

Na⁺/K⁺-ATPase α 3 mediates sensitivity of hepatocellular carcinoma cells to bufalin

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Abstract. Bufalin, a major bioactive component of the Chinese medicine *Chansu*, has been reported to exhibit significant antitumor activity against various cancer cell lines. However, the exact mechanism remains unclear. In this study, we demonstrated that bufalin inhibited the growth of hepatocellular carcinoma (HCC) cells in a dose-dependent manner, which correlated with the expression level of Na⁺/K⁺-ATPase α 3 in HCC cells. The IC₅₀ of bufalin markedly increased when Na⁺/K⁺-ATPase α 3 was silenced by RNA interference. Furthermore, we show that bufalin increased the phosphorylation of Akt and ERK1/2 while inhibited FoxO3a expression. Thus, our study suggests that Na⁺/K⁺-ATPase α 3 might serve as a therapeutic target for bufalin in HCC, and its expression status may help predict sensitivity of HCC cells to bufalin treatment.

Introduction

Bufalin is a major ingredient of Chinese medicine *Chansu*, which is obtained from the skin and the parotid of venom glands of toad. Bufalin has been found to inhibit the growth of various tumor cells, such as leukemia (1-3), prostatic cancer (4), liver cancer (5), and osteosarcoma (6) by inducing apoptosis and cell cycle arrest. It is also implicated that the anti-tumor effect of bufalin is through increasing Bax/Bcl-2 ratio, activating capase-3, capase-8 and inhibiting Tiam1 (3). However, the exact mechanism remains unclear.

Numerous studies have shown that cardiac glycosides, including bufalin are capable of suppressing the proliferation

of cancer cells (7). The role of cardiac glycoside as an inhibitor of Na⁺/K⁺-ATPase, a member of P-type ATPase family, is well appreciated. Recently, this particular enzymes has been found to play a key role in cardiac glycoside elicited anticancer activity (reviewed in ref. 7). Na⁺/K⁺-ATPase is an important receptor to transducer ligand binding into the activation of protein kinase cascades (8), and changes in Na⁺/K⁺-ATPase activity are also indicated in the course of malignant transformation, with evidence showing that these occur at the very early stages of tumorigenesis (9-12). A recent study from our college showed that oleandrin, another type of cardiac glycoside, could selectively inhibit human cancer cell growth, which is mediated by the relative level of Na⁺/K⁺-ATPase subunit expression (13).

Hepatocellular carcinoma (HCC) remains one of the most common cancers worldwide (14). Despite advances in therapy for HCC such as the recently approved tyrosine kinase inhibitor, Sorafenib, as standard care, overall outcome has not been substantially improved (15). Therefore, it is critical to search for novel agents that can inhibit the growth of HCC cells. Cardiac glycosides have been found to be novel cancer therapeutic agents, including bufalin, for HCC (7). The use of bufalin containing agent Huachansu, a Chinese medicine that comes from dried toad venom from the skin glands of *Bufo gargarizans* or *B. melanostictus*, have yielded promising effect for the treatment of HCC patients in our pilot study (16). Less well known, however, is the exact function and the potential mechanism of bufalin in the treatment of HCC.

In this study, we examined the effect of bufalin on the proliferation of HCC cells. We also evaluated the expression profile of Na⁺/K⁺-ATPase subunits in different hepatocellular carcinoma (HCC) cell lines and investigated the role of Na⁺/K⁺-ATPase subunit expression on the sensitivity of HCC cells to bufalin treatment.

Materials and methods

Reagents. Bufalin was purchased from Sigma Corporation (St. Louis, MO). It was dissolved in 100% DMSO (Sigma) and diluted with RPMI-1640 (Gibco-BRL, Gaithersburg, MD) to desired concentration with a final DMSO concentration of

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Table I. Primers for RT-PCR and their product size.

Gene	Primer sequence	Product size (bp)
Na ⁺ /K ⁺ -ATPase α 1	F: 5'-AGTACACGGCAGTGATCTAAAGG-3' R: 5'-CAGTCACAGCCACGATAGCAC-3'	155
Na ⁺ /K ⁺ -ATPase α 2	F: 5'-GGAGATGCAAGATGCCTTTCA-3' R: 5'-GCTCATCCGTGTCGAATTTGA-3'	131
Na ⁺ /K ⁺ -ATPase α 3	F: 5'-GACCTCATTTGACAAGAGTTCGC-3' R: 5'-GGGCAGACTCAGACGCATC-3'	143
Na ⁺ /K ⁺ -ATPase α 4	F: 5'-CTGTAAGCAACTGTTCCGGAGG-3' R: 5'-GTAGAGGTTGTCTTTGGTAGGC-3'	118
GAPDH	F: 5'-ATGAATTCAGGTGAAGGTCGGAGTCAACG-3' R: 5'-ATGGATCCAGGCTGTTGTCATACTTCTC-3'	353

0.1% (v/v) for *in vitro* studies. DMSO was added to cultures at 0.1% (v/v) as control.

Cell lines. The human hepatocellular carcinoma cell lines SMMC7721, HepG2 and PLC/PRF/5 were obtained from the American Type Culture Collection. SMMC7721 and PLC/PRF/5 cells were grown in RPMI-1640 containing 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL). HepG2 cells were grown in DMEM containing 10% FBS. All the cells were cultured at 37°C, 5% CO₂.

Cell survival assays. Cells were plated at 5,000 per well in 96-well microtiter plates and incubated overnight at 37°C in a humidified incubator containing 5% CO₂. The following day, various concentrations of bufalin were added to wells and cultures were incubated for an additional 24 h. The measurement of viable cell was performed with a Cell Counting Kit (Dojindo Laboratories, Tokyo, Japan) to count living cells by WST-8. Absorbance was determined at 490 nm.

Reverse transcription-polymerase chain reaction analysis. The expression of Na⁺/K⁺-ATPase α subunit was detected by RT-PCR with specific primers of α 1, α 2, α 3, α 4 isoforms, keeping expression of housekeeping GAPDH gene as control. Total RNA was isolated from cultured cells using the TRIzol® Reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions. Semiquantitative real-time RT-PCR using SYBR-Green I to compare the relative expression of specific gene mRNA was done as described previously (17). The primer sequences are described in Table I.

RNA interference-mediated gene knockdown. PLC/PRF/5 and HepG2 cells were pretreated with Stealth siRNA to Na⁺/K⁺-ATPase α 3 (Invitrogen) for 48 h. RT-PCR analysis was performed to confirm Na⁺/K⁺-ATPase α 3 knockdown. The anti- α 3 siRNA with sense (5'-UAAUAAUGGCAGAAA CCAAGCACGC-3') and anti-sense (5'-GCGUGCUUGGUU UCUGCCAUAUAUA-3') showed the most effect of inter-

fering and was selected for further study. In brief, HCC cells were plated at 3,000 per well in 96-well microtiter plates and incubated overnight at 37°C for 24 h. They were then transfected with α 3 siRNA and Scramble Stealth RNAi™ siRNA duplex (Invitrogen) using Lipofectamine 2000 transfection agent (Invitrogen) according to the protocols of the manufacturer for 48 h. Bufalin was added and further incubated for 48 h. CCK-8 assay (Dojindo, Molecular Technologies, Gaithersburg, MD) was used to evaluate the proliferation of the cells.

Western blot analysis. Western blot analysis was performed as we described previously (18). Briefly, cells were washed with cold PBS and lysed with cell lysis buffer [10 mM Tris-HCl pH 7.4, 1.5 mM EDTA, 130 mM NaCl, 1% Triton, 10 mM NaF, 10 mM NaPi, 10 mM NaPPi, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 100 μ g/ml PMSF and 0.25 mM Na₃V0₄] containing protease inhibitors. Protein (75 μ g) of HCC cell lysates was loaded on 10% SDS-PAGE, electrophoresed and transferred onto PVDF membranes. The levels of Na⁺/K⁺-ATPase α 1 and Na⁺/K⁺-ATPase α 3 were determined with specific primary antibodies (1:1,000), followed by treatment with the appropriate peroxidase conjugated secondary antibodies (1:2,000). All blots were developed by ECL Western Blotting Detection Reagents, and analyzed using Gel-Pro Analyzer software (Media Cybernetics).

Statistical analysis. Student's t-test was used to determine the statistical significance of differences between experimental groups. Differences were considered significant at $p < 0.05$.

Results

Bufalin inhibits proliferation of HCC cells in a dose-dependent manner. Different HCC cell lines, including HepG2, PLC/PRF/5 and SMMC7721, were treated with bufalin (0.01, 0.1 and 1 μ M) for 24 h. Bufalin inhibited cell proliferation in a dose-dependent manner with an IC₅₀ of 182.30 \pm 13.78 nM, 52.20 \pm 14.16 nM and 97.74 \pm 8.83 nM for

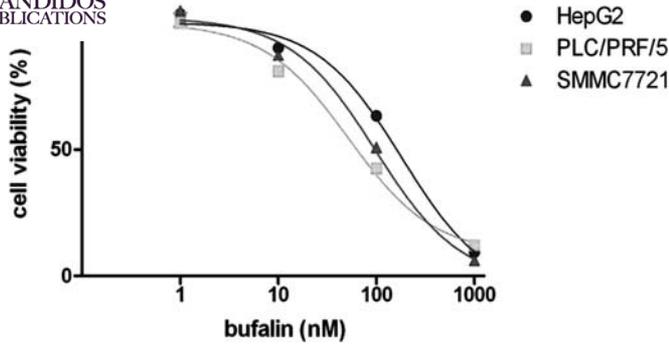


Figure 1. Inhibition of cell proliferation in HCC cells after bufalin treatment. Cells were exposed to various concentrations of bufalin (0.1 to 1 μ M) for 24 h. Cell proliferation was determined with Cell Counting Kit-8 assay. Bufalin inhibited cell proliferation in a dose-dependent manner in all three cell lines tested.

HepG2, PLC/PRF/5 and SMMC7721 cells, respectively (Fig. 1).

The inhibitory effect of bufalin on HCC cells correlates with the expression of Na⁺/K⁺-ATPase isoform α 3. We tested the mRNA expression of four Na⁺/K⁺-ATPase α isoforms in these three HCC cell lines. The levels of mRNA of α 1, α 2 or α 4 were relatively similar in HepG2, PLC/PRF/5 and SMMC7721 cells while the level of Na⁺/K⁺-ATPase α 3 was much different in these three cell lines (Fig. 2A). The level of Na⁺/K⁺-ATPase α 3 was the highest in the PLC/PRF/5 cell line that is sensitive to bufalin, and the lowest in the HepG2 cell line that is relatively insensitive to bufalin. To confirm

whether the levels of Na⁺/K⁺-ATPase α 3 subunit is associated with the differential sensitivity of HCC cells to bufalin, the PLC/PRF/5 and HepG2 cells were treated with siRNA of Na,K-ATPase α 3 subunit. As shown in Fig. 3A, expression of Na,K-ATPase α 3 subunits were markedly reduced in the siRNA transfected cells comparing to that of control siRNA transfected cells in both PLC/PRF/5 and HepG2 cells. The reduction of expression of this particular enzymes was more pronounced in the HepG2 cells. Intriguingly, the anti-proliferative activities of bufalin in Na,K-ATPase α 3 subunit siRNA treated cells were reduced in both PLC/PRF/5 and HepG2 cells evidenced by increased IC₅₀ of bufalin in Na⁺/K⁺-ATPase α 3 down-regulated cell lines, from 29.29 \pm 4.75 nM (control siRNA transfected) to 42.10 \pm 4.45 nM (siRNA transfected) PLC/PRF/5 cells and from 84.53 \pm 13.96 nM (control siRNA transfected) to 200.10 \pm 10.75 nM (siRNA transfected) HepG2 cells (Fig. 3B), suggesting that anticancer activity of bufalin on HCC cells may correlate with the expression level of Na⁺/K⁺-ATPase α 3 subunit.

Bufalin treatment increases the phosphorylation of Akt and ERK1/2 and down-regulates FoxO3a expression in HCC cell lines. To study whether the bufalin-induced HCC cell inhibition is mediated through PI3Kinase and MAPK pathways, we tested the effect of bufalin on regulation of phosphorylation of ERK1/2 and Akt. As shown in Fig. 4, phosphorylation of Akt and ERK1/2 was markedly increased in the bufalin-sensitive PLC/PRF/5 cells when they were treated with a lower concentration (0.1 μ M), while pronounced increased ERK1/2 and Akt phosphorylation was observed only when cells were treated with high concentration (1 μ M)

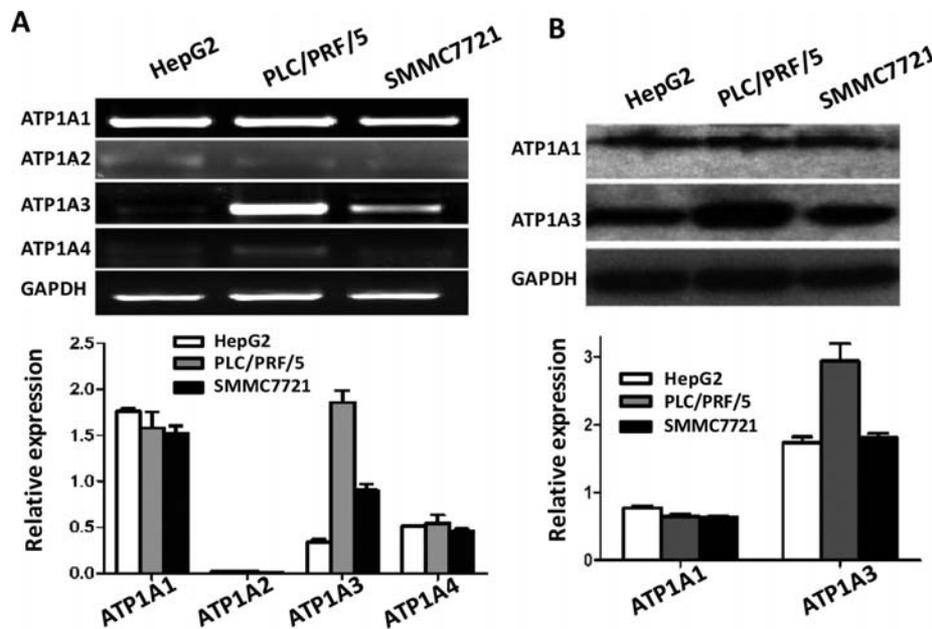


Figure 2. Expression of Na⁺/K⁺-ATPase α subunit in human HCC cell lines. (A) the mRNA expression of Na⁺/K⁺-ATPase α subunit was detected by RT-PCR (top panel) with specific primers of α 1, α 2, α 3, and α 4 isoforms. GAPDH was used as a loading control. Comparison of the relative levels was tested by semiquantitative real-time RT-PCR (bottom panel). (B) The protein expression of Na⁺/K⁺-ATPase α 1 and α 3, was detected by Western blot analysis (top panel). GAPDH was used as a loading control. The relative protein level was analyzed using Gel-Pro Analyzer software (bottom panel). Data are presented as the mean \pm SD of three independent experiments.

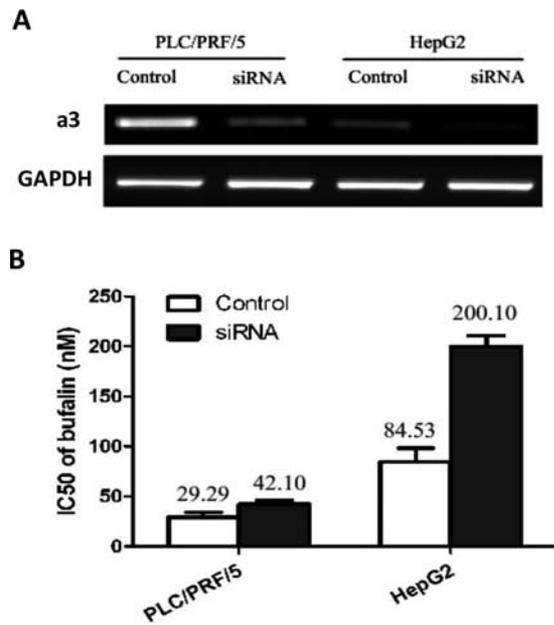


Figure 3. Down-regulation of Na⁺/K⁺-ATPase α 3 reduces the sensitivity of hepatocellular carcinoma cells to bufalin. (A) HCC cells were treated with either α 3 siRNA or control siRNA for 48 h, total RNA was isolated and the mRNA expression of Na⁺/K⁺-ATPase α 3 was detected by RT-PCR. (B) HCC cells were transfected with siRNA for 48 h followed with treatment of bufalin for 48 h. The growth inhibition was analyzed by CCK-8 and IC₅₀ for each cell line was calculated. The IC₅₀ of bufalin increased as Na⁺/K⁺-ATPase α 3 was down-regulated in both cell lines. Data are presented as the mean \pm SD of three independent experiments.

of bufalin in bufalin insensitive HepG2 cells. Our data indicated that bufalin can induce higher activation of Akt and ERK1/2 in bufalin-sensitive cell lines than that in bufalin-insensitive cell lines. As it has been reported that cardiac glycoside has the ability to increase reactive oxygen species (ROS) and that FoxO3a regulates the ROS generation, we further tested the expression of FoxO3a in both PLC/PRF/5 and HepG2 cells. The expression of FoxO3a markedly declined in PLC/PRF/5 cells treated with bufalin at both 0.1 and 1 μ M, whereas the reduction of FoxO3a was only

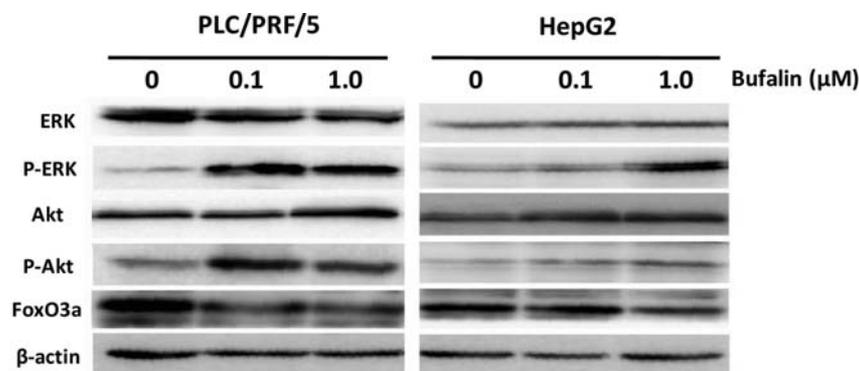


Figure 4. Bufalin increases phosphorylation of Akt and ERK1/2 while down-regulates FoxO3a levels in HCC cell lines. PLC/PRF/5 and HepG2 cells were treated with bufalin for 2 h. The protein levels of Akt, ERK1/2 and FoxO3a were detected by Western blot analysis. β -actin was used as loading control. Bufalin increased phosphorylation of Akt and ERK1/2 and decreased expression of FoxO3a in both cell lines, especially in relatively bufalin-sensitive PLC/PRF/5 cells.

observed in HepG2 cells treated with higher concentration of bufalin (1.0 μ M) (Fig. 4). Taken together, our data suggested that bufalin inhibited HCC cell proliferation partly through regulating multiple cell signaling proteins.

Discussion

The major finding of this study is that bufalin, a cardiac glycoside, can inhibit the proliferation of hepatocellular carcinoma cells, which might be mediated by the expression of Na⁺/K⁺-ATPase α 3 subunit.

Emerging evidence has suggested that cardiac glycosides might have a great potential in the context of treatment of malignant diseases. Previously, an epidemiological study revealed that very few patients maintained on cardiac glycoside treatment for heart problems died from breast cancer (19). More recently, number of studies demonstrated that cardiac glycoside can inhibit the growth of various cancer cells and induce apoptosis (1-6). As the important mechanism for pharmacological effect of cardiac glycosides in treating cardiac malfunction is associated with inhibition of Na⁺/K⁺-ATPase, this particular enzyme appears to play an important role in cardiac glycoside-elicited inhibition of cancer cell proliferation (13,20).

Na⁺/K⁺-ATPase is composed of three subunits in equimolar ratios, include α catalytic subunit, the multipass transmembrane protein containing the binding sites for Na⁺/K⁺-ATPase and the cardiac glycoside, β regulatory subunit, required for the biogenesis and activity of the enzyme complex, and γ subunit, another regulator of the Na⁺/K⁺-ATPase that can increase the apparent affinity of the enzyme for ATP (21). Na⁺/K⁺-ATPase α has four different isoforms, namely α 1, α 2, α 3, α 4. Recent studies on the anti-cancer effect of cardiac glycoside were mainly focused on Na⁺/K⁺-ATPase α subunit (13). Our data showed that bufalin inhibited the growth of liver cancer cells which correlated with Na⁺/K⁺-ATPase isoform α 3, suggesting that Na⁺/K⁺-ATPase isoform α 3 may be an important therapeutic target for bufalin to treat liver cancer.

Cardiac glycoside binds with the Na⁺/K⁺-ATPase which then activates Src (22-24). The activated Src transactivates



osine kinases, such as ERK1/2, which results in activation of protein kinase cascades (22,24). We have observed similar phenomenon which is that bufalin activate both PI3Kinase and MAPKinase pathways evidenced by increased phosphorylation of Akt and ERK1/2. However, it is also well known that Akt and ERK1/2 are highly expressed and persistently activated in most cancers, and inhibiting the activation of Akt can induce apoptosis in some cancer cell lines (25). The deviation between bufalin induced Akt and ERK1/2 phosphorylation and inhibition of cell proliferation in HCC cell lines may partly reflect the possibility that apart from Akt and ERK1/2 signaling, there must be another signaling pathway that mediate bufalin-induced proliferation inhibition, which needs to be further identified. The discrepancy indicates the most evolutionarily conserved function of Akt, that is the control of energy metabolism, which in mammalian cells is coupled to its ability to inhibit apoptosis and to promote cell-cycle progression (22,24,26-28). The attenuated insulin signaling through PI3k and its down-stream effector, Akt, is associated with a decline in energy metabolism (26). Negoeira *et al* (29) have provided genetic evidence that Akt determines replicative senescence of mammalian cells in culture and mediates premature senescence induced by activated Ras or oxidative stress, and Akt activation is sufficient to induce premature senescence. They also showed that Akt exerts its effect by increasing intracellular levels of ROS (Reactive oxygen species) through an increase in oxygen consumption and inhibition of FoxO transcription factors. In other words, increasing sensitivity of the cell to H₂O₂-mediated cell death is likely due to the activation of Akt and the subsequent phosphorylation and inactivation of FoxO transcription factors, suggesting that Akt can sensitize the cells to oxidative stress-induced cell death. Much interest has been paid to the development of inhibitors to target the PI3K/Akt signal pathway, however, existing drugs against this pathway have shown limited clinical success (30). Perhaps our study which showed that bufalin sensitizes liver cancer cells to death via activating Akt and inhibiting FoxO, suggests a new strategy for cancer therapy sensitizing ROS-inducing cell death via Akt activation.

In this study we noted that ERK1/2 was also activated after the cells were treated by bufalin. Several explanations are possible. First, bufalin combining with Na⁺/K⁺-ATPase isoform α 3 will activate Src which can activate the Ras/Raf/MEK/ERK1/2 signal pathway (22-24). Second, the active ROS itself also mediates the activation of ERK1/2 (31,32). At this point, it is suggested that activated ERK1/2 and Akt team up for bufalin to induce cell death in HCC cell lines. We can not yet evaluate whether activated ERK1/2 involved with bufalin-induced HCC cell death, since ERK activation delivers a survival signal in many cancers (33). The discrepancy needs to be further studied.

In conclusion, our results indicated that bufalin inhibited the growth of hepatocellular carcinoma (HCC) cells in a dose-dependent manner, which correlated with expression level of Na⁺/K⁺-ATPase α 3 in HCC cells. Bufalin increased the phosphorylation of Akt and ERK1/2 while inhibited the FoxO3a, which suggested a new mechanism underlying bufalin-induced HCC cell death, i.e. sensitizing ROS inducing

cell death via Akt activation. It should be noted that the association between bufalin-induced Akt activation, FoxO3a inactivation and cell growth inhibition in HCC cell lines needs to be further clarified. We can not conclude whether Akt and ERK1/2 activation mediated bufalin-induced HCC cell death, neither can we rule out the possibility of other undiscovered bufalin-induced signaling pathways which hold growth inhibitory properties that predominate over the growth promotion induced by Akt and/or ERK1/2 activation. However, our data suggest that Na⁺/K⁺-ATPase α 3 subunit may serve as a therapeutic target for bufalin to treat HCC, and its expression status may help predict sensitivity of HCC cells to bufalin treatment.

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