Metformin synergistically enhances antitumor activity of cisplatin in gallbladder cancer via the PI3K/AKT/ERK pathway

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Received: 23 July 2017 / Accepted: 26 October 2017 / Published online: 6 November 2017
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Abstract Metformin (Met) is a widely used antidiabetic drug and has demonstrated interesting anticancer effects in various cancer models, alone or in combination with chemotherapeutic drugs. The aim of the present study is to investigate the synergistic effect of Met with cisplatin (Cis) on the tumor growth inhibition of gallbladder cancer cells (GBC-SD and SGC-996) and explore the underlying mechanism. Cells were treated with Met and/or Cis and subjected to cell viability, colony formation, apoptosis, cell cycle, western blotting, xenograft tumorigenicity assay and immunohistochemistry. The results demonstrated that Met and Cis inhibited the proliferation of gallbladder cancer cells, and combination treatment with Met and Cis resulted in a combination index < 1 , indicating a synergistic effect. Co-treatment with Met and Cis caused G0/G1 phase arrest by upregulating P21, P27 and downregulating CyclinD1, and induced apoptosis through decreasing the expression of p-PI3K, p-AKT, and p-ERK. In addition, pretreatment with a specific AKT activator (IGF-1) significantly

neutralized the pro-apoptotic activity of Met + Cis, suggesting the key role of AKT in this process. More importantly, in nude mice model, Met and Cis in combination displayed more efficient inhibition of tumor weight and volume in the SGC-996 xenograft mouse model than Met or Cis alone. Immunohistochemistry analysis suggests the combinations greatly suppressed tumor proliferation, which is consistent with our in vitro results. In conclusion, our findings indicate that the combination therapy with Met and Cis exerted synergistic antitumor effects in gallbladder cancer cells through PI3K/AKT/ERK pathway, and combination treatment with Met and Cis would be a promising therapeutic strategy for gallbladder cancer patients.

Keywords Metformin · Gallbladder cancer · Cisplatin · AKT · ERK

Introduction

Gallbladder carcinoma (GBC), most frequently diagnosed incidentally after a cholecystectomy for symptomatic diseases such as gallstones, is the fifth most common neoplasm of the digestive tract (Reid et al. 2007). It is also the most aggressive cancer of the biliary tract with the shortest median survival from the time of diagnosis (Zhu et al. 2010). The only potentially curative therapy for GBC is surgical

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resection, however, at initial presentation, only 10% of patients are candidates for surgery with a curative intent (Zhu et al. 2010). Additionally, chemotherapy and radiotherapy have limited efficacy on GBC, which was evidenced by low response rates and no demonstrated survival benefit (Liu et al. 2013a, b). The outcome of advanced GBC is extremely poor, and the overall 5-year survival rate is less than 5% (Goetze and Paolucci 2010). Therefore, novel therapeutic agents are urgently needed for the treatment of advanced GBC.

Cisplatin (Cis) is an inorganic platinum agent (cis-diamminedichloroplatinum) with antineoplastic activity. It has been widely used for its potent cytotoxic effects upon a variety of human malignancies including gallbladder carcinoma (Piulats et al. 2009). However, cisplatin resistance is common among gallbladder cancer patients with cisplatin treatment as adjuvant therapy (Jurado et al. 2007). Thus, new strategies to overcome chemotherapeutic resistance are under exploration and it is necessary to design new drugs for a more selective tumor therapy.

Metformin (1,1-dimethylbiguanide hydrochloride; Met), a widely prescribed drug for treating type II diabetes, is one of the most extensively recognized metabolism modulators. It has attracted much attention because of its low toxicity and wide pharmacological effects. According to a large number of recent observational studies, diabetic patients treated with Met show a reduced incidence of neoplastic disease. In addition, Met has been extensively studied, for their cancer chemopreventive potential against various cancers, such as renal (Liu et al. 2013a, b), liver (Miyoshi et al. 2014; Cai et al. 2013), lung (Qu et al. 2012), pancreas (Li et al. 2014a, b), ovary (Nair et al. 2013) and gastric cancer (Shank et al. 2012). However, there is no information about the chemosensitization effect of Met on GBC until now.

In the present study, we utilize the GBC-SD, SGC-996 cells, and SGC-996 xenograft mouse model to explore the possible chemosensitization effect of Met to potentiate the antitumor effect of Cis in vitro and in vivo, and found that these two agents act synergistically anticancer activity. The findings may offer a promising new approach in the effective treatment of GBC.

Materials and methods

Reagents and antibodies

Met was purchased from Sigma-Aldrich (St. Louis, MO, USA), and diluted across a range of concentrations in a complete cell culture medium. Cis was purchased from Selleck Chemicals (Houston, TX, USA), and dissolved in dimethyl sulfoxide (DMSO). Met and Cis solution were sterilized through 0.22 µm filter for use in subsequent experiments and stored at − 20 °C. Stock solutions were diluted to the desired final concentrations with growth medium just before use.

Materials used included 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Company, St. Louis, MO, USA), Apoptosis and cycle Detection kit (MultiSciences Biotech, Shanghai, China), Hoechst 33342 staining assay kit (Molecular Probes, Beyotime Institute of Biotechnology, Shanghai, China), anti glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Kangchen Bio-tech, Shanghai, China), anti phosphatidylinositol 3-kinase (PI3K), anti phospho-phosphatidylinositol 3-kinase (p-PI3K), anti total-AKT, anti p-AKT (Thr308), anti total-ERK, anti phospho-ERK, anti CyclinD1, anti P21, and anti P27 antibodies and horseradish peroxidaseconjugated sheep anti-mouse immunoglobulin G (IgG) and sheep anti-rabbit IgG antibodies (Cell Signaling Technology, Danvers, MA, USA) and mouse anti-Ki67 antibody (GeneTex Inc, Irvine, CA, USA).

Cell lines and culture

The human GBC cell lines GBC-SD and SGC-996 were purchased from the Shanghai Cell Institute Country Cell Bank. The GBC-SD cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM). Another cell line SGC-996 was maintained in RPMI-1640 medium, with all media containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 mg/mL streptomycin and 100 units/mL penicillin (Hyclon, Logan, UT, USA), and incubated at 37 °C in an atmosphere of 95% air and 5% CO₂.

Cell viability assay

Cell viability was measured by MTT assay and colony formation test. For MTT assay, GBC-SD and SGC-996 cells were seeded into 96-well plates at a density of 1×10^4 cells/well and cultured overnight. Then they were exposed to Cis (0, 0.5, 1, 2, 4, 8, 16, 32 μ M), Met (10, 20, 40 mM) or Cis + Met for 48 h. Untreated cells served as controls. Thereafter, 20 μ L MTT (5 mg/mL) was added to each well followed by 4-h incubation. Then the medium was removed and 150 μ L of DMSO was added to dissolve the resulting formazan crystals. The absorbance values at 490 nm were measured by a micro plate reader. Cell viability was calculated as follows: (1-average absorbance of treated group/average absorbance of control group) $\times$ 100%.

Colony formation test was performed to evaluate the long-term proliferative potential of GBC-SD and SGC-996 cells following Cis and/or Met treatment. GBC-SD (800 cells/well) and SGC-996 (600 cells/well) cells were plated into 6-well plates and cultured at 37 °C with 5% CO₂. The medium was replaced with fresh culture medium every 2–3 days. After 10 days, the plates were fixed with 4% paraformaldehyde at 4 °C for 15 min and stained using Giemsa for 30 min. The number of stained colonies that contained 50 cells was manually counted. Proliferation potential was assessed as: relative colony formation rate (%) = number of colonies in the treatment group/number of colonies in the control group $\times$ 100%.

Combination index analysis

After detection the single and combination inhibitory effects of Cis and Met, the combination index (CI) was calculated according to Chou's CI model (Chou 2010) using CalcuSyn software program. The value of CI is a quantitative measure of the degree of interaction between different drugs. CI > 1, denotes antagonism; CI = 1, denotes additive effects; CI < 1, indicates synergism.

Cell apoptosis and cycle analysis

The flow cytometry analysis was performed as previously described (Liu et al. 2016a). For cell apoptosis assay, SGC-996 cells were treated with Cis and/or Met for 48 h, harvested, and prepared for flow cytometry

analysis immediately (Becton Dickinson, Franklin Lakes, NJ, USA). After treated with Cis and/or Met for 48 h, cells were harvested and tested by Becton Dickinson FACScan. The ratio of cells in the G0/G1, S, and M phases of the cell cycle was established by their DNA content.

Hoechst staining analysis

Hoechst 33342 staining was used to confirm the alterations of nuclei morphology of SGC-996 cells after Cis and/or Met treatment. Briefly, cells were stained with Hoechst 33342 (10 μ g/ml) for 15 min after incubation for 48 h, then washed with phosphate-buffered saline (PBS) and observed using an inverted fluorescence microscope. Cells from 5 randomly selected microscopic fields were counted. The apoptosis index (AI) of cells was calculated using the following formula: AI (%) = apoptotic cells/total cells $\times$ 100%.

Western blotting analysis

Western blotting analysis was carried out as we described previously (Liu et al. 2016a, b). Proteins (40 μ g) were separated by SDS-PAGE and then transblotted onto a polyvinylidene difluoridex membrane (Beyotime Institute of Biotechnology). The membranes were incubated with primary antibodies overnight and then with appropriate secondary antibodies conjugated to horseradish peroxidase. Signals were visualized by ECL chemiluminescence. The GAPDH expression was used as reference band. The bands were semi-quantified using Image J software.

Animal experiment

Animal studies were carried out as we described previously (Liu et al. 2015). Exponentially growing SGC-996 cells (4×10^6) were suspended in 100 μ L PBS and subcutaneously injected into the right axillary fossa of each nude mouse. On day 7, a total of 32 nude mice whose tumors were similar in size were chosen and equal numbers were assigned to 4 groups (n = 8/group). Group I was given sterile physiological saline intraperitoneally injected every other day as the control group; Group II was given 5 mg/kg Cis; Group III was injected with 25 mg/kg Met and Group IV was given 5 mg/kg Cis + 25 mg/

kg Met intraperitoneally every other day. All mice were sacrificed on day 26 after these drugs had been administered seven times, and tumors were dissected and weighed. The tumor volume and inhibition ratio were detected as described previously (Liu et al. 2016b). The animal studies were approved by the Wujiang No. 1 People's Hospital Ethics Committee, and the principles of laboratory animal care were followed in all animal experiments.

Then formaldehyde-fixed, paraffin-embedded tissue blocks were prepared from xenograft tissue and cut into serial sections (5 μm) to examine the expression of p-AKT and Ki67 by immunohistochemistry using the streptavidin–peroxidase kit (Fuzhou Maixin Biotechnology Development Co., Fuzhou, China). Each slice was enumerated under 5 fields of medium magnification (400 $\times$) to determine the proportion of positive cells.

Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD) and analyzed by the SPSS 17.0 software (SPSS Inc., Chicago, USA). One-way ANOVA followed by the appropriate post hoc test (Bonferroni) was used to establish whether significant differences existed among groups. The *P* value of less than 0.05 was considered statistically significant. All experiments presented here are derived from at least three independent experiments.

Results

Met synergistically enhances the anti-proliferative effect of Cis in human GBC cells

Firstly, we evaluated the potential role of Cis and Met on cell growth of GBC-SD and SGC-996 cells using MTT assay. As shown in Fig. 1a, SGC-996 cells performed more sensitive than GBC-SD cells to Cis treatment. When the concentration exceeded 8 μM , GBC-SD and SGC-996 cells showed somewhat resistance to Cis treatment. However, Met inhibited GBC-SD and SGC-996 cells proliferation in a dose-dependent manner. For cells simultaneously co-treated with Cis (1, 2 μM) and Met (10, 20, and 40 mM), a stronger inhibitory effect on cell proliferation was observed than for either drug alone (Fig. 1b, c). To further

validate the synergistic feature of Cis and Met, the CalcuSyn software was used to analyze the cell viability inhibition effects of the single and combined treatment. The CI values for Cis (1 μM) + Met (10 mM) combination dose are shown in Fig. 1b, c. The CI value for Cis (1 μM) + Met (20 mM) was 0.62 ± 0.11 for GBC-SD and 0.53 ± 0.09 for SGC-996 cells; the CI value for Cis (1 μM) + Met (40 mM) was 0.46 ± 0.07 for GBC-SD and 0.40 ± 0.08 for SGC-996 cells; the CI value for Cis (2 μM) + Met (10 mM) was 0.65 ± 0.12 for GBC-SD and 0.56 ± 0.07 for SGC-996 cells; the CI value for Cis (2 μM) + Met (20 mM) was 0.52 ± 0.07 for GBC-SD and 0.43 ± 0.06 for SGC-996 cells; the CI value for Cis (2 μM) + Met (40 mM) was 0.41 ± 0.09 for GBC-SD and 0.37 ± 0.07 for SGC-996 cells. In our previous study, we explored many concentrations of Cis and Met, and we selected the representative concentrations of both Cis and Met used in this experiment. For SGC-996 cells, the combination of Cis (1 μM) and Met (10 mM) displayed the best synergistic inhibition capacity, which was selected for further investigations.

To further explore the effect of Cis and Met on GBC cell tumorigenicity, colony formation test was performed. As shown in Fig. 1d–f, we found that single-agent treatment with Cis or Met decreased both the size and number of colonies, and the combination of both agents could further attenuate the colony-forming ability compared with single-drug treatment.

Met enhanced Cis-induced apoptosis in human GBC cells

In the present study, cell apoptosis was checked by two approaches (Hoechst staining was used to reveal the morphological changes of apoptotic cells and Flow cytometry was used to quantify apoptosis of cells). Results from Hoechst staining showed that SGC-996 cells treated with Cis or Met alone exhibited morphological features of early apoptotic cells, such as bright nuclear condensation or fragments, whereas in Cis + Met group, apoptotic bodies began to appear and the number of late apoptotic cells increased (Fig. 2a, b). Next, we quantified apoptosis of SGC-996 cells by flow cytometry assay. As shown in Fig. 2c, d, both Cis and Met induced apoptosis in SGC-996 cells, and the combination treatment resulted in the greatest apoptosis rate ($P < 0.01$ vs. Cis or Met alone).

Fig. 1 Met synergistically enhances the anti-proliferative effect of Cis in GBC cells. **a** Cell viability was measured by MTT assay after Cis treatment in GBC-SD and SGC-996 cells. **b, c** Cell viability was measured by MTT assay at 48 h after Cis and/or Met treatment. Combination index (CI) value was

analyzed using CalcuSyn software. **d–f** Colony formation of GBC-SD and SGC-996 cells after treatment with Cis and/or Met. Each experiment was conducted in triplicate. * $P < 0.05$, versus the control group; ** $P < 0.01$, versus Cis or Met alone group

Fig. 2 Combination treatment with Cis and Met induced cell apoptosis in SGC-996 cells. **a** The nuclei were stained by Hoechst 33342 and visualized under fluorescence microscope (original magnification $\times 200$). **b** The percentage of apoptotic cells was calculated as apoptosis index (AI) (%) and shown in a

histogram. **c** Cell apoptosis was detected after Cis and/or Met treatment in SGC-996 cells. **d** Flow cytometric analysis of apoptotic cells stained with annexin V-FITC/PI. * $P < 0.05$, versus the control group; ** $P < 0.01$, versus Cis or Met alone group

Met and Cis treatment induced cycle arrest of GBC cells

Flow cytometry analysis in Fig. 3a, b indicated that compared with untreated controls, Cis and Met

treatment significantly increased the number of cells in G₀/G₁ phase ($P < 0.05$). Co-administration of the two drugs resulted in a significantly greater proportion of cells in G₀/G₁ phase compared with either drug alone. To further elucidate the mechanisms accounting

Fig. 3 Effects of Cis and/or Met on cell cycle distribution

a, b Cell cycle analysis was performed in SGC-996 cells after Cis and/or Met treatment for 48 h as indicated. **c** The expression level of CyclinD1, P21, and P27 in SGC-996 cells was detected by western blotting analysis. **d** The expression of CyclinD1, P21, and P27 protein levels in every group were analyzed by densitometry normalized to GAPDH density. The values represent the mean $\pm$ standard deviation of triplicates. * $P < 0.05$, versus the control group; ** $P < 0.01$, versus Cis or Met alone group

for the cell cycle arrest, we examined the expression of regulators of the G1/S phase transition. Western blotting analysis showed that the protein level of Cyclin D1 was significantly decreased after co-treatment with Cis and Met. In contrast, the levels of P21 and P27 were significantly increased in Cis + Met group compared with the control groups (Fig. 3c, d; $P < 0.01$).

Met potentiated the anti-tumor activity of Cis through PI3K/AKT/ERK pathway

To investigate whether the PI3K/AKT/ERK pathway is involved in Cis and Met induced apoptosis in SGC-996 cells, we detected the protein expression of PI3K, p-PI3K, AKT, p-AKT, ERK, and p-ERK by western blotting analysis. As shown in Fig. 4a, b, the expression levels of PI3K, AKT, and ERK in the Cis and/or Met treatment groups were not significantly different from that in the control group, while the expression levels of p-PI3K, p-AKT, and p-ERK were significantly decreased compared with the control group. It is important to note that these effects were more pronounced when Cis and Met were used together ($P < 0.01$ vs Cis or Met alone). Furthermore, we investigated whether activation of AKT could neutralize the inhibitory effect of Cis + Met by using

IGF-1, an activator of AKT. One hundred nanograms per milliliter of IGF-1 partially decreased the apoptosis of SGC-996 cells, and the combination of IGF-1 and Cis + Met resulted in a significantly higher proliferation index than Cis + Met alone ($P < 0.01$; Fig. 4c).

Met treatment improved the anti-tumor effect of Cis in vivo

We future investigated the effect of Cis and Met on GBC cell growth in vivo. The xenograft model was established in BALB/c nude mice following subcutaneous transplantation of SGC-996 cells. Results showed that treatment with Cis (5 mg/kg) or Met (25 mg/kg) alone had little effect on the growth of tumors, whereas the combination of both agents resulted in a significant reduction in the tumor weight and volume. The mean tumor weights in different groups were also calculated, and results showed that the tumor weight inhibition rates were 25.12, 32.36 and 75.12% for Cis, Met, and Cis + Met treatment groups, respectively (Fig. 5c). The trends of tumor volume inhibition in different groups were consistent with tumor weight inhibition (Fig. 5b).

We next examined the subcutaneous tumor tissues by H&E staining. The tumor tissue from control mice

Fig. 4 Met potentiated the anti-tumor activity of Cis through PI3K/AKT/ERK pathway. **a** Expression of PI3K, p-PI3K, AKT, p-AKT, ERK and p-ERK was analyzed by Western blotting assay. Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) was used as the sample loading control. For one experiment, three assays were carried out, but only one set of gels is shown. **b** The expression levels of PI3K, p-PI3K, AKT, p-AKT, ERK and p-ERK protein in every group were analyzed by

densitometry normalized to GAPDH density **c** Effects of IGF-1 on Cis + Met induced apoptosis detected by AnnexinV-FITC/PI staining assay. Apoptotic cells were measured after treatment with Cis + Met in the presence or absence of IGF-1 (100 ng/mL) for 36 h. The values represent the mean $\pm$ standard deviation of three independent experiments. * $P < 0.05$, versus control group; ** $P < 0.01$, versus the Cis + Met group

Fig. 5 Combination treatment with Cis and Met inhibited tumorigenicity in vivo. **a** Representative images of the subcutaneous tumors in each group. **b**, **c** The final tumor weight and volume in each group after treatment. **d** H&E staining analysis of the pathological features of the tumors from the four groups. **d**, **e** Expression of p-AKT was quantified in percentages of positive cells within five medium-power fields under a microscope and shown in a histogram. **d**, **f** The proliferation index as determined by the percentage of Ki67 stained nuclei was calculated for the different groups. All data are expressed as mean $\pm$ standard deviation. ** $P < 0.01$, versus versus Cis or Met alone group

showed compact tumor cells with blue-purple nuclei and pink cytoplasm. In the Cis or Met treatment group, the tumor cells were sparse and separated from each other and the ratio of nucleus to cytoplasm was reduced. In Cis + Met treatment group, the structure of tumor tissue was more seriously damaged than that in the Cis or Met alone groups and the nuclei were polygonal and slightly stained (Fig. 5d). In addition, the expression of p-AKT and Ki67 in Cis or/and Met treated tumors were examined by immunohistochemistry. Results showed that co-treatment with Cis and Met significantly decreased the expression of p-AKT and Ki67, compared to the Cis or Met alone groups ($P < 0.01$).

Discussion

Met, a widely used anti-diabetic drug now draws much attention since its anti-tumor activity in vitro and in vivo was described (Miyoshi et al. 2014). The basis of molecular anticancer mechanism of Met is activation of AMPK, which is closely associated with tumor cell metabolism. In recent years, advances in cancer metabolism research increased the clinical interest to target aberrant metabolic pathways for treatment of malignant tumors (Tennant et al. 2010; Teicher et al. 2012; Peng et al. 2016). However, whether Met can become a good chemo-sensitizer to amplify the effectiveness of chemotherapy in GBC is not clear. In this study, we found that Met significantly suppresses cell proliferation, induces cell apoptosis, and enhances the efficacy of Cis in GBC cells in vitro and in vivo. To the best of our knowledge, the present study is the first preclinical research that assesses the anti-tumor effect on GBC cells of using Cis and Met in combination.

Apoptosis is a pivotal homeostatic mechanism that balances cell division and cell death to maintain the appropriate cell number in the body. During the process, apoptosis is characterized by cell shrinkage, chromatin condensation, blebbing of the plasma membrane, and nuclear condensation without cell lysis (Liang et al. 2012; Liu et al. 2014). Since deregulation of apoptosis is the hallmark of all cancer cells, the induction of apoptosis has been described as a standard and best strategy in anticancer therapy (Kelly and Strasser 2011). Also, it is reported that cell cycle deregulation is one of the major hall-mark traits

of cancer cells (Wang et al. 2011; Liu et al. 2016c). Clearly, an agent which could efficiently induce apoptosis and cycle arrest of cancer cells would be a hopeful candidate to suppress cancer progression and thus could reduce mortality. In our present study, the MTT assay and colony formation test demonstrated that the cell viabilities of GBC-SD and SGC-996 decreased significantly after co-treated with low doses of Cis and Met in comparison to either single treatment. Combination index data analysis showed that this combination is highly synergistic at inhibiting cell viability of GBC cells. Furthermore, Annexin V-FITC/PI staining assay showed that the combination of Cis and Met was synergistic at inducing apoptosis in SGC-996 cells. Additionally, there was an accumulation of cell population in G0/G1 phase after Met treatment, and Met co-treatment significantly enhanced the Cis caused cell cycle arrest in G0/G1 phase by increasing p27, p21 and decreasing Cyclin D1.

Cell apoptosis is induced and controlled by polygenic pathways, such as blockage of cell cycle and expression changes of correlative apoptosis genes. Some signaling pathways, such as PI3K/AKT and ERK/MAPK, play a critical role in the control of tumor cell growth, progression, apoptosis, invasiveness, and metastasis formation (Zhang et al. 2012b). The PI3K/AKT signaling pathway has been well documented to play a major role in breast cancer, cervical cancer, lung cancer (Citro et al. 2015; Wu et al. 2013; Kim et al. 2015), and this pathway represents an attractive target for anticancer therapeutics. The activation of AKT was positively correlated with cancer stages, indicating that p-AKT is an independent prognostic marker for cancer patients (Zhang et al. 2012a). ERK is a subfamily of the MAPK family, which can be activated by many kinds of growth factors and cytokines, and participates in regulating proliferation and apoptosis of the cells (Wang et al. 2014). Activated ERK causes activation of many kinds of genes, including Cyclin D1, which results in malignant cell transformation. Blockage of the AKT or ERK signaling pathway results in programmed cell death and growth inhibition of tumor cells (Li et al. 2012). In this study, we found that Met and Cis all inhibited the expression of p-PI3K, p-AKT, and p-ERK. Moreover, combined treatment with Met and Cis decreased these proteins in comparison to single agent treatment, indicating synergistic

suppression of PI3K/AKT and ERK pathways. Furthermore, specific activation of AKT (IGF-1) inhibited apoptosis and significantly neutralized the inhibitory effect of Met + Cis, which further confirmed that Met + Cis induced apoptosis, which can be attributed, at least partially, to AKT inactivation.

It has been reported that Cyclin D1 is relevant with abnormal proliferation, invasion, and prognosis of tumor cells (Alao 2007). Instead, P21 and P27 are implicated in the negative regulation of cell cycle progression from G1 to S phase (Lu and Hunter 2010). It is reported that AKT and ERK could positively regulate the expression of Cyclin D1 and negatively regulate P21 and P27 expression (Li et al. 2014a, b). Therefore, in this study, we can conclude that co-treatment with Cis and Met induced cell cycle arrest at G0/G1 phase by down-regulating Cyclin D1 and up-regulating p21, p27 through inactivation of AKT pathway.

In addition, our *in vivo* study found that both Cis and Met could induce tumor inhibition, especially when combination treatment was applied. Furthermore, H&E staining analysis of the tumors from mice treated with Cis or Met revealed morphological features characteristic of apoptotic cells and the structure of the tumor tissue was more seriously damaged in the co-treatment group. More importantly, co-treatment with OMT and OXA significantly decreased the expression of p-AKT and Ki67, which was consistent with our findings *in vitro*.

In conclusion, our present investigation confirms that the combination of Cis and Met could synergistically inhibit human GBC cells proliferation, induce cell apoptosis and G0/G1 phase arrest, and reduce tumorigenicity *in vitro* and *in vivo*. The synergistic effects of Cis and Met may be associated with the inactivation of the AKT and ERK pathways. Thus, we propose that the combination of Cis and Met may be as a highly efficient way to achieve antitumor synergism in the clinical treatment of GBC, which warrants further investigation in a clinical setting.

Acknowledgements This study was supported by the Program for Young Scientist in Science and Education of Suzhou City (No. KJXW2014053), the Program for Young Scientist in Science and Education of Wujiang No. 1 People's Hospital (No. 201724).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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