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Effect of berbamine on invasion and metastasis of human liver cancer SMMC-7721 cells and its possible mechanism

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Berbamine is a bisbenzylisoquinoline alkaloid extracted from Berberis poiretii of Berberis of Berberidaceae. It has been reported that it can significantly inhibit the proliferation of a variety of malignant tumor cells, including liver cancer. However, the effect of berbamine on the invasion and metastasis of liver cancer has not been reported. The present study demonstrated that berbamine inhibited the migration and invasion of SMMC-7721 cells in a concentration-dependent manner and obviously increased the gap junction function and the expression of Cx32 in SMMC-7721 cells compared with control group. However, after silencing Cx32, berbamine had no significant effect on cell invasion and metastasis. Before silencing Cx32, the expression of PI3K and P-AKT were decreased after berbamine treated on SMMC-7721 cells for 24 h. After silencing Cx32, the expression of PI3K and P-AKT were increased in SMMC-7721 cells. The expression of PI3K and P-AKT had no significant effect after berbamine treated on SMMC-7721 cells for 24 h with silencing Cx32. In conclusion, the results of the present study suggest that berbamine could inhibit the SMMC-7721 cell migration and invasion, and its mechanism may be related to the regulation of PI3K/AKT signaling pathway by enhancing the expression of Cx32.

Anti-Cancer Drugs 2022, **33**:e178–e185

Keywords: berbamine, Cx32, migration and invasion, PI3K/AKT

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Received 24 February 2021 **Revised form accepted** 12 July 2021

Introduction

Liver cancer is one of the most serious malignant tumors in the world and seriously threatens human health. About half of the new cases occur in China each year [1]. The reason for the high mortality rate of liver cancer is due to its strong ability to invade and metastasize. Even in small liver cancers with a diameter of less than 2 cm, about 20% have developed microvascular invasion and metastasis [2]. Therefore, it is of great significance to find therapies to inhibit invasion and metastasis. Berbamine is a bisbenzylisoquinoline alkaloid extracted from Berberis poiretii of Berberis of Berberidaceae. It has been reported that it can significantly inhibit the proliferation of a variety of malignant tumor cells, including ovarian cancer, rectal cancer, pancreatic cancer, lymphoma and prostate cancer [3–5]. It also has a certain inhibitory effect on the invasion and metastasis of some tumors, such as breast cancer and melanoma [6,7]. The study of Meng *et al.* has shown that berbamine can also inhibit the growth of liver cancer cells and induce apoptosis [8]. However, the effect of berbamine on the invasion and metastasis of liver cancer has not been reported.

Studies have shown that there is a special kind of protein channel, gap junction (GJ), which is closely associated with tumor development, invasion and metastasis [9]. GJ function is weakened in tumor cells, and restoration of GJ function will significantly inhibit tumor invasion and metastasis [10]. In liver cancer cells, the enhancement of GJ function inhibits the growth of tumor cells or enhances the antitumor efficacy of chemotherapeutic drugs [11]. GJ is made of connexins. The major connexins expressed in the liver are Cx26, Cx32 and Cx43, 90% of the GJ in hepatocytes is composed of Cx32 [12]. The effect of berbamine on GJ function in liver cancer cells has not been reported.

Based on our previous studies [13], in this study, we investigate the effects of berbamine on the invasion and metastasis of human hepatocellular carcinoma and the GJ function as well as their correlation in SMMC-7721 cells and explore its possible mechanisms.

Materials and methods

Materials

The SMMC-7721 cells were acquired from the Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China). 1640 medium, fetal bovine serum were purchased from Gibco (Grand Island, Nebraska, USA). RIPA cell lysate, SDS-PAGE gel configuration kit were purchased from Beyotime Institute of Biotechnology, China. The ECL plus kit were purchased from Millipore (Bedford, Massachusetts, USA). Matrigel was purchased from BD Biosciences (San Jose,California, USA).

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Berbamine was purchased from Shanghai Shidande Biotechnology Co. Ltd and was stored in dimethyl sulfoxide (DMSO) at 100 mmol/L for ready use. Antibodies and other reagents were purchased from Sigma Company. Three groups of siRNA sequences targeting Cx32 gene were synthesized by Shanghai Jima Pharmaceutical Co. Ltd. Sequence is as follows:

siRNA1: 5′-GCAACACAUAGAGAAGAAATT-3′ 5′-UUUCUUCUCUAUGUGUUGCTT-3′

siRNA2: 5′-GCCGUCUUCAUGUAUGUCUTT-3′ 5′-AGACAUACAUGAAGACGGCTT-3′

siRNA3: 5′-GCUCCCUGAAAGACAUACUTT-3′ 5′-AGUAUGUCUUUCAGGGAGCTT-3′

Methods

Cell culture

SMMC-7721 cells were cultured in 1640 medium containing 10% fetal bovine serum in a 37°C, 5% CO2 incubator. The cells were passaged every 2–3 days, and cells in logarithmic growth phase were harvested for subsequent experiments.

Scratch assay

SMMC-7721 cells were spread in a 6-well plate at a concentration of 1×10^5 cells/mL (2 mL per well). The back of the plate was marked before cell seeding. A horizontal line was drawn every 0.5 cm and ensure more than five lines per well. The next day, when the cells achieved 80% confluency, vertical lines were drawn using a pipette in a 6-well plate. Detached cells were washed using PBS and mitomycin was added to each well at the concentration of 1.0 μg/mL in order to eliminate the effect of cell proliferation on cell invasion and metastasis [14]. After that, 2 mL serum-free 1640 medium containing different concentrations of berbamine was added to the six-well plate. The cells were observed and photographed under an inverted microscope at 0, 24 and 48 h. Five fields (100×) were randomly selected for each group.

Transwell assay

A 24-well transwell chamber with gelatin-coated polycarbonate membrane filters was used to assess the migration ability of SMMC-7721cells. The invasive capacity of SMMC-7721cells was assessed using transwell inserts with Matrigel (BD Biosciences). After incubation for 48 h, the cells in the upper surfaces of the transwell chambers were removed with cotton swabs, and the migrated and invaded cells were fixed with 4% paraformaldehyde, and then stained with purple crystal solution. The stained cells were photographed and counted under a light microscope in five randomly selected fields. Five visual fields were randomly selected for counting (100×).

Fluorescent tracing assay

SMMC-7721 cells were seeded at a concentration of 1×10^5/mL in a 6-well plate with 2 mL per well and treated with a related reagent when the cell confluency reached 80%. Berberamine treated cells for 24 h. And then the cells were incubated with fluorescent indicator calcein for 30 min to form 'donor cells' containing calcein. After that, the 'donor cells' were incubated with drug-treated 'recipient cells' for further 4 h to allow the formation of stable intercellular GJ (calcein which emits green fluorescent can enter adjacent cells through GJ). Finally, the number of 'recipient cells' containing calcein around 'donor cells' was counted under fluorescence microscopy as an indicator of GJ function (200×).

Western-blot assay

See reference [14].

Silencing Cx32 expression

The cells were seeded on a 6-well plate at a concentration of 1×10^5/mL, and cultured for 24 h, followed by siRNA experiments. The siRNA reaction system, 10 μL siRNA +250 μL Opti-MEM, as well as 5 μL Lipofectamine2000 + 250 μL Opti-MEM was placed at room temperature for 5 min. Then, siRNA and Lipofectamine 2000 were mixed and stayed at room temperature for 20 min. 500 μL of the above mixture was added to each well of a 6-well plate and made up to 2 mL with Opti-MEM. After 6 h, the culture medium was changed to normal culture medium and incubated for further 24 h for subsequent experiments.

Statistical analysis

All of the experiments have a minimum of three determinations. The statistical analysis between groups was performed by unpaired Student's *t* test with spss20. Data were presented as mean ± standard deviations. Differences with $P < 0.05$ were considered significant.

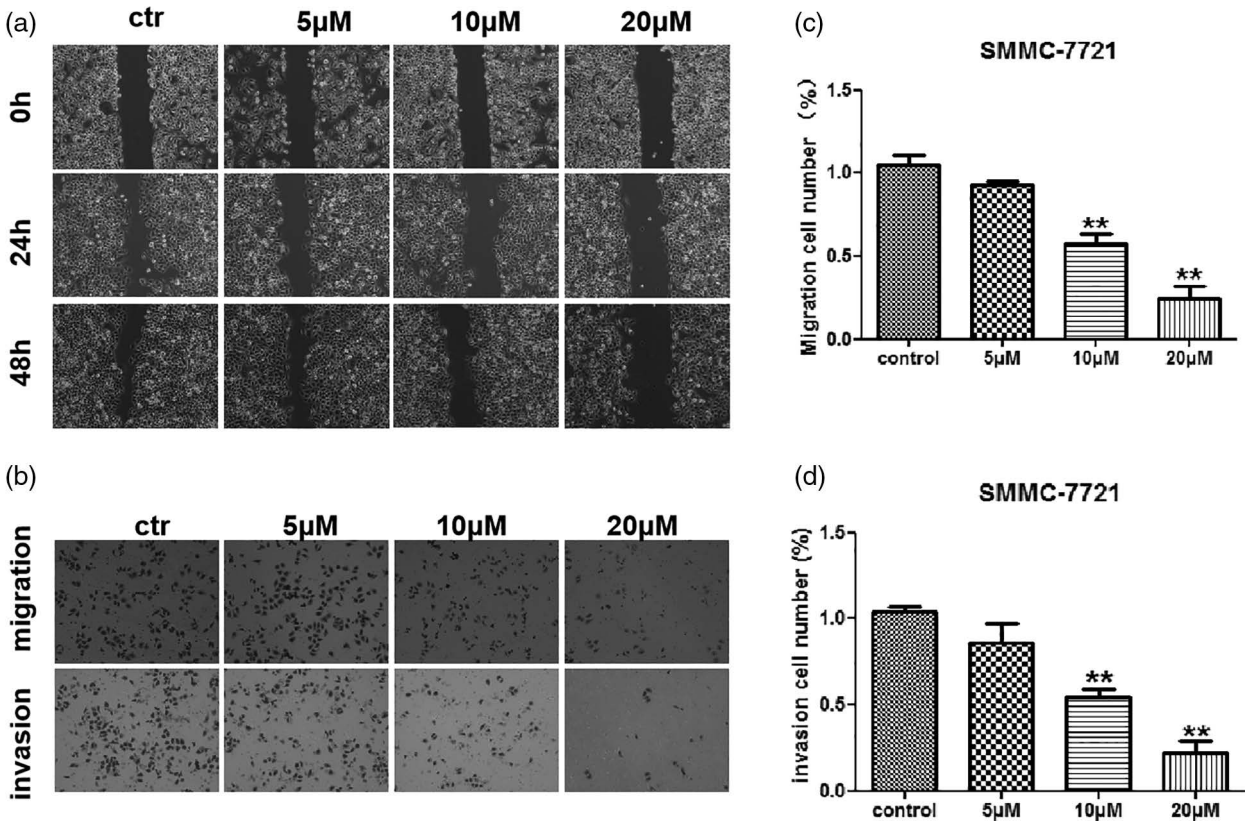
Results

Our previous studies found that the treatment of berbamine lower than 20 μmol/L did not significantly affect the viability of SMMC-7721 cells. Therefore, we selected 5, 10, 20 μmol/L of berbamine to conduct the following experiments.

Effect of berbamine on migration and invasion of SMMC-7721 cells

Scratch test showed that after 24 and 48 h treatment of berbamine, compared with the blank control group, the rate of 'scratch' healing rate in the treatment group was slower. With the drug concentration increased, the 'scratch' area became large. It suggested that the cell migration ability was weakened (Fig. 1a). Transwell assay result showed, compared with the blank control group, after 24 h treatment

Fig. 1



The effect of berbamine on cell migration and invasion of SMMC-7721 cells. (a) Wound healing assay ($n = 3$). (b) Transwell assay ($n = 4$). (c) The percentage of migration cells in transwell assay. (d) The percentage of invasion cells in transwell assay. Data are expressed as mean ± SE. $^{**}P < 0.01$ vs. control group.

of berbamine, cell migration and invasion decreased in the treatment group and the effect was dose-dependent (Fig. 1b). The inhibitory rates of cell migration and invasion of 10 μmol/L berbamine on SMMC-7721 cells were (57.35 ± 8.84)% ($P < 0.05$) and (64.40 ± 6.23)% ($P < 0.05$) respectively. And the inhibitory rates in 20 μmol/L concentration were (24.40 ± 10.47)% ($P < 0.05$) and (22.00 ± 9.90)% ($P < 0.05$) respectively, which further verified the dose-dependent inhibitory effect of berbamine on the invasion and metastasis of SMMC-7721 cells.

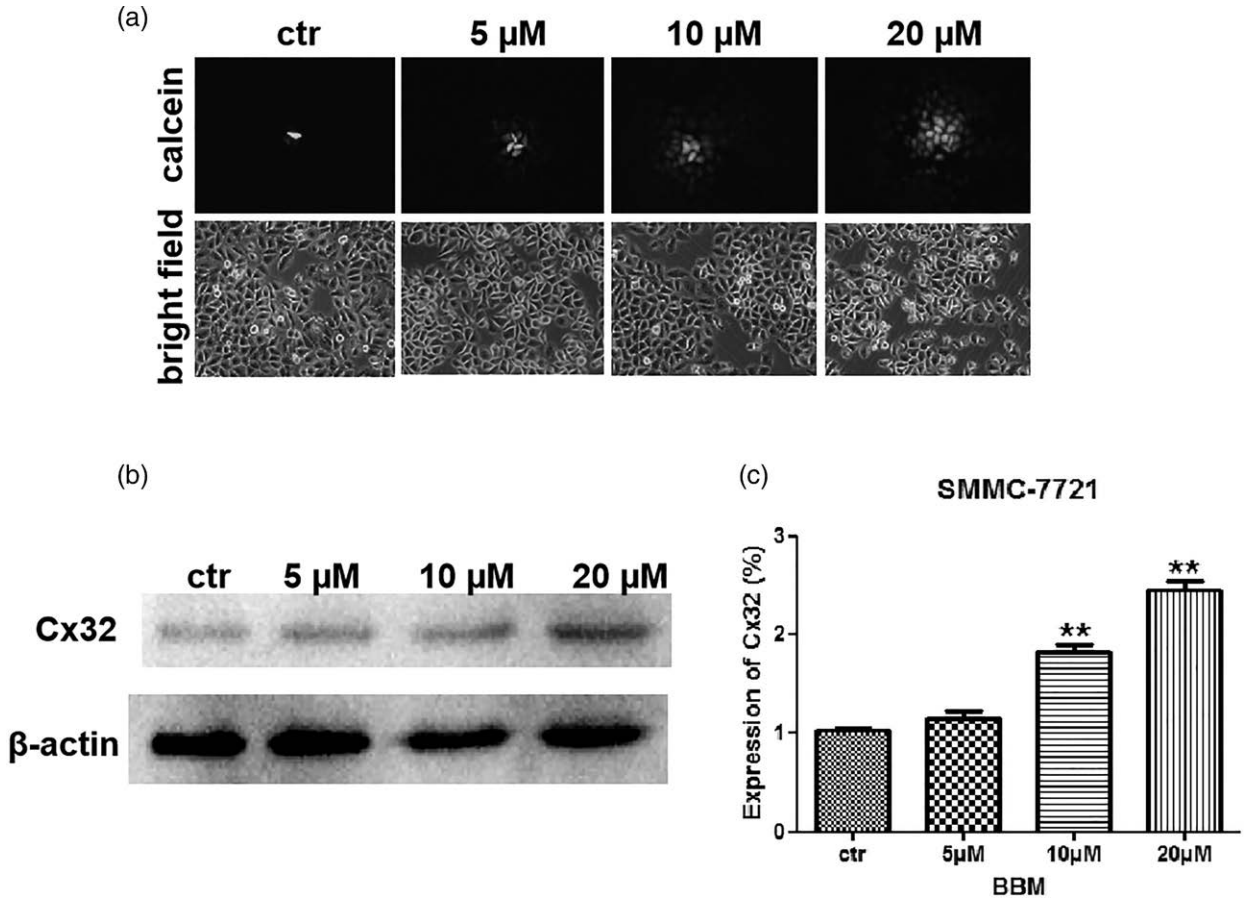
Effect of berbamine on gap junction of SMMC-7721 cells

After SMMC-7721 cells were treated with 5, 10 and 20 μmol/L berbamine for 24 h, GJ-mediated intercellular fluorescence transmission was significantly enhanced. Compared with the control group, the number of transmitted cells were significantly increased in the drug-treated group (Fig. 2a). Western blot showed that the expression of Cx32 protein were significantly increased after 24 h treatment of 5–20 μmol/L of berbamine (Fig. 2b). Compared with the control group, the expression level of Cx32 increased by (14.50 ± 13.79)%, (82.44 ± 13.27)% ($P < 0.05$) and (146.01 ± 14.72)% ($P < 0.01$) in the treatment group for different concentrations.

Inhibition of Cx32 expression by siRNA reversed the migration inhibitory effect of berbamine

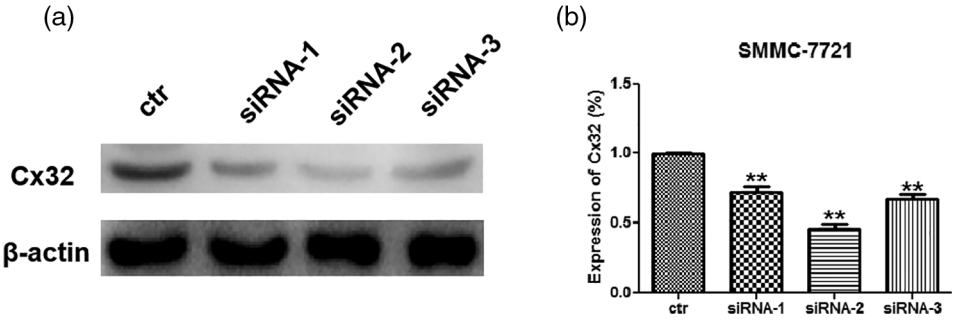
In order to explore the relationship among berbamine, SMMC-7721 cell invasion and metastasis, and GJ, we used siRNA to silence the expression of Cx32 from the gene transcription level. As shown in Fig. 3, compared with the control group, the expression of Cx32 protein in the three siRNA treatment groups was (71.80 ± 6.46)% ($P < 0.01$), (45.13 ± 6.18)% ($P < 0.01$) and (67.37 ± 3.69)% ($P < 0.01$). Cx32-siRNA 2 has the best inhibitory effect, so siRNA 2 was selected for subsequent experiments. After silencing the expression of Cx32 in the cells, scratch test showed that berbamine did not have significant effect on cell migration ability compared with the control group (Fig. 4a). The result of transwell assay also showed that the cell migration and metastatic ability of SMMC-7721 cells in treatment group were not significantly different compared with the control group.

Fig. 2



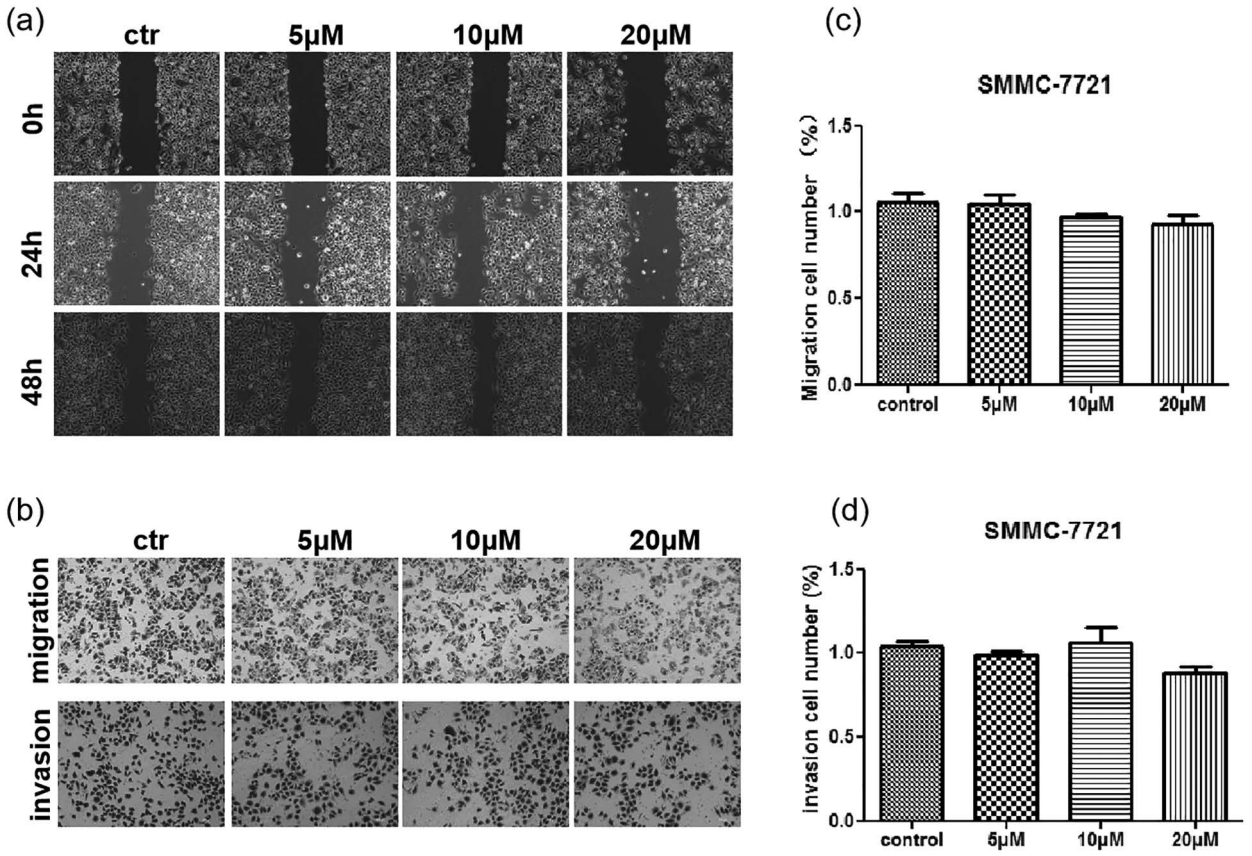
The effect of berbamine on the dye spread through GJ and the expression of Cx32 in SMMC-7721 cells. (a) Fluorescence images show the dye coupling by the 'parachute' dye-coupling assay in SMMC-7721 cells ($n = 4$). (b) Western blotting was used to detect the expression of Cx32 in SMMC-7721 cells ($n = 3$). (c) Bar graphs derived from the densitometric scanning of the blots. Data are expressed as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control group.

Fig. 3



Selecting the optimal silencing sequences of Cx32 si-RNAs in SMMC-7721 cells. (a) Western blotting was used to detect the expression of Cx32 in SMMC-7721 cells. (b) Bar graphs derived from the densitometric scanning of the blots. Data are expressed as mean ± SE ($n = 3$). $^{**}P < 0.01$ vs. control group.

Fig. 4



the effect of berbamine on cell migration and invasion of SMMC-7721cellls after transfection. (a) Wound healing assay ($n = 3$). (b) Transwell assay ($n = 4$). (c) The percentage of migration cells in transwell assay. (d) The percentage of invasion cells in transwell assay.

Possible mechanism of berbamine on invasion and metastasis of SMMC-7721 cells

Effect of berbamine on PI3K, p-AKT and AKT protein expression in SMMC-7721 cells

Western blot was used to detect the expression of PI3K, p-AKT and AKT in SMMC-7721 cells after different concentrations of berbamine. Compared with the control group, the relative expression of PI3K in the 20 μmol/L berbamine group decreased by (42.52 ± 3.63)%. The expression of p-AKT/AKT decreased by (64.30 ± 3.17)%. And the differences were statistically significant ($P < 0.05$). The results are shown in Fig. 5.

Silencing Cx32 expression, the effect of berbamine on the expression of PI3K, p-AKT and AKT protein in SMMC-7721 cells

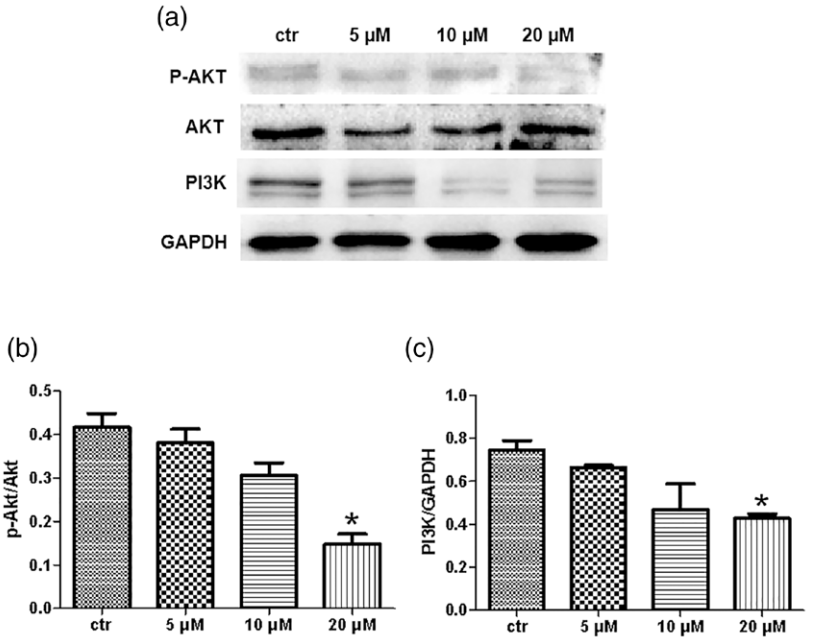
Compared with the control group, the expression levels of PI3K and p-AKT/AKT in the interference group were increased by (44.40 ± 3.65)% ($P < 0.05$) and (141.08 ± 6.15)% ($P < 0.05$). After silencing Cx32 expression, 20 μmol/L berbamine acted on SMMC-7721 cells for 24 h. Compared with the interference group, the expression levels of PI3K and p-AKT/AKT were no significant difference in the 20 μmol/L berberamine group. The results are shown in Fig. 6.

Discussion

Tumor invasion and metastasis is a complex process involving activation/inactivation of multiple genes and alteration of various signaling pathways [15]. During metastasis, cancer cells are detached from the primary site, invade the surrounding tissues, enter the blood and lymphatic system, and then spread to distant tissues and organs to form new metastatic tumors. In order to minimize the effect of drug on the invasion and metastasis by inhibiting cell proliferation, we selected concentrations with a small effect on the survival rate of SMMC-7721 cells, namely 5, 10 and 20 μmol/L berbamine. The results of scratch and transwell assay showed that berbamine significantly inhibited the invasion and metastasis of SMMC-7721 cells.

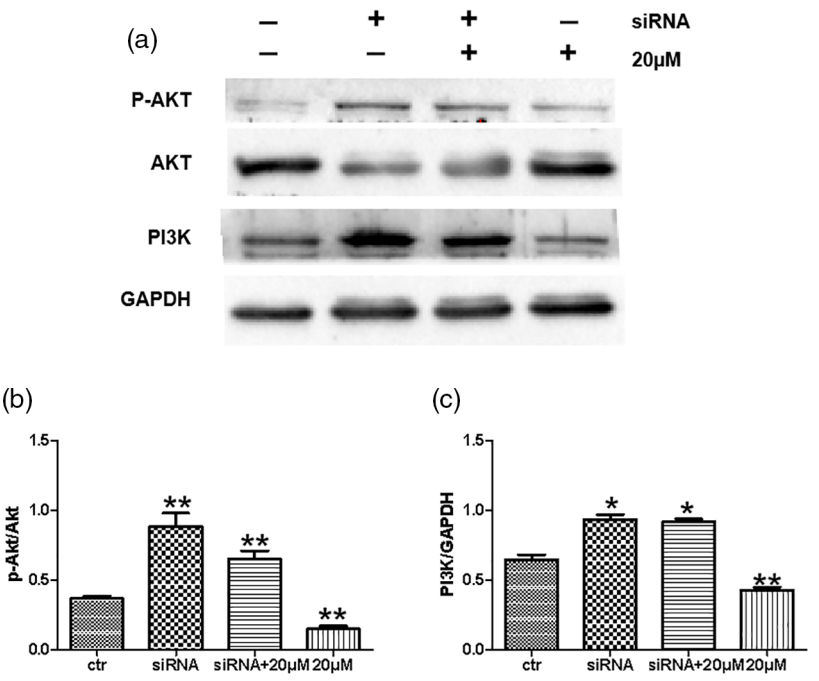
GJ plays an important role in regulating homeostasis, cell differentiation and growth. GJ is composed of connexins, allowing small molecules (molecular weight less than 1 kDa) to pass through and transport the molecules to

Fig. 5



The effect of berbamine on the expression of PI3K, p-AKT and AKT in SMMC-7721 cells. (a) Western blotting was used to detect the expression of PI3K, p-AKT and AKT in SMMC-7721 cells. (b, c) Bar graphs derived from the densitometric scanning of the blots. Data are expressed as mean ± SE ($n = 3$). *$P < 0.05$, vs. control group.

Fig. 6



The effect of berbamine on the expression of PI3K, p-AKT and AKT in SMMC-7721 cells after Cx32 silencing by siRNA. (a) Western blotting was used to detect the expression of PI3K, p-AKT and AKT in SMMC-7721 cells. (b, c) Bar graphs derived from the densitometric scanning of the blots. Data are expressed as mean ± SE ($n = 4$). *$P < 0.05$, **$P < 0.01$ vs. control group.

adjacent cells [16]. A number of studies have shown that GJ function is weakened in tumor cells, and recovering GJ function can inhibit tumor growth or increase the antitumor effect of chemotherapeutic drugs. After enhancing the GJ function in cervical cancer Hela cells, the antitumor effect of etoposide can be increased. Studies by Tong *et al*. found that enhancing GJ function in testicular cancer cells increased the cytotoxic effect of oxaliplatin, whereas, in normal testicular cells, the cytotoxicity of the drug was diminished [17]. The major connexins expressed in the liver are Cx26, Cx32 and Cx43, 90% of the GJ proteins in hepatocytes is composed of Cx32 [12]. Studies have shown that increasing the expression of Cx32 in liver cancer cells enhances the sensitivity of cancer cells to doxorubicin [18]. To understand whether berbamine can regulate GJ function, we examined the effect of berbamine on Cx32 expression and intercellular GJ function in liver cancer cells. Our results showed that berbamine could enhance the GJ function in SMMC-7721 cells and increase the expression of Cx32, the effect showed a concentration-dependent manner.

It has been reported that abnormalities of GJ function in cells are often accompanied by tumor invasion and metastasis [19]. For example, GJ composed of Cx43 can inhibit the invasion and metastasis of cisplatin-resistant testicular cancer cells [11]. In the early stage of liver cancer, the expression of Cx32 is significantly reduced. The expression levels of Cx32 mRNA and protein in human liver cancer tissue samples are significantly lower than those in normal liver tissue adjacent to cancer [20]. And the recovery of GJ function could reverse or decrease the malignancy of tumor cells to some extent. Zhu Qian *et al*. also confirmed that the overexpression of Cx32 can enhance the GJ function of human liver cancer cell lines, thereby reduce the invasive ability of cancer cells. If the expression of Cx32 and GJ function in liver cancer cells was inhibited, and the invasive ability of liver cancer cells would promote [21]. To investigate whether the inhibitory effect of berbamine on liver cancer is associated with GJ function, we silenced the expression of Cx32 in SMMC-7721 cells, which weakened the GJ function. It was found that the ability of berbamine to inhibit the invasion of SMMC-7721 cells was significantly weakened or basically disappeared. It can be seen that the ability of berbamine to inhibit the invasion and metastasis of SMMC-7721 cells is related to its enhanced GJ function.

To explore its possible mechanism, we measured the changes in PI3K/AKT signaling pathway activity of berberamine-treated SMMC-7721 cells and found that berberamine reduced the expression of PI3K and p-AKT protein in the cells. After the expression of Cx32 was silenced in SMMC-7721 cells, the cells were treated with berbamine, and it was found that the expression of PI3K and p-AKT protein in the cells increased, and the activity of the PI3K/AKT signaling pathway was significantly increased. According to the report, the PI3K/AKT signaling pathway can participate in the development of tumor invasion and metastasis through a variety of mechanisms [9, 22, 23]. (1) After the PI3K/AKT signaling pathway is activated, it increases the expression of nuclear transcription factors such as snail, slug, twist and directly inhibits the expression of E-cadherin. (2) The PI3K/AKT signaling pathway induces the expression of matrix metalloproteinase and degrades the expression of E-cadherin. (3) PI3K/AKT and other signaling pathways such as wnt/β-catenin and Notch signaling pathway induce EMT indirectly or synergistically, and promote tumor invasion and metastasis. In this study, after interfering with the expression of Cx32 in SMMC-7721 cells, the activity of the PI3K/AKT signaling pathway increased significantly, suggesting that Cx32 may regulate the activity of the PI3K/AKT signaling pathway.

In summary, we used scratch assay, transwell assay, fluorescent tracing assay, siRNA knockdown and western blot to study the effect of berbamine on the invasion and metastasis ability of SMMC-7721 cells and its possible mechanism. The results suggest that berbamine can inhibit the invasion and metastasis of liver cancer SMMC-7721 cells, and its mechanism is related to the enhancement of GJ function and inhibiting PI3K/AKT signaling pathway activity. These findings provide an experimental basis for the further development and utilization of berbamine, and also lay the foundation for further exploration of the molecular mechanism of the antihepatocarcinoma activity of berbamine.

Acknowledgements

This study is granted by Suzhou Municipal Science and Technology Bureau (grant no. SYSD2017160).

B.Y. helped with study design, experimental operation, data analysis and manuscript writing. L.L. helped with experimental operation, data analysis. J.Y. and J.C. helped with data collection and data analysis. Y.C. helped with conception direction and study design.

Conflicts of interest

There are no conflicts of interest.

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