Baicalein Attenuates Hepatocellular Carcinoma Cell Survival and Induces Apoptosis Through the miR-3178/HDAC10 pathway

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of hepatic malignancies with high mortality and poor prognosis and is the third most common cause of malignancies death worldwide which is more than 700,000 deaths per year. Baicalein, one of the major and bioactive flavonoid isolated from *Scutellaria baicalensis* Georgi, which is reported to have anti-proliferation effect in varying cancers, including HCC, whose underlying molecular mechanism is still largely unknown. In this study, the results showed that administration of baicalein significantly inhibited proliferation and colony formation, blocked cell cycle arrest at the S phase, and promoted apoptosis in HCC cells MHCC-97H and SMMC-7721 *in vitro* and reduced HCC tumor volume and weight *in vivo*. Increased microRNA (miR)-3178 levels and decreased histone deacetylase 10 (HDAC10) expression were found in cells treated with baicalein and in patients' HCC tissues. HDAC10 was identified as a target gene of miR-3178 by luciferase activity and western blot. Both baicalein treatment and overexpression of miR-3178 could down-regulate protein expression of HDAC10 and inactivated AKT, MDM2/p53/Bcl2/Bax and FoxO3 α /p27/CDK2/Cyclin E1 signal pathways. Not only that, knockdown of miR-3178 could partly abolish the effects of baicalein and the restoration of HDAC10 could abated miR-3178-mediated role in HCC cells. Collectively, baicalein inhibited cell viability, blocks cell cycle and induces apoptosis in HCC cells by regulating the miR-3178/HDAC10 pathway. This finding indicated that baicalein might be promising for treatment of HCC.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of hepatic malignancies with poor prognosis and is the third most common cause of malignancies death globally which is more than 780,000 deaths per year [1-2]. Several clinical interventions, including systemic chemotherapy, radiotherapy, liver transplantation, and surgical resection have been used as optimal treatments for HCC [3-4]. However, HCC is one of the most difficult cancers to treat owing primarily to its late diagnosis, low objective response rate, drug resistance, frequent recurrence after surgery, and high distant metastasis [5-6], whose 5-year overall survival rate remains less than 18% [7]. Therefore, identifying the therapeutic agents with higher efficacies and milder side effects to improve the efficacy of HCC therapy is urgently needed.

Extracting bioactive monomers from herbs and other natural products has become an attractive strategy for tumor treatment, including HCC. Baicalein, 5, 6, 7-trihydroxyflavone, is one of the major active flavonoid's monomers of the root of traditional Asian herbal medicine *Scutellaria baicalensis* Georgi (also known as Chinese Huang Qin). Growing evidence has showed that baicalein treatment inhibits the development of many types of tumors, involving gastric cancer, lung cancer, esophageal cancer and ovarian cancer [8-11]. Notably, baicalein has also have a potent anti-HCC effect, including anti-proliferation and apoptosis induction [12-13]. However, the exact molecular mechanisms of baicalein against human HCC are still vague.

In our previous study, baicalien altered the miRNA expression profiles in HCC cells. After treatment with baicalein (40 or 80 μ M) for 24 h, 13 miRNAs were up-regulated with more than 1.5-folds ($P < 0.05$) and miR-3178 was up-regulated in 40 μ M group (2.57-folds, $P = 0.005$) or 80 μ M group (2.51-folds, $P = 0.006$) [14]. Accumulated data have shown that miR-3178 serves critical roles in tumor progression. MiR-3178 exerts anti-migration and invasion function in highly metastatic prostate, lung, and breast cancer cells [15], inhibits cell proliferation and metastasis in triple-negative breast cancer [16], and ameliorates inflammation and gastric carcinogenesis [17]. Overexpression of miR-3178 could also inhibited the proliferation and angiogenesis of tumor endothelial cells of HCC [18]. However, the detailed role of miR-3178 in the progression of HCC has not yet been reported.

Herein, we demonstrated that miR-3178 was significantly decreased in hepatocellular carcinoma cell lines and patients' HCC tissues. Baicalein inhibits cancer proliferation, promotes apoptosis and blocks cell cycles in HCC MHCC-97H and SMMC-7721 cells via promoting miR-3178 expression. Inhibiting miR-3178 rescues the anti-cancer effect of baicalein, and miR-3178 exerts its anti-cancer role by targeting HDAC10. Its underlying molecular mechanism were also investigated. Collectively, our results demonstrates that baicalein has a potential ability to inhibit the development of hepatocellular carcinoma and suggests a potentially new therapeutic pathway.

Materials And Methods

Chemicals and reagents

Highly pure (>98%) baicalein and sorafenib (Raf-1 and B-Raf multi-kinase inhibitor, first-line drugs approved by the Food and Drug Administration for liver cancer) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The structures of baicalein and sorafenib are shown in Fig. 2k.

Clinical tissue specimens

A total of 36 pairs of human hepatocellular carcinoma tissues and adjacent normal tissues were collected from the Second Affiliated Hospital of Xi' an Jiaotong University (Shaanxi, China) between Jan 2015 to May 2021 with histologically confirmed. Patients who were neoadjuvant chemo- or radio therapy naïve were selected. The study was conducted with the written informed consents of all patients and the approval of the Ethics Committee of the Second Affiliated Hospital of Xi' an Jiaotong University. The clinical data was collected in Supplemental Table S1. Tissue sections are frozen and stored at -80°C until use.

Cell culture and transfection

7 different human HCC cell lines including Bel-7402, Bel-7404, SMMC-7721, MHCC-97H, HepG2, Hep3B, Huh7 and human L-O2 normal liver cell line was purchased from the American Type Culture Collection (Manassas, USA) and were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad,

USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen; Carlsbad, CA, USA). All cells were cultured at 37°C with 5% CO₂.

The human HDAC10 cDNA clone expression plasmid was synthesized by Sino Biological Inc. (Beijing, China). miR-3178 mimic, inhibitor and their negative controls were synthesized by Ribobio Co., Ltd (Guangzhou, China). All the sequences were listed in Supplementary Table S2. Primers for miR-3178, U6, HDAC10 and GAPDH were synthesized by GENEWIZ Biotech Co., Ltd (Suzhou, China) and the sequences were listed in Supplementary Table S3. Transfection was performed with Lipofectamine 3000 and supplied with Serum-free Opti-MEM® medium (Invitrogen, Waltham, USA) following the protocols of manufacturer.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) assay

Total RNA including miRNA was extracted from clinical samples and HCC cells by TRIzol reagent (Invitrogen, USA). The extracted RNA was reverse-transcribed to be cDNA by RT reagent Kit (#RR047A, TaKaRa, Japan) and to be cDNA for miRNA using miRNA First Strand Synthesis Kit (#638313, Takara, Japan). Then, qRT-PCR was implemented by SYBR® Premix Ex Taq II Kit (RR820A, Takara, Japan) on an ABI-7500 fast system (Applied Biosystems, Foster City, USA). 2^{-ΔΔCt} method was used and data were expressed as the fold change of the control group. U6 and GAPDH were used as internal normalization controls for miR-3178 and HDAC10, respectively. All sequences of specific primers were described in Supplementary Table S3. Experiments were implemented in triplicate.

Luciferase reporter assay

To verify the interaction between miR-3178 and HDAC10, wild type 3'UTR of HDAC10 containing the putative miR-3178 binding site (Site: 204-210, termed HDAC10 wt 3'UTR) and its mutant (termed HDAC10 mut 3'UTR) were cloned into PsiCheck-2 luciferase reporter vector (Genepharma Biotechnology Co., Shanghai, China). Cells were seeded in 24-well plates at a density of 5×10⁴ cells per well overnight. Then, cells were co-transfected with luciferase reporter vector with either 50 nmol/L miRNA mimics negative control (termed miR-NC) or 50 nmol/L miR-3178 mimics (termed miR-3178) (RiboBio Co., Guangzhou, China) for 48 hours. Renilla luciferase reporter plasmid (Promega, Madison, USA) was co-transfected as an internal control. After incubation, a dual-luciferase reporter assay kit (#E1910, Promega, Madison, USA) was used to examine the luciferase activities. Experiments were implemented in triplicate and renilla luciferase activity was used to normalize the firefly luciferase activity.

Cell counting kit-8 (CCK8) and colony formation assay

For CCK8 assay, cells were incubated at a density of 1.5×10³ cells per well after transfection and cultured for 1-5 days at 37°C in 96-well plates. At the indicated time points, cell viability was then measured by incubating cells with 10 µl of CCK-8 solution (1 mg/ml, Dojindo, Kumamoto, Japan) for 2 h. The absorbance was determined at 450 nm by using the Microplate reader (M20 pro, TECAN, Switzerland).

For colony formation assay, Transfected MHCC-97H or SMMC-7721 cells were counted and implanted into 6-well plates at 1500 cells/well. After cultured at 37°C with 5% CO₂ for 7-14 days until visible colony formation can be observed, cells were fixed with paraformaldehyde (4%) and dyed with crystal violet solution (0.1%) for 15 min. The colonies were photographed and counted when they contain 50 cells.

Apoptotic rate detection and cell cycle assay

As for apoptotic rate analysis, the Annexin V-FITC Apoptosis Detection Kit (Invitrogen, USA) was applied. Briefly, after transfection and/or baicalein treatment for 48h in 6-well plates, cells were harvested and stained with Annexin V-FITC conjugate (5 µl) and propidium iodide (PI, 5 µl) solution in the dark at room temperature for 20 min. Following the incubation, apoptotic rate was investigated via flow cytometer (BD Biosciences, San Jose, USA).

To determine cell cycle arrest, cells was detected by PI staining. Briefly, after transfection and/or baicalein treatment for 48h in 6-well plates, cells were harvested and fixed with 70% (v/v) ethanol at -20°C overnight. Cells were then washed and stained with cold PBS containing propidium iodide (PI, 10 µg/ml) and RNase A (1 mg/ml) in the dark at room temperature for 30 min. The cell cycle distribution was then investigated using flow cytometer (BD Biosciences, San Jose, USA) and quantified based on the DNA content of the cells using FlowJo software (Version 7.6, BD, USA).

Western blotting

Cells were lysed using cold RIPA lysis buffer with protease and phosphatase inhibitor (#04693159001 and #04906837001, Roche, USA) and quantified by Pierce BCA Protein Assay Kit (Thermo, USA). 40 µg of proteins were loaded and isolated from 10% sodium dodecyl sulfate-polyacrylamide gel and blotted on polyvinylidene difluoride membranes (Millipore, Bedford, USA). After blocking non-specific proteins with 5% non-fat milk for 2 h at 37 °C, the membranes were probed with primary antibodies at 4 °C overnight. After washed, the membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at 37 °C. Finally, after washing, the fluorescence of proteins were catalyzed by enhanced chemiluminescence (ECL) substrate (Millipore, USA) and relative fluorescence intensities were determined by Image Lab System (Bio-Rad, USA). The antibodies used in this study were shown in Supplementary Table S4.

Immunohistochemical (IHC) staining

The paraffin-embedded tumors from xenografts were cutted at the thickness of 4 µm, deparaffinized and rehydrated. After blocking endogenous peroxidase activity in 0.3% H₂O₂ and blocking nonspecific immunoglobulin binding sites with goat serum, sections were immune-stained with primary antibodies at 4 °C overnight. Then, the slides were incubated with biotinylated secondary antibodies at 37 °C for 30 min. After that, protein expression was examined by 3,3-diaminobezidine tetra hydrochloride (DAB) staining. Hematoxylin was also applied to counterstain the nucleus.

Animal experiments

All animal experiments were reviewed and performed with the approval of the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University and the methods were in compliance with the animal welfare guidelines.

Male BALB/c nude mice (3-4 weeks old, weighing 14-18 g) were fed in Xi'an Jiaotong University Experimental Animal Center. For baicalein treatment xenograft model, mice were randomly subdivided into 2 groups. 5×10^6 untreated MHCC-97H cells (in 100 μ L of normal saline) were subcutaneously injected in the armpits of nude mice ($n = 6$). Tumor formation was monitored every two days with calipers and the volumes were calculated using formula $V \text{ (mm}^3\text{)} = (\text{Length} \times \text{Width} \times \text{Width} / 2)$. When tumors reached an average volume of 50-100 mm^3 , mice were intraperitoneally injected with baicalein (80 mg/kg, dissolved in normal saline) or normal saline (100 μ L) as controls every two days. Mice were euthanized at day 28 after first baicalein injection. For miR-3178 overexpression xenograft model, mice were randomized into 2 groups. 5×10^6 MHCC-97H cells stably transfected with LV-miR-3178 or LV-miR-NC were subcutaneously injected in the armpits of nude mice (5 mice per condition). Tumor sizes were neatly excised every two days. Mice were euthanized at day 20 after cell inoculation. Tumors were stripped and tumor weights were excisely measured. A portion of tumor tissue was fixed in 10% formalin solution and then embedded in paraffin for subsequent histological examination, and other parts were subjected to total RNA or protein isolation, or froze in liquid nitrogen for later use.

Data and statistical analysis

Values were expressed as the mean $\pm$ standard deviation and were analyzed via Graphpad Prism 8.0.2 Software (San Diego, USA). A two-tailed Student's t-test was applied to analyze statistically differences between two groups. For multiple comparisons, a one-way analysis of variance (ANOVA) was used followed by Tukey's post hoc test. Pearson correlation analysis was used to explore the correlations between expression of HDAC10 and miR-3178 in tissues or cell lines. P-values less than 0.05 were thought as statistically significant (*' denotes $P < 0.05$). All experiments were separately repeated at least three times.

Results

miR-3178 expression is down-regulated in patients' liver cancer tissues and HCC cell lines and prognostic values of miR-3178 expression in liver cancer patients

The expression of miR-3178 in liver cancer tissues, paired para-carcinoma normal tissues and immortal liver cancer cell lines were determined via qRT-PCR. The results normalized to U6 suggested that miRNA-3178 was downregulated in both liver cancer tissues (HCC) and seven immortal liver cancer cell lines (HCC cell lines) compared to corresponding adjacent noncancerous tissues (HCP) (Fig. 1a). Moreover, in comparison with the normal hepatocyte line L-02, a notable reduction of miR-3178 level was observed in all seven tested HCC cells which including Bel-7402, Bel-7404, SMMC-7721, MHCC-97H, HepG2, Hep3B.

and Huh7 cells (Fig.1c). Finally, we chose SMMC-7721 (higher expression of miR-3178) and MHCC-97H (lower expression of miR-3178) for the sequent research.

Moreover, Kaplan-Meier survival analysis was performed to determine the potential prognostic power of miR-3178 (Fig. 1b). According to the cut-off expression level of miR-3178 in all samples, the 36 HCC patients recruited were divided into a high expression group and a low expression group. The low expression levels of hsa-miR-3178 was significantly associated with poor overall survival (log-rank test p-value = 0.0458).

Baicalein inhibits proliferation of HCC cells in vitro, suppresses tumor growth in mice in vivo and miR-3178 is up-regulated in baicalein-treated HCC cells

The human immortal liver cancer cell lines MHCC-97H and SMMC-7721 were treated with various concentrations of baicalein (1-100 μ M) for 24-120 h and proliferation was examined using CCK8 assay. Baicalein significantly inhibited the proliferation of both two cell lines in a dose-dependent and time-dependent manner (Fig. 2a). Colony formation assay showed that 80 μ M baicalein significantly reduced the quantity and colony size of MHCC-97H and MHCC-7721 cells after 14 days of culture (Fig. 2b). These two assays indicated that baicalein can suppress the proliferative capacities of liver cancer cells. In addition, incubating with 80 μ M baicalien for 48 h significantly triggered cell apoptosis (Fig. 2c) and resulted in an increase in the percentage of cells at S phase, which implied that cells were blocked in S phase (Fig. 2d). Sorafenib (Fig. 2k), the first targeted drug for liver cancer approved by the US FDA in 2007, was applied as a positive drug control. A similar inhibitory effect has also been observed with sorafenib (10 μ mol/L) treatment. Western Blotting analysis verified that baicalein treatment caused abnormal expression of multiple cell survival-, cycle- and apoptosis-related genes, including phosphorylated-AKT (p-AKT), p27, CDK2, Cyclin E1, p53, Bcl2, and Bax (Fig. 2e).

To investigate the anticancer ability of baicalein *in vivo*, a nude mouse xenograft model was constructed. Mice were randomly divided into two groups and were subcutaneously implanted MHCC-97H cells (5×10^6 cells/mouse) into dorsal flanks of mice. After the tumor volume is greater than 50mm³, mice received 100 μ L of intraperitoneal injections of baicalein (dissolved in normal saline) or normal saline (control) every two days. Tumor growth was measured before each drug injection. As shown in Fig. 2f, compared with those that received normal saline, tumor growth (Fig. 2g) and weights (Fig. 2h) were significantly suppressed in mice that received baicalein (50 mg/kg/day). HE staining displayed that tumor cells were loosely arranged in the baicalein group and immunohistochemical staining assay showed that Ki-67 staining was reduced after baicalein treatment (Fig. 2i). The miR-3178 level was examined in baicalein-treated MHCC-97H and SMMC-7721 liver cancer cells by qRT-PCR. Consistent with our previous microRNA chip results [14], miR-3178 was found to be increase to more than 300% in baicalein-treated cells compared with control cells (Fig. 2j).

miR-3178 mediates proliferation inhibition of HCC cells by baicalein

The loss-of-function assays were conducted to explore whether the upregulated miR-3178 mediated the proliferation inhibitory activity of baicalein. As shown in Fig. 3a, the upregulation of miR-3178 by baicalein was significantly arrested by transfection with anti-miR-3178 in SMMC-7721 cells. Down-regulation of miR-3178 in baicalein-treated SMMC-7721 cells significantly reversed the inhibitory effect of baicalein on the cell viability (Fig. 3b), colony formation (Fig. 3c). Besides, apoptosis induction and cell cycle S phase arrest by baicalein were significantly ameliorated in miR-3178-down SMMC-7721 cells (Fig. 3d and e). The above data indicated that miR-3178 played an important role in mediating the anti-tumor ability of baicalein. Apart from that, western Blotting assay verified that downregulation of miR-3178 specifically reversed the abilities of baicalein caused abnormal expression of multiple cell survival-, cycle- and apoptosis-related genes, including phosphorylated-AKT (p-AKT), p27, CDK2, Cyclin E1, p53, Bcl2, and Bax (Fig. 3f).

miR-3178 up-regulation in HCC cells delays tumor growth in vitro and in vivo

To examine whether miR-3178 up-regulation can mimic the anti-tumor ability of baicalein, MHCC-97H cells and SMMC-7721 cells were infected to overexpress miR-3178 by hsa-miR-3178 lentivirus vector. Compared with the negative control (miR-NC group), transfection with miR-3178 significantly reduced cell viability (Fig. 4a) and capability of plate colony formation (Fig. 4b), suggesting that miR-3178 inhibited liver cancer cell proliferation *in vitro*. Besides, miR-3178 up-regulation in HCC cells induced apoptosis (Fig. 4c), and blocked cell cycle at S-phase (Fig. 4d). Then, we checked the activity of miR-3178 upon the tumorigenesis of HCC cells in vivo. To establish the nude mice subcutaneous xenograft models, miR-3178-up MHCC-97H cells (infected with hsa-miR-3178 lentivirus) and negative control cells (infected with negative control (NC) lentivirus) were used. It was shown that miR-3178-up group had fewer xenografts (Fig. 4e), smaller tumor volumes (Fig. 4f), lighter tumor weights (Fig. 4g) and coupled with a marked decrease of Ki67 protein level (Fig. 4h), which indicated that miR-3178 could significantly delay the growth of MHCC-97H xenograft tumors.

miR-3178 directly interacted with HDAC10 3' UTR

The TargetScan and miRanda databases were applied to analyze potential targets of miR-3178. Among the predicted targets, HDAC10 was reported to be involved in tumor processes of lung adenocarcinoma and ovarian cancer [19-20], whose mechanism in liver cancer has not been reported yet. As shown in Fig. 5a, there was one putative binding site of miR-3178 in the 3'-UTR of human HDAC10 mRNA. Dual-luciferase reporter assay was conducted to observe the effect of miR-3178-up on relative luciferase activity of plasmid containing wild-type (WT) or mutant (MUT) HDAC10 reporter in HEK-293T cells. When miR-3178 mimic was co-transfected with HDAC10-WT, there was a notable reduction in relative luciferase activity than when co-transfected with HDAC10-MUT or the control group (Fig. 5b), suggesting that miR-3178 might interact with HDAC10 3' UTR by putative binding sites. Moreover, according to q-RT-PCR and western blotting analysis, miR-3178 overexpression triggered a marked downregulation of HDAC10 mRNA and protein levels (Fig. 5c and d), whereas the downregulation of miR-3178 induced significant HDAC10 mRNA and protein expression in both MHCC-97H and SMMC-7721 cells (Fig. 5e and f).

Moreover, there was an obvious reduction of HDAC10 mRNA level in the baicalein-treated group in comparison with the control group of the two cells in a dose-dependent manner (Fig. 5g). Analysis of clinical data showed that the mRNA expression level of HDAC10 was remarkably downregulated in HCC tissues and HCC cells (Fig. 5h). Also, Pearson correlation analysis further revealed that in 36 cases of HCC specimens, the expression level of miR-3178 was significantly inversely correlated with the expression level of HDAC10 mRNA ($r = -0.4309$, $P = 0.0014$; Fig. 5i). Generally, these results confirmed that HDAC10 was a target of miR-3178 in HCC.

miR-3178 inhibits the growth of HCC cells by targeting HDAC10

We conducted the loss-of-function assays to determine whether the upregulated HDAC10 mediated the growth inhibitory activity of miR-3178. The HDAC10 overexpression plasmid was co-transfected in MHCC-97H cells that overexpressed miR-3178. The overexpression of HDAC10 plasmid partially restored the expression levels of HDAC10 mRNA (Fig. 6a) and protein (Fig. 6f) that were reduced by overexpression of miR-3178 in miR-3178-overexpressed MHCC-97H cells. It was showed that HDAC10 overexpression significantly attenuated miR-3178-mediated inhibitory effect of proliferation and colony formation (Fig. 6b and c). Additionally, HDAC10 overexpression weakened miR-3178-induced apoptosis and cell cycle arrest in S phase in MHCC-97H cells (Fig. 6d and e). Moreover, co-expression of HDAC10 partially reversed abnormal expression of multiple cell survival-, cycle- and apoptosis-related genes, including phosphorylated-AKT (p-AKT), p27, CDK2, Cyclin E1, p53, Bcl2, and Bax caused by miR-3178 (Fig. 6f).

Effect of baicalein on miR-3178, HDAC10, phosphor-AKT, MDM2 and FoxO3a expression *in vivo*

Finally, we examined the effect of baicalein treatment on the expression of FLOT1, MAPK, NF- κ B and miR-6809-5p in subcutaneously transplanted tumors. Consistent with the results of *in vitro* studies, baicalein treatment significantly increased the expression of miR-3178 in MHCC-97H xenograft tumor tissues (Fig. 7a). Similarly, compared with control group, the mRNA (Fig. 7b) and protein (Fig. 7c) expression of HDAC10 in the baicalein-treated group was reduced, and the expression levels of phosphorylated AKT, MDM2, and FoxO3a were reduced in MHCC-97H xenograft tumors (Fig. 7c and d).

Discussion

Previous reports pointed out that decreased expression of miR-424-3p contributes to baicalein-mediated cell growth suppression, apoptosis induction and cisplatin sensitivity promotion in A549 and H460 non-small-cell lung cancer cells [21]. Baicalein inhibits cell viability and epithelial-mesenchymal transition and induces apoptosis of Hela cervical cancer cells through upregulation of miR-183 [22]. Moreover, baicalein treatment causes inhibition of proliferation, migration and invasion and induction of apoptosis in MG-63 and Saos-2 osteosarcoma cells by inducing miR-183 expression [23]. Baicalein-mediated apoptosis promotion in Panc-1 pancreatic cancer involves upregulation of miR-139-3p and downregulation of miR-196b-5p [24]. Down-regulation of miR-106 augments the antitumor effect of baicalein on T24 bladder

cancer cells [25]. These findings indicate that the anti-cancer activity induced by baicalein involves specific miRNA mediators in cells of different cancer types.

Our previous microarray results showed that the expression of miR-3178 in HCC cells was significantly up-regulated after baicalein treatment [14]. Accumulated data have shown that miR-3178 serves critical roles in tumor progression. Previous reports pointed out that miR-3178 was markedly decreased in highly metastatic prostate, lung, and breast cancer cells and overexpression of miR-3178 inhibits metastasis invasion cascade of those highly metastatic cancer cells, which can be explained by modulating downstream regulatory molecule TRIOBP [15]. miR-3178 has been reported significantly reduced in triple-negative breast cancer, which correlated with poor overall survival. The cell proliferation, invasion, and migration could be inhibited by overexpressing miR-3178 via suppressing the epithelial-to-mesenchymal transition [16]. Also, prior studies showed that miR-3178 was decreased in *H. pylori*-positive gastric tissues. During *H. pylori* infection, Tip- α , a carcinogenic factor present in *H. pylori* could promote inflammation and carcinogenesis by inhibiting miR-3178 expression in gastric mucosal epithelial cells [17]. In addition, miR-3178 could specifically inhibited the proliferation, migration, invasion, and angiogenesis of hepatocellular carcinoma tumor endothelial cells [18]. However, the detailed role of miR-3178 in the progression of HCC has not yet been reported. In our study, we first demonstrated miR-3178 mediates proliferation inhibition of HCC cells by baicalein and up-regulated miR-3178 could delays HCC tumor growth *in vitro* and *in vivo*.

In this report, a novel miRNA regulator of HDAC10 was identified. Bioinformatics analysis and luciferase reporter gene assay indicated that miR-3178 could interact with HDAC10 through the predicted binding site. Clinical data demonstrated that HDAC10 expression was notably upregulated in HCC tissues and cells and the expression of miR-3178 and HDAC10 in HCC samples was negatively correlated. Moreover, the low expression levels of miR-3178 was significantly associated with poor overall survival. In two HCC cells, ectopic expression of miR-3178 apparently down-regulated the mRNA and protein expression of HDAC10. Additionally, the overexpression of HDAC10 abrogated the effect of miR-3178 on cell proliferation and apoptosis in HCC. Multiple signaling pathways including p-AKT/FoxO3 α /p27/CDK2/cyclinE1 and p-AKT/MDM2/p53/Bcl2/Bax were inactivated by overexpression of miR-3178, which could be restored by co-expression of HDAC10. Molecular explanations may be provided for the growth effect of HCC cells mediated by HDAC10 by these findings. Overall, these results indicate that HDAC10, as a functional target of miR-3178, plays a role in regulating the proliferation and apoptosis of HCC cells.

Histone deacetylase 10 is an enzyme encoded by the HDAC10 gene in humans. The enzymatic activity of HDAC10 is involved in determining the acetylation status of histone tails, which in turn participates in the regulation of chromatin structure and gene expression [26-27]. HDAC10 has been reported to participate in tumor progression through epigenetic function or targeting certain decisive molecules or signaling pathways, and is an independent predictor of poor prognosis for various cancer types [28]. A high expression level of HDAC10 protein is positively associated with PD-L1 expression and correlated with poor overall survival and poor clinical outcome in patients with non-small cell lung carcinoma [29].

Depletion of HDAC10 suppresses cell proliferation and induces cell cycle arrest and apoptosis in lung cancer cells by inhibiting mitotic entry or regulating the phosphorylation of AKT [30-31]. Also, downregulation of HDAC10 reduces DNA repair capacity and promotes sensitization to platinum therapies in ovarian carcinoma cells [20]. Furthermore, HDAC10 has the ability to facilitate the growth of SNU-620 gastric cancer cells via altering reactive oxygen species accumulation to release cytochrome c and activate apoptotic signaling molecules [32]. Increased expression of HDAC10 is reported to contribute to promote autophagy-mediated cell survival and sensitivity to cytotoxic drug treatment in neuroblastoma cells [33]. Other miRNAs targeting HDAC10 has been documented. For instance, miR-1908 downregulates the expression of HDAC10 and leads to an inhibition of cell growth and invasion in cervical carcinoma cells [34]. Therefore, further research is needed to determine other miRNA mediators in the regulation of HDAC10 by baicalein. In addition, it is necessary to further study the important role of miR-3178/HDAC10 in other aspects of HCC progression, such as migration and invasion, and other potential targets of miR-3178 in HCC.

Previous reports pointed out that AKT-FOXO3a pathways have a central role in cancer [35-36]. Resveratrol treatment could inhibit the growth of benign prostatic hyperplasia BPH-1 cells and induce cell cycle arrest at S phase through levels of phosphor-p38 elevation and FOXO3a repression [37]. Also, adjuvin effectively suppressed small-cell lung cancer cell proliferation by inducing S phase arrest via activating the sirtuin 3 (SIRT3)-FOXO3a pathway [38]. Besides, the up-regulation of FoxO3a expression is related to the inhibition of CDK2, CDK4, Cyclin D1 and Cyclin E and the up-regulation of p21 and p27Kip1 [39-40]. Therefore, we examined whether the AKT/FOXO3a/CDK2/Cyclin E1 axis was involved in the activity of baicalein to block the cycle of liver cancer cells, and proved that baicalein can block the cell cycle by regulating the expression of miR-3178 and HDAC10, which in turn inhibited cell proliferation. Furthermore, previous studies demonstrated that AKT/MDM2 axis were related to apoptosis of tumor cells. The apoptosis-inducing effect of melatonin on gastric cancer SGC-7901 cells was mediated by blocking the intracellular pathway of AKT/MDM2 [41] and FBXO31 was confirmed to downregulate cervical cancer progression by inducing apoptosis via inactivate PI3K/AKT-mediated MDM2/p53 axis [42]. Hence, we investigated the role of AKT/MDM2/p53 axis in these two HCC cells and confirmed that baicalein can regulate the AKT/MDM2/p53 axis by regulating the expression of miR-3178 and HDAC10 to induce apoptosis. However, how does HDAC10 directly or indirectly affect the expression of phosphorylated AKT remains to be further studied.

In this report, we demonstrated that baicalein inhibits the growth and induces apoptosis of hepatocarcinoma MHCC-97H and SMMC-7721 cells *in vitro* and *in vivo*, and also up-regulates the expression of miR-3178. miR-3178 was lowly expressed in clinical hepatocarcinoma cancer tissues and cells, which was consistent with previous study in HCC patients' blood and serum [43](Pascut et al., 2019). In line with the effects of baicalein treatment, overexpression of miR-3178 inhibited the growth and induced the apoptosis of HCC cells *in vitro* and *in vivo*. Whereas, miR-3178 deficiency resulted in hepatocellular carcinoma cells becoming more resistant to baicalein-mediated proliferation inhibition and apoptosis induction. Therefore, miR-3178 mediates the growth-inhibiting and apoptosis-inducing activity of baicalein on hepatocarcinoma.

Conclusions

Collectively, our data disclosed that miR-3178 was down-regulated in HCC tissues and cells, and the low expression of miR-3178 was associated with HCC progression and poor prognosis. We firstly demonstrated that baicalein could inhibit the expression of HDAC10 by up-regulating miR-3178, and then inactivating p-AKT/FoxO3 α and p-AKT/MDM2 signals, resulting in a reduction in the proliferation capacity and an increase in the rate of apoptosis of HCC cells *in vitro* and *vivo*. Fig.7e shows the interaction between baicalein, miR-3178, and the two key pathways. This data suggests that baicalein and miR-3178 have potential and might be promising for treatment of hepatocellular carcinoma.

Declarations

Author Declarations

Ethics approval and consent to participate The animal study was approved by Medical Laboratory Animal Welfare and Ethics Committee of Xi'an Jiaotong University Health Science Center and the methods carried out based on the animal welfare guidelines. The clinical study was approved by the Ethics Committee of Second Affiliated Hospital of Xi'an Jiaotong University and informed consent was obtained from.

Consent for publication All authors agree to publish.

Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests All authors declare that they have no competing interests. Junan Qi declares that he has no conflict of interest. Jun Li declares that she has no conflict of interest. Beibei Bie declares that she has no conflict of interest. Mengjiao Shi declares that she has no conflict of interest. Mengchen Zhu declares that she has no conflict of interest. Jing Tian declares that she has no conflict of interest. Kai Zhu declares that he has no conflict of interest. Jin Sun declares that he has no conflict of interest. Yanhua Mu declares that she has no conflict of interest. Zongfang Li declares that he has no conflict of interest. Ying Guo declares that she has no conflict of interest.

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Authors' contributions All authors contributed to the study conception and design. Z. L. and Y. G. designed the experiments, reviewed & edited the manuscript, and funded the project. J. Q. performed experiments and wrote the manuscript. J. L., B. B., M. S. and M. Z. performed experiments. J. T., K. Z., J. S., and Y. M. performed the statistical analysis. All authors read and approved the final manuscript.

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• **Compliance with Ethical Standards**

Disclosure of potential conflicts of interest Not applicable.

Research involving Human Participants and/or Animals Male BALB/c nude mice and 36 pairs of human hepatocellular carcinoma tissues and adjacent normal tissues were used in this study. All animal experiments were reviewed and performed with the approval of the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University and the methods were in compliance with the animal welfare guidelines. The clinical study was conducted with the written informed consents of all patients and the approval of the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Figures

Figure 1

miR-3178 expression in liver cancer (HCC) tissues and HCC cell lines and prognostic values of miR-3178 expression in liver cancer patients. **a** Compared with corresponding adjacent noncancerous tissues (HCP), miR-3188 expression was markedly decreased in HCC tissues and HCC cells. Student's t-test, Means $\pm$ SD, * $P < 0.05$ and ** $P < 0.01$. **b** Estimated Kaplan-Meier curves of overall survival for has-miR-3178 in hepatocellular carcinoma (HCC) patients. **c** qRT-PCR analysis of miR-3178 expression in 7 HCC

cell lines and immortalized human normal liver cell line L-02. * $P < 0.05$ and ** $P < 0.01$. Values are shown as Means $\pm$ SD. Student's t-test was conducted.

Figure 2

Baicalein inhibits cell proliferation and clone formation, promotes apoptosis, blocks cell cycles in MHCC-97H and SMMC-7721 cells in vitro, suppresses tumor growth in mice and miR-3178 is up-regulated in baicalein-treated HCC cells. a MHCC-97H and SMMC-7721 cells were treated with 0 - 100 μ mol/L

baicalein for 0-120 h and CCK-8 assay was performed to measure cell proliferation. **b** Clone formation assay was used to detect number of colonies after baicalein treatment. **c** Annexin V-FITC/PI double staining and flow cytometry were used to detect apoptosis in MHCC-97H and SMMC-7721 cells treated with 0, 80 $\mu\text{mol/L}$ baicalein for 48 h. **d** PI staining and flow cytometry were used to detect cell cycle distribution MHCC-97H and SMMC-7721 cells. 10 $\mu\text{mol/L}$ sorafenib was used as positive control. **e** Western blotting was performed to examine expression levels of HDAC10, phospho-AKT and proteins involved in the FOXO3 α /p27/CDK2/cyclinE1 and MDM2/p53/Bcl2/Bax pathway 48 h after baicalein treatment. Xenograft mice were divided into two groups (vehicle control and baicalein) of randomly six each after cell injection. **f** The tumor formation of subcutaneous xenograft in baicalein-treatment group was significantly inhibited than that in negative control group. **g** Compared to the negative control group, tumor volumes of baicalein-treatment group were virtually smaller. **h** The tumor weights of baicalein-treatment group were also significantly reduced compared with the negative control. **i** Immunohistochemical staining of Ki67 in xenograft tumor tissues (bar scale 50 μm) suggested that the protein level of Ki67 was downregulated in baicalein-treatment group. **j** miR-3178 was found to be increase in baicalein-treated cells compared with control cells. **k** The chemical structure of baicalein and sorafenib.

Figure 3

The growth-suppressive activity of baicalein was restored by anti-miR-3178 in SMMC-7721 cells. **a** The upregulation of miR-3178 by baicalein was significantly arrested by transfection with anti-miR-3178 in SMMC-7721 cells. **b-e** The inhibited cell proliferation and clone formation, induced apoptosis and arrested cell cycle in S phase by baicalein are restored by transfection with anti-miR-3178. **f** Western blotting was applied to analyze the protein levels (left) and Image J software was used to determined relative expression levels (right). GAPDH was used as loading reference. * $P < 0.05$, ** $P < 0.01$, compared

to the anti-miR-NC group; $^{\#}P < 0.05$, $^{##}P < 0.01$, compared to baicalein+anti-miR-NC group. Values are shown as Means $\pm$ SD. Student's t-test was conducted.

Figure 4

Overexpression of miR-3178 inhibited liver cancer tumorigenesis in vitro and vivo. **a** Cell viabilities of miR-3178-overexpressed MHCC-97H and SMMC-7721 cells were suppressed relative to their negative control cells. **b-d** The overexpression of miR-3178 inhibited cells' capability of plate colony formation, induced

apoptosis, and blocked cell cycle at S-phase. **e** miR-3178 was overexpressed in hsa-miR-33178 lentivirus-infected MHCC-97H cells. The tumor formation of subcutaneous xenograft in miR-3178-overexpression group (LV-miR-3178 group) was significantly inhibited than that in negative control group (LV-miR-NC). **f** Compared to the negative control group, tumor volumes of miR-3178-overexpression group were virtually smaller. **g** The tumor weights of miR-3178-overexpression group were also significantly reduced compared with the negative control. **h** Immunohistochemical staining of Ki67 in xenograft tumor tissues (bar scale 50μm) suggested that the protein level of Ki67 was downregulated in miR-3178-overexpression group. * $P < 0.05$ and ** $P < 0.01$. Values are shown as Means $\pm$ SD. Student's t-test was conducted.

Figure 5

miR-3188 directly regulates HDAC10. **a** miR-3178 and its putative binding sites in the 3'-UTR of HDAC10, predicted by TargetScan software. A mutation that binds to the miR-3178 seed region was generated in the complementary site. **b** Luciferase reporter assay was used to determine miR-3178 directly targeting the 3'UTR of HDAC10. **c-d** The mRNA and protein expression levels of HDAC10 in miR-3178-overexpressed HCC cells were examined by qPCR or Western Blotting. **e-f** The mRNA and protein expression levels of HDAC10 in miR-3178-downregulated HCC cells were examined, too. * $P < 0.05$ and ** $P < 0.01$. Values are shown as Means $\pm$ SD. Student's t-test was conducted. **g** HDAC10 was reduced in baicalein-treated cells compared with control cells. **h** The expression levels of HDAC10 were determined by qPCR in HCC and HCP tissue specimens and HCC cells, normalized to the GAPDH. Values are shown as Means $\pm$ SD. $P = 0.039$, $P = 0.044$. Student's t-test was conducted. **i** Correlations between the expression levels of miR-3178 and HDAC10 were calculated. Values are shown as Means $\pm$ SD. $P = 0.0014$. Two tailed Spearman's correlation analysis was conducted.

Figure 6

The growth-suppressive activity of miR-3178 was partially reversed by overexpression of HDAC10 in MHCC-97H cells. **a** The downregulation of HDAC10 by upregulated miR-3178 was significantly arrested by transfection with HDAC10 in MHCC-97H cells. **b-e** The inhibited cell proliferation and clone formation, induced apoptosis and arrested cell cycle in S phase by upregulated miR-3178 are partially restored by transfection with HDAC10. **f** Western blotting was applied to analyze the protein levels (left) and Image J software was used to determined relative expression levels (right). GAPDH was used as loading

reference. * $P < 0.05$, ** $P < 0.01$, compared to the miR-NC group; # $P < 0.05$, ## $P < 0.01$, compared to HDAC10+miR-NC group. Values are shown as Means $\pm$ SD. Student's t-test was conducted.

Figure 7

Effect of baicalein on miR-3178, HDAC10, and AKT expression in vivo. **a-b** Compared with the normal saline (NS) group, miR-3178 was significantly increased and HDAC10 mRNA was significantly decreased in the MHCC-97H xenograft tissue residues of baicalein treatment group. **c-d** Western blotting and

immunohistochemistry assay found that baicalein treatment reduced the expression of HDAC10, phosphorylated AKT and MDM2, and increased the expression of FoxO3a in MHCC-97H xenograft tumors. * $P < 0.05$ and ** $P < 0.01$. Values are shown as Means $\pm$ SD. Student's t-test was conducted. **e** Schematic diagram of the potential molecular mechanism of miR-3178 mediating the anti-cancer effect induced by baicalein.

Supplementary Files

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