Introduction

Cholangiocarcinoma (CCA), the second most common primary liver malignancy, is an epithelial cell malignancy arising from varying locations in the biliary tree (1,2). CCA is highly malignant and generally fatal with extremely poor survival rates (3). Owing to the lack of specific symptoms and signs, most CCA patients are diagnosed at advanced stage and missing the optimal time for operation (4). Although several new-targeted therapies have been developed, surgical resection remains the only potential curative treatment. Unfortunately, only a few tumors are resectable and curable at diagnosis and the 5-year survival rate following resection is only about 20% (5). Furthermore, the epidemiological studies showed a global trend of rising morbidity and mortality of CCA over the past decade, especially in China (6). Thus, the development of novel and promising therapeutic approaches is an urgent task.

Chansu is a Traditional Chinese Medicine used in China for hundreds of years, extracted from parotid glands and skin glands of the Chinese toad (Bufo gargarizan) (7-9). In China and some Asian countries, it has been extensively used to treat a variety of diseases, including inflammation,
CNS respiratory diseases, cardiac disease, and cancer. Cinobufagin is one of the major active components in Chansu, isolated and purified in the past few decades (10,11). It has reportedly been used for anti-cancer actions including inhibition of prostate cancer cells by inducing apoptosis and migration proliferation of human hepatic cells (12,13). Furthermore, cinobufagin could also restrain other cancer rapid progression, such as non-small cell lung cancer, breast cancer, osteosarcoma (14-16). Additionally, the toxicity of cinobufagin to normal cells is low and it is suitable as a therapeutic agent.

However, whether cinobufagin affects CCA remains unclear. This current study investigated the antitumor effects of cinobufagin on CCA. Results illustrated that cinobufagin exhibited significant suppression effects on CCA cell proliferation as well as inducing cellular apoptosis, both \textit{in vitro} and \textit{in vivo}. As is well-known, the Notch signaling pathway plays a vital role in the fundamental processes of cancer cell proliferation and apoptosis. In the present study, we found that cinobufagin may inhibit tumor growth by inducing apoptosis through the activation of Notch signaling pathways in CCA.

**Methods**

**Cell culture**

Human CCA cell lines QBC939 (extrahepatic) and RBE (intrahepatic) were provided by Department of General Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China (17,18). QBC939 and RBE were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL). Cells were maintained at 37 °C with 5% CO$_2$. Cinobufagin was purchased from Chinese Materials Research Center (Beijing, China) and dissolved in dimethyl sulfoxide.

**Cell-counting kit-8 (CCK-8) and colony formation assays**

For CCK-8 assay, 1,000 cells were seeded and cultured in a 96-well plate. Viability of cells was detected with CCK-8 assay, according to instructions, at indicated time points. OD$_{450}$ values were proportional to the viability of cells. For colony formation assay, 1,000 cells were seeded and cultured in 6-well plate. The colony number was counted after 14 days of culturing. All experiments were performed in triplicate.

**Flow cytometric apoptosis assay**

After respective treatment, cells were collected and washed with PBS. Next, apoptotic cell death rates were examined by Annexin V-FITC and PI double staining using the Annexin V-FITC Apoptosis Detection Kit, according to manufacturer instructions.

**Quantitative real-time PCR (qPCR)**

Total RNA was extracted with TRIzol Reagent. Reverse transcription was performed using a TOYOBO Reverse Transcription Kit. qPCR systems were set up in triplicate and run on the 7900 PCR machine with SYBR Green PCR Master Mix. Relative gene expression was calculated using the 2$^{-ΔΔCT}$ method and normalized to GAPDH. Primers used are listed in Table 1.

**Western blot**

Protein was extracted with RIPA buffer. Protein lysates were then separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel and transferred to PVDF membranes. Membranes were then blocked in 5% skim milk for 2 hours. They were then incubated with primary antibodies overnight at 4 °C. After 2 hours of

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| GAPDH     | GGAGCGGAGATCCTCCAAAAT | GGCTGTGTGCATATCTCTCATGG |
| Notch1    | GAGGCGTGGCAGACTATGC | CTTGTACTCCGTCAGCGTGA |
| Hes-1     | TCAACACGACACCGGATAAAC | GCCCGGAGCTATCTTTTCCTCA |
| Hes-5     | GAAAAACGACTGCGGAAGC | GACGAAGGTTTGCTGTGCT |
| Hey-1     | ATCTGCTAAGCTAGAAAAAGCCG | GTGCCGCGTCAAAGTACCT |

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incubation with secondary antibodies at 37 °C, membranes were detected with enhanced chemiluminescence. Primary antibody of NICD1 was purchased from Abcam and GAPDH was from ProteinTech. Caspase-3, cleaved Caspase-3 and Bax were obtained from Cell Signaling Technology.

Xenograft study

The xenograft study was approved by the Experimental Animal Ethics Committee of Ruijin Hospital and carried out according to the Guide for the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiaotong University. Ten four-week-old nude mice were subcutaneously injected with 5×10⁶ QBC939 cells. Two weeks after injection, the mice were randomized into 2 groups (n=5 mice/group) for the following treatments: vehicle control and cinobufagin (10 mg/kg, i.p. injection, twice a week). Tumor sizes and body weight were monitored and measured every 3 days. After 2 weeks of treatment, the mice were euthanized and tumor tissue was collected.

Statistical analysis

Statistical analyses were performed with SPSS16.0. Data are presented as mean ± standard deviation. Student’s t-test or one-way ANOVA were used for statistical analysis when appropriate. P values <0.05 were considered statistically significant.

Results

Cinobufagin inhibited CCA cell proliferation in vitro

As one previous study has demonstrated, cinobufagin has the ability to suppress cancer cell growth in several tumors. This present study investigated the cytostatic and cytotoxic effects of cinobufagin in two human CCA cell lines. As shown in Figure 1A, CCK-8 assay indicated that proliferation of QBC939 cells was significantly inhibited by cinobufagin in a dose-dependent and time-dependent manner. A similar effect was also observed in RBE cells (Figure 1B). And the half-maximal inhibitory concentration of cinobufagin at 48 h was 2.08 and 1.93 μM in QBC939 and RBE cells, respectively. Then we established an optimum concentration of cinobufagin in 2 μM for the subsequent experiments. To further validate the role of cinobufagin, colony formation assay was conducted with the same phenomenon observed. The colony formation ability of QBC939 and RBE cells was dramatically suppressed by cinobufagin (Figure 2A,B,C,D). Taken together, these results suggest that cinobufagin could inhibit CCA cell growth in vitro.

Cinobufagin induced apoptosis of human CCA cells

Cell apoptosis is one of the major causes of cell growth inhibition. It has been reported that cinobufagin can induce apoptosis in osteosarcoma cells. To determine whether cell apoptosis contributes to cinobufagin-induced cell growth
Cinobufagin inhibits colony formation of human CCA. (A,B) Cell colony formation assay in human CCA cell line QBC939 treated with cinobufagin is shown; (C,D) cell colony formation assay in human CCA cell line RBE treated with cinobufagin is shown. Mean ± SD of triplicate experiments were plotted. **P<0.001. CCA, cholangiocarcinoma.
cinobufagin could attenuate tumor growth by inducing apoptosis through Notch signaling pathways in a xenograft model of CCA.

**Discussion**

Incidence and mortality of CCA have been increasing in recent years (19–21). Surgery is still the most effective treatment option. CCA is typically diagnosed at an advanced stage, making surgical treatment ineffective. Development of a novel helpful treatment is an urgent task. Cinobufagin is an important cardenoidal steroid, extracted from skin secretions of the Chinese giant toad. It has been reported that cinobufagin had antitumor effects in several tumors,
including hepatocellular cancer, lung cancer, pancreatic cancer, and colon cancer (12-14,16,22). Experimental studies have suggested that cinobufagin has crucial effects on tumor developing processes including cell proliferation, cell differentiation, apoptosis, cell cycle arrest, angiogenesis, and immune escape (13,14,16).

Previous studies suggested that cordycepin triggered osteosarcoma cell apoptosis via the intrinsic mitochondria-dependent apoptosis pathway by the accumulation of ROS and the loss of ΔΨm (23). In addition, cordycepin has been reported to inhibit breast cancer cell growth and triggers apoptosis by affecting the expression of Bax and Bcl-2 in vitro (15). These are consistent with our present finding that cinobufagin effectively inhibits CCA growth, both in vitro and tumor xenograft experiment in vivo. Furthermore, our mechanistic studies demonstrated that cinobufagin may exert antitumor effects by induction of apoptosis. Notably, it was also found that cinobufagin-mediated inactivation of Notch pathways may play an important role in the induction of apoptosis in CCA, possibly providing a new therapy. In addition, the combination of gemcitabine and cisplatin is the current first-line chemotherapy for patients with advanced-stage CCA not amenable to locoregional and surgical options, irrespective of anatomical disease subtype. However, chemotherapy response rate in CCA is low, cinobufagin might be expected to improve the therapeutic effect of these chemotherapeutic agents.

Various signaling pathways could transmit biological information from the outer to inner of cells, however, Notch signaling is the only pathway that conveys the signal into a transcriptional response through cell-cell communication via a ligand-receptor interaction (24). Recent studies have shown that Notch signaling plays an important role in CCA cell proliferation and it contributes to human CCA pathogenesis (25). The Notch signaling pathway is also involved in the genesis and progression of CCA, the activation of Notch plays an important role in bile duct ontogenesis during the steps of ductal plate remodeling and tubulogenesis (26-28). Inhibition of the Notch pathway by γ-secretase inhibitor IX prevents CCA growth in vitro, and in vivo overexpression of the intracellular domain of Notch together with an inactivation of p53 significantly increased tumor growth (29). Hence, the inhibition of Notch signaling may have vital therapeutic effects on CCA treatment. Due to the central role of Notch in CCA development and progression, we hypothesized that this pathway is a target

Figure 4 Cinobufagin inactivates Notch 1 signaling pathways. (A,B) The mRNA levels of Notch1, Hes-1, Hes-5, and Hey-1 in human CCA cell line QBC939 and RBE treated with cinobufagin were detected by qPCR; (C,D) protein levels of NICD1 in cell lines QBC939 and RBE treated with cinobufagin were detected by Western blot. **P<0.01, ***P<0.001. CCA, cholangiocarcinoma.
Figure 5 Cinobufagin attenuates growth of CCA in vivo. (A) Growth curves of tumors in nude mice. Tumor diameters were assessed every three days; (B) representative xenograft tumor; (C) protein levels of Caspase-3, Bax, and NICD1 in tumor tissues treated with cinobufagin and detected by Western blot. ***P<0.001.

CCA, cholangiocarcinoma.

for cinobufagin. Notch signaling pathway is conserved and responsible for cell-fate determination, differentiation, development, tissue patterning, cell proliferation, and death (30,31). Interaction of Notch ligands with their receptors promotes the release of NICD1 resulting in activation of the pathway. NICD1 translocates to the nucleus and induces target gene transcription like Hes-1, Hes-5, and Hey-1. It has been reported that Notch signaling is frequently overactivated in many cancers, contributing to the survival advantage of tumors (32-35). Thus, Notch activation leads to poorer survival. Because the role of Notch signaling remains tissue and context dependent, alterations within this pathway may lead to tumor suppressive or oncogenic phenotypes. Understanding context-specific effects of Notch pathway will be important for the development of anticancer therapeutics. In the current study, it was found that cinobufagin significantly inactivated Notch signaling pathway.

In conclusion, cinobufagin can suppress the tumorigenicity of CCA, both in vitro and in vivo. Cinobufagin-mediated inactivation of Notch pathway may play an important role in the induction of apoptosis in CCA, possibly providing a new therapy.

Acknowledgments

Funding: This study is supported by the Interdisciplinary Program of Shanghai Jiao Tong University (ZH2018QNA49).

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.10.06). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee(s) and with the Helsinki Declaration (as revised in 2013). The institutional ethical approval and written informed consent were waived. The xenograft study was approved by the Experimental Animal Ethics Committee of Ruijin Hospital.

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Cite this article as: Ren J, Wang S, Jin L, Ma F, Zhou D, Cai Q. Cinobufagin inhibits tumor growth by inducing apoptosis through Notch signaling pathways in human cholangiocarcinoma. Transl Cancer Res 2019;8(6):2461-2469. doi: 10.21037/tcr.2019.10.06
**Figure S1** The side effect of cinobufagin is minimal *in vivo*. Growth curves of body weight of nude mice. Body weight was assessed every three days. Mean ± SD of triplicate experiments were plotted. ns, not statistically significant.