Effects of ΔNp63 Gene Down-expression on Invasion of Bladder Carcinoma Cells in Vitro

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Purpose: This work aims to investigate the effects of ΔNp63 gene down-expression on invasion of bladder carcinoma cells in vitro.

Materials and Methods: Bladder carcinoma cell lines UM-UC-3 and 5637 were cultured. The expression plasmids encoding ΔNp63 were constructed and transfected into UM-UC-3 and 5637 cells. The migration and adhesion of cells were detected. The expressions of ΔNp63 and invasion-related zonula occludens protein-1 (ZO-1) in cells were determined by real-time polymerase chain reaction (PCR) and western blot analysis. Confocal microscopy was used to observe the location of ZO-1 in cells.

Results: Results showed that the down-expression of ΔNp63 reduced the migration of UM-UC-3 and 5637 cells, decreased the heterogeneity adhesion, and increased homogeneous adhesion. After transfection with ΔNp63, the ZO-1 expression in cell membrane and cell cytoplasm was inhibited, also the ZO-1 mRNA and protein levels in cells were significantly decreased.

Conclusion: This study indicates thatΔNp63 gene down-expression can reduce the invasion of bladder carcinoma cells in vitro.

Keywords: ΔNp63; ZO-1; bladder cancer; invasion

INTRODUCTION

Bladder cancer is a multi-factor mixed and multiple genes involved disease. Accumulation of abnormal genotypes and the role of external environments eventually leads to the occurrence of this disease. A previous study showed that p63 is present in all cell layers of papillary urothelial neoplasm[(1)], and other studies showed that ΔNp63 is expressed in some invasive carcinomas using immunoblotting and quantitative reverse transcriptase-polymerase chain reaction assays[(2,3)]. ΔNp63 is an important member of p53 family, and the p63 gene located at chromosome 3q27-29 shows strong homology with the tumor suppressor gene p53[(4)]. Although p63 owes high sequence and structural similarities with p53, their function and expression profiles are different. Wei et al[(5)] found that ΔNp63 is the predominant isoform during bladder development. Castillo-Martin et al [(6)] have characterized its role for bladder tumor progression by a p63 positive basal/intermediate cells and "umbrella" cells. However, the role of ΔNp63 in bladder cancer cell lines is not clear.

ΔNp63 isoform is selectively highly expressed in cell compartments of stratified and glandular epithelia[(4,7)]. Our previous stud[y(8)] found that ΔNp63 is located in the nucleus. Silence of ΔNp63 suppressed the invasion and metastasis of UM-UC-3 cells and reduced claudin-1 expression. Claudin-1 is located in the cell membrane, especially in tight junctions. In this study, we focused on another tight junction associated protein-zonula occludens protein-1 (ZO-1) and investigated the effects of ΔNp63 gene down-expression on ZO-1 expression and invasion of bladder carcinoma cells in vitro.

MATERIALS AND METHODS

Cell culture and transfection assay

The human bladder carcinoma cell lines, UM-UC-3 and 5637, were purchased from the Institute of Cell Research of Chinese Academy of Sciences (Shanghai, China). The study was approved by the ethics committee of North Sichuan Medical College, Nan Chong, China. UM-UC-3 Cells were cultured in MEM medium (Gibco Inc., CA, USA) supplemented with 10% fetal bovine serum (FBS; Sijixin Inc., Beijing, China) and 1% penicillin-streptomycin (Invitrogen, Shanghai, China); 5637 cells were cultured in RPMI-1640 medium (Gibco Inc., CA, USA) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were cultured at 37 oC with 5% CO2. The sh-ΔNp63 plasmid was kindly provided by Dr Yunfeng He (The First Affiliated Hospital, Chongqing Medical University, Chongqing, China) . The 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'-GATCCGTGCCCAGACTCAATTTAGTTTCAAGACGACTAAATTGAGTCT-

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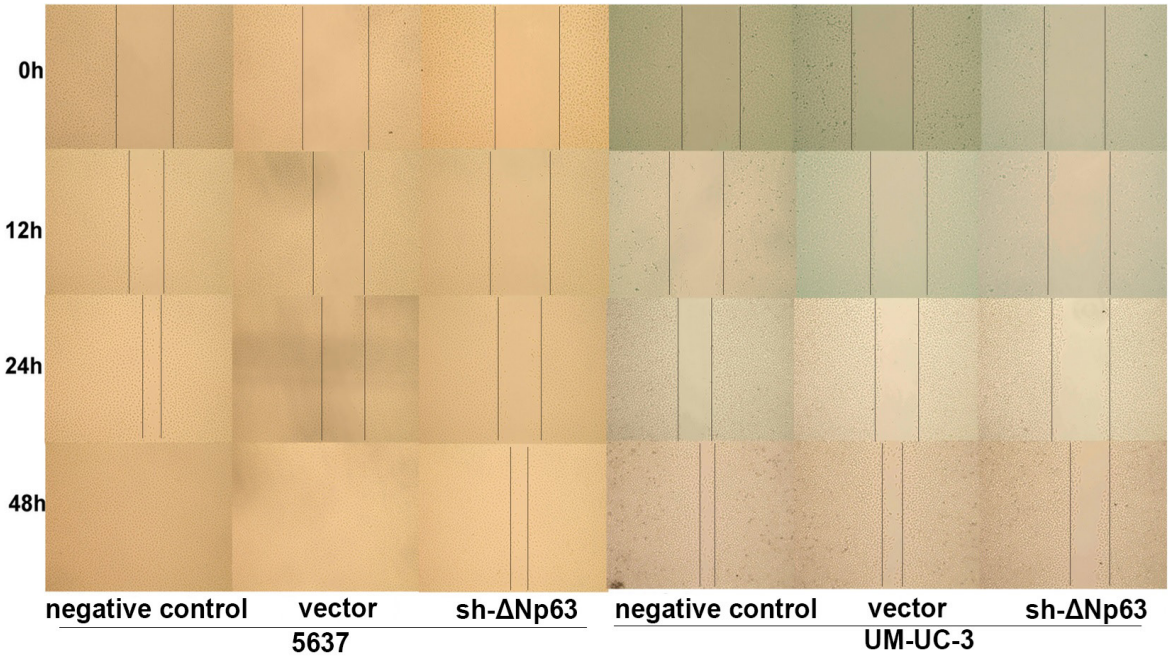

Figure 1. Down-expression of ΔNp63 reduced the migration of cells. 5637 and UM-UC-3 cells were cultured and transfected with vector plasmid or sh-ΔNp63 plasmid for 48 hr, respectively. Transfected cells were used for scratch assay. Cells migrating to the unit length area after 0, 12, 24, and 48 hr scraping were counted (magnification×20).

GGGCATTTTGTCTTCA AGACGACTAAATT-GAGTCTGGGCATTTTTTGTCGACA-3' and reverse, 5'-AGCTTGTCGACAAAAAATGCCCAGACT-CAATTTAGTCGTCTTGAAACTAAATTGAGTCT-GGGCACG-3'. The sequences of the vector plasmid were as follows: forward, 5'-GATCCGACTTCATAA-GGCGCATGCTTCAAGACGGCATGCGCCT-TATGAAGTCTTTTTTGTCGACA-3' and reverse, 5'-AGCTTGTCGACAAAAAAGACTTCATAA-GGCGCATGCCGTCTTGAAGCATGCGCCT-TATAAGTCG-3'. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell wound healing assay

Cells were plated in six well plates for the wound healing assay. A wound was created on the monolayer cells when the cells reached 90% confluence by scraping a gap using a micropipette tip. The 5637 cells plate was then washed with serum-free RPMI-1640 medium to clean the dissociated cells, and UM-UN-3 cells were washed by serum-free MEM medium. 5637 cells were then incubated with serum-free RPMI-1640 medium at 37 oC in 5% CO_2, and UM-UN-3 cells were then incubated with serum-free MEM medium at 37 oC in 5% CO2. Cells that migrated into the unit length area were counted five times for each group at 0, 12, 24 and 48 h following scraping.

Cell homogeneous adhesion assay

Cell homogeneous adhesion assay could indicate the adhesion ability of 5637 and UM-UC-3 cells, which could indirectly reflect the invasion ability of tumor cells. Cells were plated in 48 well plates for the homogeneous adhesion assay. The culture medium was sucked out, followed by twice washing with phosphate-buffered saline (PBS) to remove the suspended cells, then the cells reached 90% confluence. 5637 cells were re-suspended with RPMI640 medium and UM-UN-3 cells with DMEM medium. The cell re-suspension concentration in each group was 1×10^5/mL. 200 ul cells were added to a 48-well plate incubated at 37 oC in 5% CO_2 for 8 h. The non-adherent cells were sucked out, followed by washing with PBS twice. All non-adherent cells were counted. The number of homogeneity adherent cells was equal to seeded 200 ul cells minus non-adherent cells. Each group was repeated for four times.

Cell heterogeneity adhesion assay

Cell heterogeneity adhesion assay could verify the adhesion ability between tumor cells and matrix, which indirectly reflected the invasion of cells. Cells (1×10^5 / mL) were added into a 96-well plate covered with collagen IV and incubated at 37 oC in 5% CO_2 for 120 min. The plate was washed with PBS to clean the dissociated cells. Approximately 20 μl of 5 mg/mL MTT (Sigma Aldrich Inc., MO, USA) was 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 to each well. Absorbance (A value) was measured at 570 nm. Each sample was assayed four times.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using an RNeasy mini kit (Qiagen Inc., Hilden, Germany) and treated with DNase I (Qiagen Inc., Hilden, Germany). Real-time PCR was conducted using an iCycler Bio-Rad Laboratories, Inc., PA, USA) with an iQ SYBR-Green Supermix (Bio-Rad), according to the manufacturer's instructions. The ΔNp63 primer and β-actin as described previously (8). The ZO-1 primer was as follows: ZO-1, 5'-TC-CAGTCCCTTACCTTTCGC-3' (sense) and 5'-CCC TGGGTGACTAACGGC-3' (antisense). The PCR con-

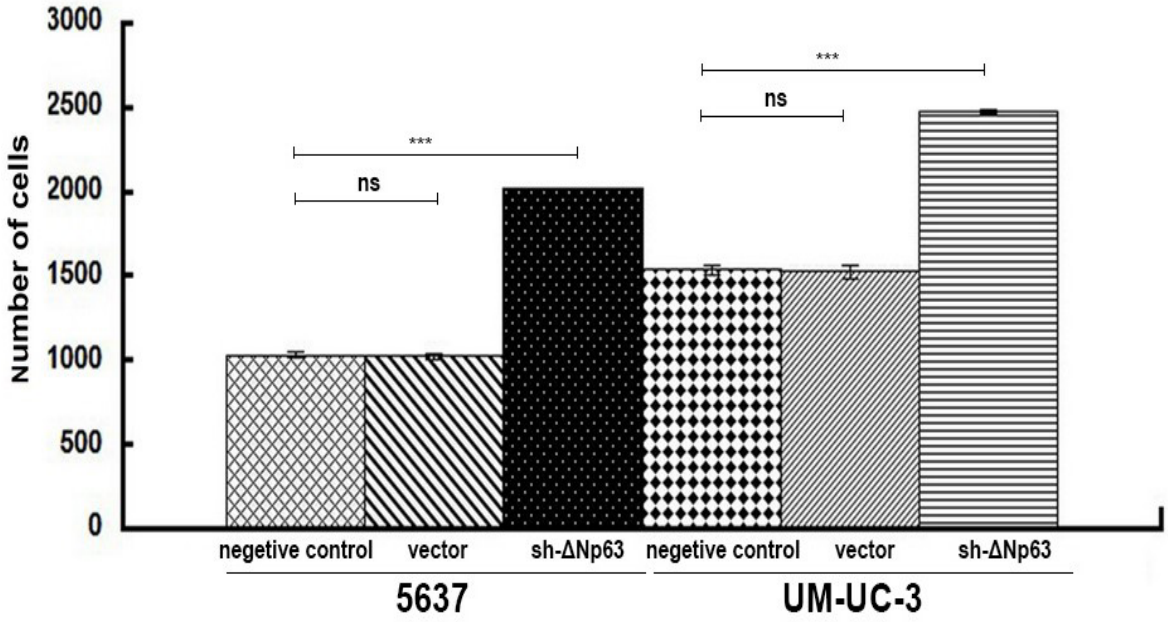

Figure 2. Down-expression of ΔNp63 increased homogeneous adhesion of 5637 and UM-UC-3 cells. 5637 and UM-UC-3 cells or Cells transfected with vector plasmid or sh-ΔNp63 plasmid were used for homogeneous adhesion assay. The adherent cells were calculated. The data are shown as the mean ± SD (n = 4). ***, $p < .001$; ns, not significant.

ditions were as follows: 94 oC for 4 min, followed by 35 cycles at 94 oC for 20 sec, 60 oC for 30 sec and 72 oC 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

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 oC for 5 min. The protein was transferred onto a polyvinylidene difluoride membrane (Millipore Inc., MA, USA) and the membrane was then blocked for 1 h at room temperature with 5% BSA in Tris-buffered saline containing 0.05% Tween-20 (TBST). Then, the blots were washed

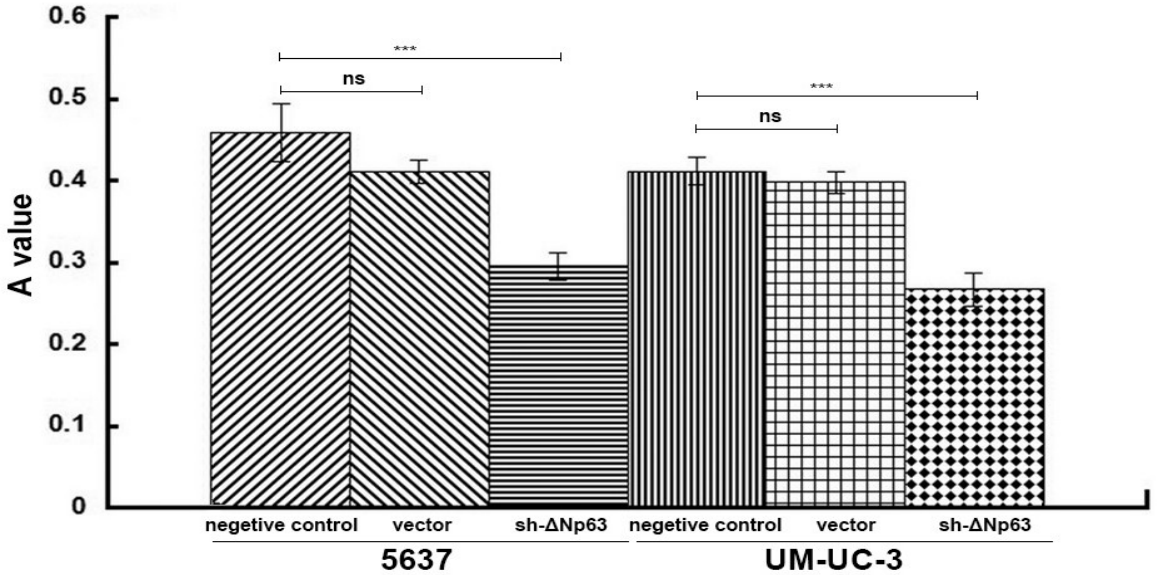

Figure 3. Down-expression of ΔNp63 reduced the heterogeneity adhesion of 5637 and UM-UC-3 cells. 5637 and UM-UC-3 cells or Cells transfected with vector plasmid or sh-ΔNp63 plasmid were used for heterogeneity adhesion assay. MTT assays were used to determine the adherent cells. The data are shown as the mean ± SD (n = 4). ***, $p < .001$; ns, not significant.

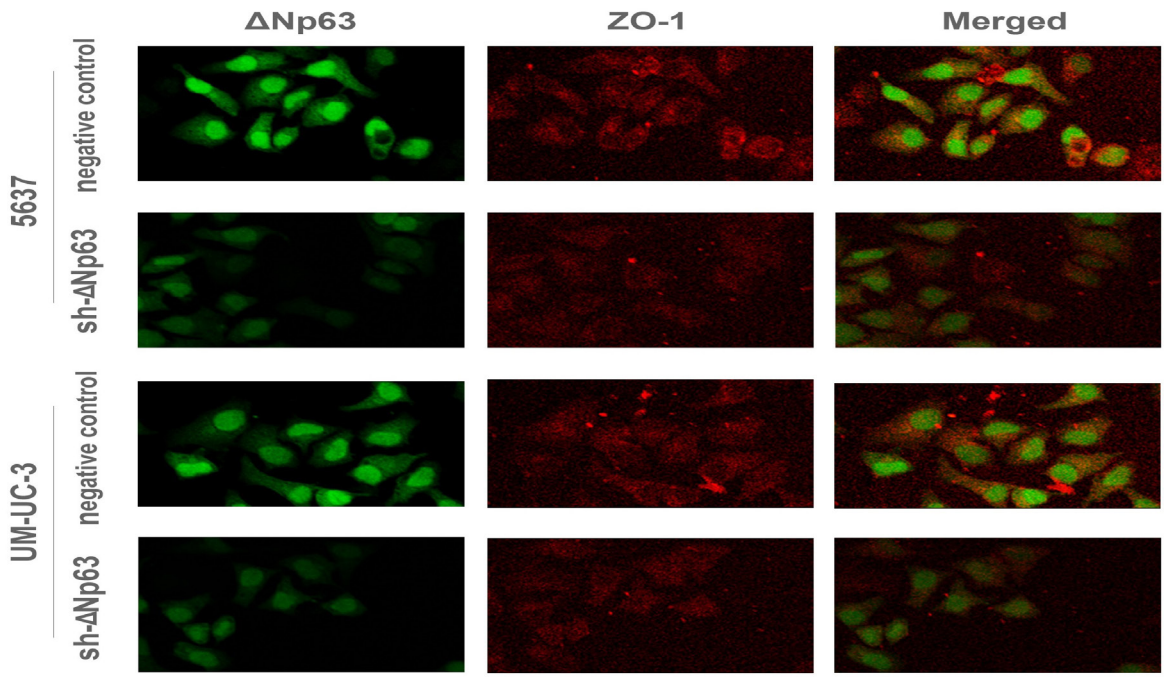

Figure 4. ZO-1 expression was inhibited in 5637 and UM-UC-3 cells transfected with sh-ΔNp63. ZO-1 and ΔNp63 expression were analyzed by immunofluorescence assays. Representative pictures of three independent experiments with consistent outcome are shown.

and incubated overnight at 4˚C in TBST containing 1% BSA with primary antibodies against ΔNp63 (1: 200), ZO-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

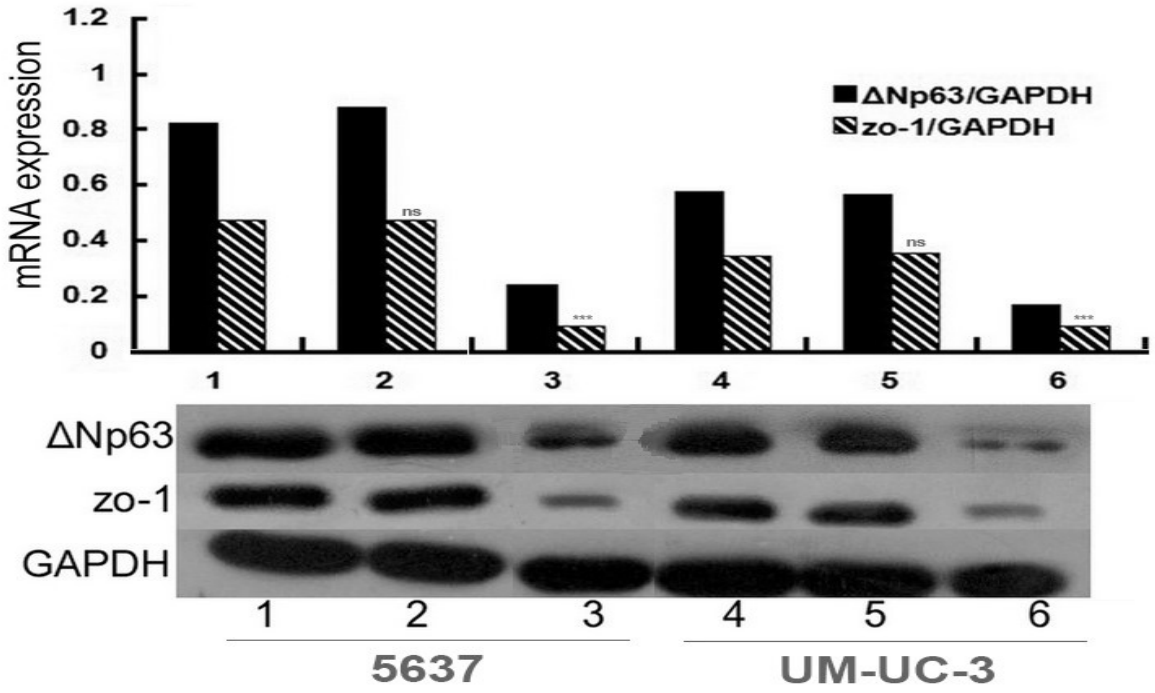

Figure 5. ZO-1 expression both in mRNA and protein levels was decreased in cells transfected with sh-ΔNp63. ZO-1 and ΔNp63 expression were analyzed by PCR and western blot assays. For 5637 cells: 1, negative control; 2, vector plasmid; 3, sh-ΔNp63 plasmid; For UM-UC-3 cells: 4, negative control; 5, vector plasmid; 6, sh-ΔNp63 plasmid. For western blot analysis, representative blots of three independent experiments with consistent outcome are shown. The data are shown as the mean ± SD (n = 3). ***, $p < .001$; ns, not significant.

quantitative analysis, bands were detected and evaluated densitometrically with UVP Gelatin image processing system Labworks 4.6 software and normalized against GAPDH density.

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 Corp., MO, USA) for 4 min. Cells were then incubated with 5% bovine serum albumin (BSA; Sigma-Aldrich Corp., MO, USA) and immunolabeled with anti-ΔNp63 (1: 500; Santa Cruz Biotechnology Inc., CA, USA) and anti-ZO-1 antibodies (1: 500; Santa Cruz Biotechnology Inc., CA, USA) at room temperature for 1 h. Normal goat IgG instead of anti-ΔNp63 antibody was used 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., CA, USA) and Cy3-conjugated anti-ZO-1 antibodies (1: 500; Santa Cruz Biotechnology Inc., CA, USA) for 1 h. Additional washes were performed and the cells were mounted using fluorescent mounting medium (Applygen Technologies, Inc., Beijing, China). Cells were viewed with a Leica SP2 upright microscope and the images were captured in LCS Light (Leica Science Lab, Berlin, Germany).

Statistical analysis

All statistical analysis was carried out using SPSS17.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±SD. One-way ANOVA was used to determine the levels of difference between all groups. P <.05 was considered as statistically significant.

RESULTS

Down-expression of ΔNp63 reduced the migration of cells

Cell wound healing assay showed that, at 12 h, the densities of UM-UN-3 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 14.2±3.7, 13.9 ± 3.3 and 6.2 ± 2.3 cells/mm2, respectively. At 24 h, the densities of UM-UN-3 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 22.0 ± 1.2, 18.2 ± 2.1 and 12.6 ± 1.4 cells/mm^2, respectively. At 48 h, the densities of UM-UN-3 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 35.2±1.7, 33.5±1.3 and 27.2±2.3 cells/mm2, respectively. At 12h, the densities of 5637 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were11.8 ± 3.7, 11.2 ± 3.3 and 8.2 ± 3.3 cells/mm^2, respectively. At 24 h, the densities of UM-UN-3 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 19.0±3.2, 16.2±3.1 and 11.6±2.4 cells/mm^2, respectively. At 48 h, the densities of UM-UN-3 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 30.2 ± 1.2, 27.5 ± 2.3 and 26.2 ± 2.1 cells/mm2, respectively. At each time point, the densities of UM-UN-3 and 5637 cells in sh-ΔNp63 plasmid group were significantly lower than that in other groups (P < .05). This indicated that the down-expression of ΔNp63 could reduce the migration of bladder cancer cells (Figure 1).

Down-expression of ΔNp63 reduced the heterogeneity adhesion, but increased homogeneous adhesion of cells Cell homogeneous adhesion assay showed that the numbers of adherent 5637 cells in the negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 1020.25 ± 20.25,1025.5 ± 17.48, and 2012.75 ± 9.54 cells/ml, respectively. The numbers of adherent UM-UC-3 cells in the vector plasmid, negative control and sh-ΔNp63 plasmid groups were 1521.95 ± 35.45, 1536.35 ± 20.65, and 2475.45 ± 15.35 cells/ml, respectively (**Figure 2**). The cell heterogeneity adhesion assay showed that the A values of 5637 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 0.459 ± 0.035, 0.412 ± 0.014 and 0.295 ± 0.017, respectively. The A values of UM-UN-3 cells in negative control, vector plasmid, and sh-ΔNp63 plasmid groups were 0.412 ± 0.017, 0.398 ± 0.013 and 0.267 ± 0.021, respectively (**Figure 3**). This indicated that, after transfection with ΔNp63, the heterogeneity adhesion capacity of bladder cancer cells was decreased. Taken together, these results indicated that down-expression of ΔNp63 inhibited the invasion ability of 5637 and UM-UC-3 cells.

Location and expression of ZO-1 in cells

Laser confocal microscopy showed that ZO-1 protein was mainly located in the cell membrane and cell cytoplasm. Our results showed that, after transfection with sh-ΔNp63, ZO-1 expression was inhibited (**Figure 4**). Real-time PCR and western blot analysis demonstrated that, after transfection with sh-ΔNp63, both ZO-1 mRNA and protein expression in 5637 and UM-UN-3 cells were significantly decreased (Figure 5).

DISCUSSION

Approximately 90% of cancers occur in epithelial original cells (9), so understanding the events that allow epithelial cells to progress towards tumorigenic pathways is required. Usually, ΔNp63 is over-expressed in epithelial cancers, showing correlation with poor prognosis[11]. Some studies[7,9,11,12] have focused on the signaling pathways regulated by ΔNp63 and studied p63 levels in the mature epidermis. ΔNp63 was the main isoform detected and expressed mainly in the basal layers. Its expression was down regulated in well differentiated layers[13,14]. In addition, ΔNp63 opposes the tumor suppressive effects of cellular senescence suggesting a role in oncogene initiation[15,16]. Molecular mechanisms about the role of ΔNp63 in cell migration and invasion to date mainly comprised the identification of specific genes known to influence cell motility, including N-cadherin, E-cadherin, epithelial cell-cell adhesion molecule, and so on[17-19]. Further research is still needed.

Tight junctions proteins are important in effecting invasive phenotype of cancer cells, also important in influencing intracellular signaling pathways of these cells. Our previous study[8] found that the down-expression of ΔNp63 changed the cell adhesion, and there was correlation between ΔNp63 and claudin-1. Whether other tight junction associated proteins are involved in this process is still unknown. In this study, we further proved that ΔNp63 influence the invasion ability of bladder cancer cells partially through regulating the expression of ZO-1. Down expression of ΔNp63 leaded to decreased expression of ZO-1, which contributed to

the impaired adhesive and invasive ability of bladder cancer cells transfected with sh-ΔNp63 plasmid.

ZO-1 is membrane-associated guanylate kinase-family proteins presenting in tight junctions. In epithelial cells, ZO-1 is exclusively located at the zonula occludens which is composed of tight junctions. ZO-1 could promote tumor cell invasion. Reduced expression of ZO-1 correlated with decreased proliferation and/or transformation of epithelial cells[20-22]. The depletion of ZO-1 in cultured epithelial cells resulted in a delay in barrier formation[23,24], and ZO-1 gene deletions were embryonic lethal in mice[25]. ZO-1 has been reported to accumulate transiently in the nucleus of proliferating cells[26], playing a role in cell differentiation rather than cell proliferation[27]. How ΔNp63 influences cell-cell adhesion is still not well defined. We tried to demonstrate this by confocal microscopy and western blot analysis. Our results indicated that ZO-1 is located both in the cell membrane and cell cytoplasm. Confocal microscopy and western blot analysis showed that ZO-1 expression reduced in cells transfected with sh-ΔNp63. ΔNp63 silence in the human bladder carcinoma cell lines, UM-UC-3 and 5637, was confirmed by PCR and western blot assays. To date, we have demonstrated the down expression of ZO-1 and claudin-1 in ΔNp63-silenced cells.

The regulatory role of ΔNp63 in cell adhesive ability was explored by wound healing and adhesion assays in vitro. It may also promote cell migration during tumor invasion and metastasis. In addition, ΔNp63 modulated extensive adhesive gene spectrum, including N-cadherin, β4-integrin, and tight junction-associated protein [17,28]. Although the role of p63 in tumor formation and progression has been well studied, as a member of p53 gene family, its role in tumors' metastasis is complex and remains unclear. The role of ΔNp63 expression in urothelial carcinomas still remains to be elucidated [2,29]. Because of rare mutations or allelic deletions of p63 gene in human bladder carcinomas[30], the loss of ΔNp63 mRNA may attribute to epigenetic alterations. Based on recent researches, ΔNp63 expression correlates with the severity of bladder cancer. In conclusion, ΔNp63 regulated the invasive ability of tumor cells partially through tight junction associated proteins, especially ZO-1 in bladder cancer cells. This study lays the basis for further understanding on the role of p63 in tumors.

CONCLUSIONS

This study indicates thatΔNp63 gene down-expression can reduce the invasion of bladder carcinoma cells in vitro, laying the basis for further understanding of the role of p63 in tumors.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

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