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microRNA-608 inhibits human hepatocellular carcinoma cell proliferation via targeting the BET family protein BRD4

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ABSTRACT

Over-expression of the bromodomain and extraterminal (BET) family protein BRD4 is associated with hepatocellular carcinoma (HCC) progression. In the present study, we indentified a novel putative anti-BRD4 microRNA: microRNA-608 ("miR-608"). In HepG2 cells and primary human HCC cells, over-expression of miR-608, using a lentiviral construct, induced BRD4 downregulation and proliferation inhibition. Conversely, transfection of the miR-608 inhibitor increased BRD4 expression to promote HepG2 cell proliferation. Our results suggest that BRD4 is the primary target gene of miR-608 in HepG2 cells. shRNA-mediated knockdown or CRSIPR/Cas9-mediated knockout of BRD4 mimicked and overtook miR-608's actions in HepG2 cells. Furthermore, introduction of a 3′-untranslated region (3′-UTR) mutant BRD4 (UTR-A1718G) blocked miR-608-induced c-Myc downregulation and proliferation inhibition in HepG2 cells. *In vivo*, HepG2 xenograft tumor growth was significantly inhibited after expressing miR-608 or BRD4 CRSIPR/Cas9-KO construct. Importantly, BRD4 mRNA was upregulated in human HCC tissues, which was correlated with downregulation of miR-608. Together, we conclude that miR-608 inhibits HCC cell proliferation possibly via targeting BET family protein BRD4.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and second leading cause of cancer-associated human mortalities [1,2]. The current curative treatments, including HCC resection, liver transplantation, local ablation, can be only applied to a very limited proportion of HCC patients [3,4]. Identification of novel biomarkers for HCC prevention and treatment is desperately needed [3,5].

At least four bromodomain and extraterminal (BET) family proteins have been identified, including Bromodomain-containing protein (BRD)2, BRD3, BRD4 and the testis-specific isoform BRDT [6,7]. BRD4 is one abundant and primary BET family protein [8–10]. Existing studies have shown that BRD4 binds to acetylated-histones, acting as an epigenetic regulator [8–11]. In the process of mitosis, BRD4 regulates chromatin structure in the daughter cells [8–10]. Additionally, BRD4 recruits P-TEFb (the positive transcription elongation factor b), and phosphorylating RNA polymerase II, which are essential for transcription elongation and mRNA transcription [10]. Many BRD4-dependent genes, including *Bcl-2*, *c-Myc* and *cyclin D1*, are oncogenic and pro-cancerous [8–13]. It has been previously shown that BRD4 expression level is upregulated in human HCC, which is associated with cancer cell progression [14–16]. microRNAs can silence targeted mRNAs via binding to 3′-untranslated region (UTR) [17–19]. In the current study, we identified a putative anti-BRD4 microRNA: microRNA-608 ("miR-608"). Our results show that miR-608 expression inhibits human HCC cell proliferation possibly via targeting BRD4.

2. Methods

Chemicals and reagents. The antibodies were all purchased from Cell Signaling Tech (Beverly, MA). The reagents for cell culture were provided by Hyclone Co. (Logan, Utah). All other reagents were provided by Sigma-Aldrich (St Louis, Mo) unless mentioned

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Cell culture. HepG2 human HCC cells, provided by the Cell Bank of Chinese Academy of Biological Science (Shanghai, China), were maintained in RPMI-1640 medium plus 10% FBS. For culture of primary human HCC cells, the surgical-isolated human HCC tissues were digested via collagenase I and hyaluronidase. Afterwards, the cell suspensions were washed, filtered through a 75-μm nylon cell strainer, and seeded onto collagen-coated Petri dishes. The fibroblasts and blood vessels were abandoned. The primary cancer cells were cultured in the medium for the primary human cells, as previously described [20]. The primary cells at passage 3–10 were utilized for *in vitro* experiments. All investigations of primary human cells were according to Declaration of Helsinki. The procedures were approved by the Ethics Review Board (ERB) of Nanjing Medical University.

Human tissues. Eight pairs of human HCC tissues and adjacent non-tumor liver tissues were obtained from patients undergoing HCC hepatectomy. The tissue samples were collected between October 2017 and February 2018 at the First Affiliated Hospital of Nanjing Medical University, after informed consent and ERB approval. Patients enrolled received no prior chemotherapy and radiotherapy before surgery. Tissues were minced into small pieces, which were incubated with the tissue lysis buffer (Beyotime Biotechnology, Wuxi, China). Tissue lysates were then stored in liquid nitrogen for further analysis.

Quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) assay. RNA was extracted by using the miRNeasy Mini Kit (QIAGEN) with DNase I (QIAGEN, Shanghai, China) digestion. First-Strand Synthesis Kit (SABiosciences, Frederick, MD) was applied for cDNA synthesis. The qRT-PCR was performed in Quant studio 3 (Applied Biosystems, Shanghai, China) using the SYBR GREEN kit (TaKaRa, Beijing, China). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* was tested as the internal control. The relative expression of target mRNA was calculated by the $2^{-\Delta\Delta Ct}$ method. The mRNA primers for *BRD4*: 5′-ACCTCCAACCCTAACAAGCC-3′ and 5′-TTTCCATAGTGTCTTGAGCACC-3′, the mRNA primers for *GAPDH*: 5′-GCACCGTCAAGGCTGAGAAC-3′ and 5′-TGGTGAAGACGCCAGTGGA-3′ were synthesized by Genepharma (Shanghai, China). The primers for detecting miR-608: F: 5′-AGGGGTGGTGTTGGGACAGCTCCGT-3’; Probe: 5′-ACGGAGCTGTCCCAACACCACCCCT-3’. miR-608 expression was normalized to *U6 mRNA* using the descried primers [21].

Expression of miR-608. miR-608 was amplified using the described sequence [21], inserting into the lentiviral PGLV3/U6/GFP/Puro vector (“LV-miR-608”, Genepharma, Shanghai, China). LV-miR-608 was transfected to HCC cells. Puromycin (2.5 μg/mL, Sigma) was added to select the stable cells for 4 passages. Expression of miR-608 in stable cells was tested by qRT-PCR assay. Control cells were infected with lentiviral PGLV3/U6/GFP/Puro control vector with non-sense microRNA sequence (“miR-C”).

miR-608 inhibition. The miR-608 inhibitor oligo (5′-ACGAGCUGUCCCAACACCACCCCU-3′, “antagomiR-608”, 500 nM) and the inhibitor negative control oligo (5′-CAGUACUUUUGUGUAGUACAA-3′, “antagomiR-C”, 500 nM) were transfected to HCC cells via Lipofectamine 2000 (Invitrogen, Shanghai, China). miR-608 knockdown was verified via qRT-PCR assay.

Clonogenicity assay. HepG2 cells were plated onto the six-well plates at 5000 cells per well. The cell culture medium was renewed every two days for a total 10 days. The number of remaining HepG2 colonies was counted manually.

BrdU assay of proliferation. HepG2 cells were plated onto the twenty-four well tissue culture plates at 10,000 cells per well. HCC cell proliferation was tested by BrdU incorporation, which was analyzed by a commercial available BrdU ELISA kit (Cell Signaling Tech). BrdU ELISA optical density (OD) value at 450 nm was recorded.

Western blotting assay. The protein lysates (30 μg per treatment of each lane) were separated by 10–12% SDS-PAGE gels. Protein samples were then transferred to PVDF membrane (Millipore, Wuxi, China). Each protein membrane was blocked in PBST with 10%-milk, incubating with the designated primary and secondary antibodies. Signal detection was performed via ECL reagents (Roche, Shanghai, China). Image J software (NIH) was utilized to quantify the protein band.

***BRD4 mRNA* luciferase assay.** The 3′-UTR of BRD4 with the putative miR-608 binding site (position 1715–1722) was inserted into the pmIR-ReporterTM firefly luciferase reporter vector (provided by Dr. Cao [22]). The construct was co-expressed with miR-608 in HepG2 cells, and luciferase activity was measured using a luciferase assay kit (Promega, Shanghai, China). Its activity was quantified on a luminescence microplate reader LUMIstar Galaxy [22]. The luciferase activity was normalized to β-galactosidase (as the internal standard).

BRD4 shRNA. BRD shRNA lentivirus was provided by Santa Cruz Biotech (sc-43639-V, Shanghai, China), which was added to HCC cells for 48 h. Puromycin (2.5 μg/mL, Sigma) was added to select the stable cells for 4 passages. BRD4 knockdown in stable cells was confirmed by Western blotting assay.

CRISPR/Cas9 knockout of BRD4. The BRD4-lentiCRISPR/Cas9-GFP-KO plasmid was provided by Dr. Zhao [23], which was transfected to HepG2 cells, followed by puromycin selection [24]. GFP-positive cells were thereafter FACS-sorted, and subjecting BRD4 knockout screening.

BRD4 3′UTR mutation. An *in vitro* site-directed mutagenesis system was utilized to generate a miR-608-binding 3′UTR-mutant BRD4 (BRD4-UTR-A1718G, Flag-tagged) by Genepharma (Shanghai, China). The construct was sub-cloned into the GV248 lentiviral vector, which was transfected to HepG2 cells. Stable cells were selected by puromycin for 48 h. Expression of the mutant BRD4 (Flag-tagged) in stable cells was confirmed by the Western blotting assay.

Xenograft assay. CB17 severe combined immunodeficiency (SCID) mice (5–6 week old, 18 g weight, all female) were purchased from Nanjing Medical University Laboratory Animal Centre (Nanjing, China). Mice were maintained in a 12-h light/12-h dark cycle with free access to food/water. For each mouse, six million (6×10^6) HepG2 cells were injected subcutaneously (*s.c.*) to the left flanks. Within 2–3 weeks, the volume of each tumor was close to 100 mm^3. The bi-dimensional tumor volumes were measured every 6 days. Tumor volume was measured as described [25]. The animal procedure was in accordance with the Institute's Animal Care and Use Committee (IACUC) of Nanjing Medical University.

Statistical analysis. The statistical significance of differences among groups were determined by one-way analysis of variance (ANOVA) followed by the Tukey's post hoc multiple comparison tests. $\boldsymbol{p} < 0.05$ was considered significant.

3. Results

3.1. Over-expression of miR-608 downregulates BRD4, inhibiting HepG2 cell proliferation

It has been shown that BRD4 expression level is elevated in human HCC, which is important for HCC cell progression [14–16]. The current study aims to indentify an anti-BRD4 microRNA. The miRNA database TargetScan Human (V7.1) was consulted. Screening results show that microRNA-608 (“miR-608”) could be an anti-BRD4 microRNA (Fig. 1A). miR-608 putatively targets 3′ UTR (at position 1715–1722) of *human BRD4* (Fig. 1A), showing an

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