Original Article
A Notch1-regulated lncRNA, AK022798, contributes to the baicalein-induced apoptosis in hepatocellular carcinoma

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Abstract: Hepatocellular carcinoma (HCC) is one of the most universal carcinomas in the world, especially in Asia. Although baicalein has been used as an anticancer reagent in HCC, the further molecular mechanism is non-elucidated. In this study, we provide the mechanistic link between baicalein and its anticancer activity. Primarily, baicalein inhibits the cell proliferation and induces apoptosis. During this process, seven vital cancer-related genes show differential alteration in which Notch1 presents a lowest expression. We subsequently screened out Notch1-regulated long non-coding RNA (lncRNA) AK022798 which is a central downstream target of baicalein. Consistently, we demonstrate that expression of AK022798 positively correlate with anticancer action of baicalein. Collectively, we have identified a potential mechanism by which AK022798 regulated by Notch1 signaling contributes to the baicalein-induced apoptosis in HepG2 cell. These findings also give evidence of the feasibility of lncRNA as potential downstream targets for therapeutic intervention in HCC.

Kewords: HCC, baicalein, Notch1, lncRNA, AK022798

Introduction

Cancer formed by liver cells includes two main types, hepatocellular carcinoma (HCC), a most frequent subtype, and hepatoblastoma. HCC is one of the most prevalent carcinomas throughout the world, especially in Asia. Although the prominent risk factors involved in HCC have been identified [1], including infection with chronic hepatitis B and C virus (HBV and HCV), the molecular mechanisms of development of HCC remain largely unknown, therefore impeding effective therapy.

It is well-known that cell proliferation, differentiation, and apoptosis during cell development are mediated by Notch signaling pathway. Out of 4 Notch receptor, Notch1 signaling functions in many biological processes of numerous tissues or cell types, and kinds of developmental defects may result from its dysfunction [2]. Given that inactivation of Notch1 has been shown to cause nodular regenerative hyperplasia in mouse liver [3], it is therefore rational to suggest that Notch1 signaling may be related to the pathogenesis of liver diseases, even of HCC. Consistent with this viewpoint, previous studies demonstrated that activation of Notch1 signaling contributes to HCC tumor growth and proliferation [4, 5]. However, in some circumstances, Notch1 is found to play a suppressive role. Recent reports revealed the promotion of HCC growth by suppressing the Notch1 pathway [6, 7]. Thus, it is an intriguing concept that Notch1 signaling may be both oncogenic and tumor suppressive in HCC, which is supported by the event that Notch signaling also have a dual role in breast tumor [2, 8]. This dual action of Notch1 is generally determined by several following factors including different cell type, individual cytokines or growth factors in the cellular microenvironment and heterogenous population of cells [2, 9]. Taken together, a becoming possibility is that the drug for treatment of Notch1-related HCC may be developed through distinguishing which kind of Notch1 signaling-derived HCC.

Accumulating reports have revealed that mammalian genomes contain thousands of long noncoding RNA (lncRNA) genes [10, 11], which have been shown to be crucial in diverse cell development [12, 13] and identified as mediators of disease [14]. Recent evidence of dysregulated lncRNA expression across numerous cancer types suggests that aberrant lncRNA expression may be a major contributor to tumorigenesis [15]. A first lncRNA, which positively correlated to HCC, was detected by utilizing cDNA array [16]. Another lncRNA, MEG3, was implanted as tumor suppressor in HCC [17]. In addition, nonoverlapping signatures of a small number of lncRNA were also identified in HBV-related HCC [18]. Although above-mentioned results have presented the functional characterization of lncRNA, the overall pathophysiological contributions of lncRNAs to human HCC remains extremely limited.

Besides the Notch1 and lncRNA, baicalein, one of bioactive component of *Scutellaria*, is also associated with growth inhibition of cancer cell. Baicalein can induce the cell death of human cancer [19], including HCC [20], in vitro. Although the potential anticancer activity of baicalein has been speculated in other cancers [19], molecular mechanism by which baicalein acts is poorly understood in HCC. Here we attempted to investigate whether the Notch1 signaling and lncRNA are involved in the anticancer effect of baicalein in HCC HepG2 cells.

Materials and methods

Cell culture

The human hepatoma cell line HepG2 was cultured at 37°C, 5% CO_2 incubator in Dulbecco's modified eagle medium (DMEM, Hyclone) supplemented with 10% (V/V) FBS (Hyclone), 100 U/ml penicillin G and 100 U/mL streptomycin. Cells grown in 60 mm cell culture dish were allowed approximately to reach 90% confluence. Then cells were rinsed and removed from the dishes by incubating them with a trypsin-EDTA solution (Hyclone), and harvested in a 15 mL centrifuge tube for subsequent study.

Cell proliferation assay

HepG2 cells were directly cultured at a density of $1×10^5$ cells/well in 6 well plates. After culturing overnight, the cells were then incubated with DMSO (negative control) and baicalein (prepared in DMSO) respectively for indicated time at 37°C. Cell proliferation was first investigated by immunofluorescence. For immunofluorescence observation, we incubated HepG2 cells with anti-fade mounting medium containing carboxyfluorescein diacetate (CFDA, Invitrogen) for cytoplasmic staining and DAPI (Invitrogen) for nuclear staining, and followed by analysis with a laser scanning confocal microscope (Leica 224).

Cell proliferation was further analyzed by using CCK-8 assay kit (Dojindo) according to manufacturer specifications. Briefly, HepG2 cells were respectively incubated in the medium containing DMSO or baicalein in 96-well plates. 24 or 72 h later, 5 μl CCK-8 reagent was added to each well and incubated at 37°C for 1 h. The cell numbers were assessed by measurement of absorbance at 450 nm. Proliferation assay for cells incubated with baicalein in the absence or presence of siRNA against long non-coding RNA (LncRNA) AK022798 was also assessed by applying above-mentioned methods. All the experiments were performed in triplicate.

Flow cytometric analysis of apoptosis

Exposure of phosphatidylserine (PS) was assessed to detect early stage apoptosis by analysis of annexin V-FITC binding. Increased propidium iodide (PI) was a correlate for increased secondary necrosis. In brief, $2×10^5$ cells were plated onto 6 well plates and then were respectively treated with DMSO and baicalein for indicated time at 37°C. After that, cells were manipulated by sequentially harvesting, washing in PBS and resuspending in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin V-FITC was added to a final concentration of 200 ng/ml prior to incubation in the dark at room temperature (RT) for 10 min, then washed in PBS and resuspended in 190 μl of binding buffer. 10 μl of PI was loaded to each sample before flow cytometric analysis. Stained cells were analyzed using a FACStar plus flow cytometer (Becton Dickinson). The ratio of fluorescence intensities excited at 488 nm was monitored at an emission wavelength of 515 nm for FITC and 560 nm for PI. Data analysis was performed with standard Cell Quest software (Becton Dickinson). Apoptosis induction in HepG2 cells treated with baicalein,

AK022798 siRNA or baicalein plus AK022798 siRNA was also determined by identical condition.

PCR array screening

HepG2 cell RNA was converted to cDNA using RT² first strand kit (SABioscience). Real-time PCR was done using SuperArray RT² SYBR Green qPCR Master Mix (SABioscience) and RT² Profiler™ PCR Array contains gene-specific PCR arrays and three RNA quality control elements. PCR cycling conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of amplifications at 94°C for 15 s, 60°C for 60 s. Data were analyzed by PCR Array Data Analysis Web Portal using the default set format.

Western blotting

For immunodetection of Notch1, HepG2 cells were lysed directly in Laemmli's sample buffer and boiled for 10 min. After the removing of the insoluble fraction by centrifuge, 50 μg of total protein extracts was resolved on 10% SDS-PAGE, which was then transferred to nitrocellulose membranes for western blotting. The membranes were first stained with Ponceau S to confirm the transfer efficacy. After blocking with 3% bovine serum albumin (BSA) dissolved in Tris-buffered saline (TBS), containing 0.05% Tween-20 (TBST), for 1 hour at RT, membranes were incubated with the primary antibody at a dilution of 1:1000, followed by goat anti-rabbit secondary antibody conjugated with horseradish peroxidase at 1:2,000 dilutions. Positive band intensities were detected by using a gel documentation system (LAS-3000 Fujifilm).

Quantitative RT-PCR assay

For quantitative RT-PCR assay, RNA was isolated from HepG2 cells using the RNeasy RNA Isolation kit (Qiagen) according to the manufacturer's instructions. First-strand cDNAs were generated from 3 μg of total RNA using commercially available kits (Applied Biosystems). All subsequent PCR reactions were performed using the 7 Universal PCR Master Mix (Applied Biosystems). Amplification and detection of mRNA were analyzed by 7500 real-time PCR System (Applied Biosystems). To normalize mRNA concentrations, transcriptional levels of β-actin mRNA were identified in parallel for each sample, and relative transcriptional level of Notch1 was adjusted by standardization based on the β-actin mRNA levels. Samples for each experimental condition were run in triplicate.

RNA interference

Small interference RNA (siRNA) corresponding to AK022798 sequence was synthesized (Qiagen). HepG2 cells were transfected with siRNAs (100 nM) against AK022798 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Scrambled siRNA was used as negative control. Cells were collected at 24 hrs post-transfection for further experiment including immunofluorescence, Flow cytometric analysis of apoptosis and CCK-8 assay.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) to assess the treatment effects by using DPS 3.2 software. The Student t test was used to determine the statistically significant differences in numbers with two significant levels (0.05 and 0.01). The results are shown as mean ± standard deviation (SD).

Results

Baicalein induces the efficient apoptosis of HepG2 cell

An earlier study demonstrated that cell death of three HCC cell lines can be induced by baicalein [20]. To further understand the anticancer effect of baicalein in another HCC cell line, HepG2 cell was used to treat. Primarily, cell proliferation was measured by applying the immunofluorescence. Results showed that cell numbers are significantly reduced in baicalein-treated group in contrast to two negative control groups at day 3 although identical visible cell numbers are detected in all 3 experimental groups at day 1 (**Figure 1A**). Then, we explored the cell proliferation using CCK-8 assay, as shown in **Figure 1B**. Similarly, the HepG2 cell treated with baicalein had no difference in the cell proliferation rate compared with DMSO-treated cells at day 1 but exhibited a significant decrease at day 3 ($P < 0.01$), indicating that baicalein inhibited the HepG2 cell proliferation.
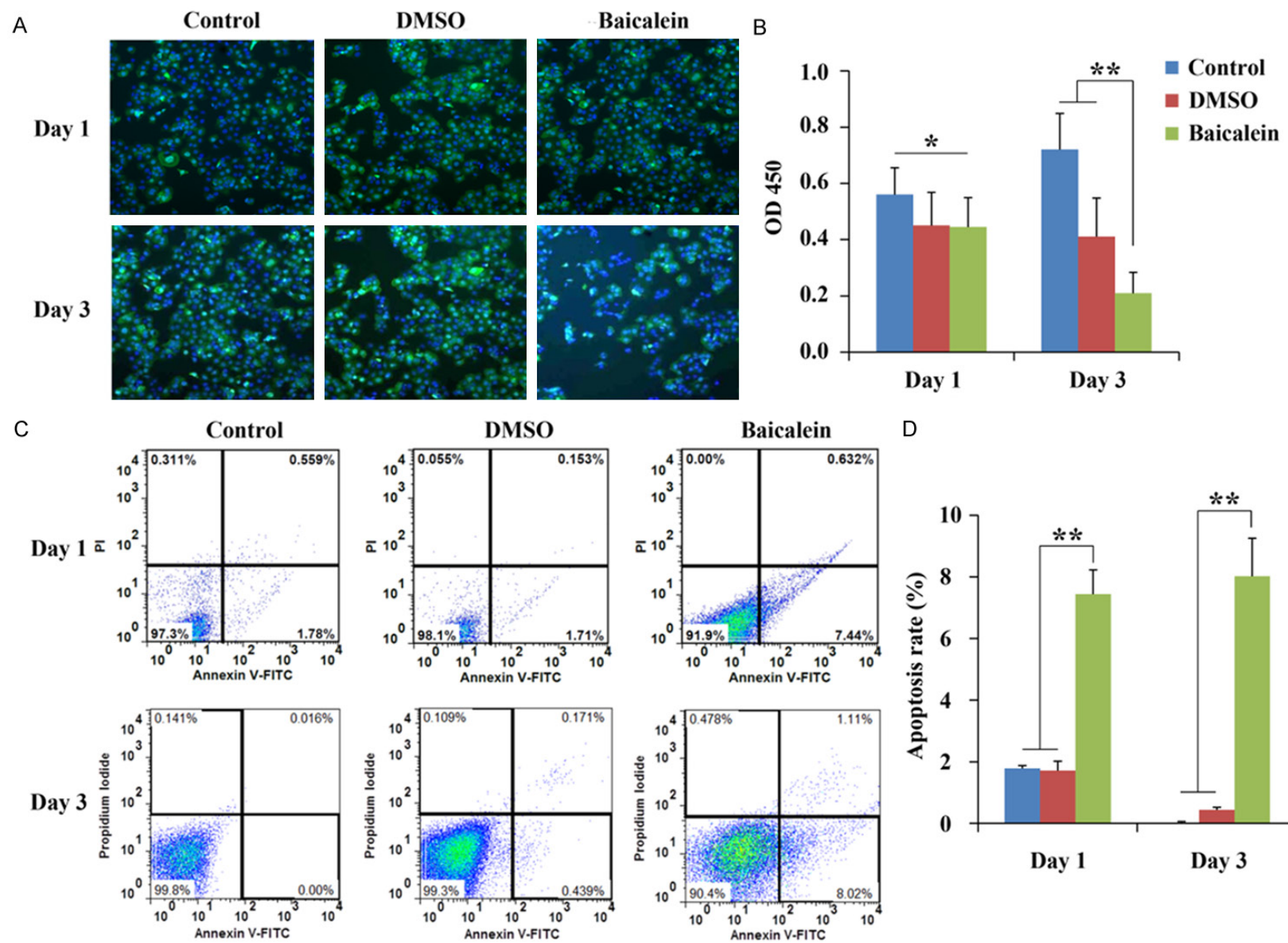
Figure 1. Baicalein inhibits HepG2 cell proliferation through apoptosis. (A) Immunofluorescence showing cell proliferation of HepG2 cell in absence or presence of baicalein at indicated time. HepG2 cells were incubated with CFDA for cytoplasmic staining and DAPI for nuclear staining. (B) CCCK-8 assay of HepG2 cell in absence or presence of baicalein at indicated time. (C) Flow cytometry analysis of apoptosis in HepG2 cells in absence or presence of baicalein. Annexin V-FITC and PI binding were applied to detect early stage apoptosis and secondary necrosis, respectively. (D) Representation of apoptosis rate from (C). Error bars ± SD, *$P < 0.05$, **$P < 0.01$.
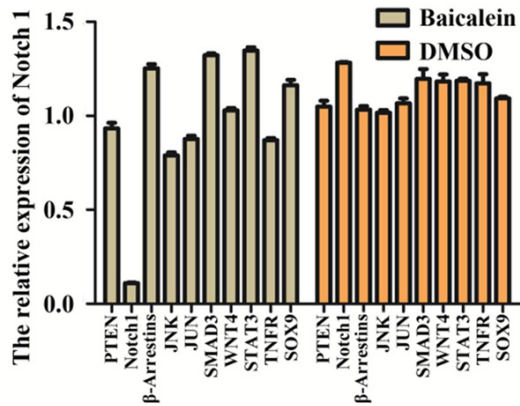

Figure 2. PCR assay screens baicalein-regulated genes in HepG2 cell. HepG2 cell was treated in absence or presence of baicalein and ten central cancer-related genes were selected to analysis. The relative transcriptional levels of these ten genes were adjusted by the transcriptional levels of β-actin mRNA.
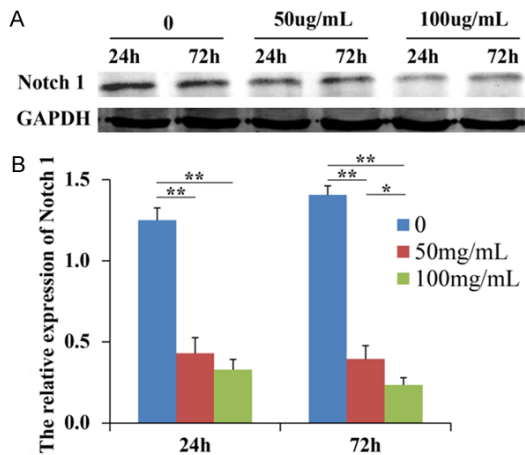

Figure 3. Baicalein reduces the expression of Notch1 in protein and mRNA levels. A. The expression of Notch1 in the presence of indicated concentration of baicalein at indicated time. B. The mRNA level of Notch1 in the presence of indicated concentration of baicalein at indicated time.

Then, we reasoned whether HepG2 proliferation suppressed by baicalein was resulted from the enhancement of cell apoptosis. Using the increase of annexin fluorescence intensity as

readout for enhanced apoptosis, we detected that the exogenous baicalein initiates the apoptosis of HepG2 cell at day 1 ($P < 0.01$) and further enhance the apoptosis at day 3 ($P < 0.01$, **Figure 1C** and **1D**). Collectively, these data suggest that baicalein is an efficient anticancer agent that inhibits HepG2 cell proliferation through apoptosis.

PCR array screen the baicalein-regulated genes

To further explore the antineoplastic molecular mechanism, we use the PCR array to identify the downstream signaling molecules of baicalein. Ten vital cancer-related genes were selected to analysis and then 7 gene showed significant differences at relative mRNA abundance in baicalein-treated cells compared with DMSO-treated cells (**Figure 2**). These seven genes express Notch1, β-Arrestins, JNK, JUN, WNT4, STAT3 and TNFR protein, respectively. Out of them, five are down-regulated in baicalein-treated cells while two are up-regulated. For above tumor-related proteins, generally, abundance was differentially increased in the cells that avail tumorigenesis (or vice versa). Given that, we primarily focus on the proteins which can be reduced by baicalein treatment. One of the five down-regulated proteins, Notch1, had a lowest expression, indicating that Notch1 is a central downstream target of baicalein.

Baicalein reduces the Notch1 expression

Next, we further confirmed the Notch 1 expression in baicalein-treated HepG2 cells from protein and mRNA levels. In protein level, untreated HepG2 cells highly expressed Notch1, while the expression of Notch1 markedly reduced in HepG2 cells treated with two concentration of baicalein (50 µg/mL and 100 µg/mL) on both 24 and 72 hours respectively (**Figure 3A**). This finding suggested that baicalein have an ability to inhibit the Notch1 expression in HepG2 cell and this suppressing effect represented a dosage- and time-dependent manner (**Figure 3A**). We also examine the Notch1 expression in
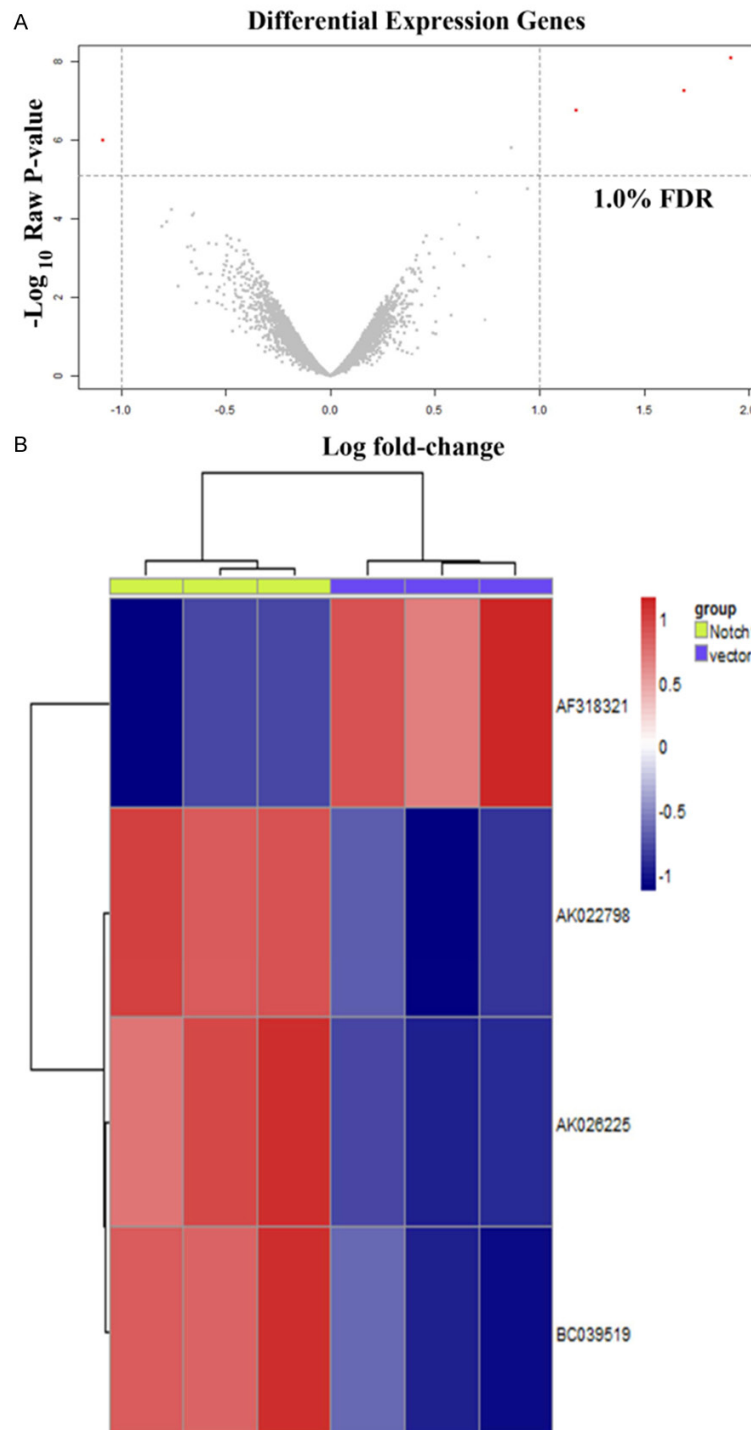
Figure 4. LncRNA can be regulated by Notch1. A. Differential expression of lncRNA in Notch1-transfected cells. After analyzing, expression of four lncRNA was altered in Notch1-transfected cells. B. Unsupervised hierarchical clustering of four Notch1-regulated lncRNA.

indicated dose of baicalein compared to the untreated HepG2 cells on both 24 and 72 hours respectively ($P < 0.01$). For 72 hour treatment, the suppressing effect of baicalein depended on the concentration by itself ($P < 0.05$).

Notch1 regulates the differential expression of lncRNA

It has been described that lncRNA is key downstream targets of oncogenic Notch1 [21]. We therefore assessed whether Notch1 can regulate the lncRNA expression in HepG2 cells. We used micro-array analysis to explore the possible lncRNA which participate in this process in the selection of differentially expressed genes, pcDNA3 vector as control. As shown in **Figure 4A**, these four Notch1-regulated lncRNA genes were AF318321, AK022798, AK-026225 and BC039519 (**Figure 4B**). Out of these four lncRNA, three were increased while the other one was decreased in Notch1-transfected cell ($P < 0.0001$). Among three lncRNA of up-regulated expression, AK022798 presented the highest fold change and lowest *p*-value (**Figure 4A** and **4B**).

A Notch1-regulated lncRNA, AK022798, is responsible for the anticancer action of baicalein

To examine the mechanism that links AK022798 to the anticancer activity of baicalein, we knocked down the expression level of AK022798 using RNA interference (RNAi) technique. Vectors encoding small interference RNA (siRNA) that target AK022789 were constructed, and their function was analyzed by
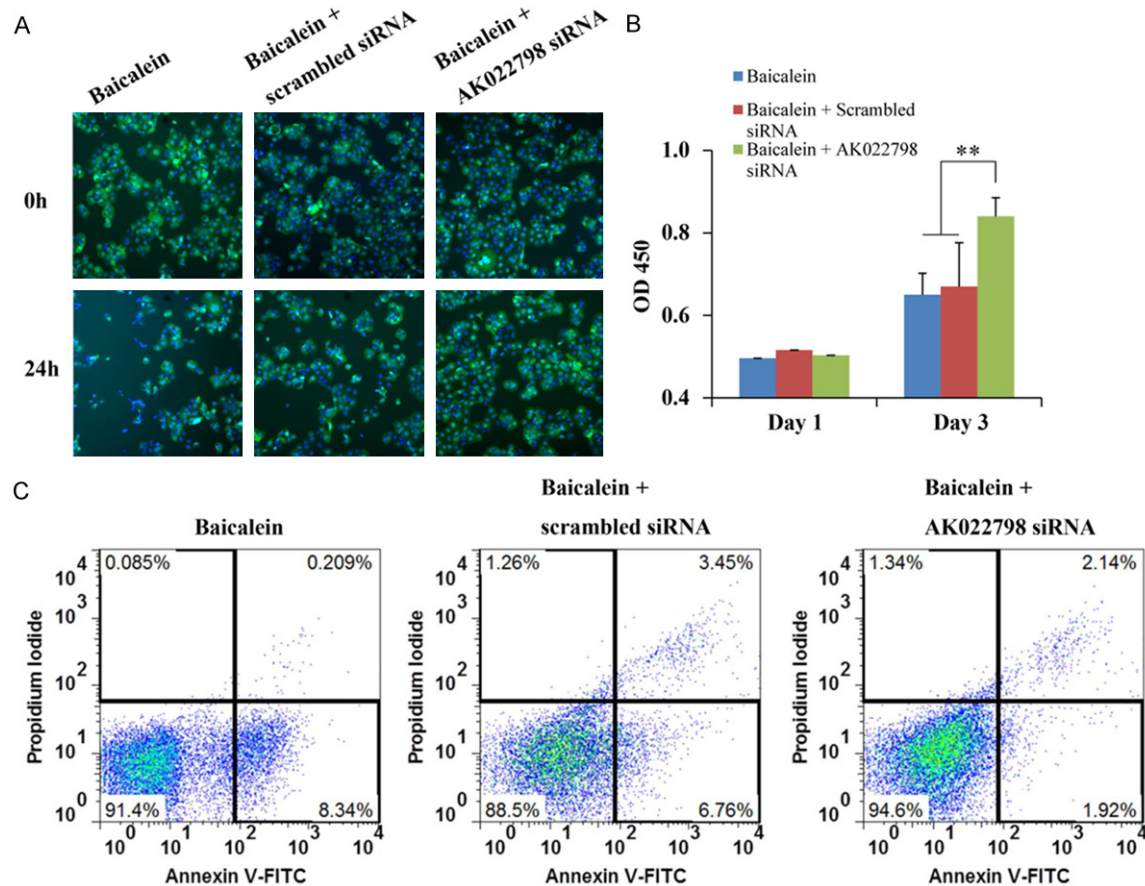
HepG2 from transcriptional level. As **Figure 3B** showed, the expression of Notch1 was significantly inhibited in HepG2 cells treated with two

Figure 5. AK022798 is responsible for the anticancer action of baicalein. A. Immunofluorescence showing cell proliferation of baicalein-incubated HepG2 cell in absence or presence of scrambled siRNA or AK022798 siRNA at indicated time. B. Representation of cell proliferation of baicalein-incubated HepG2 cell in absence or presence of scrambled siRNA or AK022798 siRNA at indicated time by using CCCK-8 assay. C. Flow cytometry analysis of apoptosis in baicalein-incubated HepG2 cells in absence or presence of scrambled siRNA or AK022798 siRNA. Error bars ± SD, *$P < 0.05$, **$P < 0.01$.

transfection into HepG2 cells. Endogenous AK022798 mRNA level was analyzed by RT-PCR and detected to be lower in AK022708 siRNA-transfected cells when compared to scrambled siRNA transfected cell, demonstrating the effectiveness of AK022708 siRNA (data not shown). Since administration of baicalein suppresses the expression of Notch1 and the Notch1 inhibition enhances the expression of AK022798, the effect of AK022798 inhibition on anticancer action of baicalein was analyzed. First, we investigate the cell proliferation of HepG2 cell with AK022798 siRNA treatment (**Figure 5A** and **5B**). Interestingly, the AK022798 siRNA-transfected HepG2 cells have a specific restoration in cell proliferation as compared to the control-transfected cells through immuno-fluorescence and CCK-8 assay. Moreover, we

also detected the apoptosis ratio of HepG2 cell with AK022798 siRNA treatment (**Figure 5C**). The apoptosis ratio was obviously prohibited in baicalein-treated HepG2 cells in treatment AK022798 siRNA when compared with the baicalein-treated HepG2 cells in absence or presence of scrambled siRNA. These observations indicate that a Notch1-regulated lncRNA, AK022798, play a significantly positive role in baicalein-induced apoptosis.

Discussion

A line of evidences has indicated that baicalein possesses the anticancer action [19, 22], and baicalein was also applied to induce the cell death in HCC cell lines very early [20]. Although the inhibition of topoisomerase II activity was detected in baicalein-treated HCC cells [20],

the detailed molecular mechanism, which accounts for the cells to apoptosis induced by baicalein, was non-elucidated. In present study, we not only enrich the baicalein as an anticancer reagent that inhibits HepG2 cell proliferation through apoptosis but also, in follow-up experiments, provide the mechanistic link between baicalein and its function of cell apoptosis.

The core finding in our study is the ability of exogenous baicalein to inhibit the expression of Notch1, and ultimately to regulate the expression of lncRNA (such as AK022798) in HepG2 cells. This is a novel discovery, because previous studies did not reveal how any lncRNA might be responsible for baicalein-induced apoptosis. In current, we show that AK022798 contributes to baicalein-induced apoptosis, demonstrating a plausible Notch1-regulated mechanism. The Notch1 regulated by baicalein and lncRNA regulated by Notch1 have been extensively documented in the literature [21, 23]. However, the ability of baicalein to promote AK022798 through inhibition of Notch1 signaling and in turn increase apoptosis in HCC was previously unrecognized.

AK022798 is derived antisense to the T3JAM (TRAF3 (TNFR (tumor necrosis factor receptor)-associated factor 3)-interacting JNK (Jun N-terminal kinase)-activating modulator) gene [24]. The T3JAM protein associates specifically with TRAF3, a downstream signaling adaptor of TNFR, and then activate JNK pathway [25]. JNK can subsequently activates JUN, a proto-oncogene, and inhibit STAT3 [26, 27]. Given the results in above literatures, baicalein treatment may lead to the inactivation of TNFR, JNK and JUN, and activation of STAT3. This is consistent with the observation that baicalein-treated cells represent up-regulated expression of STAT3, and, down-regulated expression of TNFR, JNK and JUN (**Figure 2**). JNK activating JUN is essential for the development of cancer [28] and STAT3 have a suppressive role in cancer progression [29, 30]. TNFR overexpression is a potential risk factor that transforms chronic inflammation to cancer development and progression [31, 32]. Therefore, a suitable possibility is that apoptosis induced by AK022798 is completed by altering the activity of these proteins, and certainly this inference needs to be further investigated in future.

In summary, we have identified a potential mechanism by which lncRNA regulated by Notch1 may contribute to the baicalein-induced apoptosis in HepG2 cell. These findings emphasize the inter-relationship among baicalein, Notch1 and lncRNA, and also highlight the significance and relevance of lncRNA such as AK022798 as potential downstream biomarkers for therapeutic intervention in HCC.

Disclosure of conflict of interest

None.

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