Biochemical and Biophysical Research Communications 380 (2009) 205–210

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MicroRNA-15b regulates cell cycle progression by targeting cyclins in glioma cells

Hongping Xia [a,1], Yanting Qi [b,1], Samuel S. Ng [a], Xiaona Chen [b], Shen Chen [c], Marong Fang [a,d], Dan Li [b], Yu Zhao [b], Ruiguang Ge [c], Guo Li [c], Yangchao Chen [e], Ming-Liang He [e], Hsiang-fu Kung [b,c,e], Lihui Lai [b,*], Marie C. Lin [a,b,*]

[a] Integrative Chemical Biology Laboratory, Institute of Molecular Technology, Department of Chemistry, 8/F, Kadoorie Biological Science Building, The University of Hong Kong, Pokfulam Road, Hong Kong, China
[b] Institute of Molecular and Chemical Biology, East China Normal University, Shanghai 200062, China
[c] The Laboratory of Integrative Biology, College of Life Sciences, Sun Yat-Sen University, Guangzhou, China
[d] Institute of Cell Biology, Zhejiang University School of Medicine, Hangzhou 310058, China
[e] Stanley Ho Center for Emerging Infectious Diseases, The State Key Laboratory in Oncology in South China, The Chinese University of Hong Kong, Shatin, Hong Kong, China

ARTICLE INFO

Article history:
Received 21 December 2008
Available online 9 January 2009

Keywords:
MicroRNA
MiR-15b
Cell cycle
CCNE1
Glioblastoma

ABSTRACT

MicroRNAs (miRNAs) are non-protein-coding RNAs that function as post-transcriptional gene regulators. Recent evidence has shown that miRNA plays a pivotal role in the development of many cancers including glioma, a lethal brain cancer. We have recently compared the miRNA expression profiles between normal brain and glioma tissues from Chinese patients by miRNA microarray and identified a panel of differentially expressed miRNAs. Here, we studied the function of one miRNA, miR-15b, in glioma carcinogenesis and elucidated its downstream targets. Over-expression of miR-15b resulted in cell cycle arrest at G0/G1 phase while suppression of miR-15b expression resulted in a decrease of cell populations in G0/G1 and a corresponding increase of cell populations in S phase. We further showed that CCNE1 (encoding cyclin E1) is one of the downstream targets of miR-15b. Taken together, our findings indicate that miR-15b regulates cell cycle progression in glioma cells by targeting cell cycle-related molecules.

The most common and fatal form of brain cancer, malignant glioma, occurs in the glia cells. Because these supporting cells are diffusely distributed in the brain, glioma is highly resistant to chemotherapy and radiotherapy. It is an aggressive, highly invasive, and neurologically destructive tumor considered to be among the deadliest of human cancers. In its most aggressive manifestation, glioblastoma multiforme (GBM, WHO grade IV), median survival ranges from 12 to 15 months, despite maximum treatment efforts [1,2]. About 20,000 people are diagnosed with glioblastoma in the United States each year. In addition, several research groups have reported an unexpected increase in the occurrence of glioblastomas and anaplastic astrocytomas in young Chinese in China, Taiwan, and Hong Kong [3–6]. Therefore, discovery of critical carcinogenic pathways and identification of new therapeutic targets for gliomas are crucial for global and local public health.

MicroRNAs (miRNAs) are a group of short (approximately 22-nt) non-coding RNAs that regulate target mRNAs and hence control basic cellular functions including proliferation, differentiation, and death [7]. MiRNAs are initially transcribed as primary precursor molecules (pri-miRNA) which are several hundred to thousands of nucleotides long. All pri-miRNAs contain at least one characteristic hairpin structure; these hairpins are recognized by a protein complex containing the nuclear RNase enzyme Drosha and processed into approximately 70-nt hairpin precursors known as pre-miRNAs. The pre-miRNAs are then exported to the cytoplasm, where the hairpin structure is recognized by a complex containing the RNase Dicer, which finally processes the pre-miRNA into the mature effector 22-nt miRNA molecule [8].

Emerging evidence has strongly suggested that abnormal miRNA expression is a common and important feature of human malignancies [7,9]. As one miRNA has the potential to regulate hundreds of mRNAs, it is conceivable that miRNAs are important regulatory molecules. Consistently, recent findings have suggested that miRNAs not only are important biomarkers, but also might be promising therapeutic targets for various diseases [10]. In gliomas, several miRNAs have been identified and shown to play a pivotal role in cancer progression [11–16]. To study the function of miRNAs in glioma carcinogenesis, we used miRNA microarray to identify a panel of differentially expressed miRNAs in glioma tissues as compared to normal brain tissues from Chinese patients. We then

* Corresponding authors. Fax: +86 21 62237226 (L. Lai); +852 28171006 (M.C. Lin).
E-mail addresses: lhlai@bio.ecnu.edu.cn (L. Lai), mcllin@hkusua.hku.hk (M.C. Lin).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.169

characterized the function of one significantly deregulated miRNA, miR-15b, and demonstrated that it can regulate the cell cycle distribution and proliferation of human gliomas cells by regulating its downstream cell cycle-related targets.

Materials and methods

Cell lines and cell culture, reagents. Human gliomas cell lines (U87, U373, U138, U118, SW1783, and SW1088) were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in MEM (U87, U373, and U138), DMEM (U118), or Leibovitz's L-15 medium (SW1783 and SW1088) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 U/mL). MiR-15b mimics (sense: 5′-UAGCAGCACAUCAUGGUUUACA-3′, antisense: 5′-UAAA CCAUGAUGUGC-UGCUGUU-3′), mimics control (sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense: ACGUGAC ACGUUCGGAGAA TT-3′), and miR-15b inhibitor (5′-UGUAAACCA UGAUGUGCUGCUA-3′), inhibitor control (5′-UUGUACUACA-CAA AAGUACUG-3′) were synthesized and purified by GenePharma Co. (Shanghai, China). All antibodies were bought from Santa Cruz Biotechnology (Santa Cruz, CA). NCode™ miRNA First-Strand cDNA Synthesis and qRT-PCR Kits (Cat. No. MIRQER-100) and SuperScript First-Strand Synthesis System for RT-PCR kit (Cat. No. 11904-018) were purchased from Invitrogen (Carlsbad, CA). Dual-Luciferase Reporter Assay System was bought from Promega Corporation. All primers are synthesized from Sigma–Proligo (The Woodlands, TX, USA).

Real-time RT-PCR. To quantitate miRNA expression, the isolated total RNA was polyadenylated and reverse transcribed for use in a two-step quantitative RT-PCR using the NCode miRNA First-Strand Synthesis and qRT-PCR kits (Invitrogen). The resulting cDNA was subjected to real-time qRT-PCR using the NCode universal reverse primer in conjunction with a sequence-specific forward primer for has-miR-15b (TAGCAGCACATCATGGTTTACA, 22 bps, GC = 40.92%, Tm = 56.7). A master mix was prepared for each PCR, which included SYBR GreenER™ qPCR SuperMix, Forward primer, Universal qPCR Primer, ROX reference dye and template cDNA. Let-7a was used for the standard curves. The reactions were monitored using a preheated real-time instrument (ABI 7900HT). The PCR conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 58 °C for 60 s and 95 °C for 15 s.

Flow cytometry. One day before transfection, equal numbers of U87 or U118 cells (2.0×10^5) were seeded into 6-well tissue culture plates without antibiotics. The cells were then transfected with 50 nM of various synthetic miRNAs (miR-15b mimics or mimics control for U87; miR-15b inhibitor or inhibitor control for U118) using Lipofectamine2000 (Invitrogen) or X-tremeGENE siRNA transfection reagent (Roche). Forty-eight hours after transfection, the transfected cells were fixed in ice-cold 70% ethanol and stained with the use of Coulter DNA-Prep Reagents kit (Beckman Coulter, Fullerton, CA). Cellular DNA content of 5×10^5 cells from each sample was determined by the EPICS ALTRA flow cytometer (Beckman Coulter). Cell cycle phase distribution was analyzed with the use of ModFit LT 2.0 software (Verity Software House, Topsham, ME) using data obtained from two separate experiments in which each transfection was performed in triplicate.

MTT assay. One day before transfection, 5.0×10^3 U87 or U118 cells in 80 μl growth medium were plated in each well of a 96-well plate. The cells were then transfected with 50 nM of various synthetic miRNAs (miR-15b mimics or mimics control for U87; miR-15b inhibitor or inhibitor control for U118) using Lipofectamine2000 (Invitrogen) or X-tremeGENE siRNA transfection reagent (Roche). At different time points (24 h, 48 h, and 72 h), the culture medium was removed and replaced with culture medium containing 10 μl of sterile MTT dye (5 mg/ml) to each well. The cells were then incubated for 4 h at 37 °C. After incubation, the MTT solution was removed, 150 μl of DMSO was added and thoroughly mixed for 10 min. Spectrometric absorbance at 570 nm was measured on a microplate reader.

Semi-quantitative RT-PCR. U87 or U118 cells were transfected with 50 nM has-miR-15b-mimics or miR-15 inhibitor as mentioned above. Forty-eight hours after transfection, total RNA was isolated from the cells with TRIZOL Reagent ((Invitrogen, Carlsbad, CA). RT-PCR was done using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen). Platinum® Taq DNA Polymerase (Invitrogen) PCR conditions were as follows: 2 min at 94 °C, followed by 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min for 28 cycles (CCND1, GAPDH) or 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min for 30 cycles (CCNE1). The sequences of the primer pairs were: CCND1: sense 5′-GATGCCAACCTCCTCAACGA-3′ and antisense 5′-CACTTCTGTTCCTCGCAG ACC-3′, CCNE1: sense 5′-AGCGGTAAGAAG CAGA-GCAG-3′ and antisense CGCTGCAACAGACAGAAGAG, GAPDH: sense 5′-TGCCTCCTGCACCACCA ACT-3′ and antisense 5′-CCCG TTCAGCTCAGGGATGA-3′.

Western blotting. U87 or U118 cells were transfected with 50 nM miR-15b mimics or miR-15 inhibitor as mentioned above. Forty-eight hours after transfection, the cells were washed twice with PBS and solubilized in radioimmunoprecipitation assay lysis buffer. The supernatants, which contained the whole-cell protein extracts, were obtained after centrifugation of the cell lysates at 12,000*g* for 10 min at 4 °C. The protein concentration was determined by

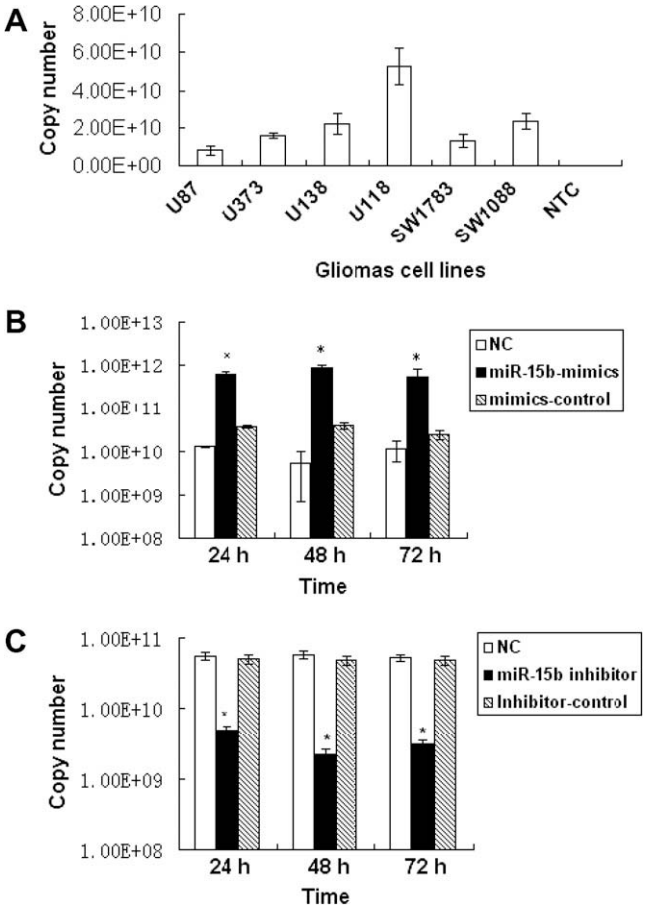

Fig. 1. (A) Quantitative PCR of miR-15b expression in six glioblastoma cell lines. The experiment was performed three times, each in triplicate. (B,C) miR-15b expression after transfection with miR-15b mimics in U87 cells or miR-15b inhibitor in U118 cells. miR-15b expression levels were detected at various time points (24 h, 48 h, and 72 h) after transfection. NC refers to the non-treated cells. $^*P < 0.05$, statistically significant difference between miR-15b mimics and NC (or mimics control), or between miR-15b inhibitor and NC (or inhibitor control).