Co-expression of ING4 and P53 enhances hypopharyngeal cancer chemosensitivity to cisplatin *in vivo*

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Abstract. Hypopharyngeal cancer is a distinct type of malignant head and neck tumor, which exhibits low sensitivity to anti-cancer drugs. The importance of developing methods for reducing chemotherapy resistance, and improving and enhancing prognosis has previously been emphasized and is considered a challenge for effective clinical treatment of hypopharyngeal cancer. The current study investigated the effects of co-expression of inhibitor of growth protein 4 (ING4) and P53, a tumor suppressor gene, on chemosensitivity to cisplatin in human hypopharyngeal cancer xenografts *in vivo*, and the potential molecular mechanisms involved. A tumor model was established by injecting athymic nude mice with FADU human hypopharyngeal cancer cells. Five days after intratumoral and peritumoral injections of an empty adenoviral vector (Ad), Ad-ING4-P53, cisplatin, or a combination of Ad-ING4-P53 and cisplatin (Ad-ING4-P53 + cisplatin) every other day for 5 days, the mice were euthanized and their tumors, livers, and kidneys were removed. The tumor weights were used to calculate the inhibition rate, and the expression levels of ING4 and P53 were detected by reverse transcription-polymerase chain reaction. Additionally, apoptotic cells were detected using terminal deoxynucleotidyl transferase dUTP nick end labeling, and immunohistochemistry determined the levels ING4, P53, B-cell lymphoma-2 (Bcl-2) and Bcl-2 associated X protein (Bax) protein expression. The results demonstrated increased expression of ING4 and P53 in the Ad-ING4-P53 groups compared with PBS and Ad groups, indicating successful introduction of the genes into the tumor cells. Notably, the Ad-ING4-P53 + cisplatin group exhibited a higher inhibition rate compared with the four other groups. The results of immunohistochemistry analysis demonstrated that Bax expression was increased and Bcl-2 was decreased in the Ad-ING4-P53 + cisplatin group. This suggested that the enhanced cisplatin chemosensitivity with Ad-ING4-P53 gene therapy in hypopharyngeal cancer xenografts may be associated with apoptosis induction through upregulation of Bax expression and downregulation of Bcl-2. The results of the present study indicated that gene therapy combined with cisplatin treatment may be a promising treatment for human hypopharyngeal cancer.

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Abbreviations: Ad, adenoviral vector; ING4, inhibitor of growth protein 4

Key words: hypopharyngeal cancer, ING4, P53, gene therapy, chemosensitivity

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common malignancies worldwide, and is characterized by high invasiveness, early metastasis, recurrence and difficult early detection (1,2). Hypopharyngeal cancer is a distinct type of malignant head and neck tumor currently treated by surgical procedures, radiotherapy and chemotherapy, which have numerous side effects, including loss of larynx function that severely affects quality of life. Despite the apparent advances in surgery in recent years, distant metastases and recurrence have remained concerns (3).

Tumor development is a multi-step process involving multiple genes, which includes the activation of proto-oncogenes and inactivation of tumor suppressor genes (4). With improved understanding of the molecular mechanisms underlying this process, gene therapy exhibits increasing potential for use as a novel cancer treatment. Hypopharyngeal cancer exhibits low sensitivity to anti-cancer drugs. Its strong resistance to various anti-tumor therapies and the unknown underlying mechanism lead to unfavorable prognosis and a low five-year survival rate for patients (5). Therefore, the importance of developing methods for avoiding chemotherapy resistance, and improving and enhancing prognosis has been emphasized, and is considered a challenge for effective clinical treatment of hypopharyngeal cancer.

Previous studies have demonstrated that gene therapy using a combination of two genes can promote tumor cell apoptosis. For example, Luo *et al* (6) reported that the adenovirus-mediated CD/TK double suicide gene, driven by a survivin promoter, specifically inhibits gastric cancer cells to a greater extent than that observed with a single suicide gene.

Therefore, the effect of combined gene therapy on hypopharyngeal tumor survival was investigated.

The inhibitor of growth protein 4 (ING4) gene was originally identified by Shiseki *et al* (7) and later recognized as an important factor in tumor growth inhibition (8). It is expressed in all cells, including normal tissues. Extensive studies have demonstrated that ING4 is critical for gene transcription, cell proliferation, apoptosis and senescence, cell contact inhibition, DNA damage repair, and tumor invasion and metastasis (9-12).

Recent studies have packaged the ING4 gene into an adenoviral vector (Ad-ING4) for introduction into various human tumor cells, including malignant melanoma (13), breast cancer (14), human lung adenocarcinoma (15) and osteosarcoma (16). These studies demonstrated significantly higher growth inhibition and apoptosis in tumor cells with Ad-ING4 compared with cells infected with the empty vector, indicating that ING4 inhibits the growth of tumor cells and induces their apoptosis. A previous study demonstrated that there were decreased expression levels of ING4 in HNSCC and concluded that it is important in cancer cell apoptosis and is anti-proliferative (17). However, its function and mechanism of action remain unknown and require further study.

P53, the first member of the P53 family to be identified, is associated with various types of cancer, including sporadic cancers, which are correlated with mutations in somatic cells (18). Although wild-type P53 inhibits tumor development and progression, a previous study demonstrated that P53 is generally mutated in tumor cells to induce and promote tumor development (19). Gene therapy with wild-type P53 and other P53 family members was observed to be effective and safe for the treatment of pulmonary metastatic tumors from hepatocellular carcinoma (20) and inhibited proliferation and apoptosis in osteosarcoma cell lines (21). Furthermore, previous investigation demonstrated that ING4 induces apoptosis through a P53-dependent pathway (22,23).

The present study used combination gene therapy with ING4 and P53 to treat hypopharyngeal cancer *in vivo*. Mutations identified in 40-70% of HNSCC tumors occur in P53 (24), providing a theoretical basis for adenovirus-mediated combination gene therapy to inhibit hypopharyngeal cancer cell growth and proliferation. Thus, it was hypothesized that combination treatment with ING4 and P53 tumor suppressors would enhance tumor chemosensitivity.

Materials and methods

Cell lines. The FADU human hypopharyngeal cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd. (Huzhou, China) and 1% antibiotic solution (100X penicillin and streptomycin; Beyotime Institute of Biotechnology, Haimen, China) at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2.

Virus propagation and purification. Adenoviral vectors with the ING4 and P53 genes were provided by Professor Jicheng Yang (Soochow University, Suzhou, China). QBI 293A cells (American Type Culture Collection) with the adenoviral vectors to amplify the recombinant virus promoter, and specific steps and titers were measured by conventional methods as described previously (16). The QBI 293A cells were cultured as described for the FADU cells, however, in RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc.) rather than DMEM.

Animals. Specific-pathogen free BALB/c nu/nu nude mice (25 males; weight, 22-25 g; age, 5-6 weeks) were obtained from the Changzhou Cavens Laboratory Animal Co. [Changzhou, China; certificate no. SCXK (Su) 2011-0003 Su regulatory certificate no. 201403734]. They were maintained and used for the experiments performed at the Laboratory Animal Center of Bengbu Medical University (Anhui, China; certificate no. Wan SYXK 2012-002). The current study was approved by the appropriate ethical review boards for the use of laboratory animals. The animals were fed as standard with access to drinking water. The temperature was maintained at 23-28˚C and relative humidity was 40-60%, with a natural light/dark cycle.

Establishment of tumor models. Each mouse was injected subcutaneously with $1x10^6$ human FADU hypopharyngeal cancer cells in the axilla of the right anterior limb. The tumor dimensions were measured 2-3 times per week with a caliper, and the tumor volume was calculated as follows: Tumor size = $axb^2/2$, where a and b represent the larger and smaller of the two dimensions, respectively.

Experimental design and preparation of cisplatin. The mice were randomly assigned to five groups (five mice per group) when the tumors developed to a mean volume of 60-80 mm^3 after ~20 days. The xenograft tumor-bearing mice were intratumorally and peritumorally injected with phosphate-buffered saline (PBS control), empty adenoviral vector [Ad; 0.1 ml, $1x10^8$ plaque forming units (pfu)], Ad-ING4-P53 (0.1 ml, $1x10^8$ pfu), cisplatin (0.1 ml, 300 μg), or Ad-ING4-P53 (0.1 ml, $1x10^8$ pfu) and cisplatin (0.1 ml, 300 μg) every other day for five days. Cisplatin (3 mg; Qilu Pharmaceutical Co., Ltd., Jinan, China) was dissolved in 1 ml normal saline and used at 3 μg/μl in all experiments.

Five days after treatment, the mice were sacrificed by cervical dislocation. The transplanted tumors were removed and weighed to calculate the inhibition rate as follows: Inhibition rate = (1-mean experimental tumor weight/mean control tumor weight)x100%. Based on the inhibition rate, the combined effect was evaluated from the Q values (25) as follows: Q value = E (A + B)/(EA + EB - EA x EB), where EA and EB are the effects of molecules A and B alone, respectively, E (A + B) is the combined effect, and the denominator represents the expected effects when the two are combined. Molecules A and B are interpreted as having additive effects when Q=1±0.15, synergistic effects when Q>1.15, and antagonistic effects when Q<0.85.

Reverse transcription-polymerase chain reaction (RT-PCR). Gene transcript levels were detected by RT-PCR analysis. Total RNA was extracted from the xenografted tumors using TRIzol according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). The RT-PCR reactions were

Table I. Reverse transcription-polymerase chain reaction primers.

Name	Primer sequence	Size (bp)
GADPH		240
F	5'-TGATGACATCAAGAAGGTGGTGAA-3'	
R	5'-TCCTTGGAGGCCATGTGGGCC-3'	
ING4		750
F	5'-TAGAGATCTACCATGGCTGCTGGGATGTATTTGG-3'	
R	5'-ACCGTCGACCCTATTTCTTCTTCCGTTCTTG-3'	
P53		259
F	5'-CCTCCTCAGCATCTTATCCG-3'	
R	5'-CACAAACACGCACCTCAAA-3'	
Bcl-2		304
F	5'-TTCTTTGAGTTCGGTGGGGTC-3'	
R	5'-TGCATATTTGTTTGGGGCAGG-3'	
Bax		257
F	5'-TCCACCAAGAAGCTGAGCGAG-3'	
R	5'-GTCCAGCCCATGATGGTTCT-3'	

ING4, inhibitor of growth protein 4; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein.

performed using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) to generate cDNA and then a PCR Master mix (2X; Thermo Fisher Scientific, Inc.) in an ABI StepOne™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) by using degenerate primers (Sangon Biotech Co., Ltd., Shanghai, China) (Table I). The thermocycling conditions were as follows: 95˚C for 3 min; 40 cycles of 95˚C for 30 sec, 61.5˚C, 52.5˚C, 59˚C or 58˚C for 30 sec (for ING4, P53, Bax and Bcl-2, respectively) and 72˚C for 1 min; followed by 72˚C for 10 min. GAPDH served as the control. The PCR products were separated on 1.5% agarose gel.

Hematoxylin and eosin (H&E) staining. The mice were sacrificed by cervical dislocation 5 days after drug administration, and the xenografted tumors, livers and kidneys were removed to observe the distribution of tumor cells, and the tumor metastasis and cytotoxicity of the livers and kidneys. Tissue sections were incubated overnight in neutral formalin buffer (10%) and then stored in ethanol and embedded in paraffin. Cross-sections (4 μm) were stained with H&E and observed under a microscope (BX43; Olympus Corporation, Tokyo, Japan), and tumor cells were identified by the following characteristics: Small volume, condensed cytoplasm, small nuclei, condensed and fractured chromatin, nuclei migrated to the cell edge, or apoptotic bodies with karyorrhexis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Sections of tumor tissue were fixed in 10% neutral formalin buffer according to the standard procedure and then stained using an *In Situ* Cell Apoptosis Detection Kit IV following the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA) using conventional methods as described previously (26). The sections were analyzed under a confocal microscope (x200). Yellow granules in the nucleus indicated apoptotic cells.

Immunohistochemistry in xenografted tumors. The expression of ING4, P53, B-cell lymphoma-2 (Bcl-2), and Bcl-2 associated X protein (Bax) in the xenografted tumors was analyzed by immunohistochemistry using an Ultrasensitive™ SP kit (Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China). Tissue sections were deparaffinized in dimethyl benzene and dehydrated by an alcohol gradient. The sections were incubated in 3% H_2O_2 for 10 min to block inactivated endogenous peroxidase. For antigen retrieval, the sections were placed in 0.01 M citrate buffer (pH 6.0) and boiled at 95˚C for 15-20 min, cooled at room temperature for 20 min, and washed with cold water to facilitate cooling. Then sections were sealed in normal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd.) for 10 min at 37˚C, and then the bovine serum was removed. The polyclonal rabbit anti-mouse antibodies were incubated for 1 h at room temperature and were as follows: Anti-ING4 (1:50; cat. no. 16188-1-AP), anti-P53 (1:100; cat. no. 10442-1-AP), anti-Bcl-2 (1:50; cat. no. 12789-1-AP) and Bax (1:50; cat. no. 23931-1-AP; all from Proteintech Group, Inc., Chicago, IL, USA) Biotinylated goat anti-rabbit secondary antibodies (1:40; Proteintech Group, Inc.; cat. no. SA00001-2) were then incubated for 20 min at room temperature. Sections were subsequently incubated in peroxidase substrate mixing liquid (Fuzhou Maixin Biotech Co., Ltd.) and washed in deionized water. Finally, the sections were counterstained with hematoxylin, dehydrated with deionized water, dried and sealed. They were observed by microscopy (BX43). Positive gene expression was indicated by the presence of yellow diaminobenzidine precipitates.

Statistical analysis. Data were analyzed by SPSS for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA) and are presented as the mean ± standard error of the mean. Differences between groups were evaluated by one-way analysis of variance (ANOVA), two-way ANOVA, and Dunnett's multiple

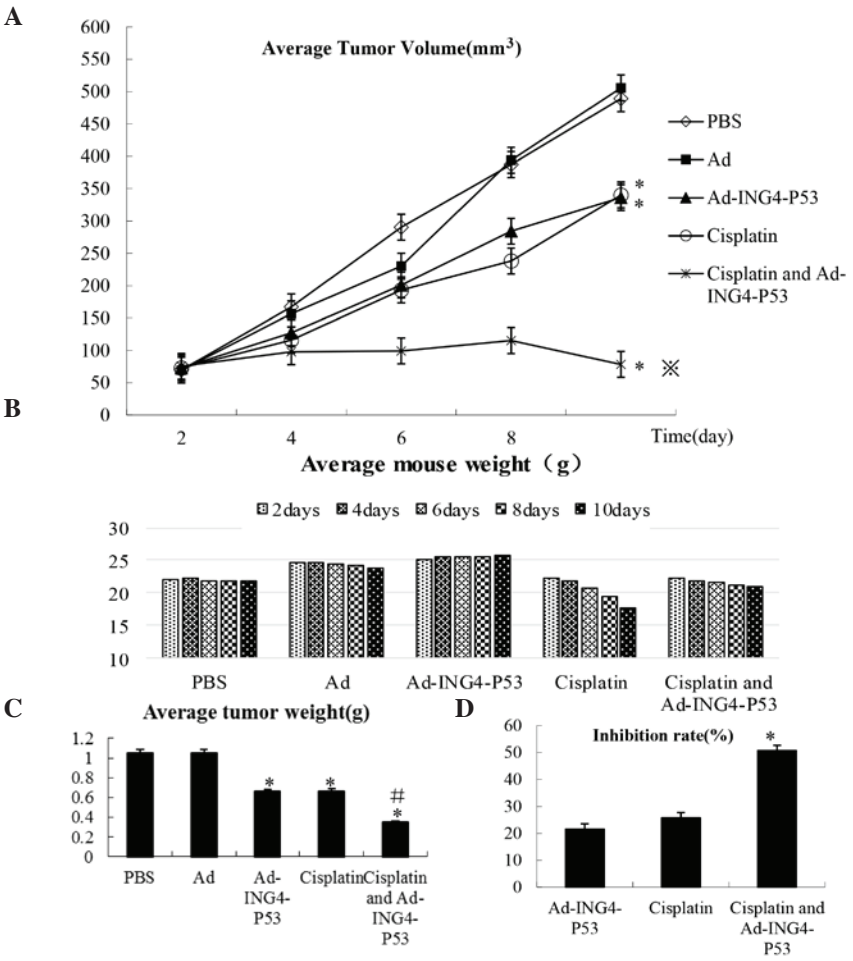

Figure 1. Effect of Ad-ING4-P53 of FADU cells. (A) FADU human hypopharyngeal xenograft tumor volumes. *P<0.05 vs. PBS and Ad groups; #P<0.05 vs. Ad-ING4-P53 and cisplatin groups. Data were analyzed with one-way and two-way analysis of variance with repeated measures and multiple comparisons (n=5 mice/condition). (B) Weights of xenograft tumor-bearing mice. The mice injected with cisplatin demonstrated weight loss, whereas the others grew normally. (C) FADU human hypopharyngeal xenograft tumor weights. *P<0.05 vs. PBS and Ad groups; #P<0.05 vs. Ad-ING4-P53 group and cisplatin group. Data were analyzed with one-way and two-way analysis of variance with repeated measures and multiple comparisons (n=5 mice/condition). The data represent three independent experiments. (D) Tumor growth inhibition rates. *P<0.05 compared with the Ad-ING4-P52 and cisplatin groups (Q=1.19). One-way and two-way ANOVA with repeated measures and multiple comparisons (n=5 mice/condition). The data shown represent three independent experiments. The values are presented as the mean ± standard error of the mean. PBS, phosphate-buffered saline; Ad, adenovirus; ING4, inhibitor of growth protein 4.

comparisons test. P<0.05 was considered statistically significant.

Results

Establishment of tumor models. Subcutaneous solid nodules developed gradually for 6-7 days post-injection. At ~20 days later, the mean tumor volume was 60-80 mm^3. All 25 nude mice were tumorigenic, with a tumor formation rate of 100%. During treatment, the mice exhibited dry skin, however there were no significant changes in their stool color, appetite or behavior. The mice treated with cisplatin and a combination of Ad-ING4-P53 and cisplatin (Ad-ING4-P53 + cisplatin) exhibited weight loss, which was most notable in the cisplatin group. Following sacrifice of the mice, no significant macroscopic or microscopic lesions were observed in the organs.

Effect of Ad-ING4-P53 + cisplatin on FADU cell inhibition. Following establishment of the tumor models, the xenograft tumor-bearing mice were intratumorally and

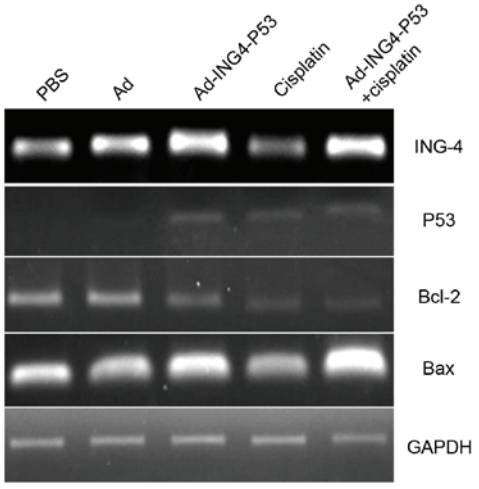

Figure 2. Gene expression in xenograft tumors. Reverse transcription-polymerase chain reaction detection of gene expression. GAPDH served as the control. The Ad-ING4-P53 group and the Ad-ING4-P53 + cisplatin group exhibit a strongly positive ING4/P53 band compared with the PBS, Ad and cisplatin groups. PBS, phosphate-buffered saline; Ad, adenovirus; ING4, inhibitor of growth protein 4; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein.

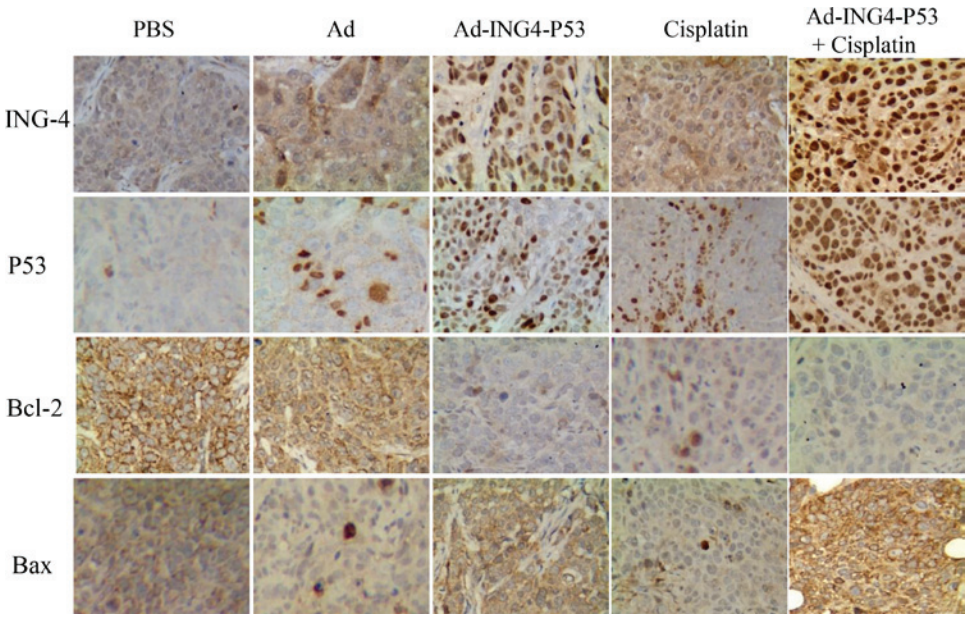

Figure 3. Immunohistochemical analysis of xenografted tumors. Immunohistochemistry was used to detect the expression levels of ING4, P53, Bcl-2, and Bax. The Ad-ING4-P53 + cisplatin group showed increased ING4, P53, and Bax expression and significantly decreased Bcl-2 expression. PBS, phosphate-buffered saline; Ad, adenovirus; ING4, inhibitor of growth protein 4; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein.

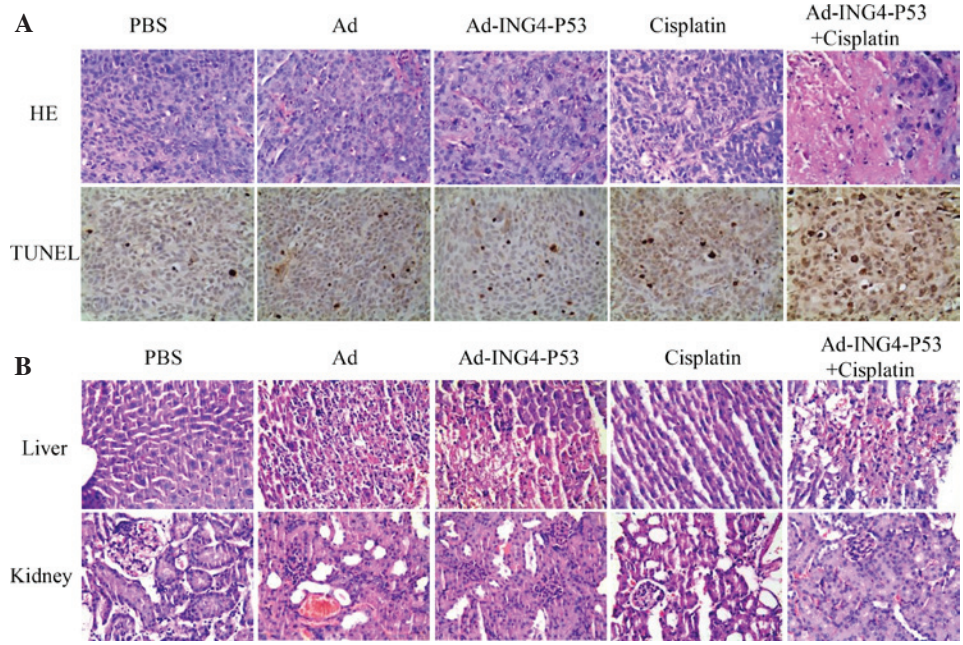

Figure 4. Effect of Ad-ING4-P53 on tumor cell apoptosis. (A) Xenografted tumors were removed to observe the distribution of tumor cells and tumor metastasis via HE staining and TUNEL assay. The results demonstrated that Ad-ING4-P53 + cisplatin exhibited an effect similar to that of cisplatin alone, while Ad-ING4-P53 enhanced tumor cell apoptosis. (B) Liver and kidney cytotoxicity. Pathological cross-sections of livers and kidneys from human tumor-bearing nude mice were stained with HE and observed under the microscope. The results demonstrated potential liver toxicity following treatment with Ad or Ad-ING4-P53. HE, hematoxylin and eosin; PBS, phosphate-buffered saline; Ad, adenovirus; ING4, inhibitor of growth protein 4; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2 associated X protein.

peritumorally injected with PBS, Ad, Ad-ING4-P53, cisplatin, or Ad-ING4-P53 + cisplatin every other day for five days. We measured the xenograft tumor volumes and weighed the mice every other day (Fig. 1A and B) and removed and weighed the tumors five days after treatment (Fig. 1C).

Compared with the cisplatin and Ad-ING4-P53 groups, Ad-ING4-P53 + cisplatin significantly inhibited the growth of FADU hypopharyngeal cancer cells in nude mice bearing transplantation tumors ($P<0.05$; Fig. 1D) and exerted a synergistic effect ($Q=1.19$).

Expression of ING4 and P53. Results of the RT-PCR analysis of ING4 and P53 gene expression levels are demonstrated in Fig. 2. GADPH was used as a reference gene. The Ad-ING4-P53 and Ad-ING4-P53 + cisplatin groups demonstrated strong positive ING4/P53 bands compared with the PBS, Ad and

cisplatin groups. Furthermore, immunohistochemical analysis demonstrated no obvious expression of ING4/P53 in the PBS, Ad and cisplatin groups, whereas the Ad-ING4-P53 and Ad-ING4-P53 + cisplatin groups demonstrated positive ING4 and P53 expression (Fig. 3).

Apoptotic gene expression changes in xenografted tumors. To determine the potential molecular mechanisms of apoptosis induction, Bcl-2 and Bax expression levels were detected in the tumor tissue using RT-PCR. Compared with the PBS group, the gene expression of the Ad group changed only marginally, whereas the Ad-ING4-P53 and Ad-ING4-P53 + cisplatin groups demonstrated strongly positive Bax expression and negative Bcl-2 expression, with a greater change in the Ad-ING4-P53 + cisplatin group (Fig. 2) compared with the PBS and Ad groups. These results were further supported by immunohistochemical analysis which demonstrated the Ad-ING4-P53 + cisplatin group exhibited increased ING4, P53 and Bax expression levels and decreased Bcl-2 expression compared to other groups (Fig. 3).

Enhanced tumor apoptosis by Ad-ING4-P53 + cisplatin. Sections of xenografted hypopharyngeal tumors from nude mice were stained by H&E (Fig. 4A), and the tumor cells exhibited a nest-like distribution and disordered arrangement. In the PBS and Ad groups, the tumor cells were closely arranged with complete and atypical structures. By contrast, the Ad-ING4-P53, cisplatin and Ad-ING4-P53 + cisplatin groups exhibited a greater degree of tumor cell apoptosis, characterized by incomplete cell membranes, condensed cytoplasm, pyknotic or cracking nuclei, and cavity-shaped organization. The Ad-ING4-P53 group and cisplatin group demonstrated similar characteristic, whereas the apoptotic effect was more obvious in the Ad-ING4-P53 + cisplatin group.

TUNEL analysis demonstrated the apoptotic cells as small, with condensed nuclei, circumscribed nuclear membranes and yellow granules in the nuclei (Fig. 4A). Comparison of the five treatment groups indicated that Ad-ING4-P53, cisplatin and Ad-ING4-P53 + cisplatin accelerated apoptosis relative to PBS and Ad, with the greatest effect exerted by treatment with Ad-ING4-P53 + cisplatin.

Toxicity of gene therapy and cisplatin. H&E-stained liver and kidney sections from human hypopharyngeal tumor-bearing nude mice were observed microscopically (Fig. 4B). The PBS and cisplatin groups exhibited normal liver tissue, with clear lobule structures and orderly hepatic cords. However, the Ad, Ad-ING4-P53, and Ad-ING4-P53 + cisplatin groups exhibited irregularly bleeding necrotic areas, and their liver cell structures did not demonstrate marked inflammatory cell infiltration. However, the kidney microstructures were in good condition for all groups, with no pathological changes, such as blood extravasation or cell necrosis. Thus, the adenoviral vector induced liver toxicity.

Discussion

Cisplatin is the most commonly used chemotherapeutic drug, particularly for the treatment of head and neck cancer. However, the development of cisplatin resistance has limited its widespread clinical use (27-29). Therefore, identification of high-efficiency chemosensitization drugs to improve the efficiency of chemotherapy is an important direction of current research.

The ING4 gene was identified as an important tumor growth inhibition factor (8). Further studies demonstrated that ING4 exerts specific anti-tumor effects through various pathways and can induce tumor cell apoptosis. Zhang *et al* (30) established several HepG-2 hepatocellular carcinoma cell lines stably expressing ING4 and observed that ING4 inhibited HepG-2 cell growth and improved the sensitivity of liver cancer cells to DNA damage reagents, including adriamycin. Another important factor, P53, is regarded as an important factor in tumor activation and an essential gene for genome integrity (31). Kraljević Pavelić *et al* (32) introduced the P53 gene into Hep-2 and CAL27 HNSCC cell lines and demonstrated that p53 overexpression at sub-cytotoxic levels enhanced the activity of low doses of cisplatin and methotrexate through changes in the cell cycle.

Resistance to multiple anti-tumor therapies arises through complex mechanisms, and tumor cell apoptosis resistance is generally understood to be a major mechanism of multi-drug resistance. The majority of chemotherapy drugs act by inducing tumor cell apoptosis. The Bcl-2 gene inhibits apoptosis caused by various factors, including carcinogens and radioactive rays, abnormally extends cell survival time, promotes the accumulation of mutations and increases resistance to immune system monitoring (33). A previous study demonstrated an association between excessive expression of Bcl-2, and expression of tumor cell drug resistance genes and inhibition of apoptosis, which leads to drug resistance (34). Bax is an apoptosis-promoting gene in the Bcl-2 family. *In vivo*, Bax/Bax cognate dimer formation promotes cell apoptosis, whereas Bcl-2/Bax heterologous dimers inhibit apoptosis (35).

The present study demonstrated that the Ad, Ad-ING4-P53, and Ad-ING4-P53 + cisplatin groups exhibited liver toxicity, however they exerted no effect on kidney cells, consistent with previous demonstrations that adenoviral vectors damage liver function (36,37). Haisma *et al* (38) demonstrated that liver toxicity arises from removal of Kupffer cells by adenoviral vectors, potentially as an immune response to the virus and its transduction gene products (39). Future studies must investigate whether changes in the adenovirus vector structure or simultaneous of administration liver-protection drugs would abrogate the observed liver toxicity.

The current study was designed to investigate the chemosensitivity of a recombinant adenovirus co-expressing ING4 and P53, and to analyze the potential mechanisms underlying this process. The results of the present study demonstrated that Ad-ING4-P53 + cisplatin significantly inhibited the growth of FADU hypopharyngeal cancer cells in nude mice bearing transplanted tumors compared with the effect of cisplatin or Ad-ING4-P53 alone ($P<0.05$) and a synergistic effect between Ad-ING4-P53 and cisplatin was observed ($Q=1.19$). Immunohistochemical analysis of the expression of associated factors in transplanted tumors further indicated that Ad-ING4-P53 + cisplatin strongly increased Bax expression and decreased Bcl-2 expression compared with the levels observed with cisplatin or Ad-ING4-P53 alone, which is consistent with previous findings by Zhu *et al* (40). Thus, the

combination therapy may increase chemosensitivity through the Bcl-2/Bax pathway; however, elucidation of the specific underlying mechanism requires further research.

In conclusion, the results of the current study demonstrated that Ad-ING4-P53 improves the chemosensitivity of transplanted human hypopharyngeal tumors, potentially by increasing and decreasing the expression levels of Bax and Bcl-2, respectively, to induce tumor cell apoptosis. The co-expression of ING4 and P53 genes via a recombinant adenovirus synergistically enhanced the anti-tumor effects with simultaneous chemotherapy for human hypopharyngeal cancer *in vivo*. Further investigation is required to elucidate the specific underlying mechanism of action *in vitro* and *in vivo*, however, this work provides an experimental basis for these future studies and for clinical applications.

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