

Enhanced antitumor effect of cisplatin in human oral squamous cell carcinoma cells by tumor suppressor GRIM-19

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Abstract. Gene associated with retinoid-interferon-induced mortality 19 (GRIM-19) is a novel candidate tumor suppressor gene located on the human chromosome 19p13.1 region. Our previous study demonstrated that the upregulation of GRIM-19 in human oral squamous cell carcinoma (OSCC) cells significantly inhibited tumor cell growth *in vitro* and *in vivo*. In the present study, the combined effects of cationic liposome (LP)-mediated GRIM-19 gene (LP-pGRIM-19) and the low-dose chemotherapeutic drug, cisplatin (CDDP), on tumor cell growth *in vitro* and *in vivo* were examined, and the molecular mechanism of their mutual action was investigated by cell proliferation, colony formation, apoptosis, migration, invasion and western blotting assays *in vitro*, and a nude tumor model. It was demonstrated that cationic LP-pGRIM-19 gene therapy sensitized the response of breast cancer cells to CDDP, and that LP-pGRIM-19 in combination with CDDP significantly induced apoptosis and inhibited proliferation, colony formation, migration and invasion of the cells, compared with CDDP treatment alone. In addition, systemic treatment with a combination of intravenous injection of LP-pGRIM-19 and intraperitoneal injection of low-dose CDDP into subcutaneous HSC3 human OSCC xenograft mice resulted in a significant inhibition of tumor growth ($P < 0.05$). Further investigations indicated that the enhancement of CDDP-mediated antitumor effects by GRIM-19 may be associated with the upregulation of phosphorylated p53 and the downregulation of B cell lymphoma-2, cyclin D1, vascular endothelial growth factor, matrix metalloproteinase (MMP)-2 and MMP-9, the proteins of which are involved in the activation of signal transducer and activator of transcription 3. The results of the present study

suggested that the combination of GRIM-19 gene therapy with low-dose CDDP-based chemotherapy may be a potent therapeutic strategy for the treatment of OSCC.

Introduction

Oral cancer, particularly oral squamous cell carcinoma (OSCC), is the most common type of head and neck cancer worldwide, with ~540,000 new cases annually worldwide (1,2). Despite surgery and chemotherapy being increasingly used to treat OSCC, the 5-year survival rate of OSCC has not improved markedly over previous years, due to late diagnosis, frequent loco-regional recurrences at the primary site and metastasis to neck lymph nodes following treatment (3,4). Therefore, it is imperative to identify novel therapeutics to improve treatment of this disease.

Cisplatin or cis-diammine-dichloroplatinum (II) (CDDP), is a DNA damage-inducing chemotherapeutic drug, and is a chemotherapeutic agent that is widely used in treatment of several types of cancer, including testicular, ovarian, cervical, head and neck, lung, oral and esophageal malignancies (5-7). Despite CDDP being a potent anticancer agent for OSCC, its application is limited due to its side effects, in particular neurotoxicity, which is dose-limiting, and inherent and acquired resistance (8-10). Therefore, novel and customized treatment strategies are required to overcome chemotherapeutic drug resistance and enhance its antitumor activity (11). Targeted therapies, which cause no or minor side effects, may compensate for the limitations of conventional chemotherapies.

It is well known that tumor suppressor genes (TSGs) are important in the pathogenesis of human OSCC and other types of cancer (12-14). The gene associated with retinoid-interferon-induced mortality 19 (GRIM-19) is one of the novel candidate TSGs located on human chromosome 19p13.1, and its overexpression significantly increases cell death (15). Our previous study demonstrated that the forced expression of GRIM-19 in OSCC cells significantly inhibited cell proliferation and colony formation, and induced cell apoptosis *in vitro*, which effectively suppressed tumor growth in mouse models of human OSCC (16). In addition, it has been demonstrated that the combination of conventional CDDP-based chemotherapy and tumor suppressor gene therapy, including LKB1, p53, NPRL2 and FUS1, may overcome cancer cell resistance

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to chemotherapeutic drugs and enhance therapeutic efficacy in cancer (17-20). However, to the best of our knowledge, no reports are available regarding the combined effects of exogenous expression of GRIM-19 tumor suppressor with any chemotherapeutic drugs, including CDDP, on tumor suppression activity. Therefore, the present study analyzed the potential correlation between the expression of GRIM-19 by cationic liposome (LP)-mediated gene transfer and the enhancement of CDDP sensitivity in human OSCC cell lines. The effects of GRIM-19 on the enhancement of low-dose CDDP-mediated antitumor activity in OSCC cell lines were examined *in vitro* and *in vivo*, and the potential molecular mechanisms of the combined treatment of cationic LP-mediated GRIM-19 gene therapy and low-dose CDDP-based chemotherapy were investigated.

Materials and methods

Cell line, plasmid and chemotherapeutic drug. The HSC3 human oral squamous cell carcinoma cell lines were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai China). The HSC3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F-12 medium (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 1% fungicide and penicillin/streptomycin (Biochrom, Ltd., Cambridge, UK) at 37°C in a humidified atmosphere containing 5% CO₂. CDDP was obtained from Sigma-Aldrich. The pVAX1-GRIM-19 (pGRIM-19) plasmid was constructed, as described in our previous study (16). The pVAX1 and pGRIM-19 plasmids were extracted and purified using an Endofree Plasmid Giga kit (Qiagen, Chatsworth, CA, USA) and were solubilized in Endo-free sterilized water for the subsequent experiments. The present study was approved (no. JL2013568B) by the ethics committee of Jilin University (Changchun, China).

Preparation of cationic LPs and plasmid DNA complexes. The cationic LPs, composed of DOTAP/cholesterol (Avanti Polar Lipids, Birmingham, AL, USA) used in the present study were synthesized, as previously described (21). For cell transfection, the pVAX/pVAX-LKB1 plasmid and LPs, diluted in equal volumes of DMEM, were mixed to form DNA:LP complexes (LP-pVAX1 or LP-pGRIM-19), according to their molecular weight ratio (1:6). For the animal experiments, the LPs and plasmid DNA were diluted and mixed in 5% dextrose, as previously described (17). The average particle size (150-300 nm) of the complexes was selected, according to previous study (17).

Cell viability. To determine CDDP sensitivities, cell viability was performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the HSC3 cells were seeded (5x10³ cells/well) onto 96-well plates and transfected with the different plasmid DNA complexes for 48 h at 37°C. Following transfection, DMEM with serial concentrations (1.0-20.0 µM) of CDDP were added and incubated for another 48 h at 37°C. The cell medium was replaced with 20 µl MTT (Sigma-Aldrich) and incubated for another 4 h, followed by the

addition of 200 µl solubilization solution containing dimethyl sulfoxide (Sigma-Aldrich) into each well. The plates were maintained in a dark room overnight, and the optical density of each sample was measured at a 570 nm test wavelength using an ELISA multi-well spectrophotometer (SpectraMax[®] M2/M2e; Molecular Devices Corporation, Sunnyvale, CA, USA). The percentages of viable cells were calculated based on the absorbency of the treated cells relative to that of the untreated cells. The half maximal inhibitory concentration (IC₅₀) values of CDDP were also calculated. To evaluate the inhibition on OSCC cell growth by the combined treatment of LP-pGRIM-19 and low-dose CDDP, the half maximal inhibitory concentration (IC₅₀) of CDDP (10 µM) was used in all the following experiments *in vitro*. The HSC3 cells were seeded (5x10³ cells/well) onto 96-well plates and transfected with either LP-pVAX1 or LP-pGRIM-19, followed by treatment with the IC₅₀ CDDP. The cell viabilities following 48 h treatment were quantified using an MTT assay, as above described. The visible colonies were then counted under an IX51 inverted microscope (Olympus Corporation, Tokyo, Japan).

Cell colony formation. The HSC3 cells were seeded into six-well culture plates at 1x10⁴ cells/well, and were transfected with either LP-pVAX1 or LP-pGRIM-19, prior to treatment with IC₅₀ CDDP. The cells were incubated at 37°C for 10 days, and the medium was replaced every 3 days. The colonies were fixed with ice methanol for 30 min at room temperature and stained with 2% Giemsa (Sigma-Aldrich) for 10 min at room temperature. The visible colonies were then counted.

Cell apoptosis. Flow cytometry was used to detect cell apoptosis. In brief, the HSC3 cells were transfected with either LP-pVAX1 or LP-pGRIM-19, and were then treated with IC₅₀ CDDP for 24 h. Following treatment, the cells were collected and fixed in 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 70% ethanol, washed with phosphate-buffered saline (PBS) and stained with propidium iodide (PI; Sigma-Aldrich) solution containing 40 µg/ml PI and 10 µg/ml DNase-free RNase A (Sigma-Aldrich). Apoptosis assays were performed, according to the manufacturer's instructions of the Annexin V-FITC Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). The rates of apoptosis were determined using CellQuest 2.7 software (BD Biosciences, San Jose, CA, USA). In addition, the induction of caspase-3, -8 and -9 activity in the HSC3 cells treated with LP-pGRIM-19 and CDDP were measured using caspase-3, -8 and -9 colorimetric protease assay kits (EMD Millipore, Billerica, MA, USA), according to the manufacturer's instructions. Briefly, the HSC3 cells were seeded into 6-well plates at a density of 4x10⁵ cells/well and cultured for 24 h, and collected by centrifugation at 1500 rpm for 5 min at 4°C. Caspase-3, -8, and -9 expression levels were measured in the cell lysates using caspase-3, -8, and -9 colorimetric protease assay kits according to the manufacturer's instructions. The optical density was read using a microplate reader (UM313565; Thermo Fisher Scientific Inc., Waltham, MA, USA) at 405 nm.

Invasion and migration assays. Cell invasion assays were performed using a QCM ECMatrix Cell Invasion Assay kit (24-well; 8 µm; EMD Millipore), according to the

manufacturer's instructions. Briefly, the HSC3 cells were treated with LP-pGRIM-19 or CDDP for 24 h, and 1×10^4 cells were seeded in the Transwell chamber (BD Biosciences). DMEM supplemented with 20% FBS was added to the lower chamber as the chemoattractant. Following incubation for 24 h, the non-invading cells in the upper chamber were gently removed using a cotton-tipped swab, and the invading cells in the lower chamber were fixed with methanol and stained with 2% Giemsa solution. The invasive ability was determined by the number of penetrating cells under a Nikon phase-contrast microscope L150 (Nikon Corporation, Tokyo, Japan) and counted in >10 fields of view at x200 magnification in each well.

The *in vitro* migration assay was similar to the invasion assay described above, however a non-Matrigel-coated 24-well Boyden Chamber (8 μ m, Millipore) was used. A total of 2×10^3 cells were added to the Transwell chamber, the incubation duration was 24 h, and the subsequent steps were consistent with the invasion assay. The number of cells migrated were counted by counting the cells in 10 randomly-selected fields per filter, under a Nikon phase-contrast microscope (Nikon Corporation, Tokyo, Japan). Triplicate assays were performed for each group of cells in the invasion and migration assays.

Western blot analysis. The cells were collected by trypsinization (5% trypsin; Sigma-Aldrich) and lysed in radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich) with the addition of protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitors (Sigma-Aldrich) for 30 min on ice. The protein quantity was analyzed using Bradford reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cell extracts (20 μ g protein) were separated by 10% SDS-PAGE (Sigma-Aldrich) and transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% dry milk in PBS, and incubated overnight at 4°C with the following antibodies: Mouse monoclonal anti-human GRIM-19 (1:2,000; cat. no. sc-365045; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), mouse monoclonal anti-human signal transducer and activator of transcription 3 (Stat3; 1:1,000; cat. no. sc-8019; Bedford, MA, USA), rabbit monoclonal anti-human matrix metalloproteinase (MMP-2; 1:1,000; cat. no. sc-13132; Cell Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal anti-human MMP-9 (1:2,000; cat. no. sc-21773; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-human vascular endothelial growth factor (VEGF; 1:2,000; cat. no. sc-53462; Santa Cruz Biotechnology), a mouse monoclonal anti-human phosphorylated (p)-p53 (1:3,000; cat. no. sc-126; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-human Bcl-2 (1:1,000; cat. no. sc-7382; Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-human cyclin D1 (1:1,000; cat. no. sc-450; Santa Cruz Biotechnology, Inc.). Mouse monoclonal anti-human β -actin (1:10,000; cat. no. sc-8432; Santa Cruz Biotechnology, Inc.) was used as a loading control. Following incubation with the primary antibodies, the membranes were washed twice in PBS and incubated with horseradish peroxidase-conjugated goat anti-mouse antibodies (1:5,000; cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) or goat anti-rabbit antibodies (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. The

proteins were detected by the enhanced protein bands, which were visualized using enhanced chemiluminescence reagent (Sigma-Aldrich). The integrated density value (IDV) was analyzed using a computerized image analysis system (Fluor Chen 2.0; Bio-Rad Laboratories, Inc.) and normalized with that of β -actin.

Tumor growth in vivo. A total of 60 female BALB nude mice aged 4-6 weeks (18-20 g) were purchased from the Institute of Laboratory Animal Science, Jilin University (Changchun, China). A total of 2×10^6 (100 μ l) HSC3 cells suspended in 100 μ l PBS were subcutaneously injected into the left abdominal wall, and the size of the resulting tumor was measured daily for 7 days following injection. The tumor volume was calculated as follows: $0.5236 \times \text{width}^2 \times \text{length}$. At ~20 days following inoculation of the HSC3 cells, the average tumor volume measured up to 100 mm³. These tumor-bearing nude mice were randomly divided into the following six groups (n=10/group): (i) control group; (ii) LP-pVAX1 group; (iii) CDDP group; (iv) LP-pGRIM-19 group; (v) CDDP+LP-pGRIM-19 group; (vi) CDDP+LP-pGRIM-19 group. In the control group, nude mice were injected with 100 μ l PBS. Other mice were administered with the LP-pVAX1 or LP-pGRIM-19 complexes by intravenous injection at a dose of 20 μ g/mouse, and/or low-dose CDDP (2 mg/kg⁻¹/mouse) by intraperitoneal injection once a week for 21 days, respectively. The mice were sacrificed 7 days following the final plasmid injection. Tumor tissue was excised, the volume measured volume and weighed. In addition, splenic tissues were collected and cultured for a splenocyte surveillance investigation using an MTT assay, as described previously (22). Briefly, the splenic tissue samples were collected from the mice, and single-cell spleen suspensions were added to serum-free DMEM by filtering the suspension through a sieve mesh with the aid of a glass homogenizer, in order to exert gentle pressure on the spleen fragments.

Statistical analysis. Data from at least three independent experiments are expressed as the mean \pm standard deviation. Statistical comparison of more than two groups was performed using one-way analysis of variance followed by Tukey's post-hoc test. GraphPad Prism 5.01 software (GraphPad Software, Inc., San Diego, CA, USA) and SPSS® 16.0 (SPSS, Inc., Chicago, IL, USA) for Windows® were used for statistical analyses. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with a combination of LP-pGRIM-19 and low-dose CDDP enhances inhibition of cell proliferation and cell colony formation. To further determine whether the exogenous expression of GRIM-19 sensitizes the response of OSCC cells to CDDP, the present study analyzed the sensitivities of the HSC3 cells to CDDP following treatment with different concentrations of CDDP alone, or combined with LP-pGRIM-19 or LP-pVAX1 transfection, respectively. Compared with CDDP treatment, the IC₅₀ concentrations of CDDP in the HSC3 cells decreased between 7.02 ± 0.46 and 3.85 ± 0.41 μ M when the cells were treated with

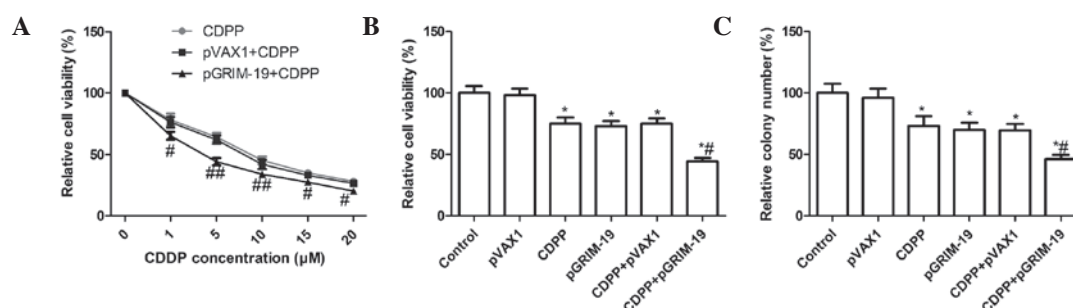


Figure 1. Combined effect of the exogenous expression of GRIM-19 and treatment with CDDP on tumor cell proliferation and clonogenicity in HSC3 cells. (A) HSC3 cells were transfected with LP-pGRIM-19 and treated with various doses of CDDP. Following 48 h treatment, cell viability was analyzed using an MTT assay, and the IC_{50} values were calculated. (B) HSC3 cells were transfected with LP-pGRIM-19 and then treated with the IC_{50} concentration of CDDP for 48 h. Cell viability was analyzed using an MTT assay. (C) Effects of LP-pGRIM-19 and low dose CDDP on colony formation of the HSC3 cells were determined following treatment with low-dose CDDP and LP-pGRIM-19, alone and in combination. Data are expressed as the mean \pm standard deviation. * $P < 0.05$, vs. control; ** $P < 0.05$, vs. CDDP. GRIM-19, gene associated with retinoid-interferon-induced mortality 19; CDDP, cisplatin; LP, liposome; IC_{50} , half maximal inhibitory concentration; p, plasmid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

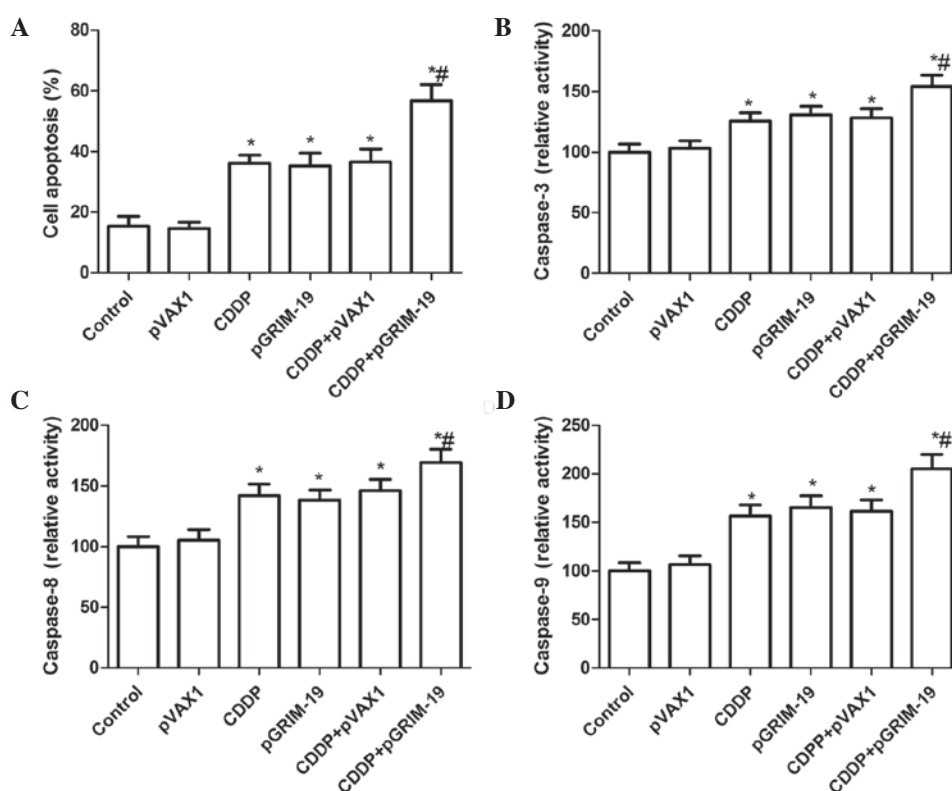


Figure 2. Combined effect of the exogenous expression of GRIM-19 and treatment with CDDP on tumor cell apoptosis in HSC3 cells. (A) Effects of LPs-pGRIM-19 and low dose CDDP on apoptosis of the HSC3 cells were determined following treatment with low-dose CDDP and LP-pGRIM-19, alone and in combination. Activities of (B) caspase-3 (C) caspase-8 and (D) caspase-9 were determined following treatment with low-dose CDDP and LP-pGRIM-19, alone and in combination. Data are expressed as the mean \pm standard deviation. * $P < 0.05$, vs. control; ** $P < 0.05$, vs. CDDP. GRIM-19, gene associated with retinoid-interferon-induced mortality 19; CDDP, cisplatin; LP, liposome; IC_{50} , half maximal inhibitory concentration; p, plasmid.

LP-pGRIM-19+CDDP (Fig. 1A), which suggested that the exogenous expression of GRIM-19 enhanced the sensitivity of the HSC3 cell lines to CDDP. Based on these results the respective IC_{50} concentrations of CDDP were selected for further treatments in the present study.

To determine whether the exogenous expression of GRIM-19 enhances low-dose CDDP-mediated cell proliferation inhibition, the HSC3 cells were transiently transfected with LP-pGRIM-19 and, following transfection, the cells were treated with low-dose CDDP at the IC_{50} concentration in The

HSC3 cells. Significantly enhanced inhibition of cell proliferation following treatment with LP-pGRIM-19+low-dose CDDP was observed in the HSC3 cells, compared with treatment with either low-dose CDDP or LP-pGRIM-19 alone ($P < 0.05$; Fig. 1B).

Subsequently, the effects of the combination of LP-pGRIM-19 and low-dose CDDP on colony formation of the HSC3 cells were analyzed. As shown in Fig. 1C, the combination of LP-pGRIM-19 and low-dose CDDP significantly inhibited the colony formation of the HSC3 cells, compared

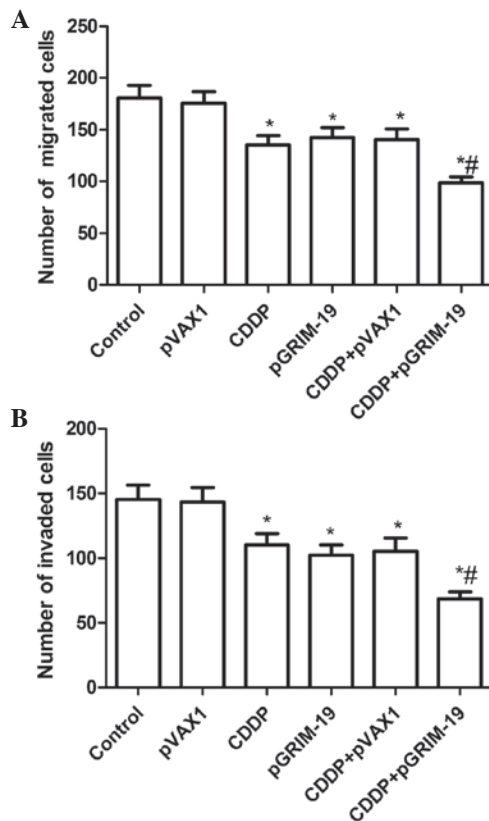


Figure 3. Combined effect of exogenous expression of GRIM-19 and treatment with CDDP on tumor cell migration and invasion in HSC3 cells. (A) Numbers of migrated cells were determined using a Transwell assay (without Matrigel) following treatment with low-dose CDDP and LP-pGRIM-19, alone and in combination. (B) Number of invaded cells were determined using a Transwell matrix penetration assay (with Matrigel) following treatment with low-dose CDDP and LP-pGRIM-19, alone and in combination. Data are expressed as the mean \pm standard deviation. * $P < 0.05$, vs. control; ** $P < 0.05$, vs. CDDP. GRIM-19, gene associated with retinoid-interferon-induced mortality 19; CDDP, cisplatin; LP, liposome; IC50, half maximal inhibitory concentration; p, plasmid.

with treatment with either low-dose CDDP or LP-pGRIM-19 alone ($P < 0.05$; Fig. 1C).

Treatment with a combination of LP-pGRIM-19 and low-dose CDDP enhances the induction of HSC3 cell apoptosis. To evaluate whether the exogenous expression of GRIM-19 enhances CDDP-induced cell apoptosis, the HSC3 cells were treated with low-dose CDDP and LP-pGRIM-19 either alone or in combination, and cell apoptosis was detected using flow cytometry. Treatment with LP-pGRIM-19+low-dose CDDP led to a significant increase in apoptosis, compared with either low-dose CDDP or LP-pGRIM-19 treatment alone ($P < 0.05$), as shown Fig. 2A. In addition, no significance different was observed between the low-dose CDDP- and LP-pGRIM-19-only treatment groups in the induction of OSCC apoptosis.

In order to examine the possible mechanism of the pro-apoptotic effect of the combination of LP-pGRIM-19 and low-dose CDDP, the activities of caspase-3, -8 and -9 were investigated. The results (Fig. 2B-D) indicated that the combination of LP-pGRIM-19 and low-dose CDDP significantly increased the activities of caspase-3, -8 and -9 in the HSC3

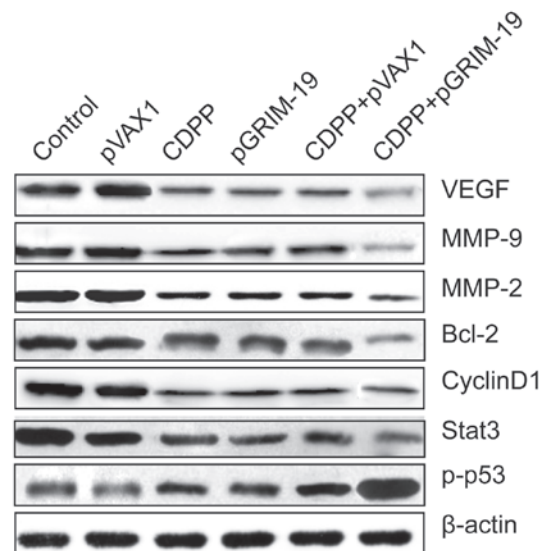


Figure 4. Combined effect of exogenous expression of GRIM-19 and treatment with CDDP on proteins associated with apoptosis and invasion. Protein expression levels of p-p53, Stat3, Bcl-2, cyclin D1, MMP-2, MMP-9 and VEGF were analyzed using western blot assay with specific antibodies following treatment with low-dose CDDP and LP-pGRIM-19, alone and in combination. β-actin was used as an internal control. GRIM-19, gene associated with retinoid-interferon-induced mortality 19; CDDP, cisplatin; LP, liposome; IC50, half maximal inhibitory concentration; p, plasmid; p-p53, phosphorylated p53; Stat3, signal transducer and activator of transcription 3; Bcl-2, B cell lymphoma 2; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth factor.

cells, relative to either CDDP or LP-pGRIM-19 treatment alone ($P < 0.05$).

Treatment with a combination of LP-pGRIM-19 and low-dose CDDP enhances the inhibition of cell migration and invasion. The present study aimed to determine whether the combination of LP-pGRIM-19 and low-dose CDDP affected cell vitality, demonstrated by migration or invasion activity. Therefore, cell migration and invasion assays were performed using a Transwell assay. As shown in Fig. 3A, the combination of LP-pGRIM-19 and low-dose CDDP significantly decreased the migration of HSC3 cells, compared with the single LP-pGRIM-19 and CDDP treatment groups ($P < 0.05$; Fig. 3A). The ability of this combination to reduce the invasiveness of HSC3 cells was subsequently investigated. The Transwell matrix penetration assay, which was coated with Matrigel revealed that the combination of LP-pGRIM-19 and low-dose CDDP significantly reduced the invasiveness of the HSC3 cells, compared with either CDDP or LP-pGRIM-19 treatment alone ($P < 0.05$; Fig. 3B).

Combination of LP-pGRIM-19 and low-dose CDDP has a synergistic effect on relevant tumor effector molecules in vitro. To elucidate the molecular mechanisms responsible for the induction of synergistic growth inhibition and apoptosis of OSCC cells by LP-pGRIM-19+low-dose CDDP, the expression levels of Bcl-2, cyclinD1, Stat3 and p-p53 were analyzed in cells treated with low-dose CDDP and LP-pGRIM-19 alone and in combination, using western blot analysis. The combination of LP-pGRIM-19 and low-dose CDDP resulted in further upregulation of the protein level of p-p53 and downregulation

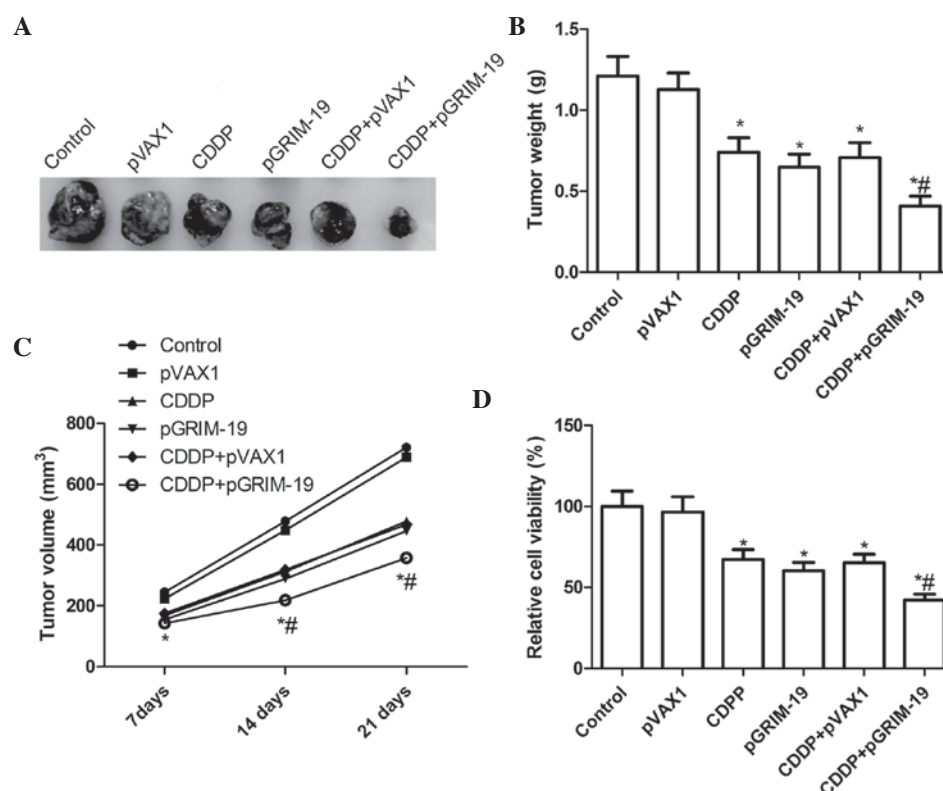


Figure 5. Inhibition of tumor growth in OSCC mouse model by combination treatment with LP-pGRIM-19 and low dose CDDP. A human HSC3 orthotopic OSCC mouse model was used to evaluate the combined effect of systemic administration of LP-pGRIM-19 and low dose CDDP on tumor growth *in vivo*. (A) Representative images of the tumors in the different treatment groups. (B) Tumor weights in each treatment group were measured on day 21. (C) Tumor volumes in each treatment group were determined at different time-points. (D) Cell viability of splenocytes from mice were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are expressed as the mean \pm standard deviation. * $P < 0.05$, vs. control; # $P < 0.05$, vs. CDDP, GRIM-19, gene associated with retinoid-interferon-induced mortality 19; CDDP, cisplatin; LP, liposome; IC₅₀, half maximal inhibitory concentration; p, plasmid.

of the protein levels of Bcl-2, cyclinD1 and Stat3 in the HSC3 cells, compared with the LP-pGRIM-19 or low-dose CDDP alone treatment groups (Fig. 4).

To further investigate the potential mechanisms underlying the effects of the combination of LP-pGRIM-19 and low-dose CDDP on cell invasion and migration, relevant effector molecules, including MMP-2, MMP-9 and VEGF were examined using western blot analysis. It was found that the protein expression levels of MMP-2, MMP-9 and VEGF were downregulated in the HSC3 cells treated with of LP-pGRIM-19+low-dose CDDP, compared with treatment with either CDDP or LP-pGRIM-19 alone (Fig. 4)

Treatment with a combination of LP-pGRIM-19 and low-dose CDDP enhances tumor growth inhibition *in vivo*. To determine whether GRIM-19 gene therapy enhances CDDP-mediated antitumor activity *in vivo*, the present study evaluated the effects of GRIM-19 combined with low-dose CDDP on tumor regression in female BALB mice bearing HSC3 tumors. The mice were sacrificed and tumor tissues were removed 21 days following treatment, and the tumor weights were measured. The tumor weights of the of LP-pGRIM-19+low-dose CDDP group were lower than those of the control group and the CDDP-only and LP-pGRIM-19-only groups ($P < 0.05$; Fig. 5A and B). In addition, the growth in tumor volume in the combination group was significantly slower in the HSC3 tumor cells, compared with the cisplatin-only and LP-pGRIM-19-only groups ($P < 0.05$;

Fig. 5C). Subsequently, MTT assays were used to examine the modulation of splenocyte proliferation and to demonstrate the antitumor activities of this combination *in vivo*. As shown in Fig. 5D, splenocyte cell proliferation in the combination treatment group decreased significantly, compared with the relative to cisplatin-only and LP-pGRIM-19-only groups ($P < 0.05$). These results suggested that GRIM-19 was responsible for the enhancement of low-dose CDDP-induced antitumor activity *in vivo*.

Discussion

Drug resistance is a major cause of treatment failure in patients with OSCC. The combination of conventional CDDP-based chemotherapy and tumor suppressor gene therapy may overcome cancer cell resistance to chemotherapeutic drugs and enhance the therapeutic efficacy in cancer (17-20). However, the role of GRIM-19, a tumor suppressor, in the response to chemotherapy, has not been reported to date, to the best of our knowledge. Therefore, the present study assessed the functional effects of GRIM-19 on the effects of CDDP in OSCC. The results of this investigation demonstrated that exogenously introduced expression of GRIM-19 sensitized the response of OSCC cells to CDDP treatment. Treatment with LP-GRIM-19 in combination with the chemotherapeutic drug, CDDP, markedly inhibited tumor cell proliferation, clonogenicity, migration and invasion, and induced apoptosis *in vitro*. The present study

also demonstrated that co-treatment with LP-GRIM-19 and CDDP synergistically suppressed tumor growth in a human OSCC tumor xenograft mouse model. To the best of our knowledge, the present study is the first to report on the combined introduction of the GRIM-19 tumor suppressor and the conventional chemotherapeutic agent, CDDP, in human OSCC cells.

Apoptosis is the primary mechanism causing several malignant cells to die when subjected to chemotherapy or radiotherapy, and it has been demonstrated that mutations in apoptotic pathways and decreased susceptibility to apoptosis are associated with cellular resistance to chemotherapeutic drugs and radiation treatment (23-25). The activation of p53 by CDDP-induced DNA damage has been reported to have various effects on cellular sensitivity towards CDDP (26), whereas downregulation in the expression of Bcl-2 in cancer cells significantly increases the sensitivity of cancer cells to CDDP (27). Our previous study demonstrated that exogenously introduced expression of GRIM-19 in OSCC cells induced cell apoptosis (16). In the present study, the result revealed that the exogenously introduced expression of GRIM-19 sensitized the response of the OSCC cells to CDDP treatment, and that co-treatment with LP-GRIM-19 and CDDP synergistically induced cell apoptosis, increased the activities of caspase-3, -8 and -9, increased the expression of p-p53 and decreased the expression of Bcl-2. These results suggested that the overexpression of GRIM-19 in CDDP-treated OSCC cells may overcome the cellular resistance and enhance the cellular response to the chemotherapy, by facilitating drug-induced apoptosis, and by upregulating and downregulating the expression levels of p53 and Bcl-2, respectively.

The altered expression of Stat3 has been reported to be key in carcinogenesis by promoting cell proliferation, differentiation and cell cycle progression, as well as inhibiting apoptosis via the incessant induction of pro-growth genes, including cyclin D1, Bcl-2, VEGF and MMP-2 (28-31). The anticancer effects of CDDP can be enhanced by downregulating the expression of Stat3 using Stat3 inhibitors or small interfering RNA (32,33). GRIM-19, a Stat3 inhibitor, has been found to upregulate the expression of GRIM-19 and inhibit Stat3 activation in different types of cancer (28,34-36), due to the binding of GRIM-19 to the Stat3 gene and inhibiting its transcription. In the present study, exogenously introduced expression of GRIM-19 sensitized the response of OSCC cells to CDDP treatment, and the combination of LP-pGRIM-19 and low-dose CDDP resulted in further upregulation of p-p53, and downregulation of Stat3, Bcl-2, cyclin D1, MMP-2, MMP-9 and VEGF at the protein levels in HSC3 cells, compared with either LP-pGRIM-19 or low-dose CDDP treatment alone. These results suggested that the enhanced sensitivity of OSCC cells to CDDP by GRIM-19 may be through inhibition of the Stat3 signaling pathway.

In conclusion, the present study demonstrated for the first time, to the best of our knowledge, that treatment with LP-pGRIM-19 in combination with a low dose of CDDP markedly inhibited OSCC tumor cell proliferation, clonogenicity, migration and invasion, and induced apoptosis *in vitro*, as well as suppressing OSCC growth *in vivo*. The enhanced sensitivity of OSCC cells to CDDP by GRIM-19 was associated with the upregulation of p-p53 and downregulation of Bcl-2, VEGF, MMP-2 and MMP-9 at the protein levels, which are involved in the activation of Stat3. These findings provide novel insight

into the molecular mechanisms of GRIM-19-mediated tumor suppression and suggest that the combination of GRIM-19 gene therapy with low-dose CDDP-based chemotherapy may be a potent therapeutic strategy for the treatment of human OSCC.

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