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ORIGINAL ARTICLE

Induction of apoptosis in renal cell carcinoma by cinobufotalin through inhibition of Notch1 signal activation*

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Abstract

Objective The aim of this study was to investigate the effect of cinobufotalin on apoptosis in renal cell carcinoma and its possible mechanism of action.

Methods The expression levels of Notch1 in renal cancer cells, as well as in adjacent and normal tissues were assessed in 64 patients with renal cellcarcinoma. The 769-P cells were treated with 0, 10, 20 and 40 mg/L cinobufotalin and the proliferation activity and apoptotic rate of the cells were measured. The expression levels of Notch1, Bcl-2, and Pro-caspase 3 were detected by RT-PCR and Western-blot.

Results (1) The rates of Notch1 expression in renal cancer cells, adjacent tissues, and normal tissues were 75.0%, 45.3%, and 9.4%, respectively. Notch1 expression had significant effects on tumor, node and metastasis (TNM) staging, Fuhrman grade, and tumor size in patients with renal cell carcinoma ($P < 0.05$); (2) The inhibition rates of cinobufotalin on 769-P cells were 0%, 6.85%, 11.37%, and 16.33% at 24 h; 0%, 13.57%, 20.14%, and 31.69% at 48 h; 0%, 19.97%, 28.53% and 51.42% at 72 h. At 24 h, the apoptotic rates were 8.2 ± 3.1%, 19.8 ± 5.6%, 33.7 ± 5.0%, and 51.5 ± 6.8%.The effect of cinobufotalin on apoptosis of 769-P cells was dose-dependent; (3) RT-PCR assay showed that protein expression levels for Notch1, Bcl-2, and Pro-caspase 3 were significantly decreased with the increase of drug concentration. Western-blot analysis also showed that Notch1, Bcl-2 and Pro-caspase 3 protein levels showed a significant downward trend with the increase of drug concentration.

Conclusion Cinobufotalin inhibits the growth of renal cancer cells and induces apoptosis in renal cell carcinoma, which may be related to the inhibition of Notch1 signal activation.

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Key words: renal cell carcinoma; cinobufotalin; cell line 769-P

Renal cell carcinoma is one of the most common malignant tumors of the urinary system in China [1]; however, its pathogenesis is not completely understood, although it is generally considered to be related to smoking, chronic urinary tract infection, and kidney stones [2]. According to traditional Chinese medicine practice, kidney cancer is caused by impaired kidney functions such asexcessive dampness or heat, qi stagnation, and blood stasis [3]. Cinobufotalinis a water-soluble extract obtained from the skins and parotid venom glands of the toad Bufo gargarizans Cantor and has been widely used in China as an effective traditional Chinese medicine to treat conditions such asswelling, pain, and heart failure for thousands of years [4]. Previous studies by the current and other authors have demonstrated that the major pharmacologic constituents of Cinobufotalinare bufodienolides (which primarily include bufalin, cinobufagin, resibufogenin, bufotalin, and lumichrome), biogenic amines, alkaloids, peptides, and proteins [5]. Currently, Cinobufotalinis widely administered through injectionin various dosagesin clinical cancer therapy in China. Cinobufotalin has been reported to have a variety of biological effects, such as immunomodulatory and antineoplastic effects [5–6]. In the treatment of liver, gastrointestinal, and esophageal cancer, as well asother malignant tumors, cinobufotalin inhibits the

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progression of cancer, improves patient symptoms such as cachexia, fever, fatigue, weight loss, and loose stools. Some researchers have reportedthat cinobufotalin has inhibitory effects on the proliferation and promotion of apoptosis in renal cancer cells *in vitro* and *in vivo*, though the mechanism is still unclear.

Materials and methods

Reagents and cell line

Human kidney cancer cell line 769-P was purchased from Wuhan Boster Biotechnology Co. Ltd. (China). The reverse transcription and RT-PCR kits were purchased from Takara Corporation (China). Monoclonal antibodies for Rabbit anti-human Notch1, rabbit anti-human Bcl-2, and rabbit anti-human Pro-caspase3, respectively,were purchased from Abcam (UK). Bicinchoninic acid (BCA) protein concentration determination and enhanced chemiluminescence (ECL) kits were purchased from Tianjin Yuyang Biological Products Technology Co. Ltd. (China).

Cell culture and 3-(4, 5) dimethylthiahiazo (-z-yl)-3, 5-diphenytetrazoliumromide (MTT) assay

The 769-P cells were inoculated into a culture medium containing 10% fetal bovine serum and penicillin and streptomycin. Cells were cultured at 37 °C with 5% CO_2 and passaged once every two days. Cells in the growth phase were used. The 769-P cells were seeded in 96-well plates at a density of approximately 2000 cells/well and cultured until the cells were adherent. The supernatant was discarded and replaced with 200 μL of cell culture medium containing 0 mg/mL, 10 mg/L, 20 mg/L, and 40 mg/L of cinnamycin according to the four group schemes. The blank group was added as control. All plates were cultured for 24 h, 48 h, and 72 h. Upon completion of the culture, 20 μL of MTT solution was added to each well, and the cells were further incubated for 4 hand the absorbance at a wavelength of 570 nm (*A* value) wasmeasured. This experiment was repeated three times.

Reverse transcription-polymerase chain reaction (RT-PCR) detection

Total RNA in the cells was extracted according to the manufacturer's protocol. The cellular RNA was reverse transcribed into cDNA, and then amplified into DNA, and all steps were performed in strict accordance with the manufacturer's instructions on the reverse transcription and the amplification kits. Notch1 primer sequences: upstream 5'-CTGTATCAAAAGGCCAACTGAA-3', d ownstream 5'-GTGTCTATCCTTATGAATCGCCA-3' , Bcl-2 primer sequences: upstream 5'-AACTGTGCTGA ACTGGATCAAA-3', downstream 5'-AGTCGTTCTCC TTATGGCATACA-3', Pro-caspase 3 primer sequences: upstream 5'-AAAAGCGCCTGCCTGAA-3', downstream 5'-GACTTCTGAATCGCTGTCTATCA-3'. All primer sequences were synthesized by Shanghai Bioengineering Technology Co. Ltd. (China). The RT-PCR assay conditions were as follows: predenaturation at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 30 s and annealing at 30 °C for 30 s, and finalextension at 70 °C for 10 min.

Protein detection by Western-blot analysis

Total cellular protein was extracted. Then 25 μg of the extracted proteins were resolved by SDS-PAGE, before transferring onto ablotting membranein 5% skim milk for 2 h. Subsequently, the membrane was incubated with primary antibodies such as Notch1 monoclonal antibody (1:2000), Bcl-2 monoclonal antibody (1:2000), and Pro-caspase 3 monoclonal antibody (1:5000) overnight at 4 °C. Then, after washing with phosphate buffer saline (PBS) for three times, secondary antibody was added in a dropwise manner to conjugate with the bound primary antibodies. The β-actin protein was used as an internal reference, incubated in a greenhouse, washed with tris buffer saline tween (TBST) buffer, exposed by chemiluminescence, and the gray color of the strip was measured by an image-J image analysis system. Transplanted apoptotic tumor cells were detected by TUNEL method. The tumor is routinely dehydrated, embedded, and sectioned. The staining process was carried out according to the kit instructions. After the filming, five fields of view were randomly selected and the number of positive cells was counted. Positive criteria: blue particles in the nucleus.

Clinical sample source and data collection

A total of 64 patients with renal cell carcinoma who underwent surgery in our hospital from October 2016 to January 2019 were enrolled. Inclusion criteria: (1) Diagnosed as patients with renal cell carcinoma by pathological evidence and for the first time; (2) Patients undergone surgical treatment, and the surgical resection specimens were preserved in our pathology department laboratory. Exclusion criteria: (1) Perioperative deaths; (2) A history of other malignant tumors; (3) Incomplete clinical data. Of the 64 patients, 48 were males and 16 were females, ranging from 38 to 81 years old with a median age of 56 years. Data on tumors were also collected, including tumor, node and metastasis (TNM) staging, Fuhrman grade, and tumor size.

Statistical analysis

All data analysis was performed using SPSS 20.0 software. Descriptive analysis of the results was performed based on frequency, rate, mean ± standard, and statistical

inference using chi-square test. The significance level is $\alpha = 0.05$.

Results

Expression of Notch1 in renal cancer cells, adjacent and normal tissues

Immunohistochemical staining showed that Notch1 was mainly expressed in the cell membrane or cytoplasm of the cells, and appeared yellowish or yellowish brown. The positive rates of Notch1 in renal cell carcinoma, adjacent tissues and normal tissues were 75.0% (48/64), 45.3% (29/64), and 9.4% (6/64), respectively, and the difference was statistically significant ($P < 0.05$; Fig. 1).

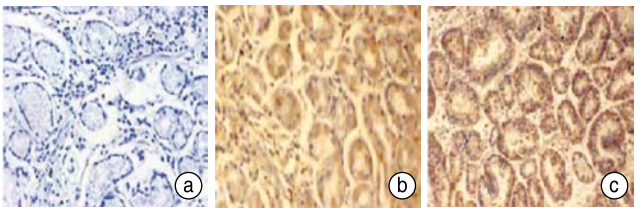

Fig. 1 Expression of Notch1 in renal cancer cells (a), adjacent (b) and normal (c) tissues

The effect of Notch1 expression on the clinical features of renal cancer cells

Table 1 showed that univariate analysis revealed that Notch1 expression was correlated to TNM stage, Fuhrman grade, and tumor size in patients with renal cell carcinoma ($P < 0.05$), but not with gender and age ($P > 0.05$).

Table 1 Effect of Notch1 expression on clinical features of renal cancer cells

Index	Negative (n = 16)	Positive (n = 48)	χ^2	P
Gender			0.444	0.505
Male	11	37		
Female	5	11		
Age (years)			2.351	0.125
< 60	10	39		
≥ 60	6	9		
TNM			7.528	0.006*
I + II	13	20		
III + IV	3	28		
Fuhrman			8.466	0.004*
1 + 2	14	22		
3 + 4	2	26		
Tumor size (cm)			6.206	0.013*
< 5	9	11		
≥ 5	7	37		

*Statistical significance at $P < 0.05$

The effect of different concentrations of cinobufotalinon the proliferation of renal cancer cells

After MTT assay, the cancer cell inhibition rates of cinobufotalin at 0 mg/L, 10 mg/L, 20 mg/L and 40 mg/L were 0%, 6.85%, 11.37%, 16.33%, 48 h after intervention of 769-P cells at concentrations of 0 mg/L, 10 mg/L, 20 mg/L and 40 mg/L, respectively. The cell inhibition rates were 0, 13.57%, 20.14%, and 31.69%, respectively, and the cell inhibition rates at 72 h were 0%, 19.97%, 28.53%, and 51.42%, respectively (Table 2). The inhibitory effect of cinobufotalin on the growth of 769-P cells was dose- and time-dependent.

Table 2 The effect of different concentrations of Cinobufotalinon the proliferation of renal cancer cells

Cinobufotalin (mg/L)	24 h (%)	48 h (%)	72 h (%)
0	0	0	0
10	6.85	13.57	19.97
20	11.37	20.14	28.53
40	16.33	31.69	51.42

Effects of different concentrations of cinobufotalin on apoptosis of renal cell carcinoma

After treatment with cinobufotalin for 0 h, 10 mg/L, 20 mg/L, 40 mg/L for 24 h, the apoptotic rate of cells was 8.2 ± 3.1%, 19.8 ± 5.6%, 33.7 ± 5.0%, 51.5 ± 6.8%. The effect of cinobufotalin on the promotion of 769-P cell apoptosis was dose-dependent (Fig. 2).

Effect of cinobufotalin on mRNA expression

After interfering 769-P cells with cinobufotalin at concentrations of 0 mg/L, 10 mg/L, 20 mg/L and 40 mg/L for 24 h, RT-PCR showed that the expression of Notch1 mRNA decreased with the increase of drug concentration (Table 3). Bcl-2 mRNA and Pro-caspase 3 mRNA increased significantly.

Table 3 Effect of cinobufotalin on mRNA expression of cell-related molecules

Cinobufotalin (mg/L)	769-P cell		
	Notch1 mRNA	Bcl-2 mRNA	Pro-caspase3 mRNA
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
10	64.7 ± 10.52	47.13 ± 9.85	26.48 ± 8.48
20	44.8 ± 6.36[a]	62.30 ± 12.03[a]	52.13 ± 14.75[a]
40	28.3 ± 5.41[ab]	75.95 ± 14.26[ab]	74.87 ± 15.94[ab]
F*	63.35	13.57	21.79
P	0.000	0.004	0.000

* One-way analysis of variance; [a] Compared with 10 mg/L, $P < 0.05$; [b] Compared with 20 mg/L, $P < 0.05$

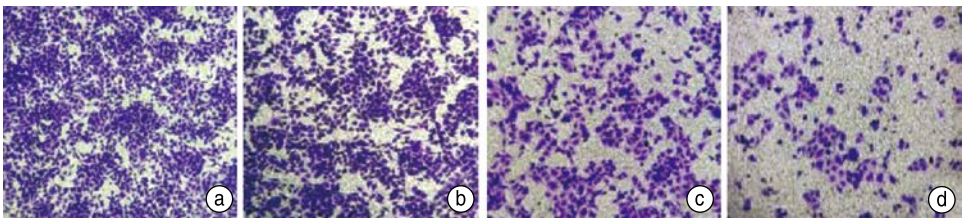

Fig. 2 Effect of different concentrations of cinobufotalin on apoptosis of renal cell carcinoma. (a) 0 mg/L; (b)10 mg/L; (c) 20 mg/L; (d) 40 mg/L

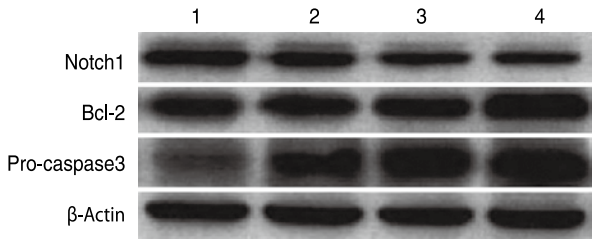

Fig. 3 Western-blot detection of Notch1 signaling pathway-associated proteins. 1: 0 mg/L; 2: 10 mg/L; 3: 20 mg/L; 4: 40 mg/L

Effect of cinobufotalin on the expression levels of proteins relative to signaling pathway

After interfering 769-P cells with cinobufotalinat concentrations of 0, 10 mg/L, 20 mg/L and 40 mg/L for 24 h, Western blot analysis showed that the expression level of Notch1 protein decreases with the increase of drug concentration. However, Bcl-2 and Pro-caspase3 proteinssignificantlyincreased.

Discussion

Renal cell carcinoma is a very common urological malignant tumor in China. Surgery is the one of the most important treatmentapproaches. At present, traditional Chinese medicine has a well-documented cancer therapy regime for the treatment of kidney cancer. In addition to the treatment efficacy, traditional Chinese medicine plays a significant role to complement Western medical practices [7]. Post operation, tumor burden in a patient's body is significantly reduced, but the risk of hidden malignancy cannot be completely eliminated, since the internal environment of tumorigenesis in the body remains. Furthermore, the host immunity is often compromisedfollowing the surgical intervention and hence, there is always a risk of cancer relapse. In view of the these reasons, traditional Chinese medicine is often sought to replenish qi and reinvigorate the spleen, dissipate the phlegm and clear the lungs, and synergistically restores the digestive functionto strengthen the host immunityand qi. When the functions of the spleen and stomach are restored, the residual disease-causing agents in the body can be removed too [8]. Thus, traditional Chinese medicine, as a treatment modality for kidney cancer, helps ward off all the disease-causing agents without affecting host immunity while also benefiting postoperative recovery.

Cinobufotalinis often made from dried mink and its main effects include detoxification, reducedswelling, and pain relief. In the treatment of malignant tumors, it can synergize with other chemotherapeutic drugs such as cyclophosphamide, cisplatin, etc. to enhance the efficacy of chemotherapy drugs. In humans, four Notch genes have been identified (Notch1, Notch2, Notch3, and Notch4), which exhibit diversity in tissue distribution and function. Gain or loss of Notch signaling is associated with multiple human disorders, including a variety of cancers [9]. The Notch gene encodes a highly conserved cell surface receptor whose signal affects multiple processes of normal morphogenesis, including differentiation of pluripotent progenitor cells, apoptosis, cell proliferation, and cell boundary formation [10]. Among these Notch genes, Notch1 is most closely related to renal cell carcinoma [11–12]. The activation of Notch1 signaling pathway is closely related to tumor formation and development. The main mechanisms include: (1) Induction of growth-promoting genes such as cyclin D1 and C-myc expression [13]; (2) Triggering anti-apoptosis such as P13 kinase/AKT pathway [14]; (3) Promotion ofgrowth and self-renewal in tissues and maintenanceof the undifferentiated state of stem cells [15]. In this study, by retrospective analysis of clinical data, Notch1 expression in renal cancer cells, adjacent tissues, and normal tissues were found to be 75.0% (48/64), 45.3% (29/64), and 9.4%, respectively; (4) Notch1 expression in renal cancer cellsis significantly higher. At the same time, Notch1 expression has a remarkablenegative impact on TNM staging, Fuhrman grading, and tumor size in patients with renal cell carcinoma.

In the *in vitro* experiment using the 769-P cell line, we found that the inhibitory effect of cinobufotalin on the growth of 769-P cells was dose- and time-dependent, and the effect on apoptosis was dose-dependent. These findings indicate that cinobufotalinhas significant inhibition ability onthe growth and apoptosis of renal cancer cells. Apoptosis is the autonomous and programmed cell death controlled by genes to maintain

homeostasis. Apoptosis is an active and complex process involving the activation, expression, and regulation of a series of genes. Defects or obstruction in apoptosis can disrupt the proliferation, differentiation, and death of normal cells, which is critical in tumorigenesis [16]. The effect of cinobufotalinon the apoptosis of renal cancer cells indicates that itcan delay the course of tumor malignancy to some extent. Both RT-PCR and Western-blot assays showed that the expression levels of Notch1 at the molecular and protein levels decreased with the increase of drug concentration, while Bcl-2 and Pro-caspase3 showed an upward trend. The main substrate for Pro-caspase-3 and poly (ADP)ribose polymerase (PARP), which is involved in DNA repair and gene integrity monitoring [17]. At the initiation of apoptosis, PARP is cleaved into two fragments by caspase-3and the two zinc finger structures bound to DNA in PARP are separated from the catalytic region at the carboxy terminus and become denatured. This indicates that cinobufotalin can induce tumor cell apoptosis by promoting the expression of Bcl-2 and Pro-caspase3.

This study found that cinobufotalinexerts anti-tumor effects by inhibiting the growth and inducing apoptosis of renal cancer cells, which may be related to the inhibition of Notch1 signaling activation.

Conflicts of interest

The authors indicated no potential conflicts of interest.

References

1. Gao Y, Wang YX. Analysis of etiology and pathogenesis of renal cancer in traditional Chinese medicine. Jilin J Tradit Chin Med (Chinese), 2013, 33: 978–979.
2. Cui HJ. A probe into the treatment of renal cancer with traditional Chinese medicine. J Pract Tradit Chin Med (Chinese), 2008, 22: 39–40.
3. Gurel A. Acute kidney injury due to abdominal compartment syndrome caused by duodenal metastases of prostate cancer. Clin Case Rep, 2015, 3: 629–631.
4. Ba X, Wang J, Zhou S, *et al*. Cinobufacini protects against paclitaxel-induced peripheral neuropathic pain and suppresses TRPV1 up-regulation and spinal astrocyte activation in rats. Biomed Pharmacother, 2018, 108: 76–84.
5. Wang Z, Qi F, Cui Y, *et al*. An update on Chinese herbal medicines as adjuvant treatment of anticancer therapeutics. Biosci Trends, 2018,12: 220–239.
6. Zhang X, Yuan Y, Xi Y, *et al*. Cinobufacini injection improves the efficacy of chemotherapy on advanced stage gastric cancer: a systemic review and Meta-analysis. Evid Based Complement Alternat Med, 2018, 4: 7362340.
7. Yuan XB, Wang P, Zhao WB, *et al*. Clinical evaluation of Yiqi Jiedu Decoction combined with cytokine adjuvant therapy after radical nephrectomy. J Shanxi Med Univ (Chinese), 2016, 47: 74–79.
8. Lv SX, Zeng FX, Shao KQ, *et al*. Overview of traditional Chinese medicine in the treatment of renal cell carcinoma. J Hebei Tradit Chinese Med (Chinese), 2017, 39: 473–476.
9. Hori K, Sen A, Artavanis-Tsakonas S. Notch signaling at a glance. J Cell Sci, 2013, 126: 2135–2140.
10. Xiao G, Li X, Li G, *et al*. MiR-129 blocks estrogen induction of NOTCH signaling activity in breast cancer stem-like cells. Oncotarget, 2017, 8: 103261–103273.
11. Zhuang Z, Lin J, Huang Y, *et al*. Notch 1 is a valuable therapeutic target against cell survival and proliferation in clear cell renal cell carcinoma. Oncol Lett, 2017, 14: 3437–3444.
12. Liu S, Ma X, Ai Q, *et al*. NOTCH1 functions as an oncogene by regulating the PTEN/PI3K/AKT pathway in clear cell renal cell carcinoma. Urol Oncol, 2011, 31: 938–948.
13. Eberl M, Mangelberger D, Swanson JB, *et al*. Tumor architecture and Notch signaling modulate drug response in basal cell carcinoma. Cancer Cell, 2018, 33: 229–243.
14. Naveed M, Imran K, Mushtaq A, *et al*. In silico functional and tumor suppressor role of hypothetical protein PCNXL2 with regulation of the Notch signaling pathway. Rsc Advances, 2018, 8: 21414–21430.
15. Hayakawa S, Yokoi Y, Hinou H, *et al*. Chemical synthesis demonstrates that dynamic O-glycosylation regulates the folding and functional conformation of a pivotal EGF12 domain of the human NOTCH1 receptor. Biochemistry, 2017, 56: 4379–4391.
16. Woo JH, Ahn JH, Jang DS, *et al*. Effect of kumatakenin isolated from cloves on the apoptosis of cancer cells and the alternative activation of tumor-associated macrophages. J Agricultural Food Chemistry, 2017, 65: 7893–7899.
17. Xanthoudakis S, Roy S, Rasper D, *et al*. Hsp60 accelerates the maturation of pro-caspase-3 by upstream activator proteases during apoptosis. Embo J, 2014, 18: 2049–2056.

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