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Original Article

Inhibition of hepatocyte carcinoma cell activity by pterostilbene via down-regulating insulin-like growth factor binding protein-2

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Abstract: Insulin-like growth factor binding protein-2 (IGFBP-2) can modulate proliferation and growth of tumor cells via specific binding onto insulin-like growth factor (IGF). Studies have shown that correlation between IGFBP-2 and hepatocyte carcinoma. Resveratrol has been demonstrated to modulate proliferation of hepatocyte carcinoma cells. As one homologous derivative of resveratrol, pterostilbene as more potent effects in inhibiting tumor cell occurrence and progression, but with unclear mechanisms in hepatocellular carcinoma cells. This study thus investigated the function of pterostilbene in hepatocellular carcinoma cells and its effect on IGFBP-2. Hepatocellular carcinoma cell line HepG2 was incubated with 100 μM or 200 μM pterostilbene. MTT assay was determined the cell proliferation while, caspase-3 assay and flow cytometry were used to describe cell apoptosis, respectively. Real-time PCR and enzyme-linked immunosorbent assay (ELISA) was used to detect mRNA and protein levels of IGFBP-2. Phosphorylation level of p38 signaling pathway modulated by IGFBP-2 was investigated by Western blotting. Pterostilbene significantly inhibited HepG2 cell proliferation, induced the apoptosis with the increase of caspase-3 activity ($P<0.05$) in a dose-dependent manner. It can also down-regulate mRNA and protein expression level of IGFBP-2 in hepatocyte carcinoma cells, in addition to depressed p38 phosphorylation level ($P<0.05$). Pterostilbene can inhibit p38 phosphorylation level via inhibiting IGFBP-2 expression, thus modulating proliferation and apoptosis of hepatic carcinoma cells.

Keywords: Hepatocyte carcinoma, IGFBP-2, pterostilbene, cell proliferation

Introduction

As one of most malignant tumors, hepatic carcinoma is the second leading cause of death among all cancers worldwide [1, 2]. The incidence of hepatic carcinoma remains at high level due to life style transition and unhealthy diet habit. Among different subtypes of hepatic carcinoma, hepatocellular carcinoma (HCC) is the most popular one [3, 4]. Due to the high frequency of hepatitis B, China is one of prevalent region of hepatic carcinoma, which is the second leading cause of death among all digestive tract tumors [5]. Primary hepatic carcinoma can be caused by hepatitis B, toxic substances, and genetic mutations. The complex pathogenesis and insidious onset make it extremely difficult to make early diagnosis, causing rapid progression [6]. Although advanced treatment techniques including surgery, liver transplantation, radio- and chemo-therapy, immune therapy and interventional treatment have been developed with individualization, no major improvements have been obtained, as most primary hepatic carcinoma patients still suffer from high recurrence rate and unfavorable prognosis, bring heavy burdens for the healthcare system [7, 8].

Insulin-like growth factor binding protein-2 (IGFBP-2) belongs to insulin-like growth factor (IGF) family [9]. IGFBP participates in modulating normal cell proliferation and malignant transformation [10, 11]. Via competitive binding with IGF against insulin-like growth factor receptor (IGFR), IGFBP exerts important role in tumor's onset and progression [12]. IGFBP-2 has been revealed to have abnormal expres-

Table 1. Primer sequence

Gome	Forward primer 5'-3'	Reversge wt5'-3'
GADPH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
IGFBP-2	CAGATCCCACCTCTGTAAG	GCTACATCTGGGGCTGTA

sion in tissue, body fluids and serum in various cancers. Most importantly, it was significantly up-regulated in serum of primary hepatic carcinoma patients, indicating close correlation with cancer progression [13]. In vitro assay demonstrated that Resveratrol played a regulatory role in tumor progression, but in vivo conditions the practical application in anticancer treatment is strongly limited due to its low bioavailability [15]. As one 3,5-dimethyl derivative of resveratrol, pterostilbene ((E)-3, 5-dimethyl-4-hydroxyl stibene) is one type of non-flavonoid polyphenol compounds that are enriched in grapes, nuts, strawberry, propolia and guangxi dragon's blood. Having similar pharmaceutical actions with resveratrol, pterostilbene has pluripotent functions including anti-fungal, anti-proliferation, anti-oxidation, anti-inflammation and decrease of blood lipids [16]. Its role in HCC, however, has not been fully illustrated. This study thus investigated the effect of pterostilbene on HCC cells and the correlation with IGFBP-2.

Materials and methods

Cell culture and treatment

HCC cell line HepG2 (ATCC cell bank, US) was resuscitated in 37°C water-bath, followed by 1,000 g centrifugation (3 min) and re-suspension in 1 mL DMEM medium (Hyclone, US). Cells were cultured in a humidified chamber with 5% CO_2 at 37°C. Cell were then inoculated in culture dish at $1 \times 10^7/cm^2$ using 90% high-glucose DMEM (containing 100 U/mL penicillin and 100 μg/mL streptomycin) plus 10% fetal bovine serum (FBS). Cells were passed every 2 or 3 days. Log-phased cells were randomly divided into three groups: normal (DMSO), 100 μM and 200 μM pterostilbene treatment groups for 72 hours [17].

MTT assay

Cells at log phase were digested and seeded into 96-well plate at 3,000 cells per well. Five repeats were set for each group. After treated with blank control, 100 μM or 200 μM pterostilbene for 72 hours, 20 μL MTT solution (5 g/L) was added into each well. The plate was further incubated for 4 hours to remove supernatants. 150 μL DMSO was added for 10-min mixture until complete dissolve of violet crystal. The membrane was measured by a micro plate reader at 570 nm for absorbance (A) value to calculate cell proliferation.

Caspase-3 activity assay

Caspase-3 activity level was determined by test kit (R&D, US) following manual instruction. Trypsin was used to digest cells, followed by centrifugation at 600 g for 5 min. Supernatants were discarded, and lysis buffer was added for 15-min lysis. After 20,000 g centrifugation for 5 min, 2 mM Av-DEVD-pNDA was added for observing optical density (OD) values at 405 nm wavelength, in an attempt to describe caspase 3 activity.

Flow cytometry

The supernatant of cell culture was discarded after treatment of DMSO or pterostilbene for 48 h, cells were digested with 0.25% trypsin and then gently pipette into single cells with DMEM medium. Cell suspension was collected for centrifugation at 300 g, 10 min. The supernatant was removed and PBS solution was added for washing. After centrifugation at 300 g, 10 min, supernatant was removed and 200 μl blocking buffer was added for 15 min incubation at room temperature. Then cells were washed with PBS and incubated with 500 μl Annexin V solution for 10 min at room temperature. After centrifugation at 300 g for 5 min, supernatant was discarded and PI dye was added for incubation at room temperature for 10 min. Cells were gently pipette for the detection of apoptosis with flow cytometry (Clolter, USA).

Real-time PCR

Total RNA was firstly extracted by Trizol reagents from all cells. Synthesis of DNA *in vitro* was performed by reverse transcription kit (R&D, US). Primers (see **Table 1**) were designed by Primer 6.0 software. RC-PCR was performed using the following conditions: 95°C pre-denature, followed by 35 cycles including 92°C denature (30 sec), 58°C annealing (45 sec) and 72°C

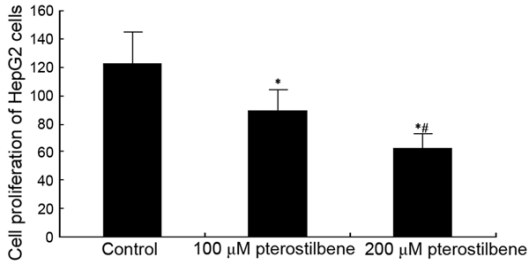

Figure 1. Effect of pterostilbene on HepG2 cell proliferation. *, P<0.05 compared to control group; #, P<0.05 compared to 100 μM group.

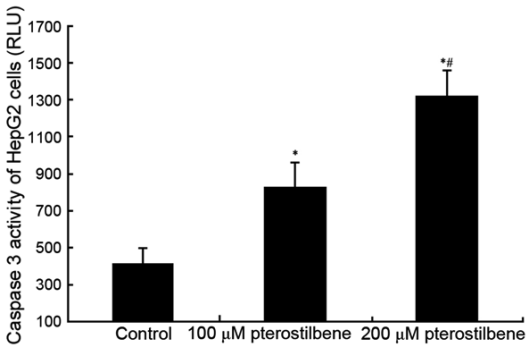

Figure 2. Effect of pterostilbene on caspase-3 activity. *, P<0.05 compared to control group; #, P<0.05 compared to 100 μM group.

elongation (35 sec). Data were collected to plot CT values of both test samples and standards. Using GAPDH as the internal reference, standard curve was plotted to obtain semi-quantitative analysis.

Enzyme-linked immunosorbent assay (ELISA) and IGFBP-2 protein expression

Cultured supernatants were collected for IGFBP-2 level assay using ELISA test kit (DSL, US). In brief, 50 μL serially diluted standards were seeded into 96-well plate. Meanwhile 50 μL test samples were added into the plate in triplicates. After gentle washing, the plate was removed and was centrifuged. 50 μL enzyme linked reagent was then added into the plate, followed by 37°C incubation for 30 min. Chromogenic substrates A and B (50 μL each per well) were mixed with samples for 37°C in dark incubation for 10 min. The reaction was then quenched by stopping solution. Using the blank group as internal reference, OD values at 450 nm were obtained within 15 min after the end point. Linear regression was developed for standard samples, on which sample concentration was illustrated.

Western blotting

Total proteins were extracted from HepG2 cells by lysis buffer, iced incubation (15~30 min), and ultrasonic rupture. Protein samples were saved by 4°C centrifugation for 15 min, and were separated by 10% SDS-PAGE. The proteins were transferred to PVDF membrane. 5% defatted milk powder for 2 hour was used to block non-specific binding site. Primary antibody against phosphorylated p38 and p38 (1:1,000, Cell signaling, US) was applied at 4°C overnight. Goat anti-rabbit IgG (1:2,000, Cell signaling, US) was then added for 30-min incubation at room temperature, followed by chromogenic substrates and X-ray exposure. Both image software and Quantity one system was used to analyze OD values of protein bands (n=4).

Statistical analysis

All data were presented as mean ± standard deviation (SD). Student t-test was employed for between-group-comparison. Analysis of variance (ANOVA) was used to analyze group difference in conjunction with Dunnett's test. SPSS 11.5 software was used to process all data. A statistical significance was defined when P<0.05.

Results

Poterostilbene inhibits HepG2 cell proliferation in a dose dependent manner

MTT assay showed that, after 72 hours of pterostilbene treatment, HepG2 cell proliferation was significantly inhibited compared to control group (P<0.05). With higher dosage of pterostilbene, the inhibition effect was further potentiated (**Figure 1**), suggesting dose-dependent inhibitory effect on HepG2 cell proliferation by pterostilbene.

Caspase-3 activity

After the treatment of pterostilbene, caspase-3 activity was significantly elevated compared to the control group (P<0.05). With higher dosage of pterostilbene, the activity of caspase-3 was further elevated (**Figure 2**), suggesting dose-

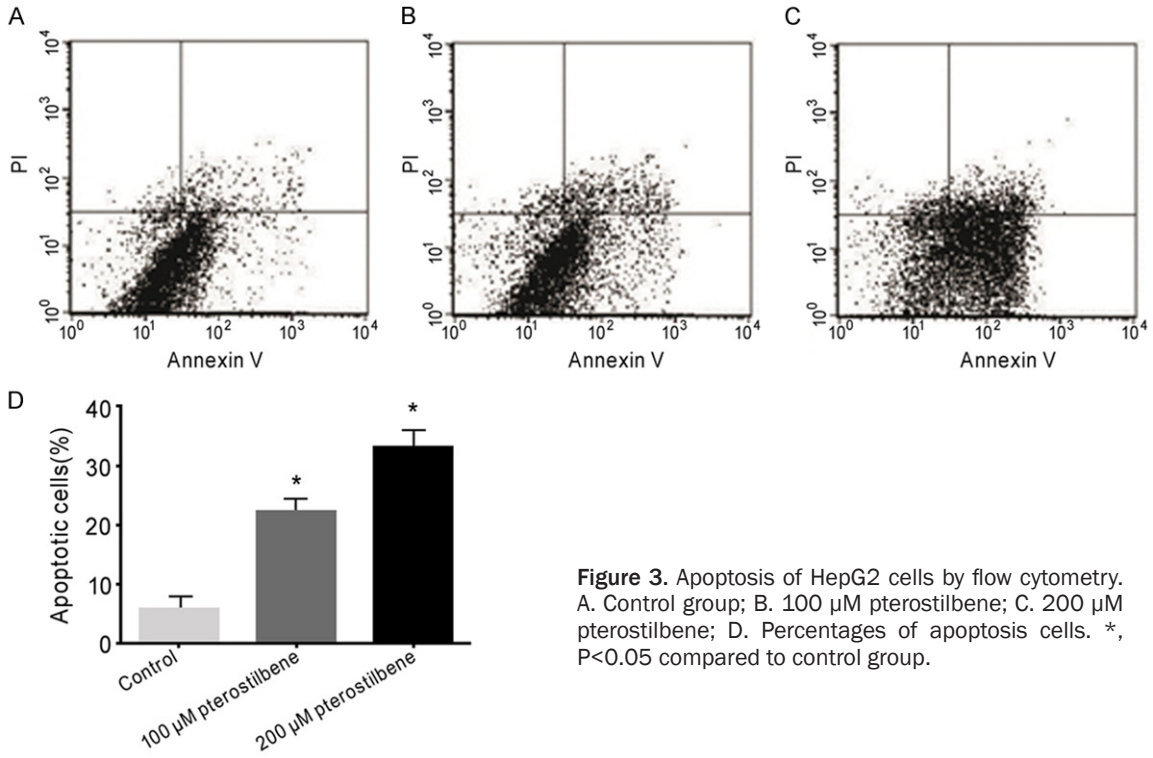

Figure 3. Apoptosis of HepG2 cells by flow cytometry. A. Control group; B. 100 μM pterostilbene; C. 200 μM pterostilbene; D. Percentages of apoptosis cells. *, P<0.05 compared to control group.

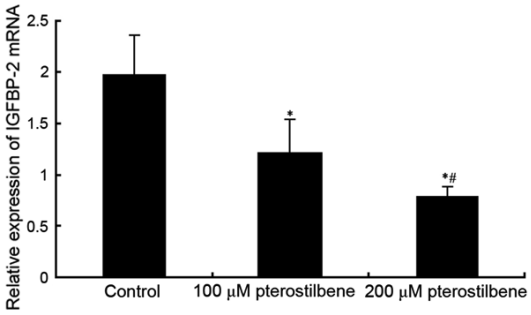

Figure 4. IGFBP-2 mRNA relative level. *, P<0.05 compared to control group; #, P<0.05 compared to 100 μM group.

dependent stimulatory effect on HepG2 cell apoptosis by pterostilbene.

The effect of pterostilbene on the apoptosis of HepG2 cells

We further studied the effect of pterostilbene on the apoptosis of HepG2 cells. The results showed that HepG2 cell apoptosis was induced, with the percentage of apoptosis 21.7% and 32.6% after being treated with 100 mol/L and 200 mol/L of pterostilbene, respectively. However, the percentage of apoptosis in DMSO treatment group was 6.2%, indicating statistical difference between DMSO and pterostilbene groups (F=21.812, P<0.05) (**Figure 3**).

IGFBP-2 mRNA level

Real-time PCR was used to detect the effect of pterostilbene on mRNA level of IGFBP-2. Results showed significant suppression of IGFBP-2 in HepG2 cells with pterostilbene as compared to control group (P<0.05). Further elevation of drug concentration further suppressed IGFBP-2 expression (**Figure 4**).

Pterostilbene decreases IGFBP-2 protein concentration in a dose dependent manner

ELISA approach was used to detect protein level of IGFBP-2. Results showed consistent patterns as those in mRNA: pterostilbene decreased IGFBP-2 in HepG2 cells in a dose-dependent manner (P<0.05, **Figure 5**). These results collectively suggested the participation of pterostilbene in regulating IGFBP-2 gene expression.

Phosphorylation level of p38

We further used Western blotting to describe the effect of pterostilbene on IGFBP-2-induced p38 signaling pathway and found inhibited ph-

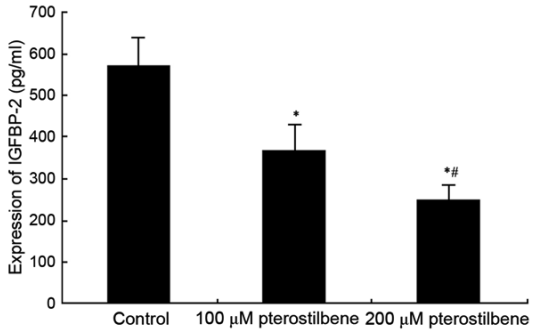

Figure 5. IGFBP-2 protein concentration. *, P<0.05 compared to control group; #, P<0.05 compared to 100 μM group.

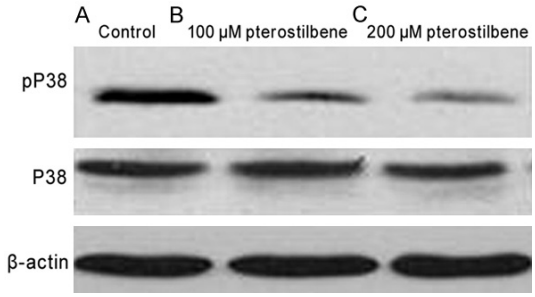

Figure 6. Phosphorylation level of p38 in HepG2 cells. A. Control group; B. 100 μM pterostilbene; C. 200 μM pterostilbene.

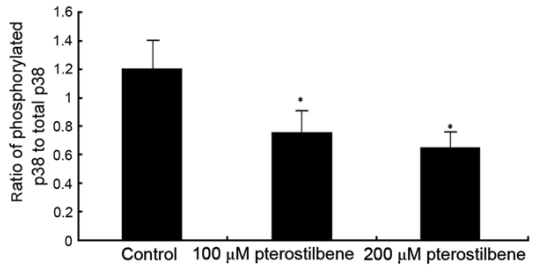

Figure 7. Effect of pterostilbene on p38 phosphorylation level in HepG2 cells. *, P<0.05 compared to control group.

osphorylation level of p38 in HdpG2 cells (P< 0.05, **Figure 6**). Elevated dosage of pterostilbene further suppressed p38 phosphorylation but not with statistical significance (**Figure 7**). There results suggest the modulation of IGFBP-2 gene expression and further p38 signal pathway by pterostilbene, in the pathogenesis and progression of primary hepatic carcinoma cells.

Discussion

The incidence of hepatic carcinoma is increasing in recent years. The pathogenesis and progression of hepatic carcinoma is a complicated process involving multiple factors and genes [18]. Therefore, the identification of precise mechanism and molecular target of primary hepatic carcinoma, for the development of effective treatment plan is the focus of current tumor therapy. Resveratrol has been demonstrated to have protective role against the intestinal mucosal barrier dysfunction, induce tumor cell apoptosis and regulate cell cycle for inhibiting tumor occurrence, in addition to the activation of body's immune response and suppress tumor angiogenesis [14, 15]. Resveratrol can be metabolized inside plant cells to synthesize pterostilbene, which has relatively higher pharmaceutical potency than resveratrol. The high specificity of pterostilbene can inhibit DNA polymerases of simian virus 40 DNA [19]. As one novel drug, pterostilbene has been shown to have efficacy in treating multiple diseases including Alzheimer's, cardiovascular disorder, brain trauma, tumor and hypercholesterolemia [20, 21]. This study found that different concentrations of pterostilbene can inhibit the proliferation of tumor cells, induced the apoptosis by elevating the activity of apoptotic proteins in cultured HCC cells, suggesting the potency of pterostilbene in suppressing primary hepatic carcinoma cell proliferation and tumor progression.

As the central organ of human GH-IGF axis, the function of liver may affect both distribution and expression of IGF family members. IGF-1 and IGFBP-2 are mainly synthesized by the liver. Therefore lower IGF-1 and/or elevated IGFBP-2 may occur under liver dysfunction or malfunction such as those in primary hepatic carcinoma patients. The abnormal expression of IGFBP-2 has been demonstrated to facilitate the growth of multiple tumors, and is thus closely related with the malignancy of tumor [22, 23]. Elevated IGFBP-2 can directly participate in the signal regulatory networks for further modulating of cell growth. As one important downstream of IGFBP-2, p38 signal pathway is the central point of multiple pathways. The activation of p38 pathway via elevated phosphorylation level may benefit cell proliferation and differentiation [24]. This study has confirmed that, pterostilbene can decrease IGFBP-2 gene expression for further inhibition of p38 phosphorylation level in HepG2 cells, which was consistent to previous study [25]. Our results provide novel molecular mecha-

nism of pterostilbene in regulating HCC cells, thus brining evidences for further illustrating precise functional pathways.

In summary, pterostilbene may inhibit p38 phosphorylation level via inhibiting IGFBP-2 expression in HCC cells, thus exerting the modulatory function non primary hepatic carcinoma cell proliferation and apoptosis.

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Disclosure of conflict of interest

None.

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