Introduction

Cholangiocarcinoma, one of the most difficult diseases to treat, is a cancer arising from bile duct epithelial cells. The incidence of cholangiocarcinoma is rising over the past decades (1, 2). However, the prognosis for cholangiocarcinoma is quite poor because of difficulties in early diagnosis, and relative resistance of the tumors to chemotherapy (3, 4), and no standard chemotherapy has been established (5, 6). Surgical resection, considered as palliative rather than curative, has been the mainstay of curative treatment for cholangiocarcinoma. However, the operative mortality is high and the 5-year survival rate remains disappointing (7). Local tumor recurrence is the major cause of death for patients who undergo resection (8).

Molecular insights into pathophysiology of the disease have been conducive to a new approach to cholangiocarcinoma, and novel therapeutic strategies are necessary for the improved clinical management of patients with cholangiocarcinoma.

Berberine is an isoquinoline alkaloid, obtained from a number of important medicinal plant species, such as Berberis aristata, Berberis vulgaris (barberry), Coscinium fenestratum, and Berberis aquifolium (9, 10). Berberine exhibits a variety of biochemical and pharmacological activities such as antibacterial (11), antihypertensive (12), anti-inflammatory (13), antidiabetic (14), and anti-hyperlipidemic activities (15). Recently, considerable evidence indicates that berberine has been shown to inhibit growth and induction of apoptosis in several human cancer cells (16 – 18). However, it remains unclear whether berberine directly inhibits proliferation or induces apoptosis in a human cholangiocarcinoma cell line (QBC939).

Thus, the aim of this study was to investigate the effect
of berberine on QBC939 proliferation and the underlying molecular mechanism, so as to provide experimental evidence for developing effective drugs for the clinical treatment of cholangiocarcinoma.

Materials and Methods

Materials

The established QBC939 was obtained from Cell Bank of Wuhan University (Wuhan, China). The primary human intrahepatic cholangiocyte cell line (HIBEC) was purchased from Sciencell (Carlsbad, CA, USA). Berberine was from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture materials were purchased from Gibco BRL (Gaithersburg, MD, USA). β-Actin, p27, CyclinD1, CyclinE, Cdk2, Bel-2, Bel-xL, and Bax antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Cdk4 and p21 antibodies were from Millipore (Billerica, MA, USA). Cdk4/CyclinD1 and Cdk2/CyclinE Kinase Activity Assay Kit were purchased from Genmed Scientifics, Inc. (Arlington, MA, USA). The enhanced chemiluminescence (ECL) western blotting detection reagents were obtained from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals were of analytical grade.

Cell culture

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in an atmosphere containing 5% CO₂. When cells reached 60% – 70% confluence, the cells were cultured in serum free DMEM for 24 h in order to reach synchronization, and then the cells were added into the DMEM (10% FCS in media) with different concentrations (10, 40, and 80 μM) of berberine for another 24 and 48 h.

Cell proliferation assay

The methylthiazol tetrazolium (MTT) reduction assay was used as a qualitative index of cell viability. After 24- or 48-h incubation with different compounds as described above, 20 μl MTT (5 mg/ml; Invitrogen Corp., Carlsbad, CA, USA) was added and cells were cultured for an additional 4 h. Subsequently, cells were lysed using dimethylsulfoxide (150 μl/well). When the formazan crystals were completely dissolved, the optical density (OD) was measured at 490 nm using a Microplate Reader Model 3550-UV Spectrophotometer (Bio-Rad Laboratories, Marnes La Coquette, France). The effect of berberine on cell viability was assessed as percent cell viability compared to berberine non-treated control cells, which were arbitrarily assigned 100% viability.

The LDH toxicity assay

The cytotoxicity was estimated by measuring lactate dehydrogenase (LDH) release into culture medium. Cultured cells were treated with or without different concentrations (10, 40, and 80 μM) of berberine for another 24 and 48 h, and LDH activity was assayed by absorbance change at a wavelength of 440 nm with an LDH assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Assessment of cell apoptosis

The cytosolic DNA–histone complexes generated during apoptotic DNA fragmentation in treated QBC939 cells were evaluated with a cell death detection enzyme linked immunosorbent assay (ELISA) kit (Cell Death Detection ELISA PLUS; Roche Applied Science, Indianapolis, IN, USA) following the supplier's instructions.

Flow cytometry

Cell cycle analysis was performed by flow cytometry. After 48-h treatment with different compounds, cells were harvested, washed twice with phosphate-buffered saline (PBS), and processed for cell cycle analysis. Briefly, 1 × 10⁶ cells were resuspended in 50 μl of cold PBS, to which cold methanol (450 μl) was added, and the cells were then incubated for 1 h at 4°C. Following two washes with PBS, fixed cells were incubated in RNase (25 μg/ml) at 37°C for 30 min, followed by staining of the DNA with propidium iodide (50 μg/ml) at 4°C for 30 min in the dark. The cell cycle distribution of the cells of each sample was then determined using a Coulter Epics XL Flow Cytometer (Beckman Coulter, Miami, FL, USA) and the proportion (percentage) of cells within the G1, S, and G2/M phases of the cell cycle were determined.

Western blotting

After being treated with different compounds for 48 h, cells were harvested and washed with ice-cold phosphate buffer. Total cellular proteins were extracted by lysing cells with buffer containing 150 mM NaCl, 0.1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 7.0), and 1 mM ethylenediaminetetraacetic acid (EDTA), and nuclear proteins were obtained using methods described previously (19). Protein concentrations were determined using the BCA method (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein were loaded onto each lane, separated by SDS – polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. After being blocked with 5% skimmed milk in Tris-buffered saline (TBS) (pH 7.6) at room temperature.
ture, the membranes were incubated overnight at 4°C with primary antibodies for p21 (1:5000), p27 (1:1000), CyclinD1 (1:1000), CyclinE (1:1000), Cdk2 (1:1000), Cdk4 (1 μg/ml), Bax (1:1000), Bel-2 (1:1000), Bel-xL (1:1000), and β-actin (1:500). After being incubated with the respective second antibody, immune complexes were detected using ECL western blotting reagents. The detected proteins were normalized to β-actin or the respective total protein as appropriate.

**Cdk2 and Cdk4 kinase activity assay**

The Cdk4/CyclinD1 and Cdk2/CyclinE Kinase Activity Assay Kit is a complete assay system designed to measure the activity of Cdk4/CyclinD1 and Cdk2/CyclinE by coupling the formation of ADP to the reaction catalyzed by PK and LDH in the presence of phosphoenolpyruvate (PEP) with the oxidation of NADH. The disappearance of NADH is detected by measuring a decrease in extinction at 340 nm. Samples were assayed according to the manufacturer’s instructions.

**Statistical analyses**

The statistical significance of a difference between the control and treatment groups was determined by simple ANOVA followed by Dunnett’s multiple comparison tests. All values are expressed as mean ± S.D. and statistical significance was defined as \( P < 0.05 \).

**Results**

*Berberine decreases cell viability and induces cytotoxic effect in human cholangiocarcinoma QBC939 cells but not in normal HIBEC*

Human cholangiocarcinoma cell proliferation was evaluated using MTT analysis. The results showed that compared with the normal group, addition of berberine for 24 and 48 h inhibited human cholangiocarcinoma cell proliferation in a dose-dependent manner at concentrations ranging from 10 to 80 μM (\( P < 0.01 \), Fig. 1B). Treatment of QBC939 cells with berberine at a concentration of 80 μM berberine for 24 and 48 h resulted in significant cell death (Fig. 1C). As shown in Fig. 1C, when compared with the control cholangiocarcinoma cells (nonberberine), treatment of QBC939 cells with berberine for 24 and 48 h resulted in a 15%–21% (\( P < 0.05 \)) increase in cell death at 10 μM, 27%–38% (\( P < 0.01 \)) increase in cell death at 40 μM, and 45%–58% (\( P < 0.01 \)) increase in cell death at 80 μM. Further, we examined whether berberine has any cytotoxic effect on normal HIBEC. For the HIBEC cells treated with 80 μM berberine for 48 h, the detached cells in the supernatant were less than 5% and did not differ from the control group (Fig. 1D). Thus berberine appears to be capable of inducing cytotoxic effects on human cholangiocarcinoma cells without incurring significant cytotoxic effect on normal HIBEC.

*Berberine induces G1 phase cell cycle arrest in QBC939 cells*

In order to further evaluate the effect of berberine treatment upon cell cycle profiles, we then performed flow cytometry. Figure 2 shows that berberine at 10, 40, 80 μM increased the number of cells in G1 to 58.20% ± 0.57% (\( P < 0.01, n = 3 \)), 68.30% ± 1.49% (\( P < 0.01, n = 3 \)), and 70.17% ± 0.35% (\( P < 0.01, n = 3 \)) and decreased that in the S phase to 23.57% ± 3.33% (\( P < 0.01, n = 3 \)), 16.07% ± 1.85% (\( P < 0.01, n = 3 \)), and 13.00% ± 1.08% (\( P < 0.01, n = 3 \)), respectively. These results showed that berberine could block cell cycle progression by inhibiting the G1–S phase transition and arresting cells in the G1 phase.

*Berberine decreases the expression of cyclins and Cdkks with a concomitant increase in p21 and p27 in QBC939 cells*

To clarify whether inhibition by berberine involves the regulation of cell cycle-related proteins, we assessed G1-checkpoint proteins in berberine-treated cell cycle arrest. As shown in Fig. 3, berberine significantly decreased the level of Cdk2, Cdk4, and CyclinD1 in a dose-dependent manner, but did not change the levels of CyclinE. In addition, the levels of p27 and p21 protein were significantly increased by berberine treatment in a concentration-dependent manner (\( P < 0.01 \)). Thus, these results indicate that berberine regulates the expression of G1-phase- and S phase-related G1-checkpoint proteins in association with its cell cycle arrest effects in QBC939 cells.

*Berberine inhibits the activity of Cdk2 and Cdk4 in QBC939 cells*

We also examined berberine’s effects on Cdk2 and Cdk4 activation, which governs the G1/S progression of the cell cycle. As shown in Fig. 4, 24-h treatment with berberine at 10–80 μM decreased Cdk2 kinase activities and Cdk4 kinase activities in a dose-dependent manner. These data suggest that the inhibitory effect of berberine on Cdk2 and Cdk4 activities that are responsible for the down-regulation of the G1/S transition may play roles in the proliferation of QBC939 cells.

*Berberine induces apoptosis and differentially affects the levels of Bel-2 family proteins in QBC939 cells*

A 24- and 48-h treatment with berberine caused a significant dose-dependent enhancement in the number of apoptotic cells. The total percent of apoptotic cells in
QBC939 cells after treatment with berberine for 24 and 48 h is summarized in Fig. 5A. However, berberine treatment of HIBEC cells for 48 h did not result in significant enhancement of apoptosis. Although a slightly higher number of apoptotic cells was observed on treatment of these cells with 120 μM berberine for 48 h, this did not reach significance and was significantly less (P < 0.05) than the levels of apoptosis of QBC939 cells induced by treatment with the same concentration of berberine for the same time period (data not shown). This suggests that at least under the experimental conditions used in this study, berberine is not toxic to normal HIBEC cells.

The proteins of the Bcl-2 family are considered as the major apoptotic signal transduction cascade associated with programmed cell death. Because more berberine-induced cell death, apoptosis, and changes in cell cycle regulatory proteins were found at 48 h after its treatment, we selected this time point for further mechanistic studies. We used western blotting to detect Bcl-xL, Bcl-2, and Bax in the cells treated with different concentrations (10, 40, and 80 μM) of berberine for 48 h (Fig. 5B). This revealed that treatment of QBC939 cells with berberine resulted in a dose-dependent decrease in the levels of anti-apoptotic proteins (Bcl-xL and Bcl-2) with a concomitant increase in proapoptotic protein Bax (Fig. 5B). Thus, berberine treatment can increase the ratio of Bax/Bcl-2, which may contribute to the susceptibility of cancer cells to berberine-induced apoptosis (Fig. 5C).
Discussion

Cholangiocarcinoma remains one of the most difficult tumors to treat in clinical practice. The inhibition of cholangiocarcinoma proliferation is considered to be one promising therapeutic strategy. Surgery offers the only opportunity to cure it. However, the majority of patients fail to qualify for such a treatment. Therefore, new therapeutic modalities are currently needed.

Natural products and their synthetic derivatives have been a continuous source of novel compounds for the treatment of cancer (20, 21). In our present investigation, we show that a naturally occurring isoquinoline alkaloid, berberine, significantly inhibits the proliferation and reduces the viability of QBC939 cells (Fig. 1), which suggested that berberine may be examined as an effective chemotherapeutic agent against cholangiocarcinoma. Importantly, we found that berberine was not toxic to HIBEC cells under the conditions used, except for a moderate reduction in cell viability at higher concentrations when cells were treated in vitro for an extended period of time.

Compared with findings with other cancer lines such as human gastric carcinoma cells and human epidermoid carcinoma cells, berberine exhibits similar mechanisms in QBC939 cells (16, 17). It has been recognized that control of cell cycle progression in cancer cells is an effective strategy to halt tumor growth (22, 23), as the molecular analyses of human cancers have revealed that cell cycle regulators are frequently deregulated in most of the common malignancies (24, 25). For this reason, cell cycle inhibition has been regarded as a promising anticancer strategy to inhibit the multiple cellular processes. Our in vitro data demonstrate that treatment of QBC939 cells with berberine induces G1 phase arrest of cell cycle progression indicating that inhibition of cell cycle progression is one of the mechanisms by which berberine inhibits the proliferation of QBC939 cells (Fig. 2). Cell cycle progression is tightly regulated through a complex network of positive and negative cell cycle regulatory molecules such as CDKs, cyclins, and CKIs (26–28). The increased expression of G1 cyclins in cancer cells promotes an uncontrolled growth advantage because the expression of Cdk4 and Cdk2, which are responsible for S phase entry, is upregulated in these cells (29–30). Consistent with these reports, further molecular mechanistic studies demonstrate that berberine treatment dose-dependently caused a marked decrease in the expressions of CyclinD1, Cdk2, and Cdk4 in QBC939 cells.
cells, suggesting the disruption of the uncontrolled cell cycle progression of QBC939 cells (Fig. 3). Additionally, berberine induced G1 arrest is mediated through the up-regulation of Cip1/p21 and Kip1/p27 proteins, which enhances the formation of heterotrimeric complexes with the G1–S Cdks and cyclins, thereby resulting in inhibition of their activity (Fig. 4).

The growth arrest of cancer cells in the G1 phase provides an opportunity for cells to undergo apoptosis. In most of the advanced malignancies, cancer cells, resistant to apoptosis, do not respond to the cytotoxic effects of chemotherapeutic agents (31). We therefore examined the effect of berberine on the induction of apoptosis in QBC939 cells. Our data indicated that treatment of QBC939 cells with berberine resulted in a significant dose-dependent induction of apoptosis (Fig. 5A). Apoptosis, considered as a fundamental cellular activity, plays a crucial role as a protective mechanism against neoplas-

**Fig. 3.** Effect of different concentrations of berberine on p21, p27, CyclinD1, CyclinE, Cdk2, and Cdk4 levels in human cholangiocarcinoma cells. A) Quiescent cholangiocarcinoma cells were exposed to different concentrations (10, 40, 80 μM) of berberine for 48 h, and total cellular protein was extracted at the indicated time. Twenty micrograms of protein was loaded for western blot analysis for CyclinD1, CyclinE, Cdk2, Cdk4, p21, and p27. Similar results were obtained from three repeated experiments. B) Quantitative analyses were performed for p21, p27, CyclinD1, CyclinE, Cdk2, and Cdk4 using a densitometer. Each bar represents the mean ± S.D. (**P < 0.05, ***P < 0.01 vs. control).

**Fig. 4.** Effect of different concentrations of berberine on Cdk2 and Cdk4 activities in cultured human cholangiocarcinoma cells. Quiescent cholangiocarcinoma cells were exposed to different concentrations (10, 40, 80 μM) of berberine for 48 h, and total cellular protein was extracted at the indicated time; then a kinase assay was performed as described in Materials and Methods. Each bar represents the mean ± S.D. (**P < 0.01 vs. control).
Berberine Inhibits QBC939 Cell Growth

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Fig. 5. Effect of different concentrations of berberine on Bcl-xL, Bcl-2, and Bax levels in human cholangiocarcinoma cells. A) In vitro treatment of QBC939 cells with berberine for 48 h results in a dose-dependent reduction in the expression of antiapoptotic proteins Bcl-xL and Bcl-2 while increasing the expression of the proapoptotic protein Bax as estimated by western blot analysis. B) Treatment of QBC939 cells with berberine significantly increases the Bax/Bcl-2 protein ratio. Quantitative analyses were performed for Bax, Bcl-xL, and Bcl-2 using a densitometer. Each bar represents the mean ± S.D. (##P < 0.01 vs. control).

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References


