Enhanced antitumor activity by combining an adenovirus harboring ING4 with cisplatin for hepatocarcinoma cells

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The inhibitor of growth (ING) family proteins have been defined as candidate tumor suppressors. ING4 as a novel member of the ING family has potential tumor-suppressive effects. In this study, we explored the combined effect of adenovirus-mediated ING4 (Ad-ING4) gene transfer plus chemotherapy drug cisplatin (CDDP) on SMMC-7721 human hepatocarcinoma cells in vitro and in vivo, and its underlying mechanism. We demonstrated that Ad-ING4 plus CDDP induced synergistic growth inhibition, enhanced apoptosis, and had an additive effect on upregulation of Fas, Bax, Bak, cleaved Bid, cleaved caspase-8, caspase-9, caspase-3 and cleaved PARP, and on downregulation of Bcl-2 and Bcl-XL in SMMC-7721 hepatocarcinoma cells. Moreover, Ad-ING4 plus CDDP synergistically suppressed in vivo SMMC-7721 hepatocarcinoma subcutaneous (s.c.) xenografted tumor growth and reduced tumor vessel CD34 expression and microvessel density (MVD) in athymic nude mice. Most importantly, Ad-ING4 plus CDDP did not have overlapping toxicities in HL-7702 normal human liver cells and normal liver tissues of mice. The in vitro and in vivo enhanced antitumor effect elicited by Ad-ING4 plus CDDP was closely associated with the cooperative regulation of extrinsic and intrinsic apoptotic pathways and synergistic inhibition of tumor angiogenesis. Thus, our results indicate that Ad-ING4 plus CDDP is a potential combined treatment strategy for hepatocarcinoma.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, and is the third highest cause of cancer-related mortality.1 The incidence is increasing worldwide because of the dissemination of hepatitis B and C virus infection.2,3 Surgical resection is the mainstay of curative treatment for early HCC, but prognosis remains unsatisfactory because of frequent recurrence.4 Liver transplantation is another potentially curative treatment for HCC, but its application is limited by the shortage of grafts.5 For unresectable HCC, various locoregional therapies may be used to palliate symptoms and prolong survival.6 Furthermore, conventional chemotherapy has not been shown to be effective in HCC, and there is no proven effective systemic therapy for HCC patients with metastatic disease.7 Hence, the search for novel therapeutic modalities for HCC is of paramount importance.

Cancer gene therapy represents a new and promising therapeutic modality for cancers, which is based on the introduction of genetic material into cells to generate a curative biological effect. A variety of gene therapy-based anticancer strategies have been effective in animal tumor models, including replacement of tumor suppressor genes, selective activation of prodrugs, genetic immunotherapy and antiangiogenic actions. Inhibitor of growth 4 (ING4), a novel member of the ING tumor suppressor family, was first isolated and characterized by Shiseki et al.8 Recently, ING4 has attracted much attention as a strong candidate tumor suppressor that has an important role in oncogenesis, DNA repair, tumor growth, angiogenesis, migration and gene transcription regulation. ING4 has a functionally conserved plant homeodomain-finger motif in the COOH-terminal region involved in chromatin remodeling and subsequent gene transcriptional regulation by its interaction with histone acetyltransferase and histone deacetylase complexes.9,11 It also has a potential bipartite nuclear localization signal domain in the middle region, which is essential for nuclear localization of ING4 and its binding and functional interaction with p53.12 Previous studies showed that ING4 was dramatically downregulated in glioblastoma,13 head and neck carcinoma,14 HCC,15 melanoma16,17 and gastric carcinoma,18 which was closely associated with tumor grade, metastasis and prognosis. ING4 can significantly
inhibit tumor cell growth and induce cell cycle alteration and apoptosis in different tumor types, such as colorectal, glioblastoma, melanoma, hepatocellular, myeloma, lung and pancreatic carcinomas, and enhance chemosensitivity to doxorubicin and etoposide in HepG2 hepatocarcinoma cells. ING4 can also suppress the activity of NF-κB and HIF-1α, leading to inhibition of tumor angiogenesis. In addition, ING4 can suppress the loss-of-contact inhibition elicited by MYCN or MYC. More recently, it has been reported that ING4 can exhibit a marked inhibitory effect on tumor cell spreading, migration and invasion. Therefore, ING4 is a potent tumor suppressor that exerts its tumor-suppressive effect via multiple pathways in a variety of tumors.

Chemotherapy is one of the most conventional therapeutic strategies for human cancers. Conventional chemotherapy drugs include cisplatin, also named cis-diaminedichloroplatinum (CDDP), adriamycin and 5-fluorouracil. CDDP is deemed to be the ‘penicillin of cancer drugs’ because of its universal, early and effective treatment for many cancers. Unlike many anticancer drugs, CDDP is an inorganic molecule with a simple structure that is often used as an attractive chemotherapy drug and broadly used for the treatment of various forms of malignant tumors. Although the mechanism has not yet been fully understood, CDDP is generally believed to kill cancer cells by binding to DNA and interfering with the cell’s repair mechanism, which eventually leads to cell death. Despite these merits, severe toxic side effects and drug resistance that limit its efficacy are major clinical obstacles associated with CDDP-based chemotherapy. Thus, it is urgent to explore novel approaches to reduce drug dosage, minimize side effects and enhance therapeutic efficacy to promote the application of CDDP in cancer chemotherapy.

Combination therapy with multiple drugs or modalities such as the combined treatment of gene therapy and conventional chemotherapy (chemo-gene therapy) or radiotherapy is a common practice in the treatment of cancers, which can achieve greater therapeutic benefit and reduce the side effects and resistance to drugs. Interestingly, the tumor suppressor ING4 can sensitize HepG2 hepatocarcinoma cells to DNA-damaging agents such as doxorubicin and etoposide, suggesting that it may be used as a chemosensitive modulator in the combination therapy with chemotherapy drugs for cancers. Therefore, we hypothesized that a combination of adenovirus-mediated ING4 (Ad-ING4) gene transfer and chemotherapy drug CDDP would exhibit an enhanced antitumor activity for human cancers. To date, the therapeutic effect of the conjugation of Ad-ING4 with CDDP on human cancers has not been reported. To enhance the therapeutic efficacy and develop a novel combination therapeutic modality for HCC, in this study, we investigated the potential combined effect of Ad-ING4 plus CDDP against SMMC-7721 human hepatocarcinoma cells in vitro and in vivo in an athymic nude mouse model, and also elucidated its underlying molecular mechanism.

### Materials and methods

Adenoviruses, cell lines, reagents and mice

The Ad-ING4 and Ad-green fluorescent protein (GFP) replication-incompetent Ad5E1- and E3-deleted adenoviruses were constructed in our laboratory. Cell and Molecular Biology Institute, College of Medicine, Soochow University (Suzhou, China). The QBI-293A human embryonic kidney cell line was kindly provided by Prof. Jiang Zhong, Fudan University (Shanghai, China). The SMMC-7721 and HepG2 human hepatocarcinoma cell lines and HL-7702 normal human liver cells were purchased from the American Type Culture Collection (ATCC) (Shanghai, China). The QBI-293A, SMMC-7721, HepG2 and HL-7702 cells were cultured in RPMI1640 (GIBCO, Shanghai, China) supplemented with 10% fetal bovine serum (HyClone, Shanghai, China), respectively. Trizol was purchased from Invitrogen (Shanghai, China). The reverse transcriptase polymerase MuMLV was purchased from MBI (Shanghai, China). The polycyonal goat anti-ING4 antibody was purchased from Abcam (Shanghai, China). The CDDP was kindly provided by Dr. Yehan Zhu, the first hospital affiliated to Soochow University (Suzhou, China). The Annexin V-PE/7-AAD apoptosis detection kit was purchased from BD Biosciences (Shanghai, China). The antibodies specific for Fas, Bel-2, Bel-XL, Bax, Bak, β-actin and CD34 were purchased from Santa Cruz (Shanghai, China). The antibodies specific for Bid, caspase-3, caspase-8, caspase-9 and PARP were purchased from Cell Signaling (Shanghai, China). The SuperEnhanced chemiluminescence detection kit was purchased from Cell Signaling (Shanghai, China). The UltraSensitiveTM SP kit was purchased from Maixin (Fuzhou, China). The mouse alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Usnc Life Science (Wuhan, China). The female athymic nude mice were purchased from Shanghai Experimental Animal Center (Shanghai, China) and maintained in the animal facility at Soochow University according to the animal research committee’s guidelines of Soochow University.

### Analysis of adenoviral infection efficiency and ING4 transgene expression

The recombinant replication-incompetent Ad5E1- and E3-deleted adenovirus with two independent cytomegalovirus promoters driving ING4 and GFP expression, Ad-ING4 and its control adenovirus Ad-GFP expressing GFP under the control of cytomegalovirus promoter were prepared as described previously. The titer of Ad-ING4 and Ad-GFP adenoviruses was determined using the gene transfer unit (GTU) method by calculating the number of the reporter gene GFP-expressing QBI-293A cells under fluorescence microscopy within 18 h after adenoviral infection. To assess the optimal ratio of infectious adenovirus (GTU) to target cells, called multiplicity of
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infection (MOI), for a maximal infection and transgene expression in SMCC-7721, HepG2 and HL-7702 cells, the SMCC-7721 and HepG2 human hepatocarcinoma cells and HL-7702 normal human liver cells were infected with Ad-GFP and Ad-ING4 at various MOIs (0, 1, 10, 25, 50, 100 and 200), respectively. The adenoviral infection efficiency was examined according to GFP expression by fluorescence microscopy. Furthermore, the ING4 transgene expression mediated by adenoviral infection in SMCC-7721 and HepG2 human hepatocarcinoma cells and HL-7702 normal human liver cells was determined by reverse transcriptase-PCR and western blot analysis. Total cellular RNAs were extracted from Ad-ING4- or Ad-GFP-infected and uninfected SMCC-7721, HepG2 and HL-7702 cells using Trizol, and first-strand complemenary DNA was reversely transcribed with RNA as template and oligo d(T)₁₈ as primer. The PCR reaction was carried out using complementary DNA as template and ING4-F (5'-GGCGTGACATGGATGTA TGGGATGTATTTGGAAC-3') and ING4-R (5'-GCAA GCTTCTAATTCTTCTCCGTTCCTGGAG-3') as primers under the conditions 1 cycle at 94 °C (2 min) and then 72 °C (10 min) followed by 35 cycles at 94 °C (50 s), 58 °C (50 s) and 72 °C (55 s). All PCR reaction products were analyzed on 1% agarose gel electrophoresis with ethidium bromide staining. Total cellular lysates derived from Ad-ING4 or Ad-GFP-infected and uninfected SMCC-7721, HepG2 and HL-7702 cells were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto a polyvinylidene difluoride membrane. The membrane was blocked by incubation with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h at 37 °C. For western blot analysis, the membrane was incubated with primary antibody polyclonal goat anti-ING4 (1:1000) in blocking solution for 1 h at 37 °C. The membrane was washed with TBST and incubated with peroxidase horseradish peroxidase-conjugated secondary antibody rabbit anti-goat IgG (1:3000) in blocking solution for another 1 h at 37 °C. The membrane was then washed and developed by use of a SuperEnhanced chemiluminescence detection kit. The protein bands were visualized after exposure of the membranes to Kodak X-ray film.

Cell viability assay
To assess the cytotoxic effect of Ad-ING4 or CDDP alone on SMCC-7721, HepG2 and HL-7702 cells, the SMCC-7721 and HepG2 human hepatocarcinoma cells and HL-7702 normal human liver cells were dispensed in a 96-well culture plate at a density of 1 × 10⁴ cells per well, respectively, and incubated at 37 °C. After 24-h incubation, the SMCC-7721, HepG2 and HL-7702 cells were treated with Ad-ING4 at the optimal MOIs, 30 (SMCC-7721), 25 (HepG2) and 25 (HL-7702), or various doses of CDDP (0.75, 1.5, 3, 6 or 12 μg ml⁻¹) for 3 days, respectively. The medium containing Ad-GFP was used as a blank adenovirus control, whereas the medium containing phosphate-buffered saline (PBS) without Ad-ING4, Ad-GFP or CDDP was used as a cell control (PBS control). At 3 days after treatment, the SMCC-7721, HepG2 and HL-7702 cells were incubated with 10 μl MTT (5 mg ml⁻¹) for 4 h at 37 °C. The formazan crystals in the cells were solubilized with stop solution and the plate was then read at 570 nm using a Microplate Reader Model 550 (BIO-RAD, Shanghai, China). To further determine the combined effect of Ad-ING4 and CDDP on hepatocarcinoma cells, the SMCC-7721 hepatocarcinoma cells, a relatively non-sensitive hepatocarcinoma cell line to chemotherapy drug CDDP, and an optimal dose of 1.5 μg ml⁻¹ of CDDP were employed in the in vitro combination therapy studies. Briefly, the SMCC-7721 human hepatocarcinoma cells were dispensed in a 96-well culture plate at a density of 1 × 10⁴ cells per well and incubated at 37 °C. After 24-h incubation, the SMCC-7721 hepatocarcinoma cells were treated with Ad-ING4 (50 MOI), Ad-GFP (50 MOI) or CDDP (1.5 μg ml⁻¹) alone, Ad-ING4 (50 MOI) plus CDDP (1.5 μg ml⁻¹), or Ad-GFP (50 MOI) plus CDDP (1.5 μg ml⁻¹) for the indicated time periods (0-4 days). The medium containing PBS without Ad-ING4, Ad-GFP, CDDP, Ad-ING4 plus CDDP, or Ad-GFP plus CDDP was used as a cell control (PBS control). To investigate whether Ad-ING4 combined with CDDP exhibits overlapping inhibitory effect on normal liver cells, the HL-7702 normal human liver cells were used as a normal liver cell control by treatment with Ad-ING4 (25 MOI), Ad-GFP (25 MOI) or CDDP (1.5 μg ml⁻¹) alone, Ad-ING4 (25 MOI) plus CDDP (1.5 μg ml⁻¹), or Ad-GFP (25 MOI) plus CDDP (1.5 μg ml⁻¹), or PBS. Before treatment and at different time points after treatment, the viability of HL-7702 normal liver cells was assessed by MTT assay as described above.

Flow-cytometric analysis of apoptosis
Ad-ING4 plus CDDP-induced apoptosis in SMCC-7721 human hepatocarcinoma cells was assessed by flow cytometric analysis using Annexin V-PE/7-AAD double staining following the manufacturer’s instructions. Briefly, the SMCC-7721 tumor cells (1 × 10⁶) were treated with Ad-ING4 (50 MOI), Ad-GFP (50 MOI) or CDDP (1.5 μg ml⁻¹) alone, Ad-ING4 (50 MOI) plus CDDP (1.5 μg ml⁻¹), or Ad-GFP (50 MOI) plus CDDP (1.5 μg ml⁻¹), or PBS. After treatment and at different time points after treatment, the viability of HL-7702 normal liver cells was assessed by MTT assay as described above.

Western blot analysis
The SMCC-7721 human hepatocarcinoma cells were treated with Ad-ING4 (50 MOI), Ad-GFP (50 MOI) or CDDP (1.5 μg ml⁻¹) alone, Ad-ING4 (50 MOI) plus CDDP (1.5 μg ml⁻¹) or Ad-GFP (50 MOI) plus CDDP.
subjected to western blot analysis as described above or Ad-GFP (1 × 10^8 GTU) or PBS alone, or i.p. injected with CDDP (1 mg kg^{-1}) alone or plus Ad-ING4 (1 × 10^8 GTU) or Ad-GFP (1 × 10^8 GTU) by intratumoral injection every other day for a total of five times. Tumor volume and body weight were monitored daily for tumor growth. Tumor progression and body weight were monitored over time. According to the preliminary CDDP-dose pilot study, an optimal dose of 1 mg kg^{-1} of CDDP was selected for the subsequent Ad-ING4 plus CDDP in vivo combination treatment. In the in vivo combination treatment, tumor-bearing mice were randomly divided into six groups (six mice each group) and intratumorally injected with Ad-ING4 (1 × 10^8 GTU), Ad-GFP (1 × 10^8 GTU) or PBS alone, or i.p. injected with CDDP (1 mg kg^{-1}) alone or plus Ad-ING4 (1 × 10^8 GTU) or Ad-GFP (1 × 10^8 GTU) by intratumoral injection every other day for a total of five times, respectively. Tumor progression and body weight were monitored daily. In addition, the tumor-bearing mice were killed 3 weeks after single or combination treatment, and the SMMC-7721 hepatocarcinoma s.c. xenografted tumors and normal liver tissues of mice were removed, weighted, fixed by 10% neutral formalin and embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry analysis, respectively. On the day of euthanization, blood samples were also collected by tail vein bleeding for measurement of liver serum enzymes such as ALT and AST by ELISA using mouse ALT and AST ELISA kits according to the manufacturer’s instructions.

**Animal studies**

Female athymic nude mice were subcutaneously (s.c.) inoculated on the armpits of their right anterior limbs with 2 × 10^6 SMMC-7721 human hepatocarcinoma cells, and then monitored daily for tumor growth. Tumor volume was measured with a caliper and calculated by the formula, tumor size = ab^2/2, where a is the larger and b is the smaller of the two dimensions. When the tumors grew up to a mean tumor volume of around 0.2–0.3 cm^3, SMMC-7721 human hepatocarcinoma s.c. xenografted tumor-bearing mice were subjected to in vivo CDDP-dose-finding study and Ad-ING4 plus CDDP combination treatment, respectively. In the CDDP-dose study, tumor-bearing mice (three mice each group) were intraperitoneally (i.p.) injected with various doses of CDDP (1, 3, 5 or 10 mg kg^{-1} body weight) or PBS every other day for a total of five times. Tumor volume and body weight were monitored over time. According to the preliminary CDDP-dose pilot study, an optimal dose of 1 mg kg^{-1} of CDDP was selected for the subsequent Ad-ING4 plus CDDP in vivo combination treatment. In the in vivo combination treatment, tumor-bearing mice were randomly divided into six groups (six mice each group) and intratumorally injected with Ad-ING4 (1 × 10^8 GTU), Ad-GFP (1 × 10^8 GTU) or PBS alone, or i.p. injected with CDDP (1 mg kg^{-1}) alone or plus Ad-ING4 (1 × 10^8 GTU) or Ad-GFP (1 × 10^8 GTU) by intratumoral injection every other day for a total of five times, respectively. Tumor progression and body weight were monitored daily. In addition, the tumor-bearing mice were killed 3 weeks after single or combination treatment, and the SMMC-7721 hepatocarcinoma s.c. xenografted tumors and normal liver tissues of mice were removed, weighted, fixed by 10% neutral formalin and embedded in paraffin for hematoxylin and eosin staining and immunohistochemistry analysis, respectively. On the day of euthanization, blood samples were also collected by tail vein bleeding for measurement of liver serum enzymes such as ALT and AST by ELISA using mouse ALT and AST ELISA kits according to the manufacturer’s instructions.

**Results**

**Ad-ING4 transgene expression**

To determine the optimal MOI for a maximal infection and transgene expression, the SMMC-7721 and HepG2 hepatocarcinoma cells and HL-7702 normal liver cells were infected with Ad-GFP and Ad-ING4 at various MOIs (0, 1, 10, 25, 50, 100 and 200), respectively, and examined by fluorescence microscopy. More than 90% of GFP expression was found in the Ad-GFP- or Ad-ING4-infected SMMC-7721, HepG2 and HL-7702 cells at MOIs of 50, 25 and 25 or above, respectively, whereas the GFP expression was not found in the uninfected SMMC-7721, HepG2 and HL-7702 control cells (data not shown). Therefore, we selected an MOI of 50, 25 and 25, respectively, as an optimal dose for adenoviral infection of SMMC-7721, HepG2 and HL-7702 cells in the following experiments. To assess Ad-ING4 transgene expression, the total cellular RNAs and lysates extracted from Ad-ING4- or Ad-GFP-infected and uninfected SMMC-7721, HepG2 and HL-7702 cells were subjected to reverse transcriptase-PCR and western blot analysis.

**CD34 immunostaining and microvessel density (MVD) counting**

The expression of CD34 in SMMC-7721 human hepatocarcinoma xenografted tumors was tested by immunohistochemistry analysis using an UltraSensitiveTM SP kit. The presence of buffy or brown diaminobenzidine precipitates is indicative of positive reactivity for CD34. MVD, detected by immunostaining for CD34, was determined as previously described by Weidner et al. Any endothelial cell cluster immunoreactive for CD34 clearly separated from adjacent microvessels was considered as a single countable vessel. Each value represents the number of vessels counted at a high-power view (×400) by microscopy. The mean value represents the average number derived from five high-power fields of each case.

**Calculation of synergistic indexes**

The interactive effects of Ad-ING4 and CDDP were evaluated by combination index (CI) calculated by the formula as described previously, 32 CI = FuAFuB/Fu(A + B), where FuA represents the fraction unaffected by Ad-ING4 alone compared with the untreated control group, FuB represents the fraction unaffected by CDDP alone, and Fu(A + B) represents the fraction unaffected by Ad-ING4 plus CDDP. A value of CI > 1 indicates a synergistic effect between Ad-ING4 and CDDP, and CI < 1 indicates a less than additive effect.

**Statistical analysis**

All data are presented as the mean ± s.d. The significance of the difference between groups was evaluated by one-way and two-way repeated-measures analysis of variance (ANOVA), multiple comparisons and Student’s t test with SPSS 10.0 software. A value of P<0.05 was considered statistically significant.
Ad-ING4 had minimal effect on HL-7702 normal liver and PBS-treated control group (35% reduction compared with viability of control cells), inhibited SMMC-7721 hepatocarcinoma cell growth. As shown in Figure 2a, Ad-ING4 treatment significantly inhibited SMMC-7721, HepG2 hepatocarcinoma cells and HL-7702 normal liver cells, but not in the Ad-GFP-infected and uninfected SMMC-7721, HepG2 and HL-7702 control cells, respectively. The first-strand complementary DNA was synthesized from RNAs using reverse transcriptase; PCRs were conducted using primer sets specific for ING4 and housekeep gene \( \beta \)-actin used as an internal control. (b) Ad-ING4 translational expression by western blot analysis. Total cellular lysates of Ad-ING4- or Ad-GFP-infected SMMC-7721, HepG2 and HL-7702 cells and uninfected control cells were analyzed by immunoblotting with anti-ING4 and anti-\( \beta \)-actin (an internal control) antibody, respectively. Data shown are representative of three independent experiments.

As shown in Figure 1, a significant amount of ING4 expression was found in the Ad-ING4-infected SMMC-7721, HepG2 and HL-7702 cells, but not in the Ad-GFP-infected and uninfected SMMC-7721, HepG2 and HL-7702 control cells, indicating that transgene ING4 mediated by adenovirus is expressed in Ad-ING4-infected SMMC-7721, HepG2 and HL-7702 cells at both the transcriptional and translational levels. In addition, chemotherapy with CDDP did not affect adenoviral infection and Ad-ING4 expression in the combination treatment (data not shown).

**Ad-ING4 or CDDP alone inhibits in vitro hepatocarcinoma cell growth**

Before initiating the experiment involving a combination of Ad-ING4 and CDDP, we assessed the effect of Ad-ING4 or CDDP alone on the viability of SMMC-7721 and HepG2 hepatocarcinoma cells and HL-7702 normal liver cells, respectively. The SMMC-7721, HepG2 and HL-7702 cells were treated with Ad-ING4 at the optimal MOIs of 50, 25 and 25, respectively, or various doses of CDDP (0.75, 1.5, 3.6, or 12 \( \mu \)g ml\(^{-1} \)) and the cell growth was examined at day 3 after treatment using MTT assay. As shown in Figure 2a, Ad-ING4 treatment significantly inhibited SMMC-7721 hepatocarcinoma cell growth (35% reduction compared with viability of control cells), whereas the moderately inhibited HepG2 hepatocarcinoma cell growth (21% reduction), compared with the Ad-GFP- and PBS-treated control group \( (P<0.05) \). However, Ad-ING4 had minimal effect on HL-7702 normal liver cells (7% reduction). These results indicated that ING4 transgene overexpression exerts selective killing activity for tumor cells such as hepatocarcinoma cells. Treatment of SMMC-7721 and HepG2 hepatocarcinoma cells and HL-7702 normal liver cells with various concentrations of CDDP resulted in a dose-dependent reduction in the number of viable cells (Figure 2b). Furthermore, SMMC-7721 and HepG2 hepatocarcinoma cells and HL-7702 normal liver cells are different in their sensitivity to chemotherapy drug CDDP. The moderate reduction in SMMC-7721 hepatocarcinoma cell viability was observed at 1.5 \( \mu \)g ml\(^{-1} \) of CDDP (19% reduction), and significant reduction was observed starting at 3 \( \mu \)g ml\(^{-1} \) (37% reduction), with maximum reduction observed at 12 \( \mu \)g ml\(^{-1} \) (69% reduction). Unexpectedly, the significant reduction in HepG2 hepatocarcinoma cell viability was observed starting at the lowest concentration of CDDP (0.75 \( \mu \)g ml\(^{-1} \)) (49% reduction), with maximum reduction observed at 12 \( \mu \)g ml\(^{-1} \) (89% reduction). However, the significant reduction in HL-7702 normal liver cell viability was observed at 6 (29% reduction) and 12 \( \mu \)g ml\(^{-1} \) (47% reduction), with slight reduction observed at 3 \( \mu \)g ml\(^{-1} \) (14% reduction). These results indicated that HepG2 hepatocarcinoma cells are much more sensitive to chemotherapy drug CDDP than SMMC-7721 hepatocarcinoma cells, and only a higher concentration of CDDP ranging from 6 to 12 \( \mu \)g ml\(^{-1} \) exhibits significant cytotoxic effect on HL-7702 normal liver cells. Therefore, we selected the relatively non-sensitive hepatocarcinoma cell line to CDDP, SMMC-7721 and the optimal dose of 1.5 \( \mu \)g ml\(^{-1} \) of CDDP in the in vitro combination treatment to minimize toxicity to normal cells and elicit only modest antitumor activity when used as single therapy, while leaving a window for observation of the additive or synergistic effects of Ad-ING4 plus CDDP combination therapy.

**Ad-ING4 plus CDDP synergistically suppresses in vitro and in vivo SMMC-7721 hepatocarcinoma cell growth**

To investigate whether Ad-ING4 plus CDDP elicits enhanced cytotoxicity to hepatocarcinoma cells in vitro, the SMMC-7721 human hepatocarcinoma cells were cultured in the presence of Ad-ING4 (50 MOI), Ad-GFP (50 MOI) or CDDP (1.5 \( \mu \)g ml\(^{-1} \)) alone, Ad-ING4 (50 MOI) plus CDDP (1.5 \( \mu \)g ml\(^{-1} \)), or Ad-GFP (50 MOI) plus CDDP (1.5 \( \mu \)g ml\(^{-1} \)). The tumor cell viability was examined daily for 4 days using MTT assay. As shown in Figure 3a, Ad-ING4 plus CDDP combination treatment synergistically inhibited in vitro SMMC-7721 hepatocarcinoma cell growth in a time-dependent manner, compared with single Ad-ING4- and CDDP-treated group \( (P<0.05; CI>1) \), whereas the phenomenon did not occur in the Ad-GFP plus CDDP combination treatment, indicating that it is Ad-ING4 expression that contributes to the combined suppressive effect on SMMC-7721 hepatocarcinoma cells. Moreover, Ad-ING4 plus CDDP exhibited minimal cytotoxic effect on HL-7702 normal liver cells, equal to that of CDDP (Figure 3a). The result further indicated that Ad-ING4 plus CDDP combination treatment selectively exerts synergistic antitumor activity.
in SMMC-7721 hepatocarcinoma cells but not in HL-7702 normal liver cells. To determine the optimal dose of CDDP employed in vivo in an athymic nude mouse animal model combination treatment, a dose-finding study was performed in which SMMC-7721 human hepatocarcinoma s.c. xenografted tumor-bearing mice (three mice each group) were treated with a range of CDDPs (1, 3, 5 or 10 mg kg\(^{-1}\)) by i.p. injection. Tumor volume and body weight were monitored over time. Mice treated with higher doses of CDDP (5 or 10 mg kg\(^{-1}\)) resulted in a significant loss of body weight and had to be euthanized within a week, whereas those treated with 1 or 3 mg kg\(^{-1}\) of CDDP showed no significant body weight losses with only minimal toxicity (data not shown). Compared with the PBS-treated control group, tumor progression was significantly retarded in the mice treated with 3 mg kg\(^{-1}\) of CDDP, but moderately retarded in those treated with 1 mg kg\(^{-1}\) of CDDP (data not shown). Therefore, an optimal dose of 1 mg kg\(^{-1}\) of CDDP was selected for the Ad-ING4 plus CDDP in vivo combination treatment. To further explore whether the combination of Ad-ING4 with CDDP would result in in vivo-enhanced antitumor effect, the athymic nude mice (six mice each group) bearing SMMC-7721 human hepatocarcinoma s.c. xenografted tumors were intratumorally injected with Ad-ING4 (1 \times 10^{8} \text{ GTU}) or PBS served as controls for 3 days, respectively. The survival cells were evaluated at day 3 point after treatment by using MTT assay. The in vitro growth of SMMC-7721 and HepG2 tumor cells treated with Ad-ING4 was significantly or moderately inhibited after infection, whereas Ad-ING4 had minimal effect on HL-7702 normal liver cells (*P<0.05, compared with Ad-GFP and PBS group, one-way repeated measures ANOVA and multiple comparisons, n = 4 replicates per condition). (b) The cytotoxic effect of CDDP on SMMC-7721 and HepG2 hepatocarcinoma cells and HL-7702 normal liver cells. The SMMC-7721, HepG2 and HL-7702 cells were treated with various doses of CDDP (0.75, 1.5, 3, 6 or 12 \mu g \text{ ml}^{-1}) for 3 days, respectively. The survival cells were evaluated at day 3 point after treatment by using MTT assay. CDDP inhibits in vitro growth of SMMC-7721 and HepG2 hepatocarcinoma cells and HL-7702 normal liver cells in a dose-dependent manner (*P<0.05, compared with PBS group, Student t test, n = 4 replicates per condition). Data shown are representative of three independent experiments.
sections of liver in different groups also showed no significant hepatic damage (data not shown). In addition, ELISA analysis of liver serum enzymes (Figure 3e) showed a slight increase in ALT and AST elicited by CDDP, but no additive effect in the Ad-ING4 plus CDDP group. These results indicated that the combina-
Ad-ING4 and CDDP also exhibits minimal toxicity in vivo without overlapping effect.

Ad-ING4 plus CDDP enhances apoptosis in SMMC-7721 hepatocarcinoma cells

To examine whether Ad-ING4 plus CDDP combination treatment elicits enhanced apoptosis in hepatocarcinoma cells, the SMMC-7721 human hepatocarcinoma cells were treated with Ad-ING4 (50 MOI), Ad-GFP (50 MOI) or CDDP (1.5 µg ml⁻¹) alone, Ad-ING4 (50 MOI) plus CDDP (1.5 µg ml⁻¹), or Ad-GFP (50 MOI) plus CDDP (1.5 µg ml⁻¹), respectively. The survival cells were evaluated at day 0, 1, 2, 3 and 4 point after treatment by using MTT assays. Ad-ING4 plus CDDP combination treatment resulted in 32.8% Annexin V single-positive cells, indicating that these SMMC-7721 hepatocarcinoma cells are in early stage of apoptosis, whereas there was 1.9, 2.6, 17.5, 10.2 and 11.9% early apoptotic cells occurring in SMMC-7721 tumor cells grown in the medium with PBS, Ad-GFP, Ad-ING4, CDDP and Ad-GFP plus CDDP, respectively. Compared with the single Ad-ING4- and CDDP-treated group, the combination of Ad-ING4 with CDDP more efficiently induces SMMC-7721 hepatocarcinoma cell apoptosis with synergistic effect (P < 0.05; CI > 1), which closely correlates with the Ad-ING4 plus CDDP-mediated in vitro and in vivo synergistic growth inhibition of SMMC-7721 hepatocarcinoma cells.

Ad-ING4 plus CDDP cooperatively regulates extrinsic and intrinsic apoptotic pathways

To further address the underlying molecular mechanism by which Ad-ING4 plus CDDP combination treatment results in enhanced antitumor effect, the expression of apoptosis-related proteins such as Fas, caspase-8, Bid, Bcl-2, Bcl-XL, Bax, Bak, caspase-9, caspase-3 and PARP in SMMC-7721 human hepatocarcinoma cells with different treatments was determined by western blot analysis. As shown in Figure 4b, the expression of Fas, Bax and Bak in Ad-ING4, CDDP, Ad-GFP plus CDDP, and Ad-ING4 plus CDDP group was significantly increased, whereas the expression of Bcl-2 and Bcl-XL was decreased, compared with the Ad-GFP and PBS control group. The activation of caspase-8, Bid, caspase-9, caspase-3 and PARP was also found in Ad-ING4, CDDP, Ad-GFP plus CDDP, and Ad-ING4 plus CDDP groups, but not in the Ad-GFP and PBS control group. Moreover, Ad-ING4 plus CDDP combination treatment elicited an additive effect on the altered expression of apoptosis-related proteins involved in the activation of extrinsic and intrinsic apoptotic pathways, indicating that Ad-ING4 plus CDDP synergistically suppresses SMMC-7721 hepatocarcinoma cell growth and induces apoptosis closely associated with the cooperative regulation of extrinsic and intrinsic apoptotic pathways.

Ad-ING4 plus CDDP synergistically reduces tumor vessel CD34 expression and MVD

The positive expression of CD34 was mainly represented as brownish yellow or brownish granules in vascular endothelial cells. In all SMMC-7721 human hepatocarcinoma s.c. xenografted tumors collected, the CD34 expression of tumor vascular endothelial cells in the Ad-ING4 plus CDDP combination treatment group was weaker or less, compared with the single Ad-ING4- and CDDP-treated group (Figure 5a), indicating that Ad-ING4 plus CDDP additively or synergistically down-regulates CD34 expression of SMMC-7721 human hepatocarcinoma xenografted tumor vessels. Furthermore, the MVD (Figure 5b) counted in the Ad-ING4 plus CDDP group was significantly less than that in the single Ad-ING4- and CDDP-treated group (P < 0.05; CI > 1), indicating that Ad-ING4 plus CDDP has an synergistic effect on reduction of MVD in SMMC-7721 hepatocarcinoma s.c. xenografted tumors, which may...
be another important mechanism responsible for the Ad-ING4 plus CDDP-mediated in vivo synergistic growth inhibition of SMMC-7721 hepatocarcinoma xenografted tumors in an athymic nude mouse model.

**Discussion**

Recent studies have shown that ING4 as a novel tumor suppressor is involved in a variety of processes including oncogenesis, cell cycle control, apoptosis, DNA repair, angiogenesis, migration and gene transcription regulation. ING4 can significantly inhibit tumor cell growth and induce cell cycle alteration and apoptosis in a variety of tumors. ING4 can also suppress brain-tumor angiogenesis by inhibiting the activity of NF-κB leading to transcriptional repression of NF-κB-responsive genes such as IL-6, IL-8 and Cox-2 by physically interacting with the p65 subunit of NF-κB. Nozell et al. confirmed that ING4 can specifically regulate the activity...
of NF-κB molecules that are bound to target gene promoters. Furthermore, Colla et al.\textsuperscript{20} reported that ING4 can suppress HIF-1α activity and its target gene NIP-3 expression in myeloma cells under hypoxic condition and consequently inhibit tumor angiogenesis. In addition, ING4 can suppress tumor-cell spreading, migration and invasion by colocalizing and interacting with liprin α1 at the lamellipodia and downregulating the expression and activity of MMP-2 and MMP-9.\textsuperscript{16,21,34} These findings revealed that ING4 as a potent tumor suppressor negatively modulates tumor growth via multiple pathways.

Chemotherapy is one of the commonly used strategies in HCC treatment, especially for unresectable patients. Conventional chemotherapy drugs often have severe side effects that limit their efficacy. Moreover, tumor cells are able to acquire resistance to chemotherapy. The increased dose of chemotherapy drug that is necessary to overcome resistance of tumor cells, even a small increase, may result in severe cytotoxicity to normal cells. Therefore, it is urgent to explore novel approaches to broaden the application of the chemotherapy drug in cancer therapy. To enhance therapeutic effect, eliminate drug resistance and reduce side effects, several conjugated strategies such as the combined treatment of gene therapy and conventional chemotherapy (chemo-gene therapy) have been applied successfully in cancer chemotherapy. Our previous studies demonstrated that Ad-ING4 gene transfer can induce growth inhibition and apoptosis in human lung and pancreatic carcinoma cells.\textsuperscript{21,22} A recent study has shown that ING4 can enhance chemosensitivity to doxorubicin and etoposide in HepG2 hepatocarcinoma cells,\textsuperscript{23} suggesting that it may be used as a chemosensitive modulator in the combination therapy with chemotherapy.

Figure 5 Synergistic inhibition of tumor angiogenesis in vivo. (a) Immunohistochemical detection for CD34 expression in SMMC-7721 hepatocarcinoma s.c. xenografted tumors. Representative pictures for different treatment groups are shown. The positive expression of CD34 was mainly represented as brownish yellow or brownish granules in tumor vascular endothelial cells. (b) The tumor MVD in different treatment groups. Ad-ING4 plus CDDP combination treatment synergistically inhibited in vivo angiogenesis of SMMC-7721 hepatocarcinoma s.c. xenografted tumors (\(P < 0.05\), compared with Ad-GFP and PBS group, one-way repeated measures ANOVA and multiple comparisons, \(n = 6\) mice per condition, \(n = 5\) observations per representative section; \(\#\text{CI in the Ad-ING4 plus CDDP combination treatment} > 1\)). Data shown are representative of three independent experiments.
Ad-ING4 plus cisplatin exerts enhanced antitumor effect
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Drugs. However, the combined effect of Ad-ING4 gene therapy and CDDP, an attractive chemotherapy drug, on human cancers, is still unknown. Therefore, in this study, we were interested in addressing the therapeutic effect of chemo-gene therapy by combining Ad-ING4 with CDDP on human hepatocarcinoma cells in vitro and in vivo in an athymic nude mouse model and its potential mechanism.

Before initiating in vitro combination therapy, we first assessed the cytotoxic effect of Ad-ING4 or CDDP alone on SMMC-7721 and HepG2 hepatocarcinoma cells and HL-7702 normal liver cells. We found that Ad-ING4 significantly inhibited SMMC-7721 human hepatocarcinoma cell growth, whereas it moderately inhibited HepG2 hepatocarcinoma cell growth. However, Ad-ING4 had only a minimal effect on HL-7702 normal human liver cells, indicating that transgene ING4 overexpression exerts selective tumor-killing activity in hepatocarcinoma cells. In addition, chemotherapy drug CDDP induced a dose-dependent growth inhibition in SMMC-7721 and HepG2 hepatocarcinoma cells or HL-7702 normal liver cells. Furthermore, the HepG2 hepatocarcinoma cells were more sensitive to CDDP than SMMC-7721 hepatocarcinoma cells. Different tumor cell lines are different in their susceptibility to drugs. In this study, the HepG2 and SMMC-7721 hepatocarcinoma cell lines are both adherent and epithelial-like cells, but from different origins. The former was established from the tumor tissue of a 15-year-old Argentine boy with HCC in 1975, which was reported to produce a variety of proteins such as alphafetoprotein, albumin, alpha2-macroglobulin, alpha1-antitrypsin and transferrin. The latter was established from the tumor tissue of a 56-year-old Chinese man with HCC in 1980. The different origins and different biological activities may be one of the reasons for the different susceptibility to Ad-ING4 and CDDP. In addition, higher concentration of CDDP (6 or 12 μg ml⁻¹) exhibited significant growth-suppressive effect on HL-7702 normal liver cells, while 3 μg ml⁻¹ of CDDP only induced slight cytotoxicity. To minimize toxicity to normal cells and produce only modest antitumor efficacy when used as monotherapy, while leaving a window for observation of additive or synergistic effects of Ad-ING4 plus CDDP combination therapy, we selected the relatively nonsensitive hepatocarcinoma cell line to CDDP, SMMC-7721, and the optimal dose of 1.5 μg ml⁻¹ of CDDP in the in vitro combination treatment. We demonstrated that Ad-ING4 plus CDDP combination treatment selectively exerted in vitro synergistic therapeutic activity in SMMC-7721 hepatocarcinoma cells, but not in HL-7702 normal liver cells. Moreover, treatment of SMMC-7721 hepatocarcinoma cells with Ad-ING4 plus CDDP resulted in enhancement of apoptosis. To further address the underlying mechanism involved in Ad-ING4 plus CDDP-induced enhanced antitumor activity, the expression of apoptosis-related proteins such as Fas, caspase-8, Bid, Bel-2, Bel-XL, Bax, Bak, caspase-9, caspase-3 and PARP in different treatments of SMMC-7721 hepatocarcinoma cells was assessed by western blot analysis. Fas as an important apoptotic marker has been shown to regulate FasL--Fas extrinsic apoptotic pathway.35 Associated with the increase in Fas expression is the cleavage of Caspase-8 and Bid, downstream targets of Fas, that have an important role in the extrinsic apoptotic pathway.35 Moreover, caspase-8-mediated cleavage of Bid, which translocates to the mitochondria, also results in the activation of intrinsic apoptotic pathway.35 Moreover, caspase-8-mediated cleavage of Bid, which translocates to the mitochondria, also results in the activation of intrinsic apoptotic pathway.35

In vivo combination treatment, a CDDP-dose-finding study was also primarily performed in SMMC-7721 human hepatocarcinoma s.c. xenografted tumor-bearing athymic nude mice by i.p. injection with various doses of CDDP. Mice treated with higher CDDP doses (5 or 10 mg kg⁻¹) caused significant toxicity, while those treated with 1 or 3 mg kg⁻¹ of CDDP showed only minimal toxicity. Moreover, tumor progression was significantly retarded in the mice treated with 3 mg kg⁻¹ of CDDP, but moderately retarded in those treated with 1 mg kg⁻¹ of CDDP. Therefore, an optimal dose of 1 mg kg⁻¹ of CDDP was selected for the in vivo combination treatment. We demonstrated that Ad-ING4 plus CDDP also synergistically suppressed in vivo SMMC-7721 hepatocarcinoma s.c. xenografted tumor growth in an athymic nude mouse model without additive toxicity in vivo. The progressive growth and metastasis of solid tumors is dependent on the process of angiogenesis.39,41 Tumor angiogenesis has a critical role in the development and progression of HCC. It has been shown that ING4 can suppress tumor angiogenesis by inhibiting the activity of NF-κB and HIF-1α.15,20 It has also been demonstrated that CDDP can exert a potent antiangiogenic activity during CDDP-based cancer chemotherapy.32,42 To explore the combined effect of Ad-ING4 plus CDDP on tumor angiogenesis in vivo, the MVD in SMMC-7721 hepatocarcinoma xenografted tumor tissues was determined by CD34 immunohistochemistry analysis. We further found that Ad-ING4 plus CDDP synergistically downregulated CD34 expression and suppressed angiogenesis in SMMC-7721 hepatocarcinoma s.c. xenografted tumors, which may be another important mechanism involved in Ad-ING4 plus CDDP-mediated in vivo synergistic growth inhibition of SMMC-7721 hepatocarcinoma xenografted tumor in an athymic nude mouse model.
Taken together, Ad-ING4 plus CDDP combined treatment resulted in in vitro and in vivo synergistic growth inhibition, enhanced apoptosis, had an additive effect on upregulation of Fas, Bax, Bak, cleaved Bid, cleaved caspase-8, caspase-9, caspase-3 and cleaved PARP, and on downregulation of Bel-2 and Bel-XL in SMMC-7721 hepatocarcinoma cells, and synergistically reduced CD34 expression and MVD in SMMC-7721 hepatocarcinoma s.c. xenografted tumors. Most importantly, the combination of Ad-ING4 with CDDP did not have overlapping toxicities in liver cells and in vivo normal liver tissues of mice. The enhanced antitumor effect in vitro and in vivo elicited by Ad-ING4 plus CDDP was closely associated with the increased Fas expression and cleavage of Caspase-8 and Bid, and decrease in the ratio of anti to pro-apoptotic molecules (Bel-2, Bel-XL; Bax, Bak) followed by activation of Caspase-9 leading to Caspase-3 activation and apoptosis by cooperatively regulating extrinsic and intrinsic apoptotic pathways, and the in vivo synergistic inhibition of tumor angiogenesis. Thus, our results indicate that Ad-ING4 plus CDDP is a potential combined treatment strategy for hepatocarcinoma.

**Conflict of interest**

The authors declare no conflict of interest.

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