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Impaired Delta Np63 Expression is Associated with Poor Tumor Development in Transitional Cell Carcinoma of the Bladder

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The oncogenic isoform of the p63 protein, delta Np63 (△Np63), plays an important role in the pathogenesis of many epithelial carcinomas, and emerging evidences suggest that △Np63 is a promising drug target. However, the functions of △Np63 in transitional cell carcinoma of bladder (TCCB) are poorly defined. In this study, a △Np63 shRNA expression vector was transfected into TCCB cell line 5637 and cell cycling, cell proliferation and protein expression were assessed by flow cytometry and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-dimethyl tetrazolium bromide (MTT) assay, and immunohistochemistry, respectively. The △Np63 shRNA expression vector was also injected into 5637 cell xenograft tumors in nude mice, and tumor size was measured, tumor tissue morphology was assessed by immunohistopathology and transmission electron microscopy. In the in vitro study, △Np63 shRNA transfection caused successful △Np63 gene silencing and resulted in significant arrest of cell cycling and cellular proliferation ($p<0.05$) as well as cyclin D1 expression. In the nude mouse xenograft model, △Np63 shRNA greatly inhibited tumor growth, induced tumor cell apoptosis ($p<0.05$) and resulted in cyclin D1 downregulation. Our data suggest that △Np63 may play an oncogenic role in TCCB progression through promoting cell survival and proliferation. Intratumoral administration of △Np63-specific shRNA suppressed tumor △Np63 expression and cellular proliferation while promoted tumor cellular apoptosis, and therefore inhibited tumor growth and improved survival of xenograft-bearing mice, which was not accompanied by significant signs of systemic toxicity.

Key Words : *Delta N p63; RNA Interference; Short Hairpin RNA; Urinary Bladder Neoplasms*

INTRODUCTION

The p63 gene is a member of the p53 gene family and it shares a high degree of homology and remarkable structural similarity with p53 gene (1, 2). Therefore, the p63 protein was originally thought to be another tumor suppressor functioning in a similar capacity to p53. However, p63 has been demonstrated to have divergent roles. It is a key transcriptional regulator of the proliferation and differentiation cascade in stratified epithelia (3), it exhibits tissue-specific roles in normal development and a complex contribution to tumorigenesis which is due to its expression as multiple protein isoforms (4). According to different amino terminals, p63 has two major isoforms: TAp63 and △Np63. TAp63 has a transactivating amino terminal while △Np63 has a dominant negative activating amino terminal (5-8). In general, the TAp63 isoform behaves like p53 because they transactivate various p53 downstream targets and induce apoptosis, mediate cell cycle control. In contrast, the △Np63 isoform has been shown to display functions opposite to that of the TAp63 isoform, including acting as oncoproteins (9-12). △Np63 overexpression is often observed in and enhances oncogenic growth of squamous cell carcinomas (11, 13). △Np63 could function as dominant-negative molecule against the p53 tumor suppressor activities (5). △Np63 overexpression induces nuclear accumulation of β-catenin and activates β-catenin signaling that promotes cell proliferation (12). Nowadays, many researchers tend to agree with the concept that TAp63 plays a more p53-like role, whereas △Np63 has an antagonistic or even oncogenic role in cancer progression (11, 14-17).

Our previous study demonstrated that △Np63 was overexpressed in human transitional cell carcinoma of bladder (TCCB) tissues at both mRNA and protein levels, so we hypothesize that △Np63 promotes the survival and proliferation of both epithelial stem cells and TCCB cells. However, the specific role of △Np63 in TCCB development remains largely unclear and the mechanisms by which △Np63 promotes cell survival remain to be elucidated. Understanding the regulation and mechanistic contributions of △Np63 in TCCB may ultimately provide new therapeutic opportunities for this disease. Thus, the objective of this study was to better understand the functions of △Np63 in TCCB with a

human TCCB cell line 5637 in vitro and in a nude mouse xenograft model where the expression of △Np63 was silenced by RNA interference (RNAi) technology.

MATERIALS AND METHODS

Plasmid construction

A pair of effective short hairpin RNA (shRNA) against △Np63 mRNA was selected from a preliminary experiment. The pair of shRNA forms a structure consisting of two 19-bp stem targeting △Np63 mRNA, a 9-bp loop and a short poly$(A)_6$ sequence. Two oligonucleotides, forward, 5′-GATCCGTGCCCAGACTCAATTTAGTTTCAAGACGACTAAATTGAGTCTGGGCATTTTTTGTCGACA-3′, reverse, 5′-AGCTTGTCGACAAAAAATGCCCAGACTCAATTTAGTCGTCTTGAAACTAAATTGAGTCTGGGCACG-3′, were synthesized and ligated directly into *Bam*HI and *Hind*III linearized genesil-1 plasmid (Jingsai Inc, Wuhan, China). The recombinant △Np63-shRNA expression construct, p△Np63-shRNA, was confirmed by using *Pst*I+*Sal*I double digestion and by gene sequencing. The negative control plasmid, termed p△Np63-cRNA has an sequence insert at the same place from the following two oligonucleotides: forward, 5′-GATCCGACTTCATAAGGCGCATGCTTCAAGACGGCATGCGCCTTATGAAGTCTTTTTTGTCGACA-3′; reverse, 5′-AGCTTGTCGACAAAAAAGACTTCATAAGGCGCATGCCGTCTTGAAGCATGCGCCTTATAAGTCG-3′.

Cell culture and transfection

Human TCCB cell line 5637 was purchased from the Institute of Cell Research, Shanghai, Chinese Academy of Sciences. The cells were cultured in RPMI 1640 medium (Gibico, 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. For transfection, cells were seeded in 6-well plates at 1×10^6 cells per well and allowed to grow overnight to approximately 80% confluence. For each well, cells were transfected with the mixture of 0.4 μg p△Np63-shRNA or p△Np63-cRNA or 0.4 μL phosphate-buffered solution (PBS) and 10 μL Effectene transfection reagent (Qiagen, Shanghai, China) in 600 μL fresh 1640 culture medium. Forty-eight hours after transfection, cells were harvested for immunocytochemistry, reverse transcription polymerase chain reaction (RT-PCR) or flow cytometry analysis as described below.

Immunocytochemistry

Cells were fixed with pre-chilled acetone at 4°C for 30 min, then incubated for 10 min with 3% hydrogen peroxide in methanol. Goat non-immune serum was used to block the non-specific binding. Cells were incubated overnight at 4°C with a 1:200 dilution of mouse monoclonal anti-△Np63 antibody (Santa Cruz, Shanghai, China) or with a 1:100 dilution of rabbit anti-cyclin D1 polyclonal antibody (Santa Cruz). Then cells were washed three times and an appropriate biotinylated-conjugated secondary antibody (Santa Cruz) with a 1:150 dilution was applied for 15 min at 37°C. Cells were washed three times and then incubated with streptavidin peroxidase (SP, Zhongshan Golden Bridge Inc., Beijing, China) for 15 min at 37°C. Then horseradish peroxidase substrate diaminobenzidine solution was then added for 15 sec and the staining was stopped by washing the coverslip with water repeatedly. Finally, cells were counterstained with hematoxylin.

Semiquantitative multiplex RT-PCR

Total RNA was extracted from tissue homogenates or cell lysates with TRIzol reagent (Invitrogen) and RT-PCR was carried out with a RNA PCR Kit Ver.3.0 (TaKaRa, Dalian, China) according to the kit's instructions. The primers used for △Np63 were: forward, 5′-TGCCCAGACTCAATTTAGTGAG-3′; reverse, 5′-TCTGGATGGGGCATGTCTTTGC-3′, which yields a product of 335 bp. The primers used for β-actin were: forward, 5′-GTGGACATCCGCAAAGAC-3′; reverse, 5′-AAAGGGTGTAACGCAATCAA-3′, which yields a product of 302 bp. The PCR condition was: 94°C for 2 min, then 35 cycles at 95°C for 1 min, 53°C for 0.5 min, and 72°C for 1 min in 1.5 mM $MgCl_2$-containing reaction buffer. Five microliters of RT-PCR products were resolved on 1.5% agarose gels. The gels were stained with ethidium bromide (EB) and were scanned for densitometric estimation of the △Np63 products with β-actin products serving as the internal control.

Cell cycle assay with flow cytometry

A total of 1×10^6 cells treated with p△Np63-shRNA vector or p△Np63-cRNA vector were trypsinized and washed with PBS twice. Then cells were transferred and fixed in pre-chilled 75% ethanol at 4°C overnight. After three times of wash, cells were digested with 1% RNase at 37°C for 30 min and stained with 50 μg/mL propidium iodide (PI, Sigma, Shanghai, China) for 1 hr at 4°C. Samples were assayed by a Coulter Epics XL flow cytometer (Beckman-Coulter Inc., NewYork, NY, U.S.A.) and data were analyzed with Multicycle DNA content and cell analysis software.

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-dimethyl tetrazolium bromide (MTT) assay for cell proliferation

Cells in logarithmic growth phase were seeded in 96-well

plates at 2×10^{4} cells per well. Then cells were transfected with pΔNp63-shRNA or pΔNp63-cRNA or PBS and continued to culture for 24, 48, 72, 96, and 120 hr respectively. Four hours before stop culturing, 20 μL of 5 mg/mL MTT (Sigma) was added to the culture medium. After incubation, the culture medium was removed and 200 μL of dimethylsulphoxide (DMSO) was added to resolve the crystal. Absorbance was measured at 490 nm. Each sample was assayed for four times.

Nude mouse xenograft model

Female BALB/c nude mice, 4 weeks of age, weighting 13 ±0.5 g, were purchased from Experimental Animal Center of Chinese Academy of Sciences, Shanghai, China. Mice were housed in microisolator cages in a specific pathogen-free (SPF) condition with 12-hr light-dark cycles. Mice were subcutaneously implanted with 2×10^6 5637 cells. Once tumors reached approximately 60 μL in volume, the mice were allocated to receive either pΔNp63-shRNA or pΔNp63-cRNA vector or PBS. Complexes of 20 μg vector (or PBS)+4 μL in vivo-jetPEI™ (polyplus-transfection Inc., Shanghai, China)+ 160 μL 5% glucose were directly injected into the tumor once a week for five weeks. Tumor dimensions were measured weekly and the tumor volumes calculated using the formula: $[1/2]\times a\times b^2$, where a and b, respectively, represented the larger and smaller tumor diameter. At the end of the five week treatment, mice were killed by overdose of ketamine (400 mg/kg) and xylazine (50 mg/kg) and necropsy was performed. Tumor tissue samples were prepared for immunohistochemistry or RT-PCR or transmission electron microscopy. Tumor growth inhibition (TGI) was calculated using the formula TGI (%)=$(1-M_T/M_C)\times100$, where M_T and M_C are the mean tumor masses in the treatment group, and control group, respectively.

Morphologic and immunohistochemical assessment of apoptosis in nude mouse xenograft model

For histological examination, tumor tissue samples were fixed with 4% paraformaldehyde for 72 hr, dehydrated in graded ethanol, and embedded in paraffin. Samples were cut into 5-μm-thick sections and stained by hematoxylin-eosin (HE).

For transmission electron microscopy, tumor tissue samples were cut into sections approximately 1×1 mm, fixed with 4% paraformaldehyde for 2 hr, and then transferred into pre-chilled 1% glutaraldehyde. The samples were dehydrated in graded ethanol, embedded in epon 812, and then cut into ultrathin or semithin sections. The sections were stained and examined under a Hitachi-800 transmission electron microscope.

For tumor tissue immunohistochemistry, sections of 5-μm-thick paraffin-embedded tissue microarrays were deparaffinized and rehydrated with xylene and ethanol. The immunostaining procedures were the same with the immunocytochemistry described above.

Statistical analysis

The results were expressed as mean±deviation. One-way analysis of variance (ANOVA) was used to determine the levels of difference between all groups. Comparisons for all pairs were made using Student-Newman-Keuls (SNK) test. $p<0.05$ was considered statistically significant.

RESULTS

ΔNp63 mRNA and protein expression were inhibited by ΔNp63 shRNA in vitro and in vivo

As previously reported (18), TCCB cell line 5637 inher-

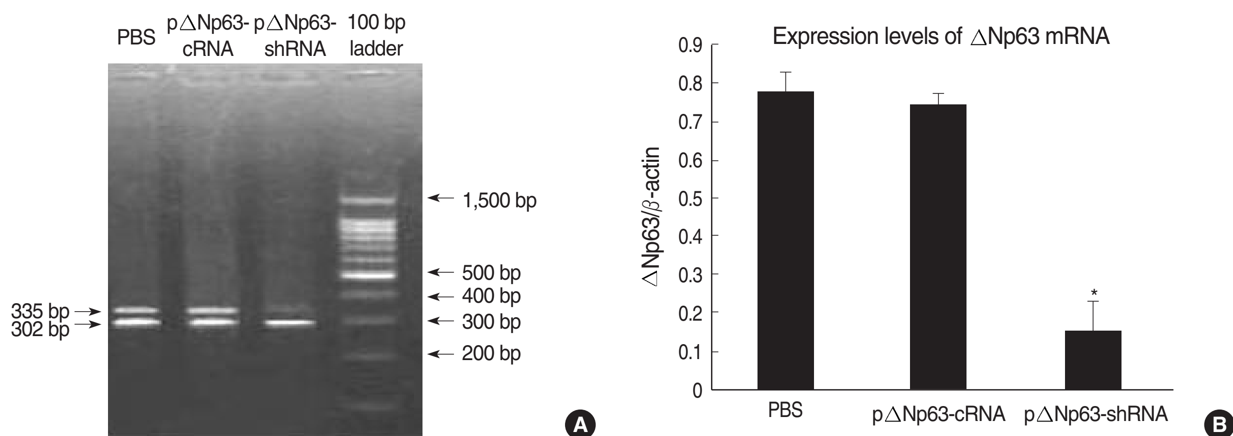


Fig. 1. The mRNA expression of ΔNp63 in 5637 cells 48 hr post-transfection, assayed by semiquantitative RT-PCR. (A) Products were analyzed on a 1.5% agarose gel. The 335 bp band is ΔNp63 mRNA. The 302 bp band is β-actin serving as the internal control. (B) Quantified expression levels of ΔNp63 as normalized to the β-actin level. *Statistically significant ($p<0.05$, mean±SD, n=10) relative to control shRNA.

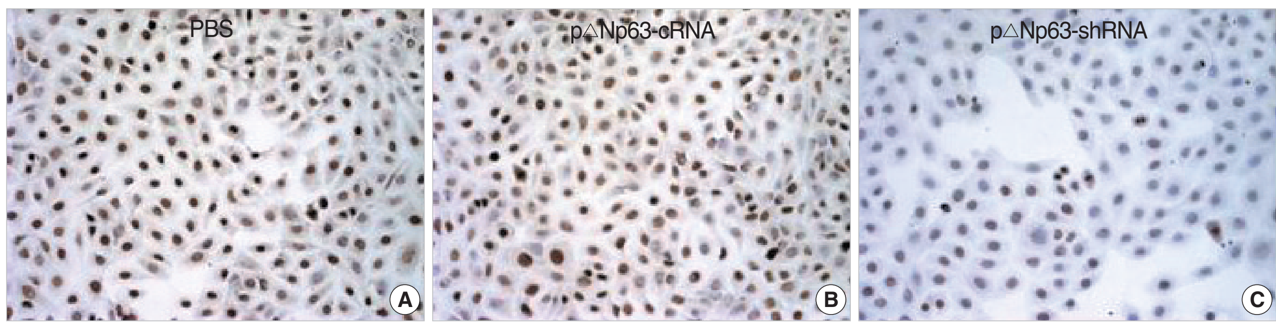


Fig. 2. Expression of △Np63 protein in TCCB 5637 cell line was assayed by immunocytochemistry at 48 hr post-transfection with the indicated vector or medium (original magnification, ×200).

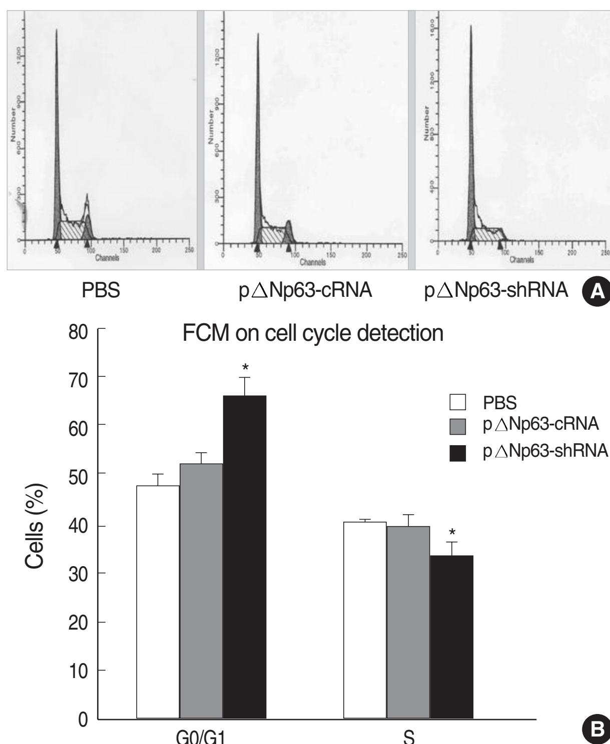


Fig. 3. Cell cycle progression of 5637 cells following different treatments as indicated. (A) Flow cytometry graphs. (B) Quantification of the percentages of cells in G_0/G_1 phase and S phase. *Statistically significant ($p<0.05$, mean±SD, n=10) relative to control shRNA.

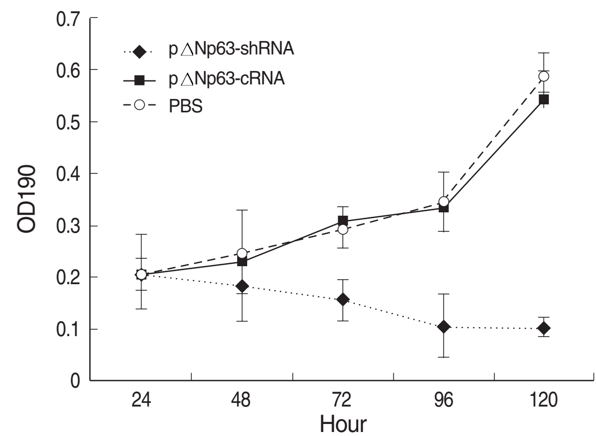


Fig. 4. Proliferation curve of 5637 cells treated with p△Np63-shRNA, control-shRNA or PBS over 120 hr. Measured by MTT assay. Data were mean±SD. n=10 per data point.

ently expresses high levels of △Np63. Following transfection of p△Np63-shRNA, at 48 hr later, the relative △Np63 mRNA expression levels (△Np63/β-actin) were 0.156±0.07 for cells transfected with p△Np63-shRNA, 0.749±0.02 for cells transfected with the control vector, and 0.786±0.04 for cells transfected with PBS. Compared with the control RNA vector, the expression levels of △Np63 mRNA were significantly inhibited by △Np63 shRNA ($p<0.05$) (Fig. 1).

The expression levels of △Np63 protein were detected at 48 hr post-transfection by immunocytochemistry. △Np63 proteins positive cells should be stained brown in the nuclei. Cells transfected with PBS or the control vector were strongly stained in the nuclei (Fig. 2A, B), while cells transfected with p△Np63-shRNA were only weakly stained in the nuclei (Fig. 2C). These demonstrated that △Np63 shRNA inhibited △Np63 protein expression significantly.

Similar suppressions of △Np63 mRNA and protein expression by △Np63 shRNA were found in the nude mouse xenograft model by semiquantitative RT-PCR and immunohistochemistry.

△Np63 shRNA caused cell cycle arrest of 5637 cells in vitro

Cell cycling of 5637 cells was assessed by flow cytometry. The percentage of cells in G_0/G_1 phase for cells transfected with p△Np63-shRNA, p△Np63-cRNA or PBS was respectively 66.38±3.08%, 52.58±2.03% and 48.27±0.84%. The percentage of cells in S phase for the three groups was 33.68±2.06%, 39.82±1.89% and 40.50±0.58%, respectively (Fig. 3). These results indicated that a significant percentage of 5637 cells were arrested in G_0/G_1 phase after treatment of △Np63 shRNA.

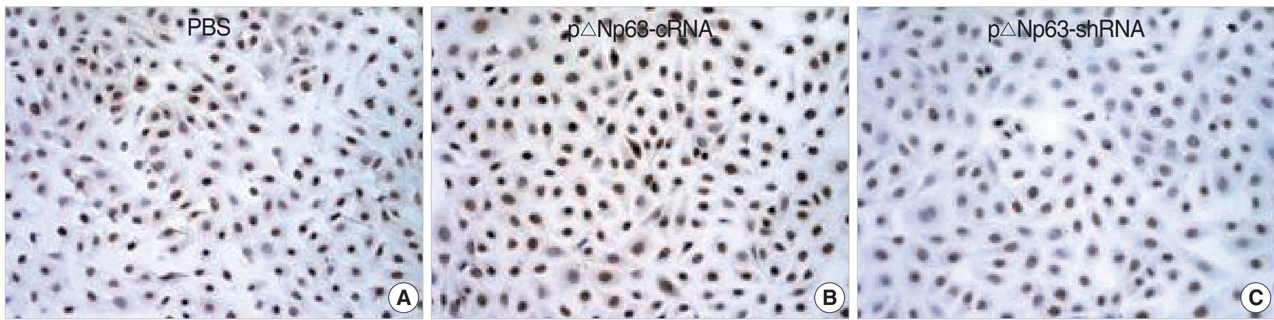


Fig. 5. Cyclin D1 protein expression in TCCB 5637 cell line at 48 hr post-transfection of △Np63 specific or control shRNA or PBS. Assayed by immunocytochemistry (original magnification, ×200).

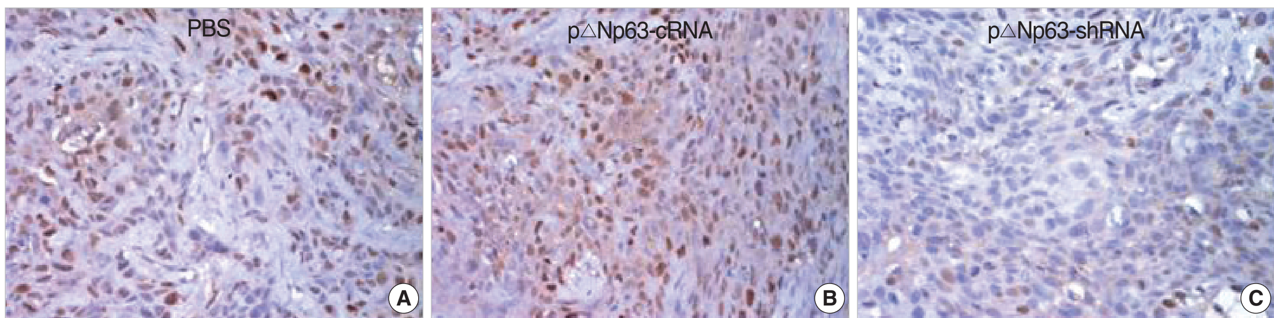
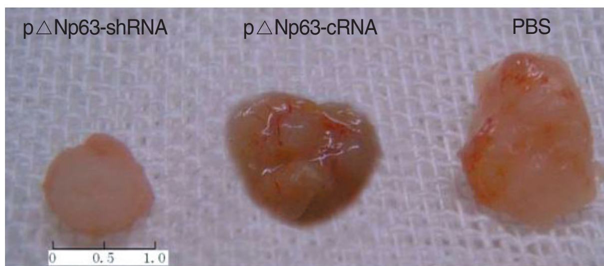


Fig. 6. Cyclin D1 protein expression in TCCB 5637 xenograft tumors from nude mice treated with PBS, control shRNA vector or △Np63 specific shRNA vector. Tumor tissues were taken at the end of the experiment and assayed by immunocytochemistry (original magnification, ×200).



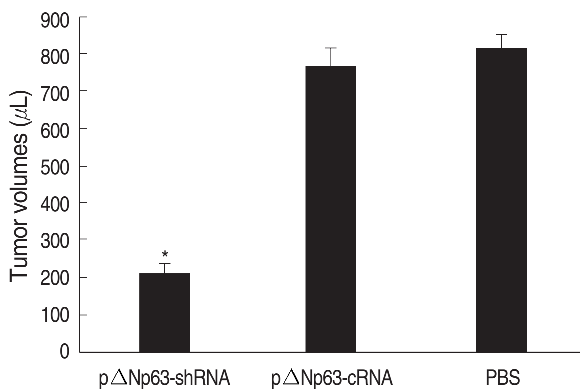


Fig. 7. Tumors were taken at 8 weeks from treatment from mice treated with p△Np63-shRNA (A1), p△Np63-cRNA (A2) or PBS (A3) and their sizes were measured and shown in B.
*Statistically significant (p<0.05, mean±SD, n=6) relative to p△Np63-cRNA group.

△Np63 shRNA inhibited 5637 cell proliferation in vitro

Proliferation of 5637 cells was evaluated with the MTT assay (Fig. 4). The absorbance-time curves showed that the absorbance of cells transfected with p△Np63-shRNA was significantly lower than that of cells treated with the control vector or PBS. The difference is especially significant at 120 hr post-transfection. The result indicated that the proliferation of TCCB cell line 5637 was significantly inhibited by △Np63 gene silencing.

△Np63 expression suppression led to down-regulation of cyclin D1 in vitro and in vivo

The effects of △Np63 gene silencing on the expression of cyclin D1 was evaluated by immunochemistry staining which was performed both in 5637 cell line in vitro and in nude mouse xenograft tumor. Cells with positive cyclin D1 protein expression should be stained brown in the nuclei. Both in 5637 cells in culture (Fig. 5) and in its nude mouse xenograft tumor (Fig. 6), cells transfected with p△Np63-shRNA were stained weakly positive in the nuclei, while cells treated with PBS or the control vector were stained strongly positive in the nuclei. These results suggested that silencing of △Np63 expression is associated with downregulation of cyclin D1 expression in 5637 cells.

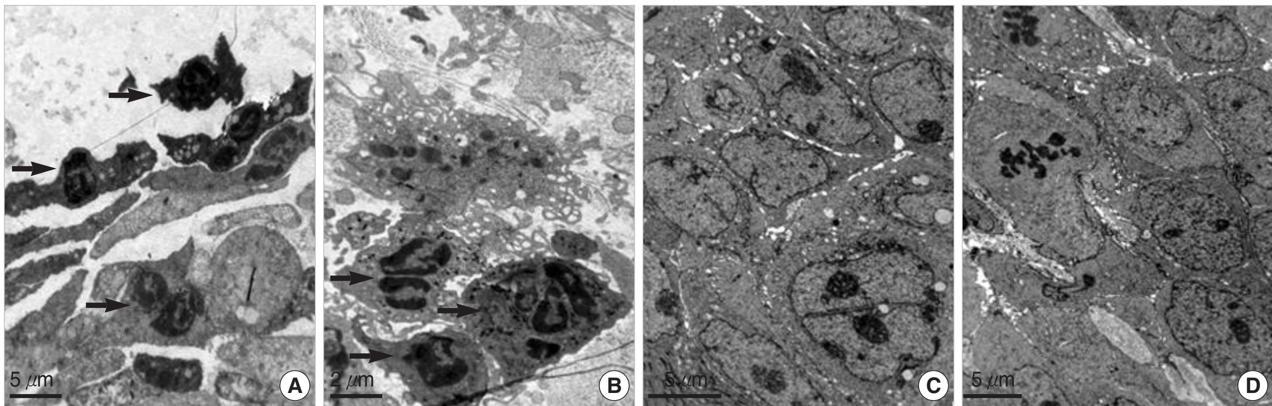


Fig. 8. Transmission electron microscopic examination of TCCB xenograft tumor tissues. Tumor tissues were from mice treated with p△Np63-shRNA (**A**, **B**), p△Np63-cRNA A (**C**) or PBS (**D**). (**A**) Tumor tissues treated with p△Np63-shRNA; the arrows indicate the apoptotic cells. (**B**) Tumor tissues treated with pNp63-shRNA; the arrow indicate the inflammatory cells. (**C**) Tumor tissues treated with p△Np63-cRNA. (**D**) Tumor tissues treated with PBS.

△Np63 shRNA transfection inhibited 5637 tumor growth in vivo

Tumor volumes were determined weekly. At the end of the experiment, mice receiving p△Np63-shRNA exhibited a 74.44% reduction in final tumor size relative to mice treated with p△Np63-cRNA and tumor-bearing mice treated with PBS had a similar tumor size as the control shRNA group (Fig. 7). Neither group exhibited signs of systemic toxicity or metastasis.

Morphologic changes in tumor

Histological assessment revealed that, compared to control shRNA and PBS treated mice, more inflammatory cells and sometimes zones of necrosis were found in tumor tissues of mice treated with p△Np63-shRNA (data not shown). Transmission electron microscopy indicated that disintegration of nucleolus, vacuoles in cytoplasm, inflammatory cell infiltration and apoptotic body formation could be observed in tumor tissues treated with P△Np63-shRNA (Fig. 8A, B) which were rarely seen in tumor tissues treated with the P△Np63-cRNA or PBS (Fig. 8C, D)

DISCUSSION

The ability of RNAi mediated by shRNA to silence individual gene expression with a high degree of specificity presents a unique opportunity to study gene functions (19, 20). Plasmid and viral vectors producing shRNA using the polymerase III promoter offer efficient and stable gene silencing (21, 22). In this study, we constructed a △Np63 shRNA expression plasmid, which achieved efficient and specific △Np63 gene silencing in human TCCB cell line 5637 following transfection both in vitro and in a nude mouse xenograft model. With △Np63 gene silencing, the cell cycle of 5637 cells was arrested in G_0/G_1 phase and cellular proliferation was significantly suppressed. △Np63 gene silencing by specific shRNA in the nude mouse xenograft tumor resulted in marked retardation of tumor growth and increase in tumor cell apoptosis. Our data suggested that △Np63 might play an oncogenic role in TCCB progression through promoting cell survival and proliferation. Therefore, △Np63 might be a new target gene for TCCB gene therapy, and △Np63 shRNA might be a novel and promising approach for silencing △Np63 expression.

One interesting new finding in our study is that cyclin D1 expression was downregulated after silencing of △Np63 expression in 5637 cells both in vitro and in vivo, indicating cyclin D1 might be a target gene of △Np63. △Np63 shares homology with the DNA-binding domain of p53, and numerous studies have reported that △Np63 proteins could bind to p53 consensus DNA sequences (23, 24). Cyclin D1 had previously been identified to have a p53 binding site. Cyclin D1 is a key regulator of cell cycle progression and a proven oncogene in several cancers, including urinary bladder carcinoma (25). It regulates cell cycle progression by activating cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), which in turn phosphorylates the retinoblastoma protein (Rb), leading to the inactivated pRB, releasing of transcription factor E2F and cell progression through G_1/S checkpoint (26-30). The strong positive correlation between △Np63 and cyclin D1 expression found in our study suggests that △Np63 might regulate TCCB cell cycle through regulating the expression of cyclin D1 which leads to less efficient phosphorylation of Rb which is a key condition for cells to pass G_1/S regulation point (31, 32). Therefore, cyclin D1 downregulation could lead to cell cycle arrest in G_0/G_1 phase, inhibition of cellular proliferation and promotion of apoptosis.

In conclusion, this study demonstrated that △Np63 plays

an oncogenic role in TCCB cells. Targeting this oncoprotein using a shRNA expressing vector induced specific silencing of the gene. Suppression of △Np63 expression impaired TCCB tumor growth in vivo and improved the survival of tumor-bearing nude mice. These effects are associated with impaired cellular proliferation and increased apoptosis. At the molecular level, our study suggests that cyclin D1 might be one of the target genes of △Np63.

REFERENCES

1. Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F, Caput D. *Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers*. *Cell 1997; 90: 809-19*.
2. Schmale H, Bamberger C. *A novel protein with strong homology to the tumor suppressor p53*. *Oncogene 1997; 15: 1363-7*.
3. Finlan LE, Hupp TR. *p63: the phantom of the tumor suppressor*. *Cell Cycle 2007; 6: 1062-71*.
4. Deyoung MP, Ellisen LW. *p63 and p73 in human cancer: defining the network*. *Oncogene 2007; 26: 5169-83*.
5. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, Andrews NC, Caput D, McKeon F. *p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities*. *Mol Cell 1998; 2: 305-16*.
6. Yang A, McKeon F. *P63 and P73: P53 mimics, menaces and more*. *Nat Rev Mol Cell Biol 2000; 1: 199-207*.
7. Augustin M, Bamberger C, Paul D, Schmale H. *Cloning and chromosomal mapping of the human p53-related KET gene to chromosome 3q27 and its murine homolog ket to mouse chromosome 16*. *Mamm Genome 1998; 9: 899-902*.
8. Osada M, Ohba M, Kawahara C, Ishioka C, Kanamaru R, Katoh I, Ikawa Y, Nimura Y, Nakagawara A, Obinata M, Ikawa S. *Cloning and functional analysis of human p51, which structurally and functionally resembles p53*. *Nat Med 1998; 4: 839-43*.
9. Wu G, Nomoto S, Hoque MO, Dracheva T, Osada M, Lee CC, Dong SM, Guo Z, Benoit N, Cohen Y, Rechthand P, Califano J, Moon CS, Ratovitski E, Jen J, Sidransky D, Trink B. *DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development*. *Cancer Res 2003 May 15; 63: 2351-7*.
10. Ratovitski EA, Patturajan M, Hibi K, Trink B, Yamaguchi K, Sidransky D. *p53 associates with and targets delta Np63 into a protein degradation pathway*. *Proc Natl Acad Sci USA 2001; 98: 1817-22*.
11. Hibi K, Trink B, Patturajan M, Westra WH, Caballero OL, Hill DE, Ratovitski EA, Jen J, Sidransky D. *AIS is an oncogene amplified in squamous cell carcinoma*. *Proc Natl Acad Sci USA 2000; 97: 5462-7*.
12. Patturajan M, Nomoto S, Sommer M, Fomenkov A, Hibi K, Zangen R, Poliak N, Califano J, Trink B, Ratovitski E, Sidransky D. *Delta Np63 induces beta-catenin nuclear accumulation and signaling*. *Cancer Cell 2002; 1: 369-79*.
13. Parsa R, Yang A, McKeon F, Green H. *Association of p63 with proliferative potential in normal and neoplastic human keratinocytes*. *J Invest Dermatol 1999; 113: 1099-105*.
14. Choi HR, Batsakis JG, Zhan F, Sturgis E, Luna MA, El-Naggar AK. *Differential expression of p53 gene family members p63 and p73 in head and neck squamous tumorigenesis*. *Hum Pathol 2002; 33: 158-64*.
15. Ratovitski EA, Patturajan M, Hibi K, Trink B, Yamaguchi K, Sidransky D. *p53 associates with and targets delta Np63 into a protein degradation pathway*. *Proc Natl Acad Sci USA 2001; 98: 1817-22*.
16. Park CK, Oh YH. *Expression of p63 in reactive hyperplasias and malignant lymphomas*. *J Korean Med Sci 2005; 20: 752-8*.
17. Dohn M, Zhang S, Chen X. *p63alpha and DeltaNp63alpha can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes*. *Oncogene 2001; 20: 3193-205*.
18. Urist MJ, Di Como CJ, Lu ML, Charytonowicz E, Verbel D, Crum CP, Ince TA, McKeon FD, Cordon-Cardo C. *Loss of p63 Expression is associated with tumor progression in bladder cancer*. *Am J Pathol 2002; 161: 1199-206*.
19. Kim VN. *RNA interference in functional genomics and medicine*. *J Korean Med Sci 2003; 18: 309-18*.
20. Filleur S, Courtin A, Ait-Si-Ali S, Guglielmi J, Merle C, Harel-Bellan A, Clézardin P, Cabon F. *SiRNA-mediated inhibition of vascular endothelial growth factor severely limits tumor resistance to antiangiogenic thrombospondin-1 and slows tumor vascularization and growth*. *Cancer Res 2003; 63: 3919-22*.
21. Brummelkamp TR, Bernards R, Agami R. *A system for stable expression of short interfering RNAs in mammalian cells*. *Science 2002; 296: 550-3*.
22. Wilda M, Fuchs U, Wossmann W, Borkhardt A. *Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi)*. *Oncogene 2002; 21: 5716-24*.
23. Di Como CJ, Urist MJ, Babayan I, Drobnjak M, Hedvat CV, Teruya-Feldstein J, Pohar K, Hoos A, Cordon-Cardo C. *p63 expression profiles in human normal and tumor tissues*. *Clin Cancer Res 2002; 8: 494-501*.
24. Harmes DC, Bresnick E, Lubin EA, Watson JK, Heim KE, Curtin JC, Suskind AM, Lamb J, DiRenzo J. *Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63-alpha contributes to differential regulation of p53 target genes*. *Oncogene 2003; 22: 7607-16*.
25. Reznikoff CA, Sarkar S, Julicher KP, Burger MS, Puthenveettil JA, Jarrard DF, Newton MA. *Genetic alterations and biological pathways in human bladder cancer pathogenesis*. *Urol Oncol 2000; 5: 191-203*.
26. Lee CC, Yamamoto S, Morimura K, Wanibuchi H, Nishisaka N, Ikemoto S, Nakatani T, Wada S, Kishimoto T, Fukushima S. *Significance of cyclin D1 overexpression in transitional cell carcinomas of the urinary bladder and its correlation with histopathologic features*. *Cancer 1997; 79: 780-9*.
27. Shin KY, Kong G, Kim WS, Lee TY, Woo YN, Lee JD. *Overexpression of cyclin D1 correlates with early recurrence in superficial bladder cancers*. *Br J Cancer 1997; 75: 1788-92*.
28. Suwa Y, Takano Y, Iki M, Takeda M, Asakura T, Noguchi S, Masu-

da M. *Cyclin D1 protein overexpression is related to tumor differentiation, but not to tumor progression or proliferative activity, in transitional cell carcinoma of the bladder. J Urol 1998; 160: 897-900.*

29. Sgambato A, Migaldi M, Faraglia B, De Aloysio G, Ferrari P, Ardito R, De Gaetani C, Capelli G, Cittadini A, Trentini GP. *Cyclin D1 expression in papillary superficial bladder cancer: its association with other cell cycle-associated proteins, cell proliferation and clinical outcome. Int J Cancer 2002; 97: 671-8.*
30. Wagner U, Süess K, Luginbühl T, Schmid U, Ackermann D, Zellweger T, Maurer R, Alund G, Knönagel H, Rist M, Jordan P, Moch H, Mihatsch MJ, Gasser TC, Sauter G. *Cyclin D1 overexpression lacks prognostic significance in superficial urinary bladder cancer. J Pathol 1999; 188: 44-50.*
31. Oya M, Schmidt B, Schmitz-Dräger BJ, Schulz WA. *Expression of G1-->S transition regulatory molecules in human urothelial cancer. Jpn J Cancer Res 1998; 89: 719-26.*
32. Monica SM, George F. *Regulation of the cell cycle. in: Mendelson J, eds. The molecular basis of cancer. 2nd ed. W. B. Saunders Company 2001; 10-7.*