

RESEARCH ARTICLE

Genistein Reinforces the Inhibitory Effect of Cisplatin on Liver Cancer Recurrence and Metastasis after Curative Hepatectomy

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Abstract

Background: The high recurrence rate after hepatic resection in hepatocellular carcinoma (HCC) is a major obstacle to improving prognosis. The objective of the present study was to explore the function of genistein, a soy-derived isoflavone, in enhancing the inhibitory effect of cisplatin on HCC cell proliferation and on tumor recurrence and metastasis in nude mice after curative hepatectomy. **Methods:** Proliferation of human HCC cells (HCCLM3) was detected by 3-(4, 5-dimethylthiazolyl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Synergistic effects of genistein and cisplatin were evaluated with the median-effect formula. Nude mice bearing human HCC xenografts underwent tumour resection (hepatectomy) 10 days post implantation, then received intraperitoneal administration of genistein or cisplatin alone or the combination of the two drugs. 33 days after surgery, recurrent tumours and pulmonary metastasis were evaluated individually. MMP-2 level in recurrent tumours was detected by immunohistochemistry and real-time PCR; MMP-2 expression in HCCLM3 was detected by immunocytochemistry. **Results:** Genistein and cisplatin both suppressed the growth and proliferation of HCCLM3 cells. The two drugs exhibited synergistic effects even at relatively low concentrations. *In vivo*, mice in the combined genistein and cisplatin group had a smaller volume of liver recurrent tumors and fewer pulmonary metastatic foci compared with single drug treated groups. Cisplatin upregulated the expression of MMP-2 in both recurrent tumours and HCCLM3, while genistein abolished cisplatin-induced MMP-2 expression. **Conclusions:** Genistein reinforced the inhibitory effect of cisplatin on HCC cell proliferation and tumour recurrence and metastasis after curative hepatectomy in nude mice, possibly through mitigation of cisplatin-induced MMP-2 upregulation.

Keywords: Hepatocellular carcinoma - genistein - cisplatin - recurrent - metastasis

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignant tumor worldwide which has an increasing incidence in the East Asia (Parkin et al., 1999; Bosch et al., 2004). Potentially curative interventions such as tumor resection, liver transplantation and thermal ablation are applicable for the 30% whose tumors or liver function meet defined criteria (Llovet et al., 2003). Even among those treated with curative intent, relapse rates are up to 50% (Yamamoto et al., 1996; Tang et al., 1998). Hepatic resection is the standard treatment for HCC, however, the survival rate is still low due to the high incidence of recurrence (Ye et al., 1995; Tang, 2000). Over the past decades, not a single method has been proved to be sufficiently effective to prevent recurrence and metastasis of HCC, which exhibits low sensitivity to most anticancer drugs. Therefore, drugs which are effective and hypotoxic, or are able to decrease chemotherapeutic doses and increase curative effect when administrated combined with chemotherapy drugs are desperately needed.

Epidemiological studies have revealed that increased intake of dietary isoflavonoids may protect against tumorigenesis (Cohen et al., 2000), suggesting the potential use of isoflavonoids in cancer prevention. Genistein, a soy-derived isoflavone, has been found to be responsible for the anticancer property of soy (Sarkar et al., 2003; Banerjee et al., 2008). Genistein exhibits significant anticarcinogenic effects on breast cancer, prostate cancer and other tumors (Lamartiniere, 2002; Li et al., 2005; Lakshman et al., 2008). It inhibits the proliferation of HCC cells (Yeh et al., 2007) and liver cancer metastasis (Gu et al., 2009). Moreover, genistein has been proved to be a relatively low toxicity agent (D'Anna et al., 2007), which makes it a promising drug in treating HCC.

Cancer chemotherapeutic strategies usually require multiple agents, among which cisplatin has been commonly used for the treatment of HCC. However, a major problem with cisplatin is the development of chemoresistance. Methods aiming at improving drug performance have been extensively explored and some experimental data suggested that the combination therapy

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of cisplatin and genistein could reduce the dose of the former drug as well as execute better anticancer activities in different types of cancers, including prostate, breast, lung, and pancreatic cancers (Li et al., 2005).

In the present study, we investigated the effect of genistein in combination with cisplatin on the proliferation of high-metastasis HCC cells (HCCLM3). We also employed a high-metastasis-and-recurrent athymic mouse model simulating post-operation human HCC (Jian et al., 2003; Wang et al., 2003) to study the impact of the combination of the two drugs on metastasis and recurrence of post-operation HCC, and the underlying mechanism.

Materials and Methods

Materials

Genistein, dimethyl sulphoxide (DMSO), MMT reagent and AP-Red substrate kit were purchased from Sigma Chemical Co, USA. Cisplatin was purchased from Dezhou Pharmaceutical Co, Ltd, China. Anti-MMP-2 monoclonal antibody was from Wuhan Boster Biological Technology, Ltd, China. PCR primers were ordered from Shanghai Sangon Biotech Co, Ltd, China. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone Co, USA.

Cell culture

Human HCC cell line with high metastatic capacity (HCCLM3) (Li et al., 2003) were obtained from the Liver Cancer Institute of Fudan University, Shanghai, China. Cells were cultured in DMEM containing 15% FBS in a humidified incubator at 37 °C with 5% CO₂.

MTT assay

HCCLM3 cells were seeded in a 96-well culture plate at a density of 4×10³ cells/well. After overnight incubation, cells were treated with DMSO (vehicle control) or various concentrations of genistein alone, or cisplatin alone, or the combination of genistein and cisplatin, respectively. Each treatment group has 3 replicates. After 48 h treatment, MTT (20 μL, 5 mg/ml) was added to each well, and plates were incubated at 37 °C for 3 h. Formazan crystals were then dissolved in DMSO and absorbance (OD value) was read at 570 nm using a multi-well spectrophotometer (Bio-Rad, USA). The IC₅₀ was defined as the concentration required for 50% inhibition of cell growth. IC₅₀ values were calculated from the regression line of the plot of percentage inhibition versus log inhibitor concentration. Survival rate of tumor cells (%) = experimental group OD value/control group OD value × 100%. Each experiment was repeated 3 times.

Determination of synergism and antagonism

Synergism or antagonism of genistein and cisplatin was quantified with the Median-effect principle, using the combination index (CI) method (Chou and Talalay, 1984). The CI for the two drugs was calculated from the following formula based on Chou and Talalay Median effect equation: $CI = (D)1/(D\chi)1 + (D)2/(D\chi)2 + (D)1(D)2 / (D\chi)1(D\chi)2$. (D)1 or (D)2 represents the dose of drug 1 (genistein) or drug 2 (cisplatin) in combination to achieve

$\chi\%$ inhibition, whereas (D χ)1 or (D χ)2 represents the dose of drug 1 or 2 to achieve $\chi\%$ inhibition when administrated alone. D χ can be calculated from the following equation: $D\chi = Dm [fa/(1-fa)]^{1/m}$. In this equation, D χ denotes the dose of drug, Dm is the median-effect dose, fa is the fraction of cells affected (killed) by the dose, and m is the exponent defining the shape of the dose-effect curve. CI < 1, CI = 1, and CI > 1 indicate synergism, additivity, and antagonism in combined drug action, respectively.

Immunocytochemistry and H-Score algorithm

HCCLM3 cells were seeded on coverslips in six-well plates. After 24 h, cells were treated with culture medium (control), genistein (40 μmol/L), cisplatin (20 μmol/L) or the combination of genistein (40 μmol/L) and cisplatin (20 μmol/L), respectively. 48 h later, cells were fixed and incubated with 10% acetic acid and nonimmune serum sequentially. Then coverslips were incubated with anti-MMP2 monoclonal antibody (1:100) at 4°C overnight. The next day, coverslips were washed and incubated with biotinylated secondary antibody for 30 min at room temperature, followed by incubation of streptavidin-Alkaline phosphate (1:200). After wash, AP-Red substrate was used for enzymatic visualization, and hematoxylin was used to stain the nuclei.

When evaluating the expression of MMP-2 in a picture, both the extent and intensity of immunopositivity were considered, using the method of the H-Score algorithm (Yin et al., 2007). The intensity of positivity was scored as 0, 1, 2, or 3 corresponding to the presence of negative, weak, intermediate, and strong brown staining, respectively. The percentage of positively stained cells for each staining-intensity was estimated. The final score was calculated by multiplying the intensity of positivity and the percentage of the corresponding intensity. For example, if 40% of tumor cells are scored 1, 30% scored 2, and 30% scored 3, the H-Score for this case is 40% × 1 + 30% × 2 + 30% × 3 = 1.9.

Construction of animal models

BALB/c nu/nu nude mice were purchased from Huaxi Animal Research Institute of Sichuan University. Mice were housed in laminar flow cabinets under specific pathogen-free conditions and used at 4-6 weeks of age. All animal procedures were approved by the Institutional Animal Care and Use Committees at Huaxi Medical Center, and conformed to the guidelines of Sichuan University statements for the care and use of laboratory animals. One nude mouse was injected subcutaneously in axilla area with 0.2ml HCCLM3 cell suspension with a concentration of 2.5 × 10⁷/ml. 35 days later, the tumor under the skin which had grown to about 2 cm in diameter was removed, cut into 1 mm³ pieces, and implanted into one single lobe of the livers of other experimental mice to establish orthotopic xenograft models as described previously (Sun et al., 1996).

Mice grouping and treatment

10 days after implantation, the tumor-bearing liver lobe was resected. 30 nude mice after hepatectomy were divided into control group, genistein treated group,

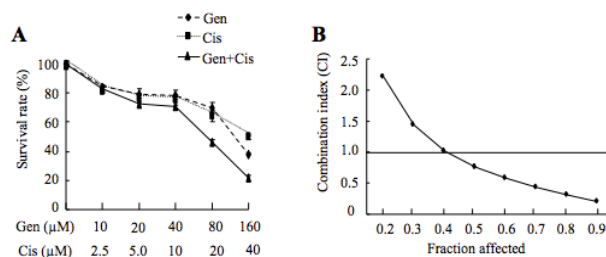


Figure 1. Genistein (Gen) and Cisplatin (Cis) Exhibited Synergistic Effect on Inhibiting the Proliferation of Human HCC Cells. (A) Dose-response curve of HCCLM3 cells after treatment of genistein and cisplatin. (B) Combination index (CI) of genistein and cisplatin in HCCLM3 cells. Fraction Affected denotes the proportion of cells affected (e.g., a Fraction Affected of 0.5 is equivalent to a 50% reduction in cell number)

cisplatin treated group and genistein combining with cisplatin treated group based on the size of resected tumor. The average sizes of the excised tumor of all groups were similar. From 3 days after liver lobe resection, those mice were given intraperitoneal injection of 0.4 ml saline containing 0.04% DMSO twice a week for 4 consecutive weeks (n=6), or genistein 2 mg/kg once every other day for 4 weeks (n=8), or cisplatin 2 mg/kg once daily for 7 consecutive days (n=8), or genistein once every other day for 4 weeks combining with cisplatin once daily for 7 days (2 mg/kg for both drugs, n=8), respectively. 33 days after liver lobe resection, all mice were sacrificed, tumor growth and pulmonary metastasis were assessed. Tumor size was measured with calipers and the volume was estimated by the formula $V=ab^2$ where “a” was the widest diameter and “b” was the smallest (Yang et al., 1992). Paraffin blocks of lungs were sectioned at the thickness of 5 μm, and stained with hematoxylin and eosin to evaluate the foci of lung metastasis. Recurrent liver tumor was also collected for immunohistochemistry study and real-time fluorescent quantitative PCR.

Immunohistochemistry

Recurrent liver tumor tissue was embedded in paraffin, cut into sections with thickness of 5 μm and stained for detection of MMP-2 expression. Briefly, sections were incubated with anti-MMP2 monoclonal antibody (1:100) at 4°C overnight, and washed with PBS for 3 times. Then sections were incubated with a goat anti-mouse IgG antibody labeled with horseradish peroxidase for 1 h at room temperature. After another 3 washes with PBS, 3-diaminobenzidine tetrahydrochloride (DAB) containing 0.01% hydrogen peroxide was used for enzymatic visualization and hematoxylin was used to stain the nuclei. The H-Score algorithm (described above) was also employed in evaluating MMP-2 expression in recurrent liver tumor tissue.

Real-time quantitative PCR

Trizol assay was used to extract total RNA from recurrent tumors, and cDNA was synthesized from 2 μg of RNA per sample. MMP-2 transcript level was determined using the following primers: mouse MMP-2 (forward, 5'-ATGCCATCCCTGATAACCT-3'; reverse, 5'-CTTCACGCTCTTGAGACTTT-3'), and GAPDH

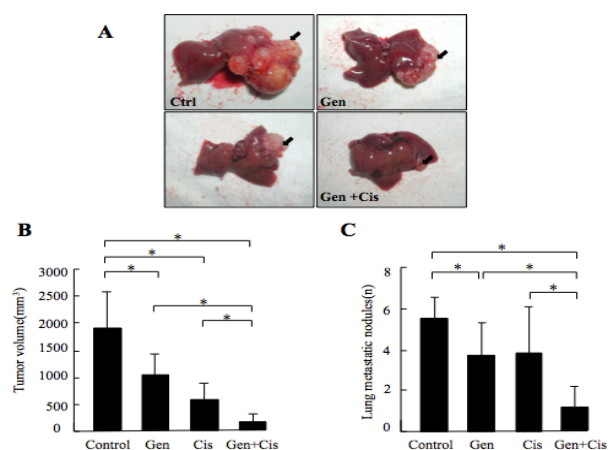


Figure 2. Genistein Reinforced the Inhibitory Effect of Cisplatin on Tumor Recurrence and Metastasis of Nude Mice after Hepatectomy. (A) Representative pictures of liver recurrent foci (arrows) 33 d after tumor resection. (B) The volume of liver recurrent focus (* $P < 0.05$). The bars indicate the means±SD. (C) The number of lung metastases (* $P < 0.05$). The bars indicate the means±SD

(forward, 5'-CCTCAAGATTGTCAGCAAT-3'; reverse, 5'-CCATCCACAGTCTTCTGAGT-3'). The Real-time PCR cycle was 94 °C for 3 minutes, following by 94 °C for 30 seconds, 55 °C for 30 seconds, and 72°C for 1 minute with 44 additional repeats, and the PCR was carried out in the Real-time PCR system 9600 (Perkin Elmer, USA). MMP-2 mRNA level was quantified by the comparative threshold cycle (CT) method (Livak and Schmittgen, 2001).

Statistical analysis

The quantitative data were presented as mean ± standard deviation (SD). Statistical analyses were performed using Student's t-test. The differences were considered significant at a p value of less than 0.05.

Results

Genistein and Cisplatin exhibited synergistic effect on inhibiting the proliferation of human HCC cells

The inhibitory effects of genistein and cisplatin on HCCLM3 cell proliferation were evaluated by MTT assay. As expected, both genistein and cisplatin dose-dependently inhibited tumor cell proliferation when administrated alone. Moreover, when the two drugs were combined, the inhibitory effect was significantly more potent than either drug used alone (Figure 1A).

The median-effect dose (IC₅₀) was calculated according to the formula mentioned above. When used alone, the IC₅₀ was 152.44 μmol/L for genistein, and was 52.75 μmol/L for cisplatin. When the two drugs were used in combination, the IC₅₀ for genistein and cisplatin were 59.30 μmol/L and 14.83 μmol/L respectively, indicating that the combined administration could effectively reduce the dose of both drugs to reach the same extent of inhibition.

We also calculated the combination index (CI) for the combined treatment. When fraction affected (Fa) was greater than 0.41, the CI value was below 1, which

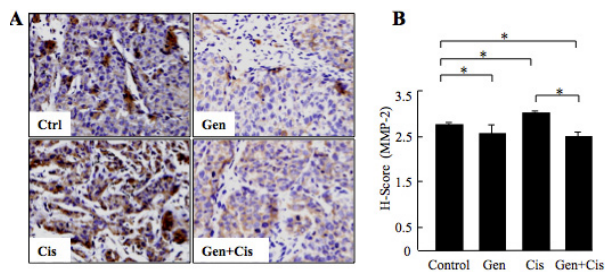


Figure 3. Cisplatin Upregulated the Expression of MMP-2, While Genistein Mitigated Cisplatin-induced MMP-2 Expression in Recurrent Tumor. Recurrent tumor tissue was fixed and sectioned, and MMP-2 expression was detected by immunohistochemistry. (A) Representative pictures of MMP-2 staining in different treatment groups (400×) (brown, MMP-2; blue, cell nuclei). (B) The H-Score evaluation (**P* < 0.05). The bars indicate the means±SD

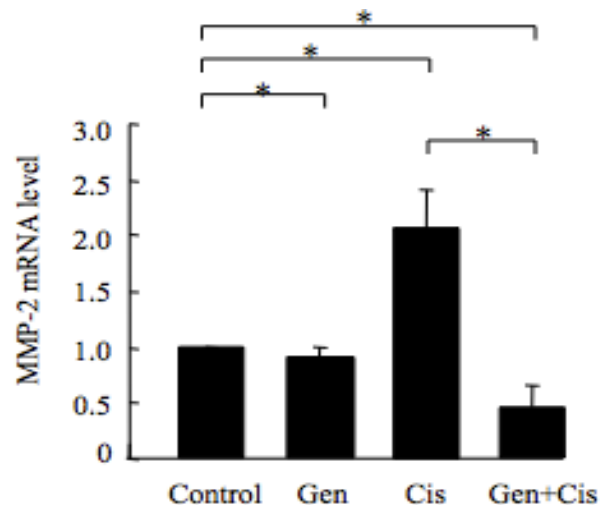


Figure 4. Cisplatin Upregulated the mRNA Level of MMP-2 in Recurrent Tumor, Which was Abolished by Genistein. Real-time quantitative PCR was employed to determine the mRNA level of MMP-2 in the recurrent tumor of different treatment groups. The bars indicate the means±SD (**P* < 0.05)

indicated that the combined treatment possessed a synergistic inhibitory effect on HCCLM3. When Fa was exactly 0.41, concentrations of genistein and cisplatin were 41.51 μmol/L and 10.38 μmol/L respectively, which were relatively low doses of both drugs (Figure 1B).

Genistein reinforced the inhibitory effect of cisplatin on tumor recurrence and metastasis of nude mice after curative hepatectomy

After hepatectomy, mice in the control group represented 100% intrahepatic recurrence and lung metastasis. The volume of the liver recurrent focus was 1917.00 ± 647.55 cm³ in the control group, and was significantly smaller in cisplatin and genistein singly treated group, in which the focus volume were 581.38 ± 290.15 cm³ and 1011.88 ± 401.14 cm³ respectively. In genistein combined with cisplatin group, however, the volume of the recurrent focus was only 163.75 ± 143.55 cm³, which was dramatically lower than either single drug treated group (Figure 2A, 2B). This was also true for tumor metastasis. In the control group, the number of lung metastasis foci was 5.50 ± 1.05 per mice. In drug treated

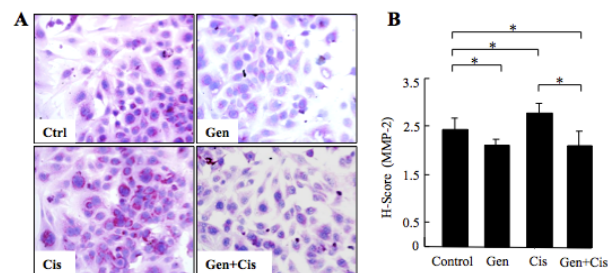


Figure 5. Cisplatin Increased the Expression of MMP-2 in Human HCC Cells, While Genistein Abolished Cisplatin-induced MMP-2 Upregulation. HCCLM3 cells were treated with genistein, cisplatin or the combination of genistein and cisplatin for 48 h, and MMP-2 expression was detected by immunocytochemistry. (A) Representative pictures of MMP-2 staining in different treatment groups (400×) (red, MMP-2; blue, cell nuclei). (B) The H-Score evaluation of different treatment groups (**P* < 0.05). The bars indicate the means±SD

group, the number of metastatic nodules in genistein group, cisplatin group and combined drug group were 3.75 ± 1.58, 3.88 ± 2.17 and 1.13 ± 0.99, respectively (Figure 2C). These results indicated that genistein in combination with cisplatin could restrain tumor recurrence and lung metastasis in nude mice after curative liver lobe resection.

Cisplatin upregulated MMP-2 expression in recurrent tumor, which was abolished by genistein

Immunohistochemistry was used to detect MMP-2 expression in liver recurrent tumor. The H-Score for MMP-2 in the control group was 2.75 ± 0.04. In cisplatin treated group, the score was 3.00 ± 0.04, which was significantly higher than the control. In genistein treated group and the drug combination group, the H-Scores were 2.56 ± 0.19 and 2.47 ± 0.10 respectively, indicating that genistein decreased endogenous as well as cisplatin-induced MMP-2 expression in liver tumor (Figure 3). To further confirm the effects of these two drugs, we detected MMP-2 transcript level in recurrent liver cancer tissue. Our results from real-time quantitative PCR revealed a 2.06 fold induction of MMP-2 mRNA by cisplatin. Consistent with the immunohistochemistry finding, genistein decreased both endogenous and cisplatin-upregulated MMP-2 mRNA level in recurrent tumor (Figure 4).

Cisplatin increased the expression of MMP-2 in human HCC cells, while genistein abolished cisplatin-induced MMP-2 upregulation.

To confirm the effects of cisplatin and genistein on MMP-2 expression in vitro, we treated HCCLM3 cells with cisplatin, genistein or the combination and detected MMP-2 level using immunocytochemistry. Representative pictures were shown in Figure 5A. The H-Score for MMP-2 in the control group was 2.44 ± 0.23. In cisplatin treated group, the score was 2.78 ± 0.19, which was significantly higher than the control. In genistein treated group and the drug combination group, the H-Scores were 2.13 ± 0.11 and 2.12 ± 0.31 respectively, indicating that genistein decreased endogenous as well as cisplatin-induced MMP-2 expression in HCCLM3 cells (Figure 5B).

Discussion

HCC has a poor prognosis with the 5-year survival rate reported below 9% (Sherman, 2005). The postoperative high recurrence rate in HCC is one of the major obstacles for improving prognosis. Therefore, effective chemotherapy is necessary for preventing tumor recurrence after curative hepatectomy. While HCC is relatively insensitive to most chemotherapeutic agents, it is still believed that the combination of drugs with different mechanisms of action may provide some benefits to overcome drug resistance and reduce the side effects. Approximately 67% of all anticancer drugs originate from plants with the bioactive compounds (Gurib-Fakim, 2006), one of which is genistein. The present study found out that genistein significantly enhanced the anti-tumor effect of cisplatin, which may be of clinical importance concerning cancer therapy.

The aim of combination chemotherapy is to achieve synergistic effect, improve treatment outcome, decrease doses of chemotherapy drugs, reduce side effects, as well as postpone resistance. When drugs are combined administrated, they exhibit synergism, additivity, or antagonism, which represent that the combined effect is greater, equal or less than the sum of individual drug effect, respectively. Our study found out that when combined administrated, the IC₅₀ for genistein was only 39% that of the drug being used alone, and the value was 28.1% when it came to cisplatin. The combination chemotherapy dramatically decreased the dose of both drugs, especially cisplatin, which had some toxicity on normal tissue. In other words, genistein significantly sensitized HCC cells to cisplatin, which would be favorable in clinical trials. Also, our results were consistent with Li Y et al., who found that genistein enhanced the anti-tumor effect of cisplatin, docetaxel and doxorubicin in various cancers (Li et al., 2005).

The animal model employed in our study was a mimic of postoperative recurrence and metastasis of human liver cancer. This model was established to have high metastatic tendency with 100% abdominal wall metastases, 80% intra-abdominal cavity metastases, 100% intrahepatic metastases, 70% diaphragm metastases, and 100% pulmonary metastases 35 days after orthotopic implantation of tumor tissue into the liver of nude mice (Li et al., 2003). In the present study, with primary tumor resected on postimplantive day 10, all mice in control group represented 100% intrahepatic recurrence and lung metastasis 33 days after resection, confirming the model to be ideal in studying tumor recurrence and lung metastasis. This model has also been used by different groups working on the same field (Jian et al., 2003; Wang et al., 2003).

Cancer invasion and metastasis is a complicated multistep process involving numerous effector molecules. The degradation of extracellular matrix is an essential step in cancer invasion and metastasis. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that degrade almost all extracellular matrix components, play important roles in cancer invasion and metastasis (Vihinen et al., 2005). Among all MMPs, MMP-2 and MMP-9 have been implicated in HCC invasion and metastasis

(Giannelli et al., 2002). Upregulation of MMPs would facilitate the degradation of extracellular matrix, thus enhance the metastatic ability of cancer cells. Compelling evidence demonstrates that resistance to chemotherapy drugs is highly related to the side effects of those drugs in promoting tumor metastasis. Chemotherapy-resistant cell lines have higher ability of metastasis, in parallel, they express higher level of MMP-2 than non-resistant cell lines (Yang et al., 2003). It has been reported that doxorubicin and 5-fluoro-2-deoxyuridine enhanced the metastatic ability of breast cancer (De Larco et al., 2001). Nevertheless, cisplatin was also found to upregulate MMP-2 in ovary cancer (Latifi et al., 2011). Our data demonstrated that both protein and mRNA level of MMP-2 in recurrent tumor were increased by cisplatin, which might be one cause of cisplatin chemoresistance. However, we also found that genistein inhibited the heightening of MMP-2 induced by cisplatin, through which genistein may exert the effect of suppressing tumor metastasis. This might account for the underlying mechanism for the synergistic repression of HCC by the combination of genistein and cisplatin.

Taken together, our data strongly supported that genistein reinforced the inhibitory effect of cisplatin in tumor recurrence and metastasis after curative hepatectomy. Our finding will hopefully shed light on the rationale of cancer chemotherapy.

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