Overexpression of osteopontin promotes resistance to cisplatin treatment in HCC

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Abstract. Osteopontin (OPN) is a multi-functional cytokine involved in cell survival, migration and adhesion. Increasing evidence has elucidated its role in tumorigenesis, progression and metastasis. However, the role of OPN in chemoresistance of human hepatocellular carcinoma (HCC) has not yet been clarified. In the present study, we examined the expression of OPN in human HCC samples before and after cisplatin-treatment, the results showed that OPN was significantly increased in cisplatin-resistant specimens. We then studied the effect of cisplatin on OPN expression in HCC cells, after exposure to cisplatin, the expression of OPN in HCC cells was elevated compared to control cells. We also found that PI3K/AKT signaling pathway was also activated by cisplatin and this effect was induced by the OPN pathway. To study the effect of OPN on chemoresistance, HCC cells were treated with cisplatin along with OPN. Incubation with OPN enchanced the chemoresistance of HCC cells to cisplatin. In contrast, blockage of OPN pathway promoted the chemosensitivity of HCC cells to cisplatin. Our results suggest that OPN enhanced chemoresistance of cisplatin in HCC cells by activating PI3K/AKT signaling pathway, blocking the OPN pathway might be a novel way to overcome the disease.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common and serious malignancies worldwide (1,2). Due to its highly aggressiveness and poor prognosis, patients with advanced HCC are not candidates for local/regional surgical resection (3-5). Cirrhosis of any etiology is a risk factor for HCC, which include infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic cirrhosis and exposure to environmental toxins such as aflatoxin (6-8).

Despite intensive therapeutic intervention, the cure rate for HCC is extremely limited due to high rates of recurrence and chemotherapy resistance. Even for those with resected disease, the recurrence rate can be as high as 50% at 2 years (9-11). Systematic chemotherapy plays an important role in HCC treatment especially for patients with advanced HCC, however, systemic therapy with cytotoxic agents provides marginal benefit, the response rates are low, and the response duration is typically short (12,13). Cisplatin is extensively used as a chemotherapeutic agent for the treatment of HCC. A major problem with cisplatin treatment of HCC is the development of cisplatin chemoresistance. Despite the rapid shrinkage in tumor mass following chemotherapeutic cycles, the acquisition of chemoresistance becomes a changllenge to oncologists (14). Currently the molecular mechanisms involved in cancer cell chemoresistance are still largely unclear (15-17). Therefore, there is an urgent requirement in revealing the underlying mechanisms responsible for HCC chemoresistance, which is indispensable for developing effective chemotherapeutic agents.

Osteopontin (OPN) is a phosphorylated multifunctional glycoprotein, which is normally secreted by activated macrophages, leukocytes, T lymphocytes and involved in tissue remodeling processes (18-20). OPN binds to either the family of αvβ integrins or the cell-surface adhesion molecule CD44 to initiate cellular signals exerting its functions (21,22). Overexpression of OPN has been reported in a variety of malignancies, including carcinomas of gastric (23,24), breast (25,26), prostate (27,28) and lung (29,30). OPN has recently emerged as a significant protein in the biology of HCC (31-33). Serum OPN was more sensitive than AFP in the diagnosis of HCC. OPN overexpression tended to be associated with the presence of tumor vascular invasion and advanced tumor grade, it was also found that interference of OPN expression inhibited the invasion and metastasis of HCC (34,35). All these data suggest that OPN plays a significant role in the development and progression of HCC. However, its role in modulating chemoresistance and molecular mechanism are relatively unexplored.

In the present study, we tested whether and how OPN participate in the cisplatin-resistance of HCC. We found that

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OPN expression are significantly increased in HCC samples that received cisplatin-based chemotherapy, and OPN was upregulated when treated with cisplatin in HCC cell lines. OPN rendered cisplatin chemoresistance through activation of PI3K/AKT pathway, while blockage of OPN pathway could regain the sensitivity to cisplatin.

Materials and methods

Patient samples. Patients were enrolled from Tongji Hospital, Huazhong University of Science and Technology. All the pathological sections were obtained with informed consent from the patients, and the study was approved by the institutional review board of Tongji Hospital, Huazhong University of Science and Technology. The diagnosis was based mainly on clinical setting including imaging (ultrasonography and computerized tomography) and biochemistry (AFP and liver function enzymes testing). A total of 8 HCC patients were enrolled, they were diagnosed between July 2009 and July 2013. All the patients were initially treated with transcatheter arterial chemoembolization (TACE) after diagnosis using cisplatin. The tissues were collected before and after TACE.

Cell culture. The human HCC cell lines HepG2, HuH-7 and PLC/PRF/5 were purchased from the Shanghai Institute for Biological Sciences of the Chinese Academy of Sciences. The cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, and 100 mg/ml streptomycin. Cells were grown in a humidified incubator at 37˚C in an atmosphere of 5% CO_2 and 95% air.

Immunohistochemistry. The slides were deparafinized, and rehydrated, then immersed in 3% hydrogen peroxide solution for 10 min, heated in citrate buffer, pH 6.0, at 95˚C for 25 min, cooled at room temperature for 60 min. The slides were blocked by 10% normal goat serum at 37˚C for 30 min, and then incubated with mouse monoclonal antibody against OPN (Abcam; 1:500) overnight at 4˚C. After washing with PBS, the slides were incubated with biotionylated second antibody (diluted 1:100) for 30 min at 37˚C, followed by streptavidin-peroxidase (diluted 1:100) incubation at 37˚C for 30 min. Immunolabeling was visualized with a mixture of DAB solution. Counterstaining was carried out with hematoxylin. Samples were scored as percentage of positive cells.

Cell cytotoxicity assay. The cells were grown in 96-well culture plates, treated as indicated and cultured for different time periods. Next, the cells in each well containing 100 μl medium were incubated with 10 μl Cell Counting Kit-8 (CCK-8) at 37˚C for 2 h. The optical density (OD) of each well was then measured at 450 nm using a microplate reader.

Quantitative real-time PCR. Total RNA was isolated and reverse transcribed. Real-time PCR was then performed using an ABI 7900 System in the presence of SYBR-Green. The following gene-specific primers were used: OPN (forward, 5'-CATCACCTGTGCCATACCAGTT-3' and reverse, 5'-TTG GAAGGGTCTGTGGGGCTA-3'; and β-actin (forward, 5'-TGGCACCCAGCACAATGAA-3' and reverse, 5'-CTAAG TCATAGTCCGCCTAGAAGCA-3'). Target sequences were amplified at 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. β-actin was used as endogenous normalization control. All assays were performed in triplicate. The fold change in mRNAs expression was determined according to the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis. Cell lysates were extracted using RIPA lysis buffer containing protease inhibitor cocktail. Protein concentrations were determined using BCA method. Cell lysates containing 40 μg of protein were loaded and separated on 10% SDS-PAGE gels and subsequently transferred to polyvinylidene difluoride membranes (PVDF). Membranes were blocked in 5% milk solution, incubated at 4˚C overnight with following primary antibodies: mouse monoclonal to OPN (Abcam; 1:500), mouse monoclonal to PI3K (Abcam; 1:1,000), rabbit polyclonal to phospho-PI3K (Abcam; 1:800), rabbit polyclonal to AKT (Cell Signaling Technology; 1:800), rabbit polyclonal to phospho-AKT (Cell Signaling Technology; 1:1,000), rabbit monoclonal to caspase-3 (Cell Signaling Technology; 1:1,000), rabbit monoclonal to actin (Santa Cruz Biotechnology; 1:2,000). They were then washed, and incubated with horseradish peroxidase conjugated secondary antibody at dilution 1:5,000 for 1 h at room temperature. Membranes were then washed and developed using ECL substrate. Densitometry was performed using ImageJ software.

FACScan analysis. The HCC cells were harvested with fresh 0.25% trypsin solution and washed using PBS. The cells were resuspended in incubation buffer with Annexin V (1 mg/ml) and propidium iodide (PI) (1 mg/ml). The cells were analyzed using a Becton-Dickinson FACSort and data were analyzed using WinMDI software. Cells that were PI negative and Annexin V negative are considered healthy cells, PI negative and Annexin V positive cells are considered apoptotic, and cells that are positive to both PI and Annexin V are considered necrotic.

Statistical analysis. Each experiment was performed independently at least three times. Values were expressed as mean ± SD. A two-tailed Student's t-test was used to estimate intergroup differences if not otherwise stated. $P<0.05$ was considered to be statistically significant.

Results

OPN is highly expressed in cisplatin resistant HCC samples. To study the underlying mechanism for the resistance of HCC to cisplatin treatment, we collected 8 patients, of whom the specimens before and after received cisplatin-based chemotherapy were available. We first focused on identifying the changes in gene expression after cisplatin-based chemotherapy. We found that OPN expression levels were significantly increased in HCC patients after chemotherapy (Fig. 1). The HCC is frequently associated with hepatitis virus infection in China, in the present study, 5 of the patients were HBV-associated HCC, and 3 of them were HCV-related HCC. We found that OPN expression levels were significantly increased after chemotherapy regardless of their serum virology status (Fig. 1A and B).

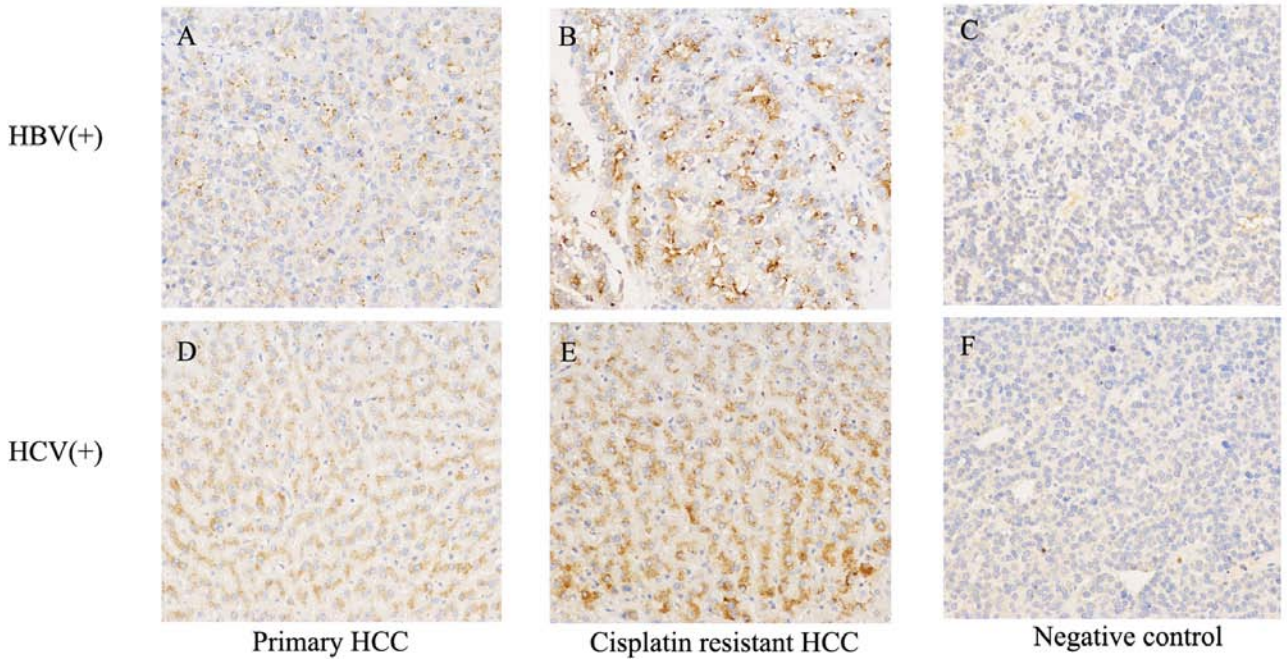

Figure 1. OPN expression in human HCC with cisplatin treatment. (A-C) Representative images of OPN expression in HBV-associated HCC samples. Left, primary HCC before chemotherapy; middle, cisplatin resistant HCC; right, negative control. (D-F) Representative images of OPN expression in HCV-associated HCC samples. Left, primary HCC before chemotherapy; middle, cisplatin resistant HCC; right, negative control.

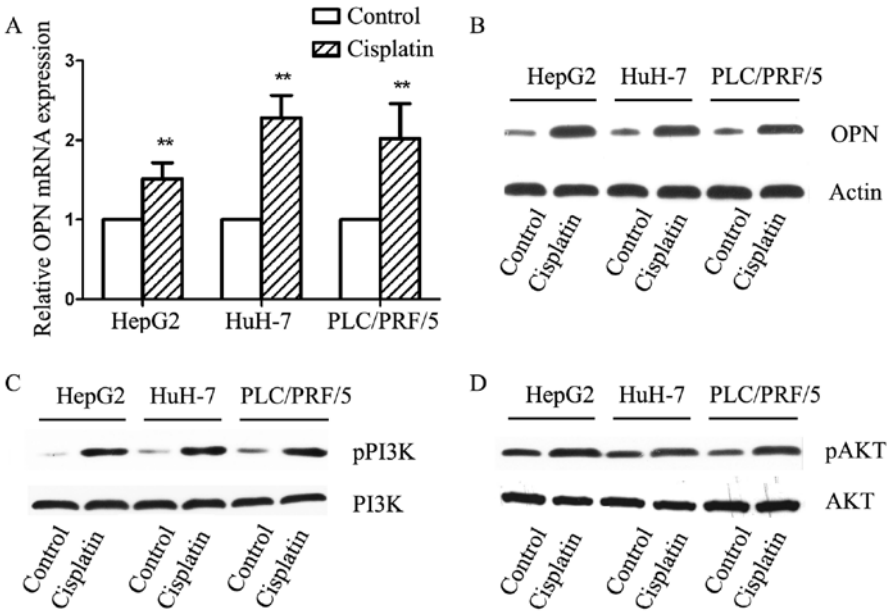

Figure 2. Cisplatin induced the expression of OPN and phosphorylation of PI3K/AKT *in vitro*. (A and B) qPCR and immunoblot data showed that OPN expression was upregulated in HCC cells after treatment by cisplatin. (C and D) The phosphorylation of PI3K and AKT was induced in HCC cells after treatment by cisplatin. $^{**}P<0.01$.

Cisplatin increases osteopotin and activates PI3K/AKT in HCC cells. We then explored the effect of cisplatin *in vitro*, we treated three HCC cell lines with 10 μM cisplatin for 24 h, which was less toxic in our preliminary study. mRNA levels were detected by qPCR, we found that in all three cell lines, OPN expression was significantly elevated after cisplatin treatment with a average fold-change of 1.94±0.44 (Fig. 2A). We further examined the protein level of OPN by immunoblotting. In agreement with qPCR data, OPN expression was statistically increased after treated by cisplatin (Fig. 2B). The involved signaling pathway in cisplatin treated HCC cells was investigated examining the phosphorylation of PI3K/AKT. PI3K activation was observed after exposure to cisplatin, which was consistent in all three cell lines (Fig. 2C). The phosphorylation of AKT was also found after exposure to cisplatin (Fig. 2D).

Osteopontin activates PI3K/AKT through CD44 and αvβ3. We further examined whether the activation of PI3K/Akt

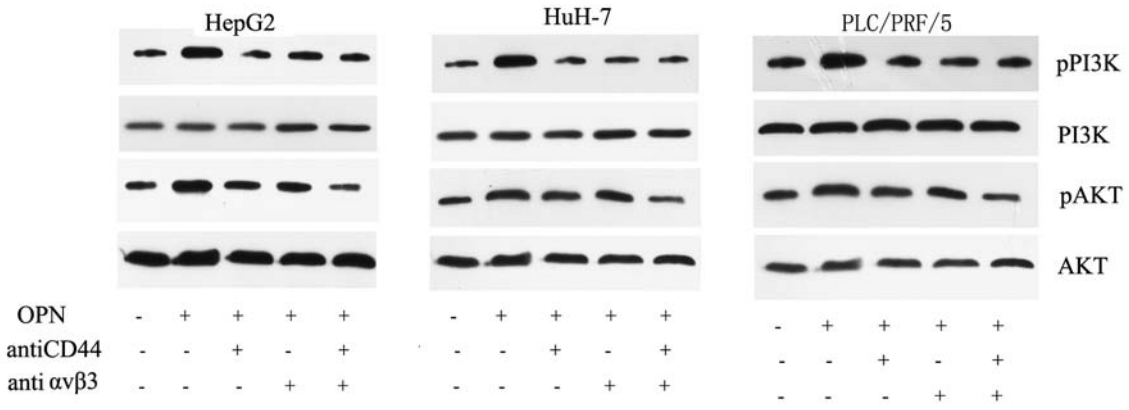

Figure 3. OPN induces the activation of PI3K/AKT signaling pathway *in vitro*. In three HCC cell lines, OPN induced the activation of PI3K/AKT signaling pathway, while the effect was significantly decreased by either anti-CD44 or anti-αβ3, and the two inhibitors showed a synergistic effect.

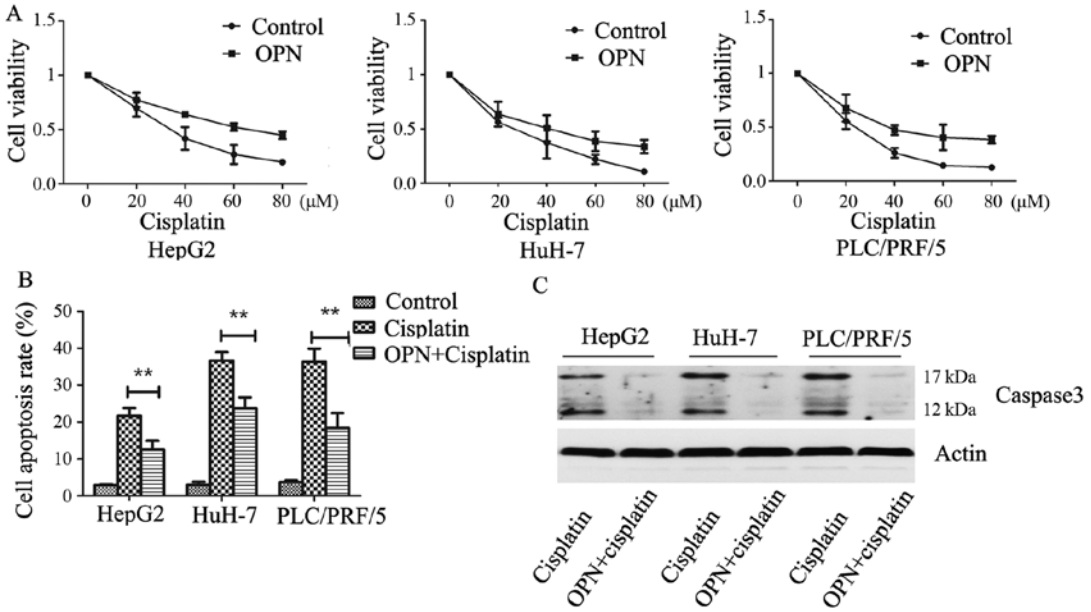

Figure 4. OPN enhances chemoresistance to cisplatin in HCC cells *in vitro*. (A) OPN increaded the Inhibitory concentration 50 (IC_{50}) of HCC cells to cisplatin. (B) OPN inhibited cell apoptosis induced by cisplatin in three HCC cell lines. (C) OPN inhibited the cleavage of caspase-3 induced by cisplatin in three HCC cell lines.

pathways was induced by OPN. We treated HCC cells with 0.5 μM OPN for 4 h. To inhibit the combination of OPN with its receptors, we incubated the cells with either anti-CD44 (20 μg/ml) or anti-αvβ3 antibody (20 μg/ml). As shown in Fig. 4, OPN strongly activated the phosphorylation of PI3K and Akt in all three HCC cell lines. In contrast, anti-CD44 and anti-αvβ3 antibody reversed the activation of the PI3K/AKT signaling pathway. While single antibody could partially reduce the phosphorylation of PI3K/AKT, the combination of the two antibodies showed a synergistic effect and could largely reverse the effect (Fig. 3). These data indicate that both the CD44 and αvβ3 participate in the OPN-induced PI3K/AKT pathway activation in HCC cells.

Osteopontin enhances resistance to cisplatin in HCC. To study the role of OPN in the resistance of HCC cells to cisplatin, the human hepatoma cell lines were treated with increasing concentrations of cisplatin plus 0.5 μM OPN and cell toxicity was assayed using cell counting reagent. We found that OPN treatment markedly increased cell resistance to cisplatin compared with PBS control in HepG2, HuH7 and PLC/PRF/5 cells (Fig. 4A). To induce HCC cell apoptosis, the cells were treated with 40 μM cisplatin and 0.5 μM OPN. The apoptosis rate was then analyzed by flow cytometry, the data showed that OPN strongly enhanced the resistance of HCC cells to cisplatin (Fig. 4B). We also detected the expression of activated caspase-3, the data showed that OPN significantly inhibited cisplatin-induced caspase-3 cleavage (Fig. 4C). These data suggest that OPN inhibited cisplatin-induced cell apoptosis.

Blockage of OPN pathway sensitizes HCC cells to cisplatin treatment. To determine whether OPN-induced cisplatin resistance could be reversed, we pre-treated the cells with anti-CD44 (20 μg/ml), anti-αvβ3 (20 μg/ml) or LY294002 (10 μM) for 24 h, followed by 40 μM cisplatin for 24 h. We

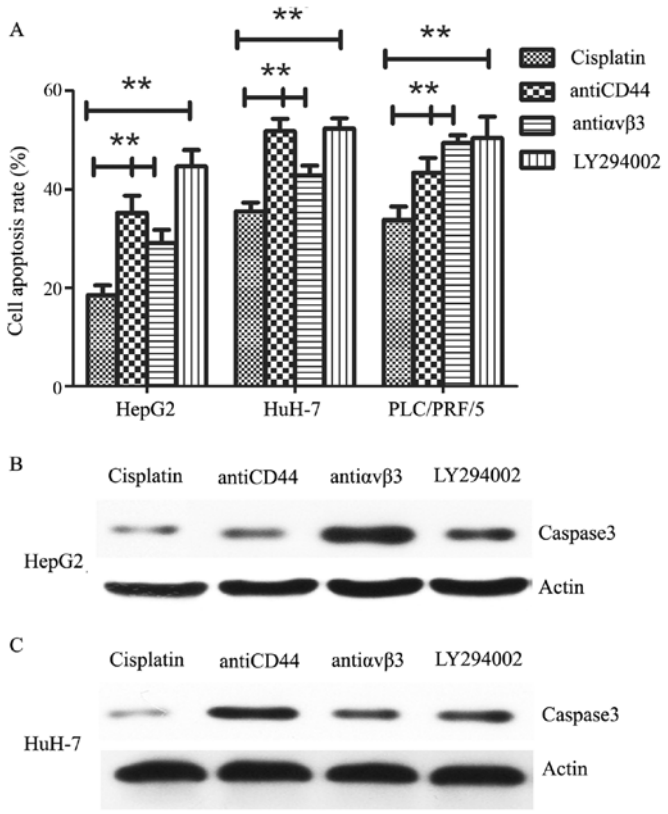

Figure 5. Blockage of OPN pathway promotes chemosensitivity of HCC cells. (A) Cisplatin-induced cell apoptosis was significantly activated by either targeting of OPN receptors or PI3K/AKT signaling pathway. (B and C) The cleavage of caspase-3 induced by cisplatin was upregulated after OPN pathway inhibiton. $^{**}P<0.01$.

found that cisplatin-induced cell apoptosis was significantly activated by targeting of OPN receptors. In consistent, cell apoptosis was also activated by blockage of PI3K/AKT signaling pathway (Fig. 5A). In addition, we tested caspase-3 expression by western blot analysis, the expression of activated caspase-3 was also shown to be upregulated after OPN pathway inhibiton (Fig. 5B). These data indicate that OPN pathway might have an important function in cisplatin resistance in HCC cells.

Discussion

Systematic chemotherapy plays important roles in HCC treatment especially for patients with advanced HCC (5). Although cisplatin is a common therapeutic agent used for chemotherapy in HCC patients, its curative effect is significantly limited due to chemoresistance of HCC (36). Much effort has been exerted in analyzing the role of OPN in the development and progression in a variety of malignancies (23,25,27,29). In the present study, we performed a retrospective analysis on the OPN gene expression before and after cisplatin-based chemotherapy in HCC patients. We found that OPN was upregulated in the HCC patients who received cisplatin treatment. The present study included both HBV and HCV-related HCC, and data showed the upregulation of OPN after cisplatin treatment was independent of virus infection status. In a study that enrolled 131 patients with HCC, plasma OPN was found to be significantly elevated compared to healthy subjects (32). It is also reported that OPN level in plasma was directly correlated with the tumor number. Elevated plasma level of OPN is thus regarded as a potential prognostic biomarker as well as a marker of early HCC detection (33). It has also been shown that oncogenic activation of the OPN is common in HCC, and overexpression of OPN is closely correlated with intrahepatic metastasis, early recurrence and a worse prognosis (37-40). Our data support the idea that OPN might participate in the chemoresistance of HCC.

To confirm the above hypothesis, we explored the effect of cisplatin on OPN expression *in vitro*. In agreement with patient-derived samples, cisplatin treatment for 24 h resulted in increased OPN expression in human HCC cells both at mRNA and protein levels. In parallel, we found that PI3K/AKT signaling pathway was activated after exposure to cisplatin. PI3K/Akt pathway is the most extensively studied and has been demonstrated to be a critical mechanism of drug resistance in HCC cells (41-43). We then ask whether OPN could activate PI3K/AKT pathway *in vitro*, we showed here that phosphorylation of PI3K/AKT occurred as early as 4 h after OPN incubation. OPN binds to the family of αvβ integrins, and the cell-surface adhesion molecule CD44, to initiate inhibition of cellular signal (18). We found that single antibody inhibitor partially reduced the phosphorylation of PI3K/AKT, the combination of the two inhibitors completely reversed the effect. A previous study showed that OPN regulates HCC cell behaviour in a CD44-dependent manner (34). Our data indicated that OPN induced PI3K/AKT signaling pathway was dependent on CD44 and αvβ3.

Elevated expression of OPN has been associated with tumor invasion, progression or metastasis in multiple cancers. Pang *et al* (25) demonstrated that OPN expression is critical for breast cancer growth and knockdown of OPN enhanced MDA-MB-231 breast cancer cells apoptosis. Research has also shown the abnormal expressions of OPN in chemoresistant cancer cells. It is reported that acquired cisplatin resistance in the small cell lung cancer line is also associated with OPN expression, which involved the maintaining of the anti-apoptotic bcl-2 protein (44). Consistent with these reports, we found that incubation with OPN inhibited cisplatin-induced cell apoptosis. Blockage of OPN by antibody targeted inhibition promoted cisplatin-induced apoptosis. Although high levels of the OPN pathway were responsible for mediating the acquired resistance to cisplatin, the downstream molecules used by OPN is still unclear. PI3K/Akt signaling is an aberrant pathway in HCC, and this pathway may be the critical target for therapeutic design (41,43), we therefore tested whether abnormal activation of PI3K/AKT signaling mediated by OPN represents a novel pathway regulating chemoresistance. Consistently, cell apoptosis was also activated by blockage of PI3K/AKT signaling pathway.

In conclusion, our results demonstrated that OPN functions as a chemoresistant gene in HCC, which involves activation of PI3K/AKT pathway. Our findings suggest that the combination of cisplatin treatment and OPN pathway blockage could be a therapeutic strategy for HCC. However, further work is needed to determine the extrapolation of *in vitro* results to an *in vivo* situation.

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