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Original Article

Exogenous OGF enhances the anti-tumor activity of cisplatin on hepatocellular carcinoma

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Abstract: Background: Hepatocellular carcinoma results in high cancer mortality and is difficult to eradicate because of its late stage at the time of diagnosis, multicentricity, and cirrhotic background. It is therefore urgent to explore effective and economical therapeutic methods to treat this disease. Objectives: We aimed to investigate the antitumor activity of exogenous opioid growth factor (OGF), as well as the effect of the combination of OGF and cisplatin on hepatocellular carcinoma. The possible underlying mechanisms were also explored. Materials and Methods: RT-PCR and immunohistochemistry were employed to determine the expression of OGF receptor (OGFr) in hepatocellular carcinoma. MTT assays were used to explore the effect of OGF on cell migration and proliferation. Animal experiments were performed to explore the effect of OGF and DDP on tumors. Results: OGFr is present in human HCC cells and was differentially expressed between HCC tumor and non-tumor tissues. OGF inhibited HCC cell proliferation and migration. The silencing of OGFr blocked the expression of p21 and p53. Treatment using OGF, DDP and OGF+DDP all suppressed the growth of HCC tumors, with the maximum effect in the OGF+DDP group. Conclusion: Our study clarified that OGF inhibits cell migration and proliferation of HCC in animal experiments and that exogenous OGF enhances the anti-tumor activity of cisplatin on HCC by upregulating p21 and p53. These findings may provide a new strategy for future HCC therapeutics.

Keywords: Opioid growth factor receptor, hepatocellular carcinoma, anti-tumor activity, cisplatin, p53

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality. HCC is a challenging disease that is hard to eradicate because of its late stage at the time of diagnosis, multicentricity, and cirrhotic background. Moreover, HCC is insensitive to conventional therapy, such as cytotoxic chemotherapy and radiotherapy. Thus, the most accepted and practiced therapy is local-regional therapy, such as ablation therapy and transarterial chemoembolization (TACE). Local-regional therapy combining biological-targeted therapy is an option for those who cannot undergo surgical treatment [1]. However, biological-targeted therapy is too expensive for most patients. Therefore, it is urgent to explore an effective and economical approach for HCC therapy.

The opioid growth factor (OGF) is an autocrine and paracrine-produced and secreted peptide that interacts with the OGF receptor (OGFr) to inhibit growth of cancer cells [2]. OGF activity is targeted to the G0/G1 phase of the cell cycle with no tissue or organ specifies [3]. Current studies have demonstrated that the OGF-OGFr axis is directed towards p21 and cyclin-dependent inhibitory kinase (CKI) pathways. It delays cells transitioning from the G1 to S phase of the cell cycle, thus inhibiting DNA synthesis [4, 5]. It is reported that the OGF-OGFr axis utilizes the p16 pathway to restrict cell proliferation [4, 7]. Thus, OGF is a potential target for treatment of cancer. Recent studies have revealed the efficacy of the OGF-OGFr axis in suppressing different kinds of cancer, such as pancreatic cancer [5, 6], head and neck cancer [7, 8], ovarian cancer [9, 10], renal cell cancer [11], colon cancer [12], breast cancer [13], thyroid cancer [14] and hepatocellular carcinoma (HCC) [3].

OGFr has been detected in surgical samples taken from human HCC reported by Avella et al.

[3]. It is reported that the OGF-OGFr axis is a native biological regulator of cell proliferation in HCC. However, little was reported on the relationship of the OGF-OGFr axis and HCC. Thus, in our investigation, we aimed to verify whether OGF inhibits cell growth in HCC by the OGF-OGFr axis, explore the impact of the OGF-OGFr axis induced in HCC, and compare OGF's antitumor activity with other cytotoxic drugs to demonstrate its possible application in HCC treatment.

Materials and methods

Cell culture and reagents

Human HCC cell lines HepG2, Huh7 cells, SK-Hep-1 cells, HCT116 cells, A2780, and HOSE cells were obtained from KeyGen Biotech (Nanjing, China). Cells were incubated with Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% L-glutamine. All the cell lines were grown at 37°C in a humidified incubator of 5% CO_2.

Rabbit polyclonal anti-OGFr antibody (Abnova, Taiwan, #PAB20975) was purchased for immunohistochemistry and western blotting. Mouse monoclonal anti-p53 (DO-1) (sc-126, Santa Cruz, USA), rabbit polyclonal anti-p16 (sc-68393, Santa Cruz, USA) and mouse monoclonal anti-p21 (sc-817, Santa Cruz, USA) were employed for in vivo studies. The peptide for OGF was obtained from the Enzo Life Sciences (#PEP-4042-M100). The reagent for cisplatin (DDP) was purchased from QILU Pharmaceutical (Jinan, China).

Tissue samples

The paired samples of the tumor and the corresponding cirrhotic non-tumor tissues were extracted from the liver specimens of HCC patients. The HCC and the non-tumor tissues were confirmed by two pathologists. The extent of the liver disease of each patient was rated according to the histological activity index (HAI). The study protocol was confirmed by the ethical guidelines of the Institutional Review Board (IRB), and written informed consent was obtained from each patient.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of tissues and cells were extracted using the Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed using 10 μg of total RNA, 50 μM decamers and 1 μL (200 units) RT-PCR Superscript II (Invitrogen, Carlsbad, CA) at 37°C for 50 min according to the protocol. The primers for qRT-PCR were as follows: OGFr forward primer, 5'-cacgccaaacctgagtttct-3'; OGFr reverse primer, 5'-ggttctcgcagaggaaacag-3'. The Quantum RNA TM 18S Internal Standard Kit (Ambion, Austin, TX) was used as the internal control according to the manufacturer's instructions. The PCR products were separated by electrophoresis on a 2% agarose gel and were visualized under ultraviolet light after ethidium bromide staining. The relative expression was normalized to the level of endogenous control GAPDH.

Immunohistochemistry

The extracted tissues were rapidly fixed in 10% cold paraformaldehyde, embedded in paraffin, and cut 4 μm thick. After rehydration, a deparaffinized section was pretreated by microwave epitope retrieval. Before applying the primary antibody, the endogenous peroxidase activity was inhibited with 3% hydrogen peroxide and blocked with biotin and bovine serum albumin. After washes, slides were incubated with rabbit polyclonal anti-OGFr (sc-85796, Santa Cruz) at 4°C overnight. The next morning, slides were washed and incubated with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase-conjugated antibody according to the manufacturer's instructions (DAKO, Glostrup, Denmark). Counter-staining was performed using Meyer's hematoxylin. The tumors were evaluated for the percentage of positive cells and the intensity of staining. The negative controls included samples incubated with preserum instead of the primary antibody.

Immunoblotting and immunoprecipitation

The cell extracts were clarified by centrifugation at 12,000 rpm, and the supernatants (1 mg/mL) were subjected to immunoprecipitation with the indicated antibodies. After an overnight incubation at 4°C, protein A agarose beads were added and incubated for an addi-

tional 3 hours. The immunocomplexes were washed 3 times with lysis buffer and then were subjected to immunoblot analysis. Thirty micrograms of each cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred to Hybond membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were blocked and then incubated with the designated primary antibodies; the signals were detected using an ECL western blotting kit (Amersham Pharmacia Biotech).

MTT assays and cell migration assay

Cell viability was measured by the methyl thiazolyl tetrazolium test (MTT, Amersham Biosciences). Cells were seeded in 24-well plates. After 24 h, cells were then incubated with complete medium containing 0.5 mg/mL MTT at 37°C. Four hours later, cells were dissolved in 200 μL of a solution containing dimethylsulfoxide plus 2.5% complete medium, and formazan absorbance was then read at a wavelength of 540 nm.

Cell migration was also measured using a cell wound healing assay performed in six-well plates in DMEM with 10% FBS. At a density of 90%, cells were rinsed with PBS and then starved for 24 h in serum-free medium. A sterile 200-μL pipette tip was used to create 3 separate parallel wounds, and migration of the cells across the wound line was assessed after 36 h. Experiments were repeated on 2 separate occasions.

Knockdown of OGFr

SiRNA sequences specifically targeting human OGFr and non-target control sequences were constructed by Sangon Biotech (Shanghai, China). The sequences used were as follows: OGFR siRNA sense, 5'-GCCCUGGACUACUUCAUGUTT-3'; anti-sense, 5'-ACAUGAAGUAGUCCAGGGCTT-3'; scrambled siRNA sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; and anti-sense, 5'-ACGUGACACGUUCGGATT-3. HEK 293 cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well and were transfected the following day with 25 nM (final concentration) siRNA using Lipofectamine RNAi MAX Transfection Reagent (Life Technologies) according to the manufacturer's instructions. Cells were collected after 48 h following transfection. OGFr knockdown was confirmed by western blot analysis with goat OGFr polyclonal antibody (Santa Cruz Biotechnology, #sc-85796).

Mouse tumorigenicity assay and drug treatment

Female athymic nude mice (BALB/cAnN, Korea) at 4 weeks of age, weighing 16-18 g, were used in all experiments. Mice were maintained in a specific pathogen-free environment. The animal room was kept at 20°C-22°C under a 12 h light/dark cycle. The cells (5×10^6 cells in 200 μL PBS) were injected subcutaneously into the right back leg of the mice. When the tumor volume reached 100 mm^3, drugs were administered to the mice. Mice were randomly divided into four groups (n = 5 of each group): PBS, OGF, DDP and OGF+DDP groups. Mice in the OGF group were intraperitoneally injected with OGF (10 mg/kg/d). The DDP group received DDP (4 mg/kg) every three days. Mice in the DDP+OGF group were given OGF (10 mg/kg/d) and DDP (4 mg/kg) every three days. The control group was injected with the same amount of physiological saline. Tumor dimensions were measured every 3 days using a digital caliper, and the tumor volume was calculated using the following formula: $V = \pi/6 \times (\text{larger diameter}) \times (\text{smaller diameter})^2$. Tumor growth was observed for at least 4 weeks. The tumorigenic experiments in vivo were performed with 5 mice in each treatment group.

Quantification and statistical analysis

Autoradiographs of the immunodot membranes were scanned using a LAS3000 system (Fuji Photo Film, Tokyo, Japan), and the densitometric data were analyzed with Quantity One software. A Chi-square test or a two-tailed Fisher's exact test was used to compare the staining results according to the independent groups, with a significance level of 5%. All data analysis was performed using GraphPad software.

Results

OGFr is expressed tumor cell lines and tissues

To explore the role of OGFr, we detected the mRNA levels of OGFr in HepG2, Huh7, SK-Hep-1, HCT116, A2780, and HOSE HCC cell lines. As demonstrated in **Figure 1A**, we found that OGFr was highly expressed in HOSE cells, A2780 cells, and HCT116 cells but had slightly lower expression in HepG2, SK-Hep1 and HUH-7 cells. Compared with non-tumor tissues, the mRNA levels of OGFr in tumor tissues were suppressed, as shown in **Figure 1B**. This data

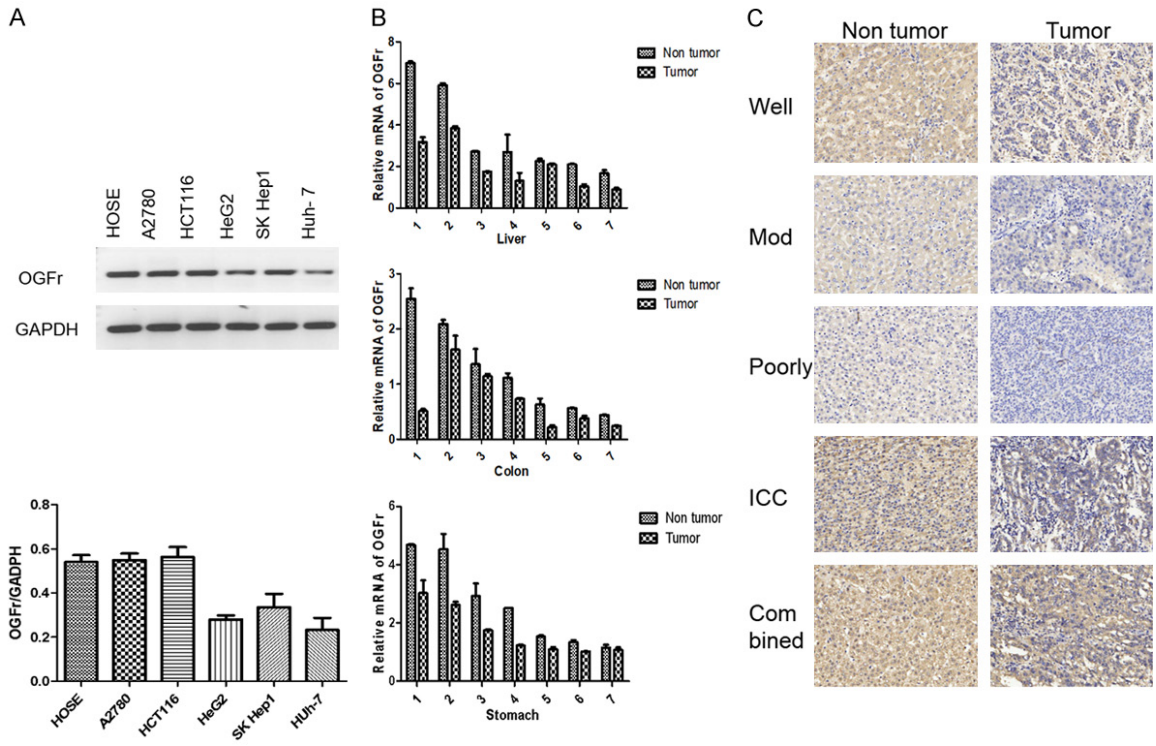

Figure 1. OGFr is expressed in HCC cells and tissues. A. OGFr mRNA expression by RT-PCR in HOSE cells, A2780 cells (ovarian cancer cells), a colon cancer cell line (HCT116 cells), and three cell lines of HCC, including HepG2, SK-Hep1 and HUH-7. B. The difference of OGFr mRNA expression levels in tumor and non-tumor tissues. Total mRNA was extracted from liver cancer, colon cancer and gastric cancer tissues for qRT-PCR. C. Hepatocellular carcinoma tissues were used to perform immunohistochemistry assays to compare the OGFr protein expression difference between tumor and non-tumor tissues. The OGFr protein expression was more pronounced and in all groups turned the non-tumor tissues a brownish color not observed in tumor tissues.

showed that OGFr is present in human HCC tissues, colon cancer tissues, and gastric cancer tissues. We also observed differential expression of OGFr between tumor tissues and non-tumor tissues in human HCC, colon cancer, and gastric cancer ($P < 0.05$).

We also performed an immunohistochemistry assay using hepatocellular carcinoma tissues in our preliminary studies. We divided these tissues into well-differentiated HCCs, moderately differentiated HCCs, poorly differentiated HCCs, intrahepatic cholangiocarcinoma (ICC), and combined hepato-cholangiocarcinoma. As seen in **Figure 1C**, the OGFr protein expression was more noticeable and resulted in a brownish color in non-tumor tissues compared to tumor tissues in all groups.

OGF inhibits HCC cell migration and proliferation

We performed the scratch assay in HepG2 cell lines. The migration of cells in the OGF group is significantly slower than control groups (**Figure 2A**), which demonstrates that OGF inhibited cell migration in HCC cell lines. We also utilized an MTT assay to observe the effect of OGF on cell proliferation in both SK-Hep1 and HepG2 cell lines. The results showed that the cell numbers of the OGF group were significantly fewer than those in the control group: approximately 20% fewer in SK-Hep1 and 33.3% fewer in the HepG1 cell line. These results indicate that OGF inhibits cell migration and cell proliferation in HCC cell lines.

OGFr inhibits cell proliferation by upregulating p21 and p53 expression

The signal pathway of the OGF-OGFr axis was evaluated on the protein level using siRNA technology. OGFr siRNA transfected SK-Hep1 cells had lower expression of p21 and p53 but higher expression of Cdk2, Cdk6, and Bcl-2 than control groups (**Figure 2C**, **2D**). As previously reported [9, 11], p21 inhibits Cdk2 by physical

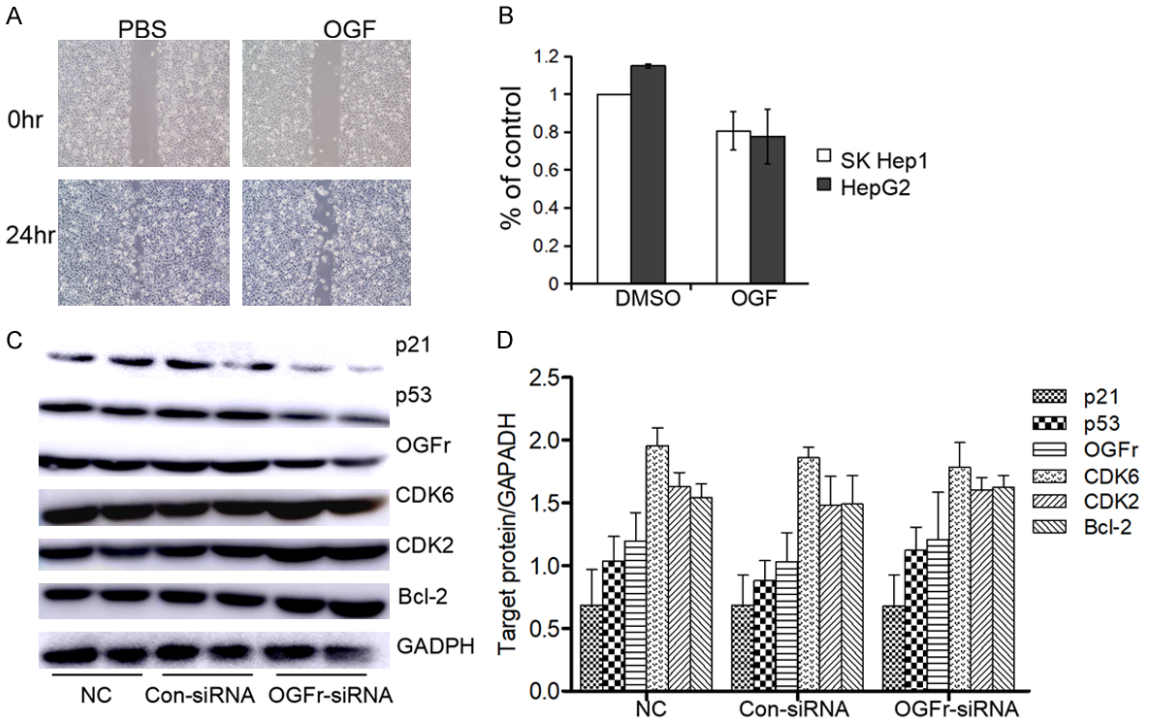

Figure 2. OGF inhibits HCC cell migration and proliferation. (A) We performed a scratch assay in a HepG2 cell line. Cells exposed to OGF migrate significantly more slowly than control cells. (B) In SK-Hep1 and HepG2 cell lines, we used a MTT assay to observe whether OGF inhibited cell proliferation. OGFr inhibits cell proliferation by upregulating p21 and p53 expression (C, D). SK-Hep1 cells were transfected with OGFr siRNA or scrambled siRNA. Proteins underwent western blotting with antibodies forp21, Cdk2, p53 and Bcl-2. GAPDH was used as the internal control. OGFr-siRNA-transfected SK-Hep1 cells showed lower expression of p21 and p53 but higher expression of Cdk2, Cdk6, and Bcl-2 than control groups.

interaction, and p21 expression is upregulated by OGF in a receptor-mediated manner. Our results further demonstrated that the OGF-OGFr axis inhibits cell proliferation by upregulating p21 expression. Since Cdk6 is inhibited by p16, higher Cdk6 expression after transfection with OGFr siRNA suggests that the OGF-OGFr axis also acts through p16. Furthermore, we also measured p53 and Bcl-2 expression in order to elucidate whether the OGF-OGFr axis reduces cell proliferation by apoptosis. In our results, p53 expression was slightly decreased and Bcl-2 slightly increased compared to the control group, which implied that the OGF-OGFr axis may have some effect on apoptosis.

OGF enhances the anti-tumor activity of cisplatin (DDP) on HCC cells

Changes in tumor volume over 28 days of the animal experiment were analyzed. The DDP, OGF and OGF+DDP groups were all different from the control group in tumor volume on day 28 ($P < 0.05$). The tumor volumes of the OGF group, DDP group and OGF+DDP group were smaller than the control group (**Figure 3A** and **3B**), which suggested that OGF and DDP inhibited HCC cell proliferation. In addition, according to the growth curve (**Figure 3C** and **3D**), the tumor growth of the OGF group, DDP group and OGF+DDP group were all slower than the control group. Tumor growth of the OGF+DDP group was also the slowest among the four groups. This implies that OGF enhances the anti-tumor activity of DDP on HCC. In addition, it is worth noting that the OGF group had similar anti-tumor activity when compared to the DDP group.

In order to explore the mechanism of OGF enhancing the anti-tumor activity of DDP, we performed immunohistochemistry and western blotting using HCC tissues derived from animal models. As shown in **Figure 3E**, immunohistochemistry results demonstrated the OGF+DDP group had higher OGFr, p16, p21, and p53

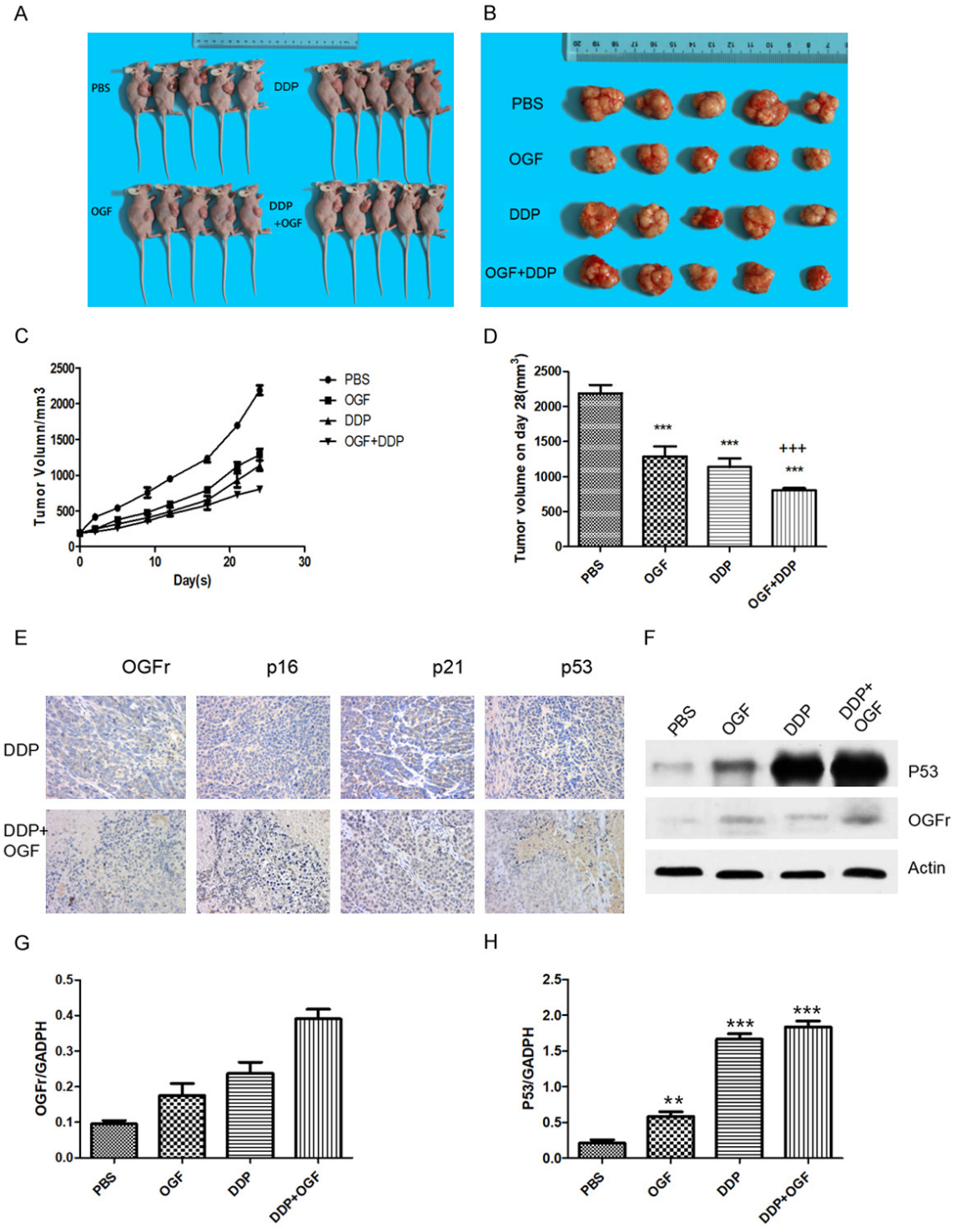

Figure 3. OGF and DDP suppressed tumor growth in animal experiments. A. We used 4 groups of HCC mice models, with 5 mice in each group. B. Here we show the resected tumors of the 4 groups of mice on day 28. The tumor volumes of the OGF group, DDP group, and OGF+DDP group are all smaller than the PBS group on day 28. C. Tumor volumes were measured every other day until the treatment ended on day 28. The tumor growth of the OGF group, DDP group and OGF+DDP group are all slower than the control group. The OGF+DDP group is the slowest. D. Tumor volume values represent means + SE for 5 mice/group. These values are significantly different from the control group at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ and from DDP at $+P < 0.05$, $++P < 0.01$ and $+++P < 0.001$. E. Immunohistochemistry results of resected HCC tissues from animal models show that the OGFr, p16, p21, and p53 expression levels are higher in the OGF+DDP group than in the DDP group. F-H. Western blot results of p53 and

OGFr of HCC tissues derived from animal models shows that OGF and DDP increased p53 and OGFr expression in HCC cells. However, OGF only increased p53 expression slightly. The DDP group had similar p53 expression compared with the OGF and DDP combination group. These values are significantly different from those of the control group at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

expression than the DDP group, implying that OGF acted by upregulating OGFr, p16, and p21. Although there is no previous evidence indicating that OGF-OGFr induces apoptosis, our results showed that OGF induced more p53 expression. We also performed a western blot of p53 and OGFr using HCC tissues derived from animal models (**Figure 3F-H**). The results showed that the OGF+DDP group had the strongest expression of p53 and OGFr among the 4 groups, although the DDP group had similar p53 expression when compared to the OGF+DDP group.

Discussion

The OGF and OGFr axis was demonstrated to function in diverse cancers by Zagon et al. [15], including hepatocellular carcinoma. Avella et al. [3] detected OGF and OGFr in surgical specimens from patients undergoing resection for HCC, which verified the presence and function of the OGF-OGFr axis in human HCC. It is reported that the OGF-OGFr axis is a physiological determinant of cell proliferation in diverse human cancers [2]. Cui et al. [16] first reported the differential expression of OGFr in tumor tissues and non-tumor tissues of human HCC in vitro with a higher expression in non-tumor tissues. In our study, we found a difference in OGFr expression between tumor tissues and non-tumor tissues of HCC, gastric cancer, and colon cancer. Thus, we speculated that we could inhibit cell growth by inducing OGFr upregulation in tumor tissues of HCC through addition of exogenous OGF.

Our study demonstrated that OGF-OGFr inhibited HCC cell growth in vivo and upregulated p21 and p16 expression, which is consistent with previous studies [4, 5, 7]. We also examined p53 expression to elucidate the relationship between the OGF-OGFr axis and cell apoptosis. Although OGF slightly increased p53 expression and decreased Bcl-2 expression, this does not appear to be its primary mechanism of action. Western blots showed that the DDP group had a similar p53 expression as the OGF and DDP combination groups. However, it is possible that OGF enhances the apoptotic effect induced by DDP since DDP has been demonstrated to induce apoptosis in HCC cells [17-19]. This possibility will need to be tested by well-designed experiments both in vitro and in vivo.

The effect of a short-term treatment with exogenous OGF on cell proliferation of HCC was comparable to that of cisplatin in our study, suggesting that OGF only has a potent antitumor effect on HCC. In addition, when OGF was combined with cisplatin in mice, the effects on cell replication were greater than in groups treated with each drug individually. These results showed that the combination of these two treatment modalities has additive action on inhibition of HCC cell proliferation in vivo, which is consistent with previous reports of pancreatic cancer [6] and squamous cell carcinoma of the head and neck (SCCHN) [8]. It is known that OGF is targeted to the G0/G1 phase of the cell cycle [3], whereas cisplatin is well known to bind to DNA and nuclear proteins to form intra- and inter-strand cross-links [20]. Therefore, the antiproliferative effects of the combination of OGF and cisplatin may be due the complementation of their respective abilities to alter the cell cycle and inhibit DNA synthesis.

Given the urgent need for advancement in the treatment of HCC, a combination of different treatment modalities has gained attention, including local-regional therapy combining biological-targeted therapy [21-23] and chemotherapy combining biological-targeted therapy [24, 25]. Although chemotherapeutic agents are not the first option considered for unresectable HCC, our results, along with previous studies, raise the exciting potential of combining chemotherapy and biotherapy into a novel treatment modality for HCC. Moreover, it introduces the possibility of combining OGF with other treatment modalities, such as local-regional therapy, which is currently the standard treatment of unresectable HCC. In addition, OGF is non-toxic, avoids problems related to drug resistance, and is easily accessible.

These factors make OGF a promising candidate for future clinical use.

As far as we know, our study is the first to demonstrate that the OGF-OGFr axis can inhibit cellular growth of HCC in animal experiments, and that OGF can enhance the antitumor activity of cisplatin. This data highlights the possibility of future clinical applications for OGF. In addition, we investigated the possibility of OGF's ability to enhance cisplatin's apoptotic effect for the first time. However, our study had some limitations. First, the sample size of animal models in our experiment is relatively small. Second, our study only employed animal models but did not explore the effect of exogenous OGF on human HCC. Further studies will need to explore whether OGF also influences p21 and p53 in vivo in humans.

Taken together, our study clarified that OGF inhibits cell migration and proliferation of HCC in animal experiments, and that exogenous OGF enhances the anti-tumor activity of cisplatin on HCC by upregulating the expression of p21 and p53. These results will provide a new strategy for HCC therapy.

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Disclosure of conflict of interest

None.

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