Downregulation of p53 promotes *in vitro* perineural invasive activity of human salivary adenoid cystic carcinoma cells through epithelial-mesenchymal transition-like changes

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Abstract. Salivary adenoid cystic carcinoma (SACC) is a malignant tumor that is characterized by perineural invasion (PNI). p53 is an essential tumor-suppressor gene and p53 mutations play a critical role in tumor occurrence and progression (e.g., pancreatic, prostate and head and neck cancer). However, the regulatory role of the p53 gene in SACC and the PNI process remains unknown. In the present study, we employed RNA interference technique to downregulate p53 gene expression in SACC-83 cells to explore the role of p53 in the PNI process. Our results showed that the downregulation of the p53 gene induced significant 'epithelial-mesenchymal transition (EMT)-like changes' in SACC-83 cells, including decreased expression levels of epithelial markers (E-cadherin, EMA and CK5) and increased expression levels of mesenchymal markers (vimentin, N-cadherin and C-cadherin). The downregulation of p53 also caused a lower apoptotic index of Annexin V-FITC/PI and a lower number of SACC-83 cells in the second G0/G1 phase of the cell cycle. Furthermore, the downregulation of the p53 gene resulted in a significant increase in PNI activity in the SACC-83 cells. Thus, our findings revealed that downregulation of p53 promoted *in vitro* PNI activity through 'EMT-like changes' in SACC-83 cells. The present study suggests the essential regulatory role of p53 in the PNI activity of SACC cells, and implies that p53 may be a new target gene for the clinical treatment of SACC.

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Introduction

Salivary adenoid cystic carcinoma (SACC) is a unique malignant tumor which is unpredictable in nature. The incidence rate of SACC is ~10% of all salivary gland tumors (1-3), 22% of all malignant salivary gland tumors (4), and 1% of all head and neck malignant tumors (2,3). SACC occurs most commonly in minor salivary glands and the oral cavity (1,3). Perineural invasion (PNI), a unique pathological entity that significantly differs from lymphatic and vascular invasion, is considered to be an essential feature of SACC (5). PNI may also act as a source of malignant tumor distant metastasis, and the range of PNI is much wider than local invasion (6-8). PNI has become an important pathological feature of many malignant tumors, including pancreatic and prostate cancer, cholangiocarcinoma and head and neck cancer. PNI is also proven to be a sign of poor prognosis and a decreased survival omen for these malignant tumors (9-13).

Epithelial-mesenchymal transition (EMT) refers to the rapid and reversible changes in cellular morphology and phenotype characterized by the transformation of epithelial features into mesenchymal features (14,15). Numerous lines of evidence have proven that EMT has the capacity of regulating cell fate and tissue morphology (16-19). In laboratory studies, EMT has also shown a close relationship with the process of tumor growth and metastasis (16,18). However, no direct evidence of EMT has been revealed in the clinic. Thus, some pathologists have conjectured that EMT exhibits no correlation with the development and progression of tumors (20). It is widely acknowledged that the narrowly defined EMT should be strictly distinguished from an EMT-like cell phenotype for exploring tumor occurrence and progression (21). Since EMT changes result from the poor differentiation of tumor cells, it seems more appropriate to use 'EMT-like changes' to describe the phenotype of tumor cells in the process of tumor genesis (21). The term 'EMT-like changes' emphasizes the

renewal of tumor cells and adaptation to a specific microenvironment instead of a positive de-differentiation process (21).

p53 is an essential tumor-suppressor gene, and p53 mutations play a critical role in tumor occurrence and progression (e.g., pancreatic, prostate, and head and neck cancer) (22,23). Our previous study showed that SACC with PNI exhibited significantly lower p53 gene expression than SACC without PNI (24). Chang *et al* found that the p53 gene could regulate the EMT process of breast cancer cells (25). Thus, in the present study, we hypothesized that p53 plays a crucial role in regulating PNI activity in SACC through a potential mechanism that is related to 'EMT-like changes'.

In the present study, the regulatory role of the p53 gene in SACC and the potential metastatic process was systematically investigated. We used the RNA interference technique to downregulate the expression of the p53 gene in SACC-83 cells (a human SACC cell line), and then examined the changes in cell phenotype markers, cell cycle, anti-apoptosis and the PNI capability of SACC-83 cells. Our data showed that downregulation of p53 gene expression promoted *in vitro* PNI activity of SACC-83 cells through 'EMT-like changes'.

Materials and methods

shRNA preparation. Four p53 shRNAs were designed according to the p53 sequence in the GenBank (NM_006500) following the rules of Tuschl (26). As shown in Table I, p53 shRNAs contained a unique 19-nt double-stranded human p53 sequence that was presented as an inverted complementary repeat and separated by a loop of 9-nt spacer. DNA oligonucleotides targeting p53 were synthesized and inserted into the linearized pGPU6/GFP/Neo shRNA expression vector according to the manufacturer's instructions (Table I). The recombinant vectors were named pGGNeo-p53-homo-1043-shRNA, pGGNeo-p53-homo-1157-shRNA, pGGNeo-p53-homo-968-shRNA and pGGNeo-p53-homo-270-shRNA. The pGGNeo-negative-shRNA contained a nonsense shRNA insert designed according to the p53-shRNA sequence and was checked by BLAST. The pGGNeo-GAPDH-shRNA was a positive control vector targeting GAPDH to test the system error.

Cell culture and shRNA transfection. SACC-83 cells are a type of human SACC cell line, which have been widely used for the investigation of the biological characteristics of SACC (27). The SACC-83 cells were cultured in 6-well plates in RPMI-1640 medium (HyClone, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37˚C in 5% CO_2. After incubation for 24 h, cells (~70% confluency) were treated with various vectors, including pGGNeo-p53-homo-1043-shRNA, pGGNeo-p53-homo-1157-shRNA, pGGNeo-p53-homo-968-shRNA, pGGNeo-p53-homo-270-shRNA, pGGNeo-negative-shRNA and pGGNeo-GAPDH-shRNA which had been precomplexed with Lipofectamine™ 2000 (Boster, Wuhan, China). Seventy-two hours post shRNA transfection, the cells were used for real-time PCR, western blotting, flow cytometry and PNI analyses.

Real-time PCR. The forward and reverse primers corresponding to the human p53 gene were: 5'-GGTCTGGCCCCT CCTCAGCA-3' and 5'-TGCCGCCCATGCAGGAACTG-3; total 178 bp. The mRNA of GAPDH was amplified with forward (5'-ctcatgaccacagtccatgc-3') and reverse (5'-ttcagctc tgggatgacctt-3') primers with a total product length of 106 bp. Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized with RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA). PCR reactions were performed in a total of 25 μl reaction mixture (1 μl of cDNA, 4 μl of forward and reverse primers, 12.5 μl of 2X SYBR-Green PCR Master Mix and 7.5 μl of ddH_2O). Data were analyzed with the comparative Ct method and normalized by actin expression in each sample.

Western blot analysis. Total proteins were extracted with a protein extraction kit (ProMab, USA). The protein extracts (30 μg/sample) were subjected to electrophoretic separation by 8 and 10% Tris-glycine SDS-PAGE and were transferred onto PVDF membranes (Millipore) after being mixed with 5X loading buffer and boiled for 8 min. The PVDF membranes were blocked in Tris-buffered saline, 0.5% Tween-20 (TBST) containing 5% BSA for 2 h and incubated overnight at 4˚C with primary antibodies to p53 (1:1,000; CST), vimentin (1:1,000), CK5 (1:1,000), N-cadherin (1:1,000), E-cadherin (1:1,000) (all from Abcam), C-cadherin (1:1,000; PTG) and GAPDH (1:1,000; Abcam) in TBST containing 5% BSA. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG was used as the secondary antibody. The membranes were then visualized by an ECL chemiluminescence system (GE ImageQuant 350; GE Healthcare). Semi-quantitative analysis was performed using the Quantity One software (Bio-Rad). The experiments were performed in triplicate.

Flow cytometric analysis. Seventy-two hours after transfection of pGGNeo-p53-homo-270-shRNA or pGGNeo-negative-shRNA, SACC-83 cells, at $2x10^6$ for each sample, were harvested by trypsinization and fixed in 75% pre-cold ethanol at 4˚C for 24 h. Cell pellets were re-suspended in 0.1 mg/ml propidium iodide (PI) solution ($3.8x10^{-2}$ sodium citrate, pH=7.0) and fixed in 10 mg/ml RNase solution (both from Sigma, USA) at 37˚C for 30 min in the dark. Cell cycle analyses were performed with a flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA). The experiments were performed in triplicate.

Apoptosis assay. Seventy-two hours after transfection of pGGNeo-p53-homo-270-shRNA, both transfected and untransfected SACC-83 cells, at 10^5-10^6 for each sample, were trypsinized, collected and washed twice with pre-cold 1X PBS. Each sample was re-suspended in 100 μl 5% Annexin V-FITC (ADL, USA) at 37˚C for 15 min in the dark and then fixed in 10 μl PI. Annexin V-FITC and PI were measured and analyzed by flow cytometry. The experiments were performed in triplicate.

In vitro PNI assay. The promoting effects of p53 downregulation on PNI activity in SACC-83 cells were investigated in modified Boyden chambers. Each Transwell invasion chamber containing polycarbonate filters (Corning, USA) was coated on the upper surface with 60-80 μl of 3.9 μg/μl basement

membrane Matrigel (BD Biosciences, USA) at 37˚C for 30 min. Cells ($5x10^4$) were suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 1% FBS and added to the upper chamber. The lower chamber contained 600 μl conditioned medium (incubating NIH3T3 cells in serum-free DMEM for 24 h) as a chemoattractant (28,29). A concentration of 25 ng/ml of nerve growth factor (R&D Systems, USA) which has been proven optimal for increasing the *in vitro* PNI activity by our previous study was added into the conditioned medium (29,30). Cells were incubated at 37˚C with 5% CO_2 for 12 h. Then, cells on the upper surface of the filter were completely removed by a cotton swab. The filter was then fixed in 95% ethanol and stained with hematoxylin. Cells that had reached the lower surface of the filter through Matrigel were counted under a light microscope at a magnification of x400. We chose eight fields of vision and counted the number of invaded cells on the lower surface of the filter. The assays were performed in triplicate.

Statistical analysis. All data presented here are expressed as the means ± standard deviation (SD). Statistical analyses were performed using computer software, Microsoft SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with Tukey's *post-hoc* analysis was used to determine the difference between every two groups. P<0.05 was considered to indicate a statistically significant result.

Results

pGGNeo-p53-homo-270-shRNA effectively downregulates the expression level of p53 in SACC-83 cells. As shown in Fig. 1A, mRNA p53 expression in the SACC-83 cells transfected with pGGNeo-p53-homo-1043-shRNA, pGGNeo-p53-homo-1157-shRNA, pGGNeo-p53-homo-968-shRNA and pGGNeo-p53-homo-270-shRNA p53 mRNA was relatively decreased by 36, 62, 51 and 78% as compared with the untransfected SACC-83 cells. Furthermore, western blot analyses showed reduced expression of p53 proteins in SACC-83 cells transfected with pGGNeo-p53-homo-1043-shRNA, pGGNeo-p53-homo-1157-shRNA, pGGNeo-p53-homo-968-shRNA and pGGNeo-p53-homo-270-shRNA (Fig. 1B). The cells transfected with pGGNeo-p53-homo-270-shRNA showed the most significant decrease in the expression of p53 protein. These results demonstrated that p53 was downregulated most specifically and effectively by pGGNeo-p53-homo-270-shRNA. Thus, cells transfected with pGGNeo-p53-homo-270-shRNA were used in the present investigation.

shRNA-mediated downregulation of p53 induces 'EMT-like changes' in SACC-83 cells. As shown in Fig. 2, expression levels of epithelial cell markers, including E-cadherin, EMA and CK5 were significantly decreased in the experimental group (p53-homo-270) as compared with the negative or blank control group (P<0.05). The experimental group also showed significant increases in the expression levels of mesenchymal cell markers, including vimentin, N-cadherin and C-cadherin (P<0.05). These results suggest that pGGNeo-p53-homo-270-shRNA-mediated downregulation of p53 induced 'EMT-like changes' in the SACC-83 cells.

Table I. Oligonucleotide sequences of the p53-specific shRNAs.

Genes	Primer sequence (5'-3')
pGGNeo-p53-homo-1043-shRNA	GCGCACAGAGGAAGAGAATCT
	AGATTCTCTTCCTCTGTGCGC
pGGNeo-p53-homo-1157-shRNA	GAAACCACTGGATGGAGAATA
	TATTCTCCATCCAGTGGTTTC
pGGNeo-p53-homo-968-shRNA	GGAAGACTCCAGTGGTAATCT
	AGATTACCACTGGAGTCTTCC
pGGNeo-p53-homo-270-shRNA	CTACTTCCTGAAAACAACG
	CGTTGTTTTCAGGAAGTAG
pGGNeo-negative-shRNA	TTCTCCGAACGTGTCACGT
	ACGTGACACGTTCGGAGAA
pGGNeo-GAPDH-shRNA	GTATGACAACAGCCTCAAG
	CTTGAGGCTGTTGTCATAC

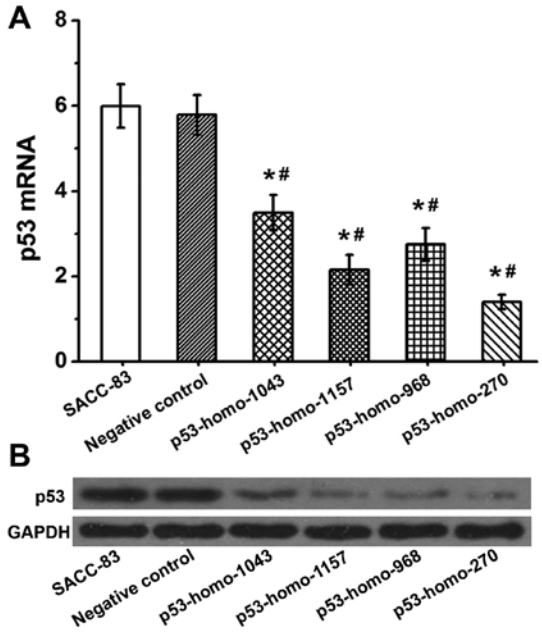

Figure 1. p53-specific shRNAs resulted in the reduction of p53 mRNA and protein expression in SACC-83 cells. The p53 mRNA level and protein expression were measured by (A) real-time-PCR and (B) western blotting, respectively. Data are presented as the means ± SD (n=3). *P<0.05 vs. the control (SACC-83) group. #P<0.05 vs. the negative control group. SACC, salivary adenoid cystic carcinoma.

Downregulation of p53 by pGGNeo-p53-homo-270-shRNA increases the survival of SACC-83 cells. Seventy-two hours after transfection with pGGNeo-p53-homo-270-shRNA, the cell cycles distribution of the SACC-83 cells was examined to evaluate cell survival. Our results showed that cells in the control and negative control group resulted in cycling of ~12 and 13% of cells in the second G0/G1 phase, respectively (Fig. 3A). The percentage of cells in the second G0/G1 phase was decreased to ~3% in the experimental group (Fig. 3A). These data suggest that downregulation of p53 promoted the anti-apoptotic ability of SACC-83 cells by modulating the second G0/G1 cell cycle regulator. As shown in Fig. 3B, the early apoptotic rates in

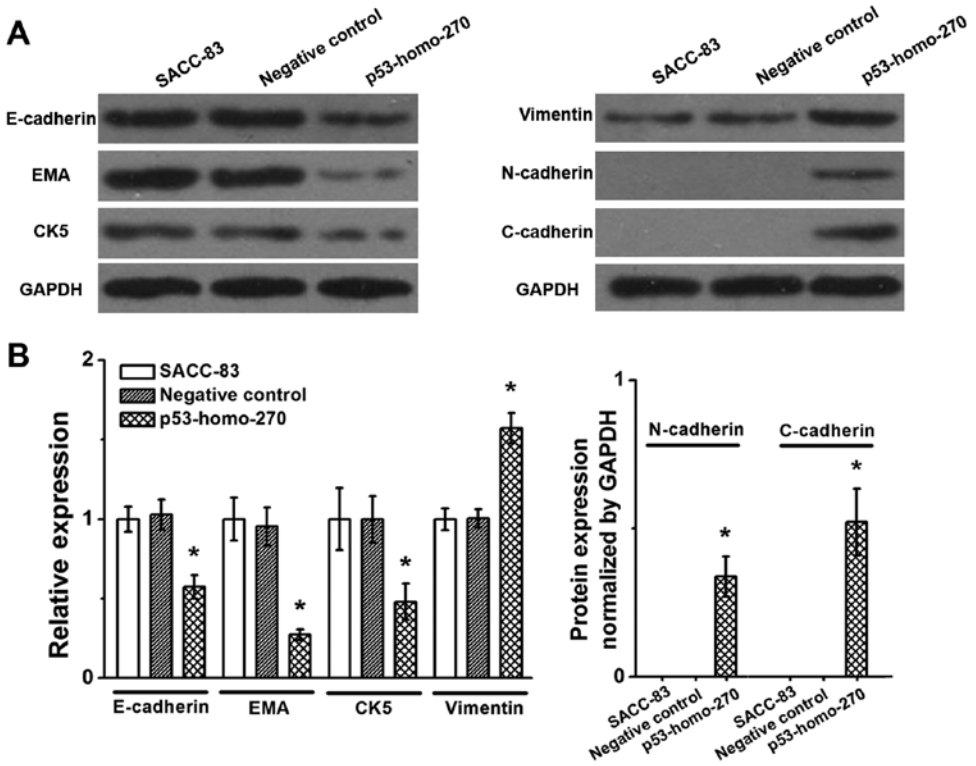

Figure 2. Downregulation of p53 induces 'EMT-like changes' in SACC-83 cells. Markers of 'EMT-like changes' were detected by western blotting. (A) The protein levels of epithelial cell markers (E-cadherin, EMA and CK5) and mesenchymal cell markers (vimentin, N-cadherin and C-cadherin) were detected by western blot analyses in the control group (SACC-83), negative control and experimental group (p53-homo-270). (B) Quantitative protein expression analyses normalized by GAPDH for epithelial cell markers and mesenchymal cell markers. Data are presented as the means ± SD (n=3). *P<0.05 vs. the control (SACC-83) group. #P<0.05 vs. the negative control group. EMT, epithelial-mesenchymal transition; SACC, salivary adenoid cystic carcinoma.

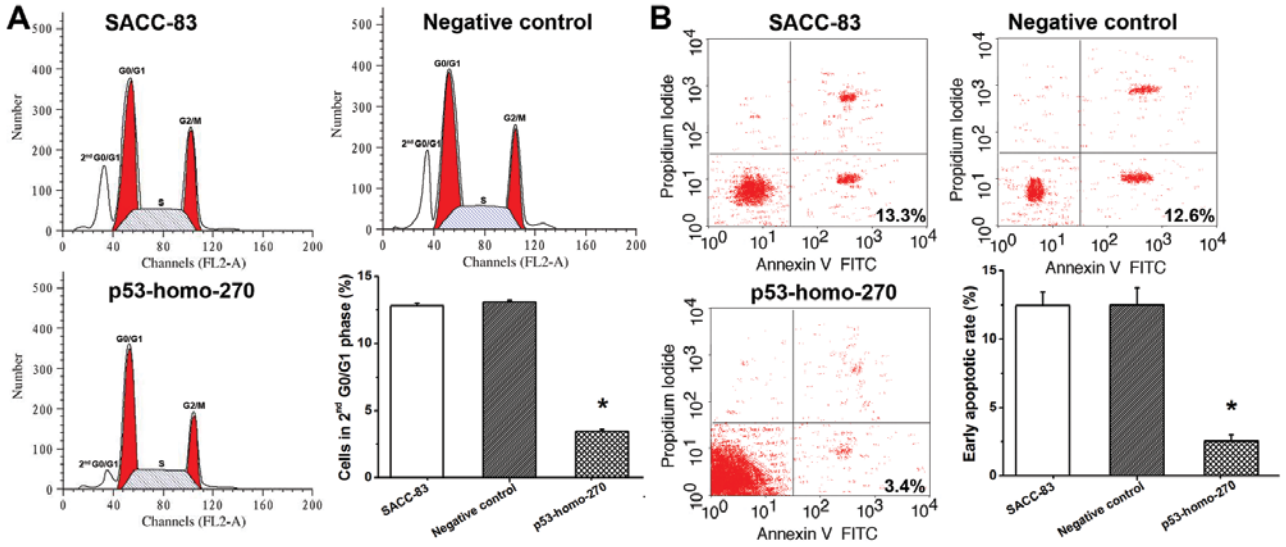

Figure 3. Downregulation of p53 increases the survival of SACC-83 cells. (A) Results of the flow cytometric analyses for the cell number in the second G0/G1 phase in the control group (SACC-83), negative control and experimental group (p53-homo-270). (B) Analyses of the early apoptosis rates of SACC-83 cells in the control group (SACC-83), negative control and experimental group (p53-homo-270). Data are presented as the means ± SD (n=3). *P<0.05 vs. the control (SACC-83) group. #P<0.05 vs. the negative control group. SACC, salivary adenoid cystic carcinoma.

the control and negative control group were 13.3 and 12.6%, respectively. In the experimental group, the early apoptotic rate was significantly reduced to 3.4% (Fig. 3B). These findings were consistent with the cell cycle results, indicating that downregulation of p53 promoted the anti-apoptotic ability and thus increased cell survival of SACC-83 cells.

Downregulation of p53 by pGGNeo-p53-homo-270-shRNA increases in vitro PNI of SACC-83 cells. PNI activity of the SACC-83 cells transfected with pGGNeo-p53-homo-270-shRNA was assayed *in vitro* using modified Boyden chambers. As compared with the control group (SACC-83) and negative control group, SACC-83 cells in the experimental group

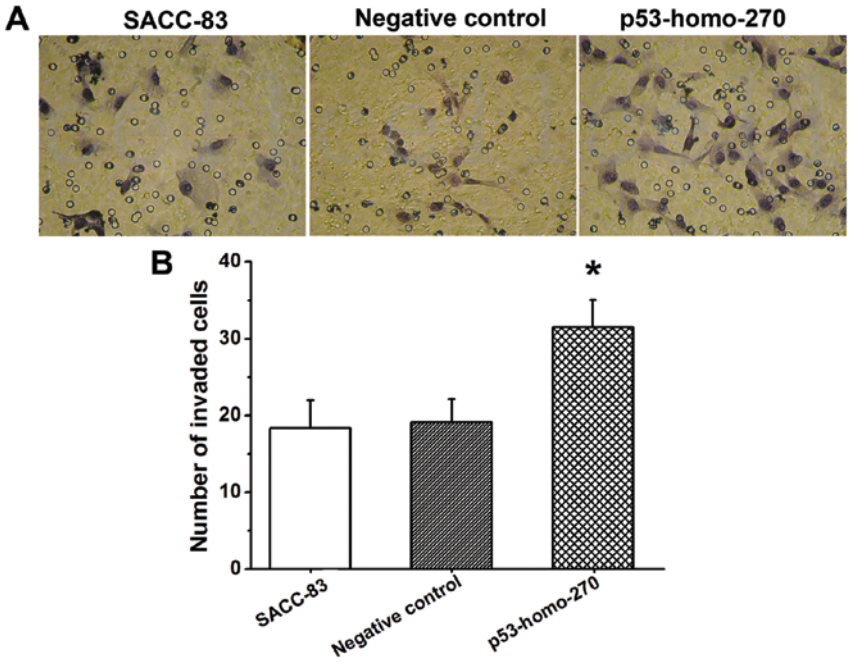

Figure 4. Downregulation of p53 increases the *in vitro* PNI activity of SACC-83 cells via the evaluation of Boyden chambers. (A) Representative images and (B) the relevant statistical analyses for the migrated cells under a light microscope in the control group (SACC-83), negative control and experimental group (p53-homo-270). Data are presented as the means ± SD (n=3). *P<0.05 vs. the control (SACC-83) group. #P<0.05 vs. the negative control group. PNI, perineural invasion; SACC, salivary adenoid cystic carcinoma.

(p53-homo-270) showed significantly higher PNI activity (P<0.01) (Fig. 4). These findings indicate that the downregulation of p53 promoted the PNI activity of SACC-83 cells.

Discussion

p53 is a recognized tumor-suppressor gene, located on the short arm of human chromosome 17p13 which shows loss of heterozygosity (LOH) status in most tumors. The p53 gene can activate the transcription of cyclin-dependent kinase inhibitor p21, and then induce G1 phase arrest of tumor cells cycling through p21. This process has been investigated and widely recognized in previous research (31). The p53 gene can also inhibit malignant growth by promoting the apoptosis of tumor cells (32). Many studies have also confirmed that the p53 gene can inhibit the process of malignant transformation and malignant proliferation of tumor cells (33-37).

EMT is considered to be closely related to tumor development, malignant tumor local invasion and distant metastasis (15-20). Generally, tumor cells always exhibit poor differentiation, which may result in various differentiation errors (e.g., EMT) during the tumor cell differentiation process. Thus, it seems more appropriate to use 'EMT-like changes' to describe the tumor cell phenotype in the process of tumor genesis (22). In addition, 'EMT-like changes' also stimulate other properties of tumor cells during tumor progression, such as enhanced migration activity, elevated anti-apoptotic capability and increased invasive activity (21,22). The present study focused on depicting the regulatory mechanisms of 'EMT-like changes' and clarifying the role of 'EMT-like changes' in tumor development and metastasis. To our knowledge, this is the first report to investigate the relationship between 'EMT-like changes' and PNI. The regulatory role of the p53 gene in EMT or 'EMT-like changes' also remains poorly understood. Chang *et al* reported that p53 acted as a key factor in the regulation of the EMT process of breast cancer (25). Considering that the mutation rate of the p53 gene in head and neck cancer is high (50-70%), we hypothesized in the present study that downregulation of p53 may induce 'EMT-like changes' in SACC and thus enhance its PNI activity.

In the present study, we used the RNA interference technique to reduce p53 gene expression in SACC-83 cells, and subsequently detected alterations in 'EMT-like change' markers. We found that pGGNeo-p53-homo-270-shRNA effectively reduced the expression of p53. Our western blot analyses demonstrated that downregulation of the p53 gene in SACC-83 cells caused significantly reduced expression levels of epithelial cell markers (E-cadherin, CK5 and EMA), and significantly increased expression levels of mesenchymal cell markers (vimentin, N-cadherin and C-cadherin). These results revealed obvious 'EMT-like changes' and suggest that downregulation of p53 induced 'EMT-like changes' in the SACC-83 cells.

Seventy-two hours after transfection with pGGNeo-p53-homo-270-shRNA, we examined the cell cycle distribution of SACC-83 cells with 'EMT-like changes'. We found that the number of SACC-83 cells in the second G0/G1 phase cell cycle was significantly reduced, indicating that downregulation of p53 promoted the anti-apoptotic activity of SACC-83 cells. Furthermore, the Annexin V-FITC and PI apoptosis experiment was performed to evaluate the anti-apoptotic activity of SACC-83 cells with 'EMT-like changes'. Our results showed that the number of early apoptotic cells was significantly decreased. Thus, these findings suggest that 'EMT-like changes' induced by p53 downregulation could increase the anti-apoptotic activity of SACC-83 cells and thus promote

cell survival. We also examined the *in vitro* PNI capacity of SACC-83 cells with 'EMT-like changes'. Our results indicated that 'EMT-like changes' induced by p53 gene downregulation enhanced the *in vitro* PNI capability of the SACC-83 cells. Together, these findings in the present study revealed that downregulation of the p53 gene induced 'EMT-like changes' in SACC-83 cells which increased the *in vitro* PNI activity in these cells.

SACC is a common malignant salivary gland tumor characterized by PNI. PNI has an important impact on clinical treatment and prognosis. Therefore, it is imperative to study the mechanism of PNI of SACC, thus providing a potentially new strategy for the clinical treatment of SACC. Since a replication-defective p53 gene virus (Ad5CMV P53) was reported in 1994 (38), p53-based gene therapy for tumors has gained extensive attention. Roth *et al* directly injected a retroviral vector containing the wild-type p53 gene into patients with non-small cell lung cancer under control of the actin promoter and achieved satisfactory therapeutic effects (39). Moreover, many clinical trials regarding p53 have also been completed, including its application in esophageal cancer (40). Several trials have reached the third stage, yet p53 gene therapy has not yet received final approval from the FDA (41). In the present study, we found that the downregulation of the p53 gene could induce 'EMT-like changes' in SACC-83 cells, and thus these 'EMT-like changes' promoted *in vitro* PNI activity of SACC-83 cells. Our findings suggest that the p53 gene may be used as a new target gene for the clinical treatment of SACC.

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