR conditions were as follows: 94˚C for 4 min, followed by 35 cycles at 94˚C for 20 sec, 60˚C for 30 sec and 72˚C for 30 sec, with data acquisition during each cycle. Melting curve analysis was conducted following PCR cycling to verify the purity and quality of the PCR product.

Western blot analysis. Cells were seeded into 100-cm^2 flasks. Confluent cell layers were washed with ice-cold PBS and lysed for 30 min at 4˚C, with 1% NP-40, 0.1% Triton X-100, 30 mM sodium phosphate (pH 7.4) containing 1 mM sodium orthovanadate, 2.5 mM Tris-HCl (pH 7.5), 100 mM NaCl and 10 μg/ml leupeptin and aprotinin 24 h after plating. The homogenate was then centrifuged at 12,000 x g for 20 min at 4˚C. The supernatant liquid was collected and the protein was quantified with the Bio-Rad protein colorimetric assay. Protein was separated using 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis following addition of the sample buffer to the cellular extract and boiling the samples at 95˚C for 5 min. The protein was transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) and the membrane was then blocked for 1 h at room temperature with 5% BSA

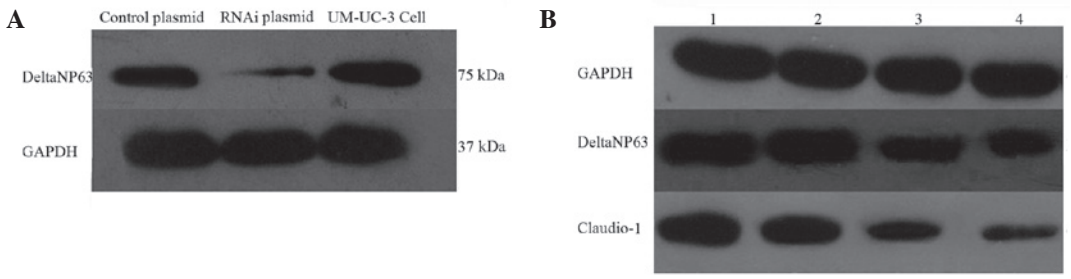

Figure 1. Western blot analysis of (A) ΔNp63 in UM-UC-3 control and siRNA-treated cells (75 kDa) and (B) ΔNp63 and claudin-1 in UM-UC-3 control and UM-UC-3 cells transfected with si-ΔNp63 plasmid, negative plasmid or empty vector. Lanes 1, UM-UC-3 cells; 2, empty vector; 3, negative plasmid; and 4, si-ΔNp63 plasmid. GAPDH was used as an internal control (37 kDa).

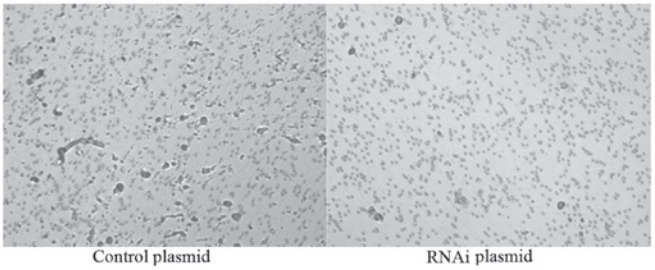

Figure 2. Cell invasion assay. Cells were added to the upper compartment of the Transwell and incubated for 24 h. The lower surface of the filter was washed and stained with Trypan blue (magnification, x40). Control had 11.25±1.2 and interfering plasmid group had 5.5±0.7 cells. (P<0.05).

in Tris-buffered saline containing 0.05% Tween-20 (TBST). Then, the blots were washed and incubated overnight at 4˚C in TBST containing 1% BSA with primary antibodies against ΔNp63 (1:200), claudin-1 (1:200) and GAPDH (1:3,000). The membranes were washed three times with TBST, incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2,500 dilution in TBST containing 1% BSA) for 120 min at room temperature and then washed three times with TBST. Following the chemiluminescence reaction, bands were detected by exposing the blots to X-ray films for the appropriate time. For quantitative analysis, bands were detected and evaluated densitometrically with UVP Gelatin image processing system Labworks 4.6 software and normalized against GAPDH density.

Statistical analysis. Results are expressed as mean ± SD. One-way ANOVA was used to determine the levels of difference between all groups. P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using the SPSS statistical software program (SPSS Inc., Chicago, IL, USA).

Results

ΔNp63 protein expression and localization. Our results showed that ΔNp63 mRNA expression is inhibited by ΔNp63 siRNA *in vitro*. In the current study, green fluorescent protein (in the UM-UC-3 cells transfected with the ΔNp63 control and ΔNp63-interfering plasmid) was observed under an inverted fluorescence microscope. ΔNp63 protein expression levels were also determined. The control and ΔNp63 siRNA-treated UM-UC-3 cells were characterized through western blot analysis, using GAPDH as the internal control. The relative ratio of the ΔNp63 protein expression with that of GAPDH in the UM-UC-3 cells was determined following stable transfection with the control or siRNA plasmid. Results indicate that ΔNp63 protein expression was inhibited by the ΔNp63-interfering plasmid *in vitro* (Fig. 1A). UM-UC-3 cells were detected under laser confocal microscopy to determine the functional position of the ΔNp63-interfering plasmid in the cells. The result indicates that ΔNp63 is largely localized to the nuclei of the UM-UC-3 cells. In addition, a sporadic distribution of ΔNp63 was revealed in the cell membrane. However, ΔNp63 protein expression was reduced and localized to the cell nucleus around the cell membrane in the ΔNp63-transfected UM-UC-3 cells. By contrast, ΔNp63 protein expression was inhibited by the ΔNp63-interfering plasmid and localized on the cell membrane of the UM-UC-3 cells.

Downregulation of invasion and metastasis. ΔNp63 knockdown in the UM-UC-3 cell line was used to examine the effect of ΔNp63-interfering plasmid on bladder cancer invasion *in vitro*. The Transwell chamber precoated with 30 μg Matrigel was used for the invasion assay. Results reveal that control exhibited 11.25±1.2 cells, whereas the interfering plasmid group had 5.5±0.7 cells following stable transfection. The invasiveness of UM-UC-3 cells transfected with ΔNp63-interfering plasmid was found to have decreased significantly (P<0.05; Fig. 2). Collagen IV-covered 96-well plates were used for cell heterogeneity adhesion and MTT assays. The result indicates that the cell adhesion capacity following stable transfection with ΔNp63 was lower compared with the control and negative plasmid groups (P<0.05).

A cell scratch experiment was performed to examine the effect of ΔNp63-interfering plasmid on cell migration *in vitro*. Cells were photographed and the number of cells that migrated per unit area was counted at 0, 12 and 24 h following scraping. At 12 h, the negative group had 14.2±3.7 cells/mm^2; control had 13.9±3.3 cells/mm^2 and interfering plasmid had 6.2±2.3 cells/mm^2. At 24 h, the negative group had 22.0±1.2 cells/mm^2, control had 18.2±2.1 cells/mm^2 and interfering plasmid had 12.6±1.4 cells/mm^2. The migration ability of the cells in the negative plasmid and control groups was higher than that of the interfering plasmid (Fig. 3)

A series of experiments demonstrated that the invasion and metastasis of bladder cancer is suppressed *in vitro* through stable transfection with ΔNp63.

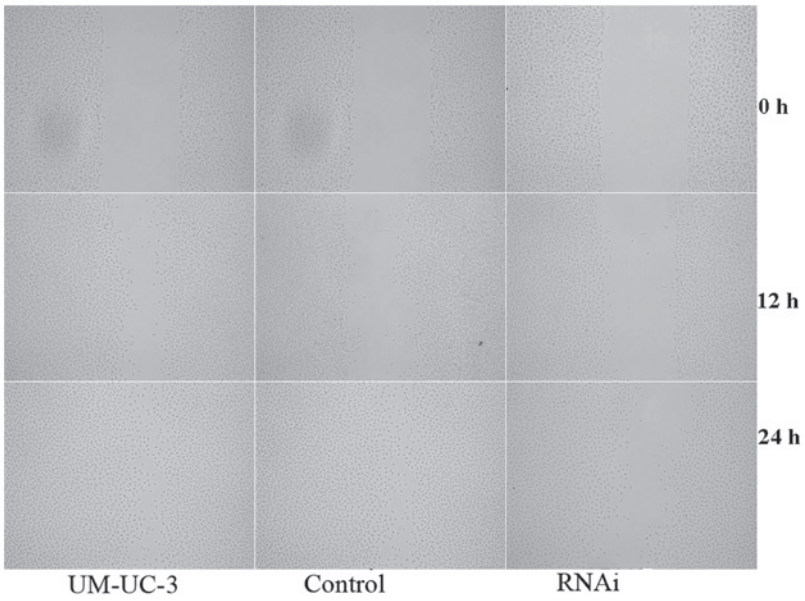

Figure 3. Migration assay to determine the number of cells that migrated to the unit length area at 0, 12 and 24 h following scraping. Counts were performed five times for each group (magnification, x200). At 12 h, negative had 14.2±3.7, control had 13.9±3.3 and interfering plasmid had 6.2±2.3 cells. At 24 h, negative had 22.0±1.2, control had 18.2±2.1 and interfering plasmid group had 12.6±1.4 cells. (P<0.05).

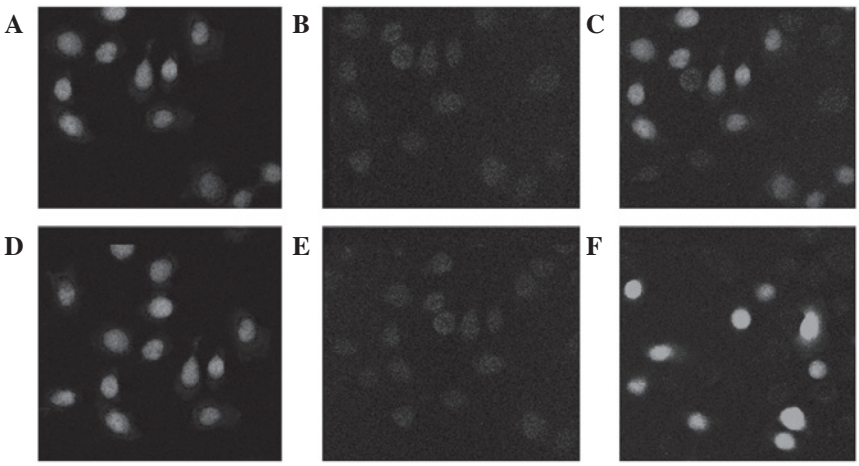

Figure 4. Double mark confocal microscopy images of ΔNp63 and claudin-1 localization in UM-UC-3 cells. Immunofluorescent antibody labeling was used to assess the changes of claudin-1 and ΔNp63. Green fluorescence was marked by FITC-anti-ΔN p63 and red fluorescence was marked by Cy3-anti-claudin-1. (A-C) Cells treated withΔNp63 control plasmid or (D-F) ΔNp63 control plasmid (magnification, x1,000). (A and D) Show ΔNp63 localization in the nucleus of UM-UC-3 cells. (B and E) Show claudin-1 localization in the membrane of UM-UC-3 cells. (C and F) Claudin-1 expression was reduced in the UM-UC-3 cells transfected with ΔNp63.

Claudin-1 expression. ΔNp63 protein expression was reduced and localized to the cell nucleus. The cell membrane did not have the same ΔNp63 protein distribution as that associated with the promotion of bladder cancer cell invasion and metastasis. However, we focused on its regulation by ΔNp63. Double mark confocal microscopy was performed to verify the binding of ΔNp63 with the claudin-1 promoter and subsequently determine its potential role as a transcriptional regulator of claudin-1. Results indicate that claudin-1 expression was reduced in the UM-UC-3 cells transfected with ΔNp63 (Fig. 4).

The ability of ΔNp63 to induce claudin-1 expression was investigated to verify whether the binding of ΔNp63 to the claudin-1 gene is associated with changes in claudin-1 gene expression. UM-UC-3 cells were transfected with si-ΔNp63 plasmid, negative plasmid or empty vector and the relative claudin-1-transcript endogenous levels were examined using real-time PCR and western blot analysis. The cells transfected with the si-ΔNp63 plasmid revealed significantly decreased claudin-1 expression at mRNA and protein levels (Fig. 1B).

In the current study, ΔNp63 was demonstrated to downregulate claudin-1 expression and promote the invasion and migration of claudin-1 in UM-UC-3 cells using a series of assays.

Discussion

Cancer development is a multi-step process through which cells accumulate genetic mutations. During the development of

human cancer, tumor cells detach and invade adjacent tissues. The tumor cells may then succeed in forming new colonies. Therefore, tumor invasion and migration are crucial steps in tumor development. The molecular mechanism of tumor invasion involves altered interactions between tumor cells and their environment, as well as intracellular and intercellular events, including cell proliferation, loss of cell-cell adhesion, acquisition of cell motility and loss of cell polarity. p63 is a member of the p53 family and a number of studies have analyzed p63 functions. However, the role of p63 in tumors is not well understood. Previously, p63 downregulation was demonstrated to increase cell migration and invasiveness of cancer cell lines (20,21). Carroll *et al* (27) reported that decreased p63 causes the downregulation of cell adhesion-associated genes. The loss of p63 expression in bladder cancer is associated with progression to more invasive and metastatic tumors (28).

The mechanism by which ΔNp63 downregulation increases the invasiveness of cancer cells still needs to be elucidated. Kommagani *et al* (24) found that the vitamin D receptor is a direct target of ΔNp63-α inhibited cell invasion in A431 human epidermoid carcinoma cell line. Fukushima *et al* (23) demonstrated that exogenous ΔNp63-α expression attenuates invasiveness by downregulating N-cadherin expression and ERK activity in bladder cancer. Decreased ΔNp63 expression accompanied by N-cadherin upregulation during muscle-invasive recurrence of bladder cancer among patients with ΔNp63 promotes the activity of bladder cancer cells. Therefore, ΔNp63 regulates cancer cell connexins. Lopardo *et al* (26) reported that claudin-1 is an important p63 target protein in epithelial cell development. Since claudin-1 is known to play a role in the formation of tight junctions, its regulation by ΔNp63 was the focus of the present study. Therefore, the expression of claudin-1 was adjusted using ΔNp63 in bladder cancer cells.

In the current study, decreased ΔNp63 expression resulted in decreased tumor invasiveness, consistent with previous studies (21-24). In addition, claudin-1 expression was downregulated by loss of ΔNp63 in bladder cancer cells. These results may provide a new mechanism of action for ΔNp63 in the invasiveness of bladder cancer. ΔNp63 is likely to promote a crucial step in invasion by affecting claudin-1 expression. Further studies must be conducted to determine the mechanism of ΔNp63 downregulation, which enables bladder cancer cells to become invasive through claudin-1.

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