

## ORIGINAL ARTICLE

# Upregulation of cell adhesion through *delta Np63* silencing in human 5637 bladder cancer cells

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Some researchs have demonstrated that the loss of *delta Np63* is associated with aggressive phenotypes and poor prognosis. However, other research indicates that *delta Np63* is considered to have oncogenic properties. *Delta Np63* overexpression is often observed in association with the oncogenic growth of squamous cell carcinomas and bladder cancer. In this study, we investigated the oncogenic role of *delta Np63* in regulating cell adhesion in transitional cell carcinoma of the bladder (TCCB). The cells were stably transfected with the *delta Np63* short hairpin RNA (shRNA) plasmid. Immunocytochemistry was performed to determine the knockdown efficiency. Tumour cells were studied for their ability to adhere to vascular endothelial cells. Confocal microscopy was used to analyse the changes in cytoskeletal F-actin. F-actin expression was measured by flow cytometry. Cell invasion ability was assessed using transwell chambers. The *delta Np63*-silenced tumour cells were shown to adhere more tightly than controls to vascular endothelial cells ( $P < 0.05$ ). The content of F-actin in the *delta Np63*-silenced cells was enhanced ( $P < 0.05$ ). The Matrigel invasion assays showed that human 5637 bladder cancer cells had a lower degree of motility when transfected with *delta Np63*-shRNA ( $P < 0.05$ ). In conclusion, silencing of the *delta Np63* expression can enhance the adhesiveness of 5637 cells by inducing F-actin cytoskeleton production, and it will possibly inhibit the TCCB invasion and metastasis.

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## INTRODUCTION

The invasion and metastasis of malignant tumour cells is a complex process that is regulated by multiple factors. In addition to the degree of neovascularisation, change in cell adhesion ability is an important factor in the progression of malignant tumours.<sup>1</sup> Tumourigenesis and the development of metastatic disease are accompanied by changes in the expression of cell adhesion molecules. In carcinomas, the cell adhesion molecules that are normally expressed are lost or expressed in a functionally altered form, which allows tumour cells to escape from contact-mediated controls and spread to remote sites. Metastatic potential in solid tumours may also be associated with the expression of new cell adhesion molecules by the tumour cells. These newly expressed adhesion molecules appear to mediate tumour cell interaction with leukocytes and endothelium and may direct dissemination of the tumour cells throughout the body.<sup>2</sup> Thus, the abnormal expression of cell adhesion molecules plays a key role in tumour development and is a common feature of malignant tumours.<sup>3</sup>

The *p63* gene is a member of the *p53* gene family. It shares a high degree of homology and structural similarity with the *p53* protein.<sup>4,5</sup> The *p63* protein was originally thought to be another tumour suppressor that functioned in a similar capacity to that of *p53*. *p63* has two major isoforms that differ at the amine terminus: *TAp63* and *delta Np63*. *TAp63* has a transactivating amino terminal, while *delta Np63* has a dominant-negative activating amino terminal.<sup>6–9</sup> In general, the

*TAp63* isoform behaves like *p53* because it transactivates various *p53* downstream targets, induces apoptosis and mediates cell cycle control. In contrast, the *delta Np63* isoform has been shown to display functions that are opposite to those of the *TAp63* isoform and may act as an oncoprotein.<sup>8–11</sup> *Delta Np63* overexpression is often observed in squamous cell carcinomas, in which context it is expected to enhance oncogenic growth.<sup>9,12</sup> *Delta Np63* may function as a dominant negative mediator in *p53* tumour suppression.<sup>5</sup> It is generally accepted that *TAp63* plays a more *p53*-like role, whereas *delta Np63* has an antagonistic or even oncogenic role in the progression of malignant tumours to cancer, including invasion and metastasis in malignant tumours.<sup>7,13–15</sup> However, research indicates that the loss of *delta Np63* is associated with increased invasive and metastatic potential.<sup>16–18</sup> Our previous study<sup>19</sup> demonstrated that *delta Np63* was overexpressed in transitional cell carcinoma of the bladder (TCCB) tissues at both the mRNA and protein levels, which suggests that *delta Np63* plays an oncogenic role in TCCB cells. Targeting this oncoprotein by using a short hairpin RNA (shRNA)-expressing vector induced the specific silencing of *delta Np63* expression. The suppression of *delta Np63* expression impairs TCCB tumour growth *in vivo* and improves the survival of tumour-bearing nude mice. No invasion or metastasis was found in tumour-bearing nude mice.<sup>19</sup> Therefore, we speculate that *delta Np63* may contribute to invasion or metastasis by affecting cell adhesion ability in TCCB.

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Because the function of different isoforms of *delta Np63* in bladder cancer remains controversial, we sought to investigate the role of *delta Np63* in human bladder cancer cell adhesion and its mechanism by using small interference RNA targeting *delta Np63* in human bladder cancer 5637 cells.

## MATERIALS AND METHODS

### Cell lines and plasmid

The 5637 human TCCB cell line was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium (Gibco, Shanghai, China) supplemented with 10% (v/v) foetal bovine serum (FBS; Sijixin Inc., Beijing, China) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% (v/v) CO<sub>2</sub>. A pair of effective shRNAs targeting human *delta Np63* mRNA were selected from among those used for preliminary experiments. The pair of shRNAs forms a structure consisting of two 19-bp stems targeting *delta Np63* mRNA, a 9-bp loop and a short polyA sequence. Two oligonucleotides (forward: 5'-GATCCGTGCCAGACTCAATTTAGTTTCAAGACGACTAAATTGAGTCTGGGCATTTTTGTGCGACA-3' and reverse: 5'-AGCTTGTGACAAAAAATGCCAGACTCAATTTAGTCTCTTGAAGTCTGAACTAAATTGAGTCTGGGCACG-3') were synthesized and ligated directly into *Bam*HI and *Hind*III linearized gesisil-1 plasmid (Jingsai Inc., Wuhan, China). The resultant *delta Np63*-shRNA expression construct, *pdelta Np63*-shRNA, was confirmed using PstI+SalI double digestions and by DNA sequencing. The negative control plasmid, termed *pdelta Np63*-cRNA, was inserted equidistant from the following two oligonucleotides: forward: 5'-GATCCGACTTCATAAGGCGCATGCTTCAAGACGGCATGCGCCTTATGAAGTCTTTTTTGTGCGACA-3' and reverse: 5'-AGCTTGTGACAAAAAAGACTTCATAAGGCGCATGCCGTCTTGAAGCATGCCCTTATAAGTCG-3'.

### Sensitivity of the 5637 cells to G418

G418 (hygromycin) is an aminoglycoside antibiotic used in molecular genetic tests. It is the dominant selection reagent that was used most often to obtain stably transfected cells. Because the plasmid contains a gene coding for resistance to aminoglycoside antibiotics, only successful transfection would result in cell survival. The surviving 5637 cells were seeded and cultured into 12-well plates and selected in the presence of different concentrations of G418 (Zhongshang Inc., Beijing, China) ranging from 100 to 1000 µg ml<sup>-1</sup>. The cell culture medium was changed every 3 days and changed to normal medium with G418 on day 14. Cells were then cultured for 7 days in normal medium without G418. The optimal concentration of G418 was chosen as the lowest concentration in a given 12-well plate without any living non-transfected cells, which was observed by fluorescence microscopy. The mean results of three experiments are reported here.

### Cell transfection and selected stable, positive cell clones

For transfection, 5637 cells were seeded in 24-well plates at 1×10<sup>6</sup> cells per well and allowed to grow overnight to approximately 80% confluence. Cells were transfected with the mixture of 0.4 µg of *pdelta Np63*-shRNA, 0.4 µg *pdelta Np63*-cRNA or 0.4 µl of phosphate-buffered saline (PBS) and 10 µl of Effectene transfection reagent (Qiagen, Shanghai, China) in 600 µl of fresh 1640 culture medium. Thirty-six hours after transfection, the cell culture medium was changed to fresh 10% (v/v) FBS with 400 µg ml<sup>-1</sup> G418, and the medium was changed every 3 days. After 21 days, cultures of clones that were positive for *pdelta Np63*-shRNA or *pdelta Np63*-cRNA as well as a PBS group

(transfected without plasmid) were maintained. Forty-eight hours after transfection, the cells were harvested for immunocytochemistry as described below. The mean results of three experiments are reported here.

### Immunocytochemistry

Cells were fixed with chilled acetone at 4 °C for 30 min, followed by incubation for 10 min with 3% (v/v) hydrogen peroxide in methanol. Goat serum was used to block nonspecific binding. The fixed and blocked cells were incubated overnight at 4 °C with a 1:200 dilution of a mouse monoclonal anti-*delta Np63* antibody (Santa Cruz, Beijing, China). Cells were washed three times, and an appropriate biotinylated-conjugated secondary antibody (Santa Cruz) was applied for 15 min at a 1:150 dilution, at 37 °C. Cells were washed three times and incubated with streptavidin-horseradish peroxidase (Zhongshan Golden Bridge Inc., Beijing, China) for 15 min at 37 °C. Horseradish peroxidase substrate diaminobenzidine solution was added for 15 s; the staining was stopped by washing the coverslip repeatedly with water. The cells were counterstained with hematoxylin. The mean results of three experiments are reported here.

### Adhesion between 5637 cells and umbilical venous endothelial cells

Human umbilical venous endothelial cells (HUVECs) were provided as a gift by the Department of Physiology of Chongqing Medical University. Then, 0.1 ml (5×10<sup>4</sup> HUVEC cells per ml) of cells was seeded into 96-well plates. When the HUVEC cells grew to confluence, 0.1 ml (5×10<sup>4</sup> bladder cancer cells per ml) of cells (*pdelta Np63*-shRNA-, *pdelta Np63*-cRNA- and PBS-treated) were added to the 96-well plate and cultured for 30 min at 37 °C. Non-adherent cells were removed. Then, 100 µl of Rose Bengal was added to each plate for 5 min, followed by the addition of 100 µl of 95% alcohol and 100 µl of PBS to the plate for 30 min at room temperature. The absorbance (A) at 570 nm was measured for each group. Notably, HUVEC cells form a monolayer, to which cells transfected with *pdelta Np63*-shRNA, *pdelta Np63*-cRNA or PBS were added for the investigation of cell adhesion ability. Rose Bengal staining was used as an index of cell adhesion ability. The mean results of three experiments are reported here.

### F-actin morphology and quantitative observations of 5637 cells

*pdelta Np63*-shRNA-, *pdelta Np63*-cRNA- and non-transfected 5637 cells at 1×10<sup>5</sup> cells per ml×0.2 ml were seeded onto coverslips in 24-well plates. The coverslips were taken out when cells were seeded; cell populations then grew to cover 70%–80% of each coverslip. The coverslips were fixed by 3.7% formaldehyde for 10 min at room temperature and stored at -20 °C in acetone for 5 min. Then, 20 µl (2 µg ml<sup>-1</sup>) of tetramethylrhodamine isothiocyanate phalloidin (Sigma, Shanghai, China) were added to each coverslip and incubated for 25 min in the dark. The coverslips were washed in deionized water and mounted. Fluorescence signals were visualized using confocal microscopy.

For flow cytometry, 0.2 ml of *pdelta Np63*-shRNA-, *pdelta Np63*-cRNA- and non-transfected cells (at 1×10<sup>5</sup> cells per ml) were seeded onto 24-well plates. Tetramethylrhodamine isothiocyanate phalloidin was added to each well after the cells had grown to confluence. The relative quantity of F-actin was determined based on the fluorescence intensity at 540 nm as detected using flow cytometry. At least 1000 cells were counted and analysed. The mean results averaged over three experiments are reported as fluorescence intensity per 10 000 cells.

### Cell invasion

Cell invasion was assessed using transwell chambers with 8  $\mu\text{m}$  pore-size membranes coated with Matrigel (Collaborative Research, Bedford, MA, USA) that were placed in 24-well plates. Cells ( $5 \times 10^4$ ) were loaded into the upper segment of the chambers and allowed to traverse the membrane for 24 h. The upper segment of the chamber contained 5% (w/v) FBS; the lower chamber contained 10% (w/v) FBS. After the cells migrate from a low to a high concentration of serum, any non-migratory cells were removed. The invaded cells were harvested by trypsinizing the lower surface of the membrane and were collected and seeded into a new 24-well plate, and 500  $\mu\text{l}$  of 0.1% gentian violet was added. The membrane was then removed after 30 min and washed with PBS. The invasive activity was then quantified by counting the number of cells that had invaded 10 randomly selected microscopic fields ( $>40$  cells per field). Each group was analysed in triplicate, and the mean was reported.

### Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA), version 13.0 for Windows. Non-parametric ANOVA was performed to compare the differences among more than two parameters. Statistical significance in this study was set at  $P < 0.05$ . All reported  $P$  values are two-sided.

## RESULTS

### The optimal G418 selection concentration for 5637 cells

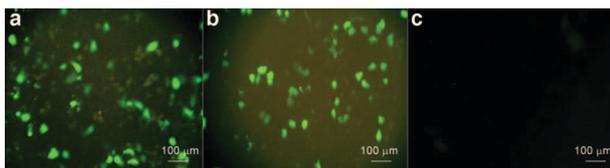
All parental 5637 cells were dead 4 days after being selected by G418 concentrations over  $500 \mu\text{g ml}^{-1}$ , while the parental cells were dead at 7 days after the application of G418 at  $400 \mu\text{g ml}^{-1}$ . There were a few surviving cells at G418 concentrations of 200 and  $300 \mu\text{g ml}^{-1}$ . Therefore,  $400 \mu\text{g ml}^{-1}$  was selected as the lethal concentration, and  $200 \mu\text{g ml}^{-1}$  was used as the selective concentration.

### Selected stable, positive cell clones

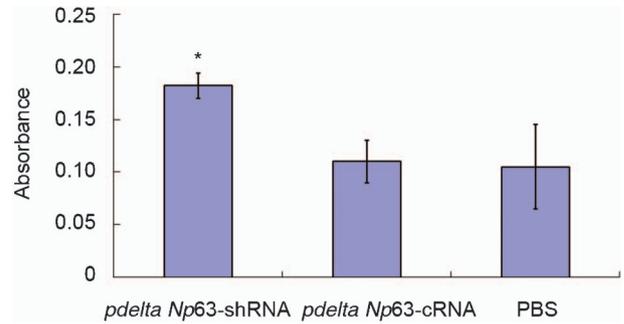
The 5637 cells were transfected with *pdelta Np63*-shRNA or *pdelta Np63*-cRNA and selected in G418 at  $200 \mu\text{g ml}^{-1}$ . The stable cell clones also expressed green fluorescent protein (GFP) and could be observed by fluorescence microscopy. There was no GFP in the PBS control group. Stable positive cell clones were those that grew in medium of  $200 \mu\text{g ml}^{-1}$  G418 (Figure 1).

### Silencing of *delta Np63* protein expression by *delta Np63* shRNA

The 5637 TCCB cell line inherently expresses high levels of *delta Np63*. The expression of *delta Np63* protein was detected 48 h post-transfection by immunocytochemistry. The nuclei of *delta Np63*-positive cells were stained brown. The nuclei of cells transfected with PBS or the control



**Figure 1** Stable cell growth of GFP cells was monitored by fluorescence microscopy. (a) *pdelta Np63*-shRNA group; (b) *pdelta Np63*-cRNA group; (c) PBS group. The stable cell clone grows with GFP which could be observed by fluorescence microscope (a, b). There is no GFP in group of PBS group (c). GFP, green fluorescent protein; PBS, phosphate-buffered saline; shRNA, short hairpin RNA.



**Figure 2** Absorbance of Rose Bengal as index of cell adhesion. \* $P < 0.05$ , compared with *Np63*-cRNA and PBS groups, respectively. PBS, phosphate-buffered saline.

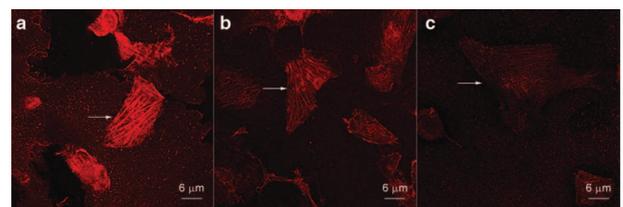
vector were strongly stained, while the nuclei of cells transfected with *pdelta Np63*-shRNA were only weakly stained.

### 5637 cell adhesion after the transfection of *delta Np63* shRNA

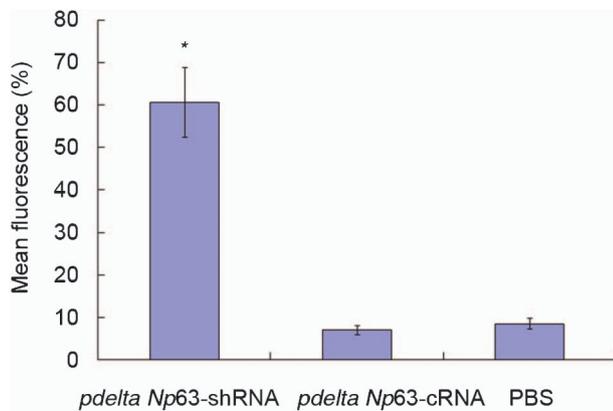
The absorbances of *pdelta Np63*-shRNA-, *pdelta Np63*-cRNA- and PBS-treated cells were  $0.182 \pm 0.012$ ,  $0.110 \pm 0.002$  and  $0.105 \pm 0.004$ , respectively (Figure 2). The level in *pdelta Np63*-shRNA-transfected cells was significantly higher than that in the *pdelta Np63*-cRNA and PBS groups ( $p < 0.05$ ). These findings suggest that silencing the expression of *delta Np63* may increase the ability of 5637 cells to adhere to HUVEC cells.

### Levels and morphology of F-actin in 5637 cells

Phalloidin specifically binds to F-actin. F-actin can be visualized by fluorescence microscopy after incubation with tetramethylrhodamine isothiocyanate phalloidin. The staining is red and distributed to the cytoskeleton and cell processes. The microfilaments had enhanced fluorescence in the *pdelta Np63*-shRNA group as compared to the control group and followed the cell's polarity, suggesting that protein levels of F-actin are increased in *pdelta Np63*-shRNA-transfected cells. Conversely, in the *pdelta Np63*-cRNA and PBS groups, the F-actin staining was diffuse and irregular (Figure 3). The mean fluorescence intensities in the *pdelta Np63*-shRNA-, *pdelta Np63*-cRNA- and non-transfected groups were  $60.71\% \pm 8.25\%$ ,  $7.00\% \pm 1.01\%$  and  $8.50\% \pm 1.26\%$ , respectively (Figure 4). The levels in the *pdelta Np63*-shRNA-transfected group were significantly higher than those in the other two groups ( $p < 0.05$ ). These results further confirm that silencing the expression of *delta Np63* increases F-actin expression in 5637 cells and thus could enhance the polymerisation of F-actin.



**Figure 3** Morphology of F-actin observation in 5637 cell. (a) *pdelta Np63*-shRNA group; (b) *pdelta Np63*-cRNA group; (c) PBS group. The protein level of F-actin in *pdelta Np63*-shRNA is obviously increased (a). But in *pdelta Np63*-cRNA and PBS groups, the F-actin is diffusion, brightness of microfilament is lower, cell polarity disappears, and cell arrangement is crumbly and irregular (b, c). PBS, phosphate-buffered saline; shRNA, short hairpin RNA.



**Figure 4** Quantitative of F-actin fluorescence intensity in each group. \* $P < 0.05$ , compared with *pdelta Np63*-cRNA and PBS groups, respectively. PBS, phosphate-buffered saline.

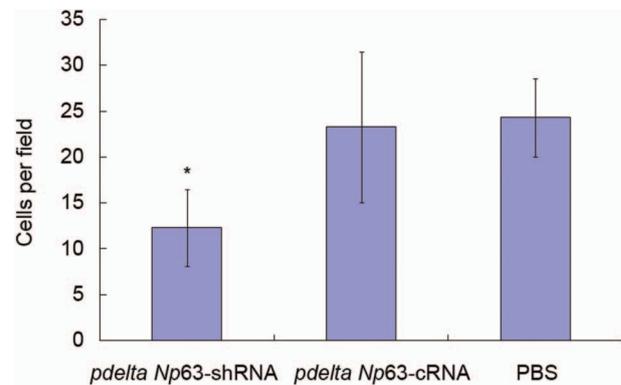
### Cell invasion

The Matrigel invasion assays showed that 5637 cells in the *pdelta Np63*-shRNA group had a lower degree of motility than those in either the *pdelta Np63*-cRNA group or the PBS group. The invasion ability of *pdelta Np63*-shRNA cells ( $12.30 \pm 4.16$  cells per field) was lower than that of cells in the *pdelta Np63*-cRNA group ( $23.27 \pm 8.23$  cells per field,  $P < 0.05$ ) or PBS group ( $24.34 \pm 4.27$  cells per field,  $P < 0.05$ ) (Figure 5).

### DISCUSSION

Despite research findings that the loss of *delta Np63* is associated with increased invasive and metastatic potential in preclinical models,<sup>20</sup> the expression of *p63* in bladder tumour cells was still higher than that in normal cells. Nevertheless, there is still some research indicating that *delta Np63* has oncogenic properties. First, *delta Np63* overexpression is often observed in squamous cell carcinomas and increases the associated level of oncogenic growth.<sup>12,21,22</sup> Second, *delta Np63* can function as a dominant-negative factor that inhibits *p53* tumour suppression.<sup>6</sup> Third, *delta Np63* overexpression induces the nuclear accumulation of  $\beta$ -catenin and activates  $\beta$ -catenin signalling, which promotes cell proliferation.<sup>13</sup> However, the role of *delta Np63* in TCCB is poorly defined, and the mechanisms underlying the effect of *delta Np63* remain to be determined. These discrepant results may be related to gene polymorphisms and to the particular isoform of *delta Np63*. Our previous study<sup>19</sup> has demonstrated that *delta Np63* transcription and translation are increased in human TCCB tumour tissues. To further explore the functions of *delta Np63* in TCCB, we utilized RNA interference technology to induce *delta Np63* gene silencing. The ability of RNA interference to silence individual gene expression with a high degree of specificity, an effect mediated by shRNA, represents a unique opportunity to study gene function.<sup>23</sup> Plasmid and viral vectors can be used to produce shRNA using the polymerase III promoter and then to achieve efficient and stable gene silencing.<sup>24,25</sup> In this study, we constructed a *delta Np63* shRNA expression plasmid, which achieved efficient and specific *delta Np63* gene silencing in the 5637 human TCCB cell line following transfection *in vitro*.

We found that when *delta Np63* expression was silenced, the ability of 5637 cells to adhere to HUVEC cells was increased significantly. Levels of F-actin and microfilament density (which followed cell polarity) were elevated in the *pdelta Np63*-shRNA group. F-actin is one of the major components of the cytoskeleton and plays an important role in many cell functions, including the regulation of cell shape,



**Figure 5** Number of migrating cells by Matrigel invasion assays. \* $P < 0.05$ , compared with *pdelta Np63*-cRNA and PBS groups, respectively. PBS, phosphate-buffered saline.

motility, secretion, intracellular transport, endocytosis and exocytosis, and cell adhesion.<sup>26–28</sup> The phenotypic changes resulting from transformation are reflected as distinct alterations in the cytoskeleton.<sup>29,30</sup> The tumour cells that traverse the blood vessels to form metastases must pass through endothelial cells and the subendothelial basement membrane. This process also involves adhesion. The reduced expression of tumour cell adhesion molecules may decrease cell adhesion, thereby permitting metastasis. Conversely, increased 5637 cell adhesion to HUVEC cells and increased levels of F-actin can inhibit tumour metastasis.<sup>31</sup>

*Delta Np63* may function as an oncogene in the TCCB progression of invasion and metastasis by downregulating F-actin expression. The actin cytoskeleton is altered during tumour cell transformation. The most notable change involves restructuring of the F-actin skeleton. F-actin has been used in human breast cancer and T-lymphatic cell carcinoma as a sensitive marker for the early detection of tumour progression.<sup>32,33</sup> Our results are consistent with Kao's research on human bladder cancer cells. When F-actin expression is increased, F-actin aligns with the cell's polarity, which results in reduced tumour metastasis.<sup>34–36</sup> Increases in the density of F-actin result in loose arrangements that are not aligned with cell polarity and result in increased tumour metastasis. Therefore, *delta Np63* may be a new drug target for TCCB therapy, especially in efforts to target invasion and metastasis. *Delta Np63* shRNA may be a novel and promising approach for silencing *delta Np63* expression. Furthermore, it is noteworthy that the migration and invasion capacities of the 5637 cells were reduced after *delta Np63* was silenced, which suggests that the *delta Np63* gene is essential for TCCB cell motility and invasion.

### CONCLUSIONS

In conclusion, our results demonstrate that targeting *delta Np63* expression, through the use of a shRNA-expressing vector, induced the specific silencing of *delta Np63* gene expression. The suppression of *delta Np63* expression may upregulate F-actin in TCCB *in vivo* and thereby change the cytoskeletal phenotype. When *delta Np63* expression was silenced among TCCB cells, the capacity for cell adhesion was increased, while levels of cell motility and invasion ability were reduced. Studies at the molecular level suggest that F-actin may be one of the genes targeted by *delta Np63*.

### AUTHOR CONTRIBUTIONS

YFH, DYT, ZJY, ZKY, XHW, CLL and WT conducted the experiments described in the study. The cell culture experiments were finished by

YFH and DYT. The plasmid was constructed by XHW and CLL. Statistical analysis was conducted by WT and ZJY. All authors read and approved the final version of the manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

### ACKNOWLEDGMENTS

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