Int J Clin Exp Pathol 2017;10(5):5712-5720
www.ijcep.com /ISSN:1936-2625/IJCEP0048042

Original Article

Targeted regulation of miR-218 on BIRC5 for hepatocellular carcinoma apoptosis and proliferation

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Received January 3, 2017; Accepted March 18, 2017; Epub May 1, 2017; Published May 15, 2017

Abstract: Baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) inhibits apoptosis via suppressing Caspase activity. Elevated BIRC5 is correlated with occurrence of hepatocellular carcinoma (HCC). Lower miR-218 expression occurs in HCC tissues, indicating it tumor suppressor role in HCC. Bioinformatics analysis showed satisfactory targeting relationship between miR-218 and 3'-UTR of BIRC5 mRNA. This study thus investigated the role of miR-218 in regulating BIRC5 expression and HCC pathogenesis. HCC tumor and adjacent tissues were tested for miR-218 and BIRC5 expression. Cultured MHCC97-H, MHCC97-L and HL-7702 cells were tested for Ki-67 expression and basal apoptosis, in addition to miR-218 and BIRC5 expressions. Dual luciferase reporter gene assay was used to confirm targeted regulation between miR-218 and BIRC5. *In vitro* cultured MHCC97-H cells were treated with miR-218 mimic and/or si-BIRC5. Cell apoptosis, proliferation and protein expression of BIRC and cleaved Caspase-3 were examined by flow cytometry, MTT and Western blot. Compared to adjacent tissues, HCC tissues had lower miR-218 and higher BIRC5 expression. Cultured HCC cells had higher BIRC5 and Ki-67 levels than normal cells, whilst miR-218 expression and basal apoptosis were decreased. MiR-218 targeted and suppressed BIRC5 expression. Transfection of miR-218 mimic and/or si-BIRC5 also decreased BIRC5 expression in HCC cells, decreased its inhibitory role on Caspase-3 activity, enhanced cell apoptosis and suppressed proliferation. MiR-218 is down-regulated in HCC tissues whilst BIRC5 expression is elevated. MiR-218 can enhance Caspase-3 activity via targeted inhibition on BIRC5 expression, thus potentiating Caspase-3 activity and facilitating HCC apoptosis whilst inhibiting proliferation.

Keywords: Hepatocellular carcinoma, microRNA-218, baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), cell apoptosis, cell proliferation

Introduction

Primary liver cancer is one common malignant tumor in clinics, and is the fifth popular cancer with third highest mortality, only next to pulmonary cancer and gastric carcinoma [1]. Inhibitor of apoptosis protein (IAPs) is one protein family with homologous structure and anti-apoptosis function [2]. Human baculoviral IPA repeated sequence includes baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), which also names as survivin as one novel member in IAPs family. BIRC5 is the most potent anti-apoptotic factor ever been discovered, as it can antagonize cell apoptosis via inhibiting apoptosis executing protein Caspase-3 and Caspase-7 [3]. Moreover, in contrast with other IAPs family members, BIRC5 has dual roles of antagonizing cell apoptosis and regulating cell cycle, as supported by facilitation of mitosis/proliferation via mediating cell cycle [4]. Elevated BIRC5 expression has been confirmed to be related with occurrence of multiple tumors including breast cancer [3], colon cancer [5] and pancreatic carcinoma [6]. Previous study showed significantly enhanced expression of BIRC5, which was closely correlated with tumor size, TNM stage, cell differentiation grade [7] and tumor proliferation index [8]. MicroRNA (miR) is one newly discovered non-coding single stranded RNA with 18-22 nucleotide acids in eukaryotes. Via complete or incomplete binding with target gene mRNA, miR can degrade mRNA or inhibit gene translation, thus playing important roles

multiple physiological processes including cell proliferation, differentiation, migration, apoptosis and cell cycle regulation, indicating the close correlation between their expression and function abnormalities and tumor occurrence [9]. Other scholars showed significantly depressed miR-218 expression in HCC tissues compared to adjacent tissues [10, 11], indicating its potential tumor suppressor role in HCC pathogenesis. Bioinformatics analysis showed satisfactory targeting complementary relationship between miR-218 and 3'-UTR of BIRC. Therefore, this study investigated if miR-218 played a role in regulating BIRC5 expression and apoptosis or proliferation of HCC cells.

Materials and methods

Reagent and material

High migrated liver cancer cell line MHCC97-H, low migrated liver cancer cell line MHCC97-L and normal human hepatocyte HL-7702 were provided by ATCC (US). DMEM culture medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (US). X-tremeGENE siRNA transfection reagent was purchased from Roche (US). ReverTra Ace qPCR RT Kit and SYBR Green dye were purchased from Toyobo (Japan). Mouse anti-BIRC5 antibody was purchased from Abcam (US). Mouse anti-cleaved Caspase-3 was purchased from CST (US). FITC labelled Ki-67 was purchased from BD Pharmigen (US). HRP labelled secondary antibody was purchased from Jackson ImmunoResearch (US). Annexin V/PI apoptosis kit was purchased from Youningwei Bio (China). Dual-luciferase reporter assay system and pGL3-promoter plasmids were all purchased from Promega (US).

Clinical information

A total of 52 HCC patients who received surgical resection of tumors in Shandong Provincial Hospital affiliated to Shandong University from December 2015 to June 2016 were collected. Tumor samples and adjacent tissues (>5 cm from tumor edge) were collected. There are 36 males and 16 females, aging between 36 and 65 years (average age =47.1 years). There were 13, 12, 15 and 12 cases at TNM stage I, II, III and IV. No patient received chemo-therapy, radio-therapy or other special treatment before treatment. Samples were snap-frozen in liquid nitrogen within 10 min, and were kept in -80°C for storage. All sample collections have obtained informed consents from patients. This study has been approved by the ethical committee of Shandong Provincial Hospital affiliated to Shandong University.

Cell culture

MHCC97-H, MHCC97-: and HL-7702 cells were kept in high glucose DMEM medium contining 10% FBS and 1% penicillin-streptomycin, in a culture chamber at 37°C with 5% CO_2. Culture medium was changed every two days. Cells at log-growth phase with good status were used in further experiments.

Dual luciferase reporter gene assay

Using HEK293 DNA as the template, full length fragment of 3'-UTR of BIRC5 gene was amplified. PCR products were collected from agarose gel, and were ligated into pGL-3M luciferase reporter plasmid after enzymatic digestion. Recombinant plasmid was then used to transform DH5α competent cells. Positive clones with primary screening were selected for further experiments. X-tremeGENE siRNA Transfection Reagent was used to co-transfect pGL3-BIRC5-3'UTR-wt (or pGL3-BIRC5-3'UTR-mut) and miR-218 mimic into HEK293 cells. After 48 h continuous incubation, dual-luciferase activity was examined. Oligonucleotide sequences used was: mimic NC, 5'-UUCUC CGAAC GUGUC ACGUU U-3'; miR-218 mimic, 5'-UUGUG CUUGA UCUAA CCAUG UAUGG UUAGA UCAAG CACAA UU-3'.

Experimental grouping and cell transfection

In vitro cultured MHCC97-H cells were divided into five groups, including scramble NC transfection group, miR-218 mimic transfection group, si-NC transfection group, si-BIRC5 group, and miR-218 mimic + si-BIRC5 group. Opti-MEM was used to dilute X-tremeGENE siRNA Transfection Reagent and oligonucleotide sequences. After 5 min culture, oligonucleotide fragments were gently mixed with X-tremeGENE siRNA Transfection Reagent. After 30 min room temperature incubation, the mixture was added into serum-free culture medium. 6 h later, original medium was replaced by normal culture medium containing serum. After 48 h continuous incubation, cells were collected for further assays. Oligonucleotide sequences used were: mimic NC,

5'-UUCUC CGAAC GUGUC ACGUU U-3'; miR-218 mimic, 5'-UUGUG CUUGA UCUAA CCAUG UAUGG UUAGA UCAAG CACA AUU-3'; si-BIRC5 sense strand, 5'-GCAUC UCUAC AUUCA AGAAdT dT-3'; si-BIRC5 anti-sense, 5'-UUCUU GAAUG UAGAG AUGCdT dT-3'; si-NC sense strand, 5'-UUCUC CGAAC GUGUC ACGUdT dT-3'; si-NC anti-sense strand, 5'-ACGUG ACACG UUCGG AGAAdT dT-3'.

qRT-PCR for gene expression assay

OMEGA test kit was used to extract total RNA. In brief, TKR Buffer was used to lyse cells, with twice centrifugation by Wash Buffer I and Wash Buffer II. RNA extracted was solved in RNase free water. Following manual instruction, ReverTra Ace qPCR RT Kit synthesize cDNA from RNA by reverse transcription. Using cDNA as the template, PCR amplification was carried under the function of TaqDNA polymerase. Primer sequences used were: miR-218P_F: 5'-AAGAC ACCCT GGACG AAGCC-3'; miR-218P_R: 5'-ACAAC CAGAG TCCAC CGGCG-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; BIRC5P_F: 5'-AGGAC CACCG CATCTC TACAT-3'; BIRC5P_R: 5'-AAGTC TGGCT CGTTC TCAGT G-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3'. In a 10 μL system, we added 4.5 μL SYBR Green Mixture, 1.0 μL forward primer, 1.0 μL reverse primer, 1.0 μL cDNA, and 2.5 μL ddH_2O. The reaction conditions were: 95°C for 5 min, followed by 95°C 5 min and 60°C 60 s.

Western blot

Total proteins were extracted and tested for quality and concentration. 60 μg protein samples were loaded and separated in 10% SDS-PAGE (3 h), and were transferred to PVDF membrane under wet condition (300 mA current, 90 min). The membrane was blocked in 5% defatted milk powder for 60 min, followed by primary antibody (anti-β-actin at 1:800, anti-BIRC5 at 1:300, or anti-cleaved caspase-3 at 1:100) incubation at 4°C overnight. By PBST washing (3 times), HRP-labelled secondary antibody (anti-mouse or anti-rabbit at 1:5,000 dilution) was added for 60 min incubation under room temperature. After PBST rinsing for three times, ECL reagent was added for 2~3 min dark incubation. The membrane was then exposure in dark and scanned for data analysis using Quantity One software.

Flow cytometry for cell apoptosis

Cells were collected in EDTA-free trypsin, and were washed twice in PBS. 100 μL Binding Buffer was used to re-suspend in 1~5×10^5 cells. The mixture was then mixed with 5 μL Annexin V-FITC and 5 μL PI, and was incubated in dark for 5~15 min. Beckman Coulter FC 500MCL flow cytometry was used to test cell apoptosis.

Flow cytometry for Ki-67 expression

Cells from all groups were collected and were rinsed twice in PBS containing 2% FBS. After fixation in 4% paraformaldehyde for 20 min, cells were treated using PBS containing 0.1% Triton X-100. FITC labelled Ki-67 antibody was added for 4°C dark incubation for 40 min, followed by twice rinsing in PBS containing 2% FBS. Cells were loaded for online testing in Beckman FC500MCL flow cytometry apparatus.

MTT assay for cell proliferation activity

MHCC97-H cells were seeded into 96-well plate at 1×10^4 per well density. After fully attached on the wall, cells were continuously cultured for 72 h. Cells at different time points (0 h, 24 h, 48 h and 72 h) were collected to test proliferation activity by MTT assay. In brief, 10 μl MTT solution was added into each test well. After 4 h continuous culture at 37°C, the original culture medium was discarded, with twice PBS washing. 150 μl DMSO was the added into each well for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 450 nm in a microplate reader.

Statistical analysis

SPSS 18.0 software was used for data analysis. Measurement data were presented as mean ± standard deviation (SD). Student t-test was used for comparison among groups. A statistical significance was defined when $P<0.05$.

Results

Lower miR-218 expression and higher BIRC5 expression in HCC tissues

qRT-PCR results showed significantly lower miR-218 expression in HCC tumor tissues, with

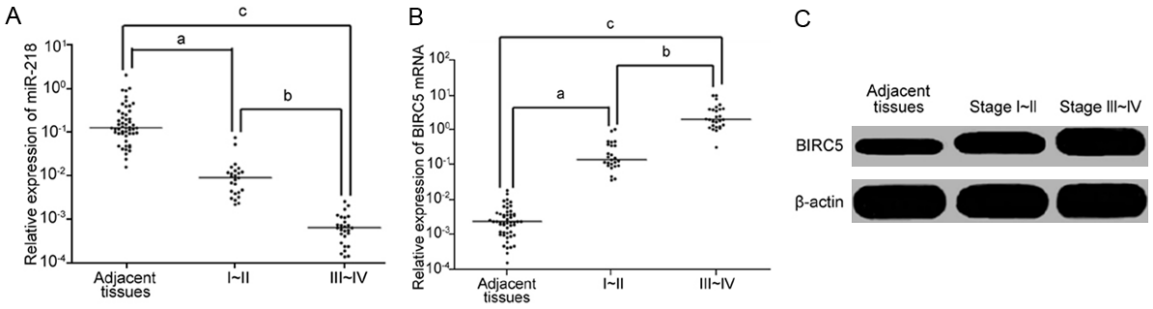

Figure 1. Increased miR-218 expression and decreased BIRC5 in HCC tissues. A. qRT-PCR for miR-218 expression; B. qRT-PCR for BIRC5 mRNA expression; C. Western blot for protein expression. a, P<0.05 comparing between stage I~II and adjacent tissues; b, P<0.05 comparing between stag III~IV; c, P<0.05 comparing between adjacent and stage III~IV tissues.

further decreased level in more advanced cases (**Figure 1A**). HCC tumor tissues also had significantly higher BIRC5 mRNA level than adjacent tissues in more advanced stage (**Figure 1B**). Western blot results showed that, compared to adjacent tissues, stage I~II HCC tumors had remarkably higher BIRC5 protein level, while stage III~IV HCC tumors had even higher expressions (**Figure 1C**).

BIRC5 as the target gene of miR-218

Flow cytometry assay showed significantly lower basal apoptosis rate in HCC cells compared to normal HL-7702 cells, with lowest basal apoptotic rate in highly-migrated cell line MHCC97-H (**Figure 2A**). Flow cytometry showed significantly higher proliferation ability in MHCC97-H cells compared to MHCC97-L cells, which had higher proliferation potency than HL-7702 cells (**Figure 2B**). Compared to normal HL-7702 cells, MHCC97-L and MHCC97-H cells had significantly lower miR-218 expression (**Figure 2C**) plus elevated BIRC5 expression (**Figure 2D**). These data indicated that miR-218 down-regulation probably played a role in up-regulating BIRC5 expression, antagonizing HCC cell apoptosis, potentiating proliferation ability and malignant phenotype. Bioinformatics analysis showed satisfactory targeted relationship between miR-218 and 3'-UTR of BIRC5 mRNA (**Figure 2E**). Dual luciferase reporter gene assay showed that elevated miR-218 expression could significantly suppressed relative luciferase activity in HEK293 cells transfected with wild type BIRC5-3'-UTR plasmid (**Figure 2F**), but had no significant effects on the relative luciferase activity of HEK293 cells transfected with mutant BIRC5-3'-UTR plasmid. Therefore, miR-218 targeted 3'-UTR of BIRC5 mRNA and inhibited its gene expression. After transfecting with miR-218 mimic, BIRC5 protein expression in MHCC97-H, MHCC97-L cells were all significantly inhibited, further demonstrating the targeted regulation of BIRC5 expression in HCC cells by miR-218 (**Figure 2G**).

Elevated miR-218 facilitated MHCC97-H cell apoptosis and inhibited proliferation

Using MHCC97-H as the research objects, transfection of miR-218 mimic significantly elevated miR-218 expression in MHCC97-H cells (**Figure 3A**) and decreased BIRC5 expression (**Figure 3A** and **3B**), significantly enhancing cleaved caspase-3 expression and cell apoptosis (**Figure 3C**) and weakening (**Figure 3D**). Transfection of BIRC5 siRNA significantly down-regulated BIRC5 expression in MHCC97-H cells, elevating cleaved caspase-3 expression, and facilitating cell apoptosis and inhibiting cell proliferation. Co-transfection of miR-218 mimic and si-BIRC5 had more potent effects for facilitating MHCC97-H cell apoptosis and inhibiting cell proliferation compared to those under single transfection scenario.

Discussion

As one of the most popular and deadly cancer in China, liver cancer has features including high malignancy, high metastatic rate, high recurrent rate and low survival rate. Therefore, the early discovery, diagnosis and treatment are of critical importance for improving patient's prognosis [12]. HCC is the most common pathological type in primary liver cancer, occupying more than 80% of all cases [13]. Currently sur-

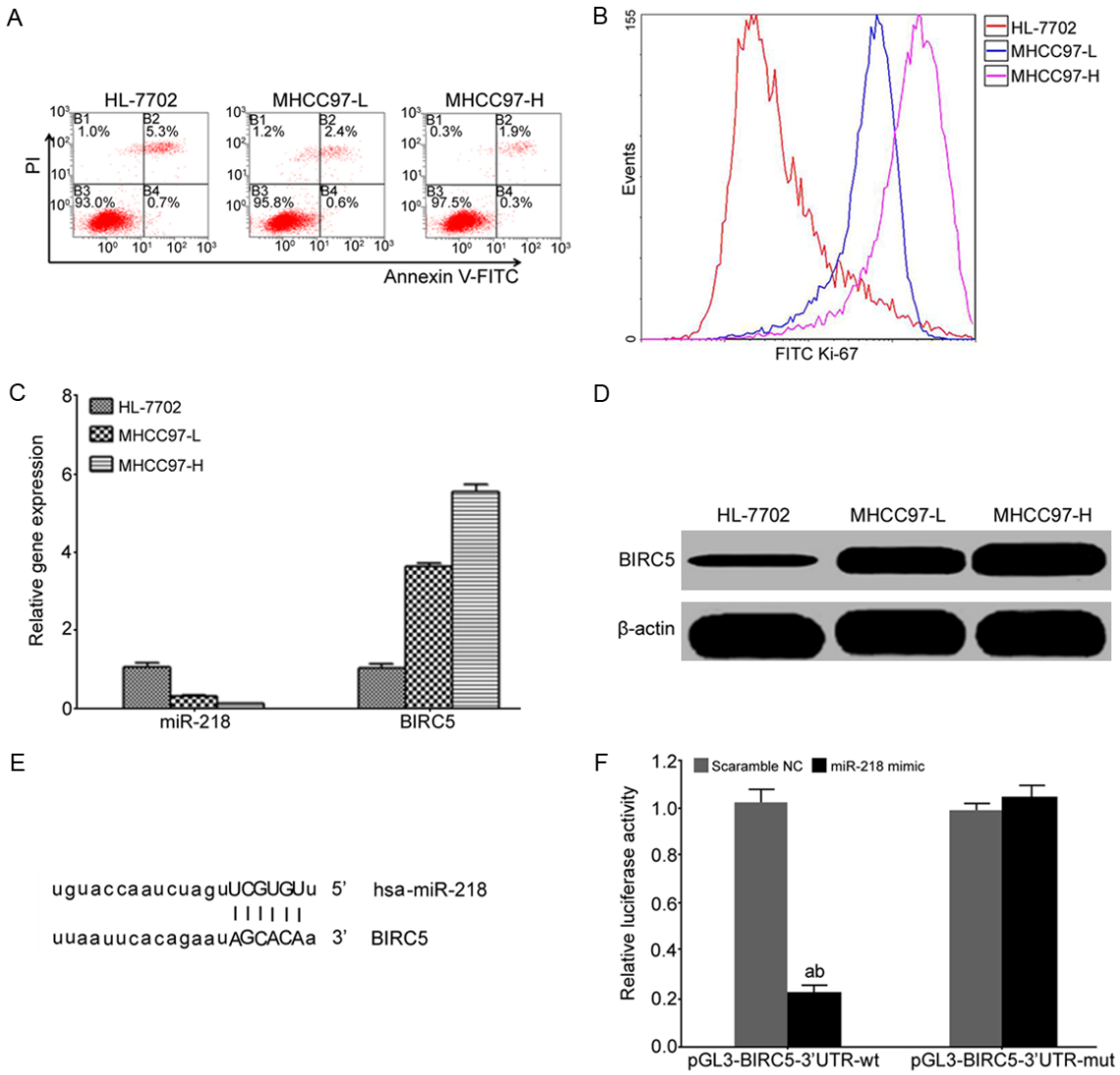

Figure 2. Targeted regulation of BIRC5 expression by miR-218 in U2OS cells. A. Flow cytometry for basal cell apoptotic rate; B. Flow cytometry for Ki-67 expression in all three cell lines; C. qRT-PCR for miR-218 and BIRC5 gene expression; D. Western blot for BIRC5 protein expression; E. Functional site between miR-218 and 3'-UTR of BIRC5 mRNA; F. Dual luciferase reporter gene assay; G. Western blot for BIRC5 protein expression in transfected cells. a, P<0.05 compared to scramble NC group; b, P<0.05 compared to pGL3-MMP-13-3-UTR-mut group.

gery is still the major approach for treating HCC. However, high metastatic rate, recurrent rate and drug resistant rate are all important factors affecting clinical treatment efficacy and patient's survival. Some studies showed that post-op recurrent rate of HCC is as high as 50%~60% [14]. Although combined treatment using surgery, radio/chemotherapy and immune therapy have made major advancement, the overall treatment efficacy was still unsatisfactory, as one-year and five-year survival rates were only 40% [15] and 10% [16], respectively.

Under the stimulus of apoptosis initiating factors, cysteine proteinase Caspase family members are sequentially activated in cascade manner, to cleave downstream apoptotic executing molecules and effector molecule for activating cell apoptotic signal pathway. As one of the most important members in Caspase

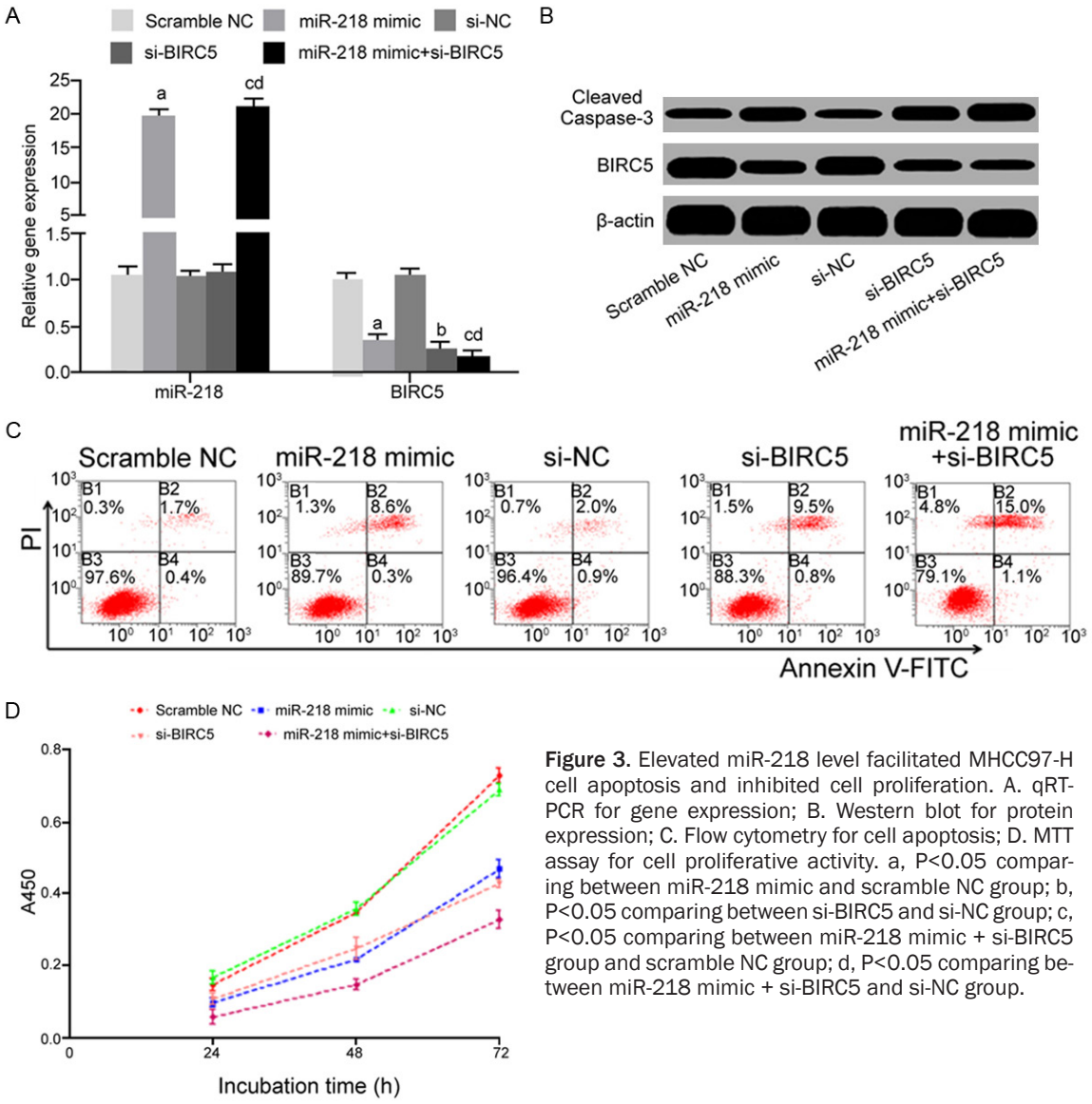

Figure 3. Elevated miR-218 level facilitated MHCC97-H cell apoptosis and inhibited cell proliferation. A. qRT-PCR for gene expression; B. Western blot for protein expression; C. Flow cytometry for cell apoptosis; D. MTT assay for cell proliferative activity. a, P<0.05 comparing between miR-218 mimic and scramble NC group; b, P<0.05 comparing between si-BIRC5 and si-NC group; c, P<0.05 comparing between miR-218 mimic + si-BIRC5 group and scramble NC group; d, P<0.05 comparing between miR-218 mimic + si-BIRC5 and si-NC group.

protein family, Caspase-3 plays an important role in mitochondrial or death receptor induced cell apoptosis signal transduction. Caspase-3 can directly degrade structural and functional proteins to induce cell apoptosis [17]. BIRC5 locates in human chromosome 17q25, has 14.7 Kb length containing 4 exons and 3 introns, and codes for a protein consisting of 142 amino acid residues [18]. BIRC5 has dual roles of inhibiting cell apoptosis and regulating cell cycle, and is the important factor connecting cell cycle and cell apoptosis. Such dual roles are critical for facilitating malignant tumor progression and inducing drug resistance. BIRC55 is the most potent anti-apoptotic factor ever been identified in IAPs family, and can directly inhibit Caspase-3 and Caspase-7 enzymatic activities in apoptosis signal pathway to block the end path of apoptosis signal transduction pathway. Such inhibitor function relied on specific binding between BIRC5 and active Caspase-3 and Caspase-7 for inactivation, in addition to the direct inhibition of spontaneous activation of Caspase-3 and Caspase-7 [19]. BIRC5 can bind with cyclin dependent kinase 4 (CDK4) at G2/M phase to form BIRC5/CDK4 complex, releasing p21 from such complex and re-locating into mitochondria for form complex with pro-Caspase-3 for inhibiting Caspase-3 activity and antagonizing apoptosis at G2/M phase [20]. Ikeguchi et al showed higher positive rate of BIRC5 in HCC tumor tissues com-

pared to adjacent tissues [21]. Fan et al also showed significantly higher BIRC5 expression level in HCC tumor tissues compared to adjacent tissues [22]. Jin et al showed remarkably higher BIRC5 expression in HCC tumor tissues, with close correlation with tumor size, peripheral infiltration, lymph node metastasis and clinical stage [23]. In patients with higher expression, their survival rates are lower with shorter median survival period. Fields et al also found the relationship between significantly elevated BIRC5 in HCC tissues with advanced pathological grade, high proliferation index, vascular invasion, high recurrence and low survival rate [24]. This study showed significantly elevated BIRC5 expression in HCC tumor tissues, with higher expression level in more advanced stage, as consistent with Fan et al [22] and Jin et al [23]. Ito performed a study and showed higher BIRC5 expression level in HCC cells compared to normal hepatocytes [25]. This study also observed higher BIRC5 expression in MHCC97-H and MHCC97-L cells compared to normal hepatocytes, as reported by Ito et al [25].

Tu et al showed that, compared to adjacent tissues, HCC tumor tissues had significantly lower miR-218 expression [26]. Lower miR-218 level indicated lower 5-year survival rates. MiR-218 expression thus may work as one independent factor affecting survival and prognosis. Yang et al showed correlations between lower miR-218 in HCC tumor tissues with lesion size, vascular invasion and clinical stage [10], with even higher diagnostic values for differentiating between HCC and benign liver lesions, and between HCC and normal controls. This study showed significantly lower miR-218 level in HCC tissues compared to adjacent tissues, as consistent with Tu et al [26] and Yang et al [10]. Dong et al showed lower miR-218 expression in liver cancer cells compared to normal hepatocytes [11]. Tu et al showed lower miR-218 expression in liver cancer cells HepG2 and SMMC-7721 compared to those in normal hepatocyte L02 [26]. This study compared liver cancer cells and normal liver cells and found lower miR-218 expression leve, indicating possible involvement of miR-218 down-regulation in HCC pathogenesis. Dual luciferase gene reporter assay showed that transfection of miR-218 mimic significantly depressed relative luciferase activity in HEK-293 cells, demonstrating that BIRC5 is one target gene of miR-218 under its regulation. Further test results showed that transfection of miR-218 mimic and/or si-BIRC5 all significantly depressed BIRC5 expression in HCC cells, suppressing the inhibitory effects on Caspase-3 activity, increasing cell apoptosis and weakening proliferation potency. Liu et al showed that interference of BIRC5 expression significantly inhibited proliferation ability of HepG2 and SMMC-7221 cells, and induced cell cycle arrest at G0/G1 phase to facilitate cell apoptosis, thus potentiating sensitivity of tumor cells against chemotherapy agent cisplatin [27]. Or et al showed PLCk3 liver cancer cells with knockout of BIRC5 gene had significantly lower proliferation ability, more arresting at S phase, and potentiating cisplatin drug sensitivity [8]. Fan et al reported that melatonin could down-regulate BIRC5 expression in liver cancer cell lines HepG2 and SMMC-7721 and facilitate their apoptosis. These studies all revealed the role of inhibiting BIRC5 expression in decreasing malignant phenotype of liver cancer cells as similar to results of this study. Dong et al showed over-expression of miR-218 could significantly inhibit E2F2 gene and protein expression in HCC cells, thus inducing cell arrest at G0/G1 phase for weakening their proliferation potency [11]. Tu et al showed that over-expression of miR-218 could inhibit HCC cell proliferation, induce cell apoptosis and cell cycle arrest via targeting BMI-1, and further slowing growth speed of tumors inside model animals [26]. This study revealed the role of miR-218 down-regulation in facilitating HCC pathogenesis from the perspective of targeted regulation on BIRC5 expression by miR-218, and provided evidences for study of anti-tumor effects of miR-218. However, it is still unclear for the down-regulated miR-218 in liver cancer tissues, probably due to genetic polymorphism at promoter region of pre-miR-218 gene [28].

Conclusion

HCC tissues have significantly lower miR-218 expression, accompanied with elevated BIRC5 expression. MiR-218 targets and inhibits BIRC5 expression, potentiates Caspase-3 activity, and facilitates HCC cell apoptosis to inhibit their proliferation.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 81373172).

Disclosure of conflict of interest

None.

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