MicroRNA-423 promotes cell growth and regulates G1/S transition by targeting p21Cip1/Waf1 in hepatocellular carcinoma

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MicroRNAs (miRNAs) are small non-coding RNA molecules that are often located in genomic breakpoint regions and can act as oncogenes or tumor suppressor genes in human cancer. Our previous study showed that microRNA-423 (miR-423), which localized to the frequently amplified region of chromosome 17q11, was upregulated in hepatocellular carcinoma (HCC). However, the potential functions and exact mechanistic roles of miR-423 in hepatic carcinogenesis remain unknown. Here, we demonstrated that miR-423 significantly promotes cell growth and cell cycle progression at the G1/S transition in HCC cells. In particular, we found that miR-423-3p contributes to these effects, whereas miR-423-5p does not. Further studies revealed that p21Cip1/Waf1 is a downstream target of miR-423 in HCC cells, as miR-423 bound directly to its 3′ untranslated region and reduced both the messenger RNA and protein levels of p21Cip1/Waf1. Moreover, enforced expression of p21Cip1/Waf1 abrogated miR-423-induced effects on HCC cell proliferation and cell cycle progression. These findings indicate that miR-423 exerts growth-promoting effects in hepatic carcinogenesis through the suppression of tumor suppressor p21Cip1/Waf1 expression. The results of this study define miR-423 as a new oncogenic miRNA in HCC.

Introduction

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules that function as critical posttranscriptional regulators of gene expression (1,2). miRNA genes are initially transcribed as long primary transcripts (pri-miRNAs) that are subsequently cleaved by Drosha into shorter hairpin-shaped precursor miRNAs (pre-miRNAs). The pre-miRNAs are then exported to the cytoplasm, where they are further processed into ~22 bp mature miRNAs by Dicer. Mature miRNAs associate with the RNA-induced silencing complex (RISC) and regulate gene expression primarily through binding to the 3′ untranslated regions (UTRs) of messenger RNAs (mRNAs). This binding results in either translational inhibition or degradation of the target transcripts (3). In human cells, there are ~1000 miRNAs that collectively regulate the expression of >60% of protein-coding genes (4). Thus, miRNAs constitute one of the largest human regulatory gene families. Increasing evidence shows that miRNAs play important roles in a variety of biological processes (5). Meanwhile, deregulation of miRNAs has been observed in a wide range of human diseases, including cancer (6). In human cancer, miRNAs are frequently located in genomic breakpoint regions and can act as oncogenes or tumor suppressor genes during tumor development and progression (7).

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Despite great advances in HCC treatment, the 5 years survival rate of HCC patients still is quite low, and HCC remains the third leading cause of cancer death (8). The risk factors for HCC, such as hepatitis B virus or hepatitis C virus infection, aflatoxin B1 intake and alcohol abuse, are well documented (9). However, the molecular pathogenesis of HCC is complicated and remains to be elucidated in a comprehensive manner. Knowledge about the molecular mechanisms underlying hepatic tumorigenesis is crucial to developing effective therapeutic strategies for HCC patients. In the past decades, studies have focused on investigating the genes and proteins underlying the development and progression of HCC (10). Recently, an increasing number of reports have shown that miRNAs play important roles in HCC progression, providing new avenues for HCC diagnostic and therapeutic application (11). In our previous study, we analyzed extensively the common recurrent chromosome aberration regions and associated specific miRNAs in HCC. We identified 22 miRNAs that are often subject to chromosome gain or loss at the genomic level (12). Of these, miR-423, which localizes to the frequently amplified region of chromosome 17q11, is upregulated in HCC tissues as compared with non-cancerous tissues. However, the potential functions and exact mechanistic roles of miR-423 remain elusive.

In this study, we found that miR-423 significantly promoted HCC cell growth and cell cycle progression at G1/S transition, mainly through miR-423-3p, but not through miR-423-5p. Further studies revealed that miR-423 downregulates both the mRNA and protein levels of p21Cip1/Waf1 expression by directly targeting its 3′ UTR. Moreover, enforced expression of p21Cip1/Waf1 blocked miR-423-induced effects on HCC cell proliferation and cell cycle progression, indicating that p21Cip1/Waf1 is a direct functional target of miR-423 in HCC cells.

Materials and methods

Cell culture

HEK-293T and HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium medium with 10% fetal bovine serum. SNU-449 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum.

Lentivirus production and infection

The pri-miR-423 sequences and open-reading frame of p21Cip1/Waf1 were amplified and cloned into the pWPXL lentiviral vector (a gift from Dr Didier Trono). The primer sequences are shown in supplementary Table S1, available at Carcinogenesis Online. The pWPXL vectors were transduced into HEK-293T cells with the packaging plasmid psPAX2 and the VSV-G envelope plasmid pMD2.G (a gift from Dr Didier Trono) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The virus particles were harvested 48 h after transfection. Cells (1 × 10⁶) were infected with 1 × 10⁵ recombinant lentivirus-transducing units plus 6 μg/ml Polybrene (Sigma, Natick, MA).

Cell proliferation and colony formation assays

Cell proliferation was measured using the CCK-8 assay kit (Dojindo Corporation, Kumamoto, Japan). Cells were plated into each well of a 96-well plate and 10 μl of CCK-8 was added to 90 μl of culture medium at the indicated time. Subsequently, the cells were incubated at 37°C for 2 h and the optical density was measured at 450 nm. For the colony formation assay, 1000 cells were placed in each well of a six-well plate and incubated at 37°C for 2 weeks. Colonies were fixed and stained in a dye solution containing 0.1% crystal violet and 20% methanol, and the number of colonies was counted.

FACS analysis

Cells were collected and fixed in ice-cold 70% ethanol overnight. The fixed cells were washed with phosphate-buffered saline and stained with a fresh-prepared solution containing 25 μg/ml propidium iodide (Sigma), 10 μg/ml RNase A, 0.05 mM ethylene diamine and 0.2% Triton X-100 tetra-acetic acid in phosphate-buffered saline for 30 min in the absence of light. For each

Abbreviations: HCC, hepatocellular carcinoma; miRNA, messenger RNA; mRNA, microRNA; RISC, pre-miRNA; precursor miRNA, RISC; RNA-induced silencing complex, UTR, untranslated region.

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sample, at least 20,000 cells were analyzed using FACS cytometry (Beckman Coulter Epics Altra, Miami, FL) and Multicycle AV for Windows 5.0 (Phoenix Flow System, San Diego, CA).

**Luciferase reporter constructs and luciferase assay**

The 3’ UTR of p21Cip1/Waf1, mutant-type, putative miR-423-3p or the miR-423-5p-binding site was cloned downstream of a cytomegalovirus (CMV) promoter-driven firefly luciferase cassette in a pCDNA3.0 vector. The primer sequences are shown in supplementary Table S1, available at Carcinogenesis Online. For the luciferase assay, SNU-449 cells were cultured in 24-well plates and cotransfected with 20 pmol of RNA (negative control, miR-423-3p or miR-423-5p mimics), 200 ng of the luciferase reporter construct and 20 ng of the pRL-CMV renilla luciferase reporter construct. After 48 h, luciferase activity was measured using the dual luciferase reporter assay system (Promega, Madison, WI).

**Oligonucleotide transfection**

miR-423-3p and miR-423-5p mimics were synthesized by Genepharma (Shanghai, China). miR-423-3p and miR-423-5p inhibitors (2′-O-methyl modification) were synthesized by Ribobio (Guangzhou, China). The sequence of the RNA duplex control was 5’-UUCUCCGAACGUGUCACGU-3’.

Oligonucleotide transfection was performed using Lipofectamine 2000 reagent (Invitrogen).

**RNA extraction and quantitative real-time polymerase chain reaction**

Total RNA was extracted using TRizol reagent (Invitrogen). The complementary DNA was synthesized using the PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan) and real-time polymerase chain reaction was performed using the SYBR Premix Ex Taq (TaKaRa). Beta-actin was measured as an internal control. Primer sequences are shown in Supplementary Table S1, available at Carcinogenesis Online. TaqMan miRNA assays (Applied Biosystems, Foster City, CA), which included specific RT primers and TaqMan probes, were used to quantify the expression of mature miR-423-3p and miRNA-423-5p. U6 snRNA was measured as an internal control.

**Western blot**

Protein were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% non-fat milk and incubated with mouse anti-p21Cip1/Waf1 monoclonal antibody (mAb) (Cell Signaling Technology, Beverly, MA) or mouse anti-β-actin mAb (Sigma). The proteins were detected with enhanced chemiluminescence reagents (Pierce, Rockford, IL).
miR-423 as a new oncogenic miRNA in HCC

Results

**miR-423 promotes liver cancer cell growth and cell cycle G1/S transition**

In an attempt to determine the functional role of miR-423 in HCC, we constructed a lentivirus vector harboring miR-423 and established two stable HCC cell lines, denoted HepG-2-423 and SNU-449-423, after lentivirus transduction (Supplementary Figure S1 is available at Carcinogenesis Online). Cell proliferation (Figure 1A and B) and colony formation assays (Figure 1C and D) revealed that ectopic expression of miR-423 led to a significant increase in cell growth of HepG-2-423 and SNU-449-423 cells; however, Transwell and Caspase 3/7 activities assays (data not shown) demonstrated that ectopic expression did not influence cell migration or cell apoptosis. These results indicate that miR-423 exerts a growth-promoting function in HCC cells. Given that miR-423 promoted HCC cell proliferation, we next sought to determine whether miR-423 has any impact on cell cycle progression of HCC cells. The cell cycle distribution of HepG2 and SNU-449 cells showed that the cell number in G1 phase was reduced in HepG-2-423 and SNU-449-423 cells as compared with vector control, whereas the cell population in S phase increased.

Statistical analysis

The results are presented as mean ± standard error of the mean. The data were analyzed using Student’s t-tests (two tailed). Statistical differences with P-values >0.05 were considered significant.
sharply (Figure 1E and F). Thus, the growth-promoting function of miR-423 may be due to its enhancement of cell cycle progression at the G1/S transition in HCC cells.

miR-423-3p, but not miR-423-5p, promotes the growth and cell cycle progression of live cancer cells

According to Sanger miRBase sequences, miR-423 includes two mature sequences: miR-423-3p and miR-423-5p (13,14) (Figure 2A). We first determined the expression levels of these two sequences in HCC tissues and cell lines using TaqMan real-time polymerase chain reaction. The results showed that both miR-423-3p and miR-423-5p are relatively abundant in HCC (Figure 2B). Although the expression level of miR-423-3p was higher than that of miR-423-5p, they showed high concordance in HCC tissues and cell lines (Figure 2B). We next investigated whether both miR-423-3p and miR-423-5p have the ability to repress gene expression. The predicted binding sites for miR-423-3p and miR-423-5p were inserted independently into the 3' UTR of the luciferase sequence in the reporter vector. After cotransfection with the miR-423 expression vector, the luciferase activity of both reporter vectors was decreased (Figure 2C), indicating that both sequences produced by miR-423 can function separately to repress gene expression. To further determine which strand of miR-423 is involved in HCC cell growth, we introduced the synthesized miRNA mimics of miR-423-3p and miR-423-5p into HepG2 cells, which have relatively low expression of miR-423 (Figure 2B). The passenger strand of the miR-423-3p mimic duplex is not equivalent to miR-423-5p, so it cannot act as miR-423-5p (and vice versa). We found that miR-423-3p, but not miR-423-5p, promoted HCC cell growth and cell cycle progression (Figure 2D and F). Furthermore, silencing of miR-423-3p led to a significant decrease in cell growth and increased cell cycle arrest at G1, whereas miR-423-5p silencing had no significant effect in SNU-449 cells (Figure 2E and G). SNU-449 cells have relatively high expression of miR-423 (Figure 2B). The activity of luciferase reporter with predicted binding sites for miR-423-3p increased in SNU-449 cells cotransfected with anti-miR-423 (Supplementary Figure S2 is available at Carcinogenesis Online), suggesting that SNU-449 cells have endogenous miR-423-3p activities. Taken together, these results indicate that miR-423-3p, but not miR-423-5p, enhances HCC cell proliferation and cell cycle progression.

miR-423 downregulates p21Cip1/Waf1 expression by directly targeting the p21Cip1/Waf1 3' UTR

It is generally accepted that miRNAs exert their function by down-regulating the expression of their downstream target genes. Thus, miR-423 may execute its growth-promoting function by inhibiting targets that are involved in the suppression of the G1/S transition. Based on Gene Ontology (15) (GO database release 2011-02-26, GO:0000082, Homo sapiens, Evidence Code: IDA), 12 potential genes (NBN, TGFB1, ACVR1B, INHBA, CDKN1A, CDKN2A, CDKN2C, CDKN1B, CDKN2D, SPDYA, BRD7 and LATS2) that are involved in negative regulation of the G1/S transition of the mitotic cell cycle were selected. However, when combined with miRNA target prediction, only ACVR1B and CDKN1A (p21Cip1/Waf1) contain a 3' UTR element partly complementary to miR-423-3p, as revealed by the RNA22 program (16). Notably, we demonstrated previously through a high-throughput luciferase reporter screen that p21Cip1/Waf1 can be directly targeted by nearly 28 miRNAs in HEK 293 cells. Interestingly, miR-423 was one of those miRNAs. Thus, we focused on p21Cip1/Waf1, as it is a master downstream effector of tumor suppressors (17) and is well known as a negative regulator of the G1/S transition (18).

To determine whether p21Cip1/Waf1 is regulated by miR-423 through direct binding to its 3' UTR, we constructed full-length
fragments of p21Cip1/Waf1 mRNA 3' UTR (either wild-type or mutant) and inserted them immediately downstream of the luciferase reporter gene. For the luciferase assays, one of the miR-423 expression plasmids, either the miR-423-3p or the miR-423-5p mimic, was cotransfected with different luciferase 3' UTR constructs into SNU-449 cells. The results showed that miR-423 decreased the relative luciferase activity with the wild-type 3' UTR of p21Cip1/Waf1. In particular, miR-423-3p, but not miR-423-5p, contributed to this effect (Figure 3B). Further analysis showed that this regulation was sequence specific because relative luciferase activity did not decrease as sharply in UTRs with mutant-binding sites as they did in wild-type counterparts (Figure 3C). In concordance with these results, we observed a clear decrease in endogenous p21Cip1/Waf1 protein and mRNA in HepG2 and SNU-449 cells with miR-423 overexpression or transfection with the miR-423-3p mimic, whereas no obvious changes were detected after transfection with the miR-423-5p mimic (Figure 4A-C). Furthermore, inhibition of miR-423-3p, but not miR-423-5p, increased significantly the expression levels of p21Cip1/Waf1 protein and mRNA in SNU-449 cells (Figure 4D and E). Taken together, these results suggest that miR-423 can downregulate p21Cip1/Waf1 expression by directly targeting its 3' UTR.

p21Cip1/Waf1 can block miR-423-induced liver cancer cell growth and cell cycle progression

To investigate whether p21Cip1/Waf1 is the direct functional mediator of miR-423-induced HCC cell growth, we introduced a p21Cip1/Waf1 lentiviral construct into miR-423 overexpressing cells to recover p21Cip1/Waf1 expression (Figure 5A). This construct contains the entire p21Cip1/Waf1 coding sequence; however, it lacks the 3' UTR fragment. Therefore, it is insensitive to miR-423-mediated repression. The results showed that p21Cip1/Waf1 overexpression could significantly abrogate miR-423-dependent effects on HCC cell proliferation and G1/S transition (Figure 5B and C). Furthermore, cotransfection experiments showed that knockdown of p21 expression by siRNA could significantly rescue anti-miR-423-induced growth suppression of SNU-449 cells (Supplementary Figure S3 is available at Carcinogenesis Online). These findings indicate that p21Cip1/Waf1 is a bona fide target of miR-423 in HCC cells.

Discussion

The miRNA miR-423 was first identified in human promyelocytic leukemia (HL-60) cells (13). The level of miR-423 expression was shown to be upregulated after 12-O-tetradecanoylphorbol...
miR-423-3p appears to be predominantly expressed because its expression level is ~5-fold higher than that of miR-423-5p. Interestingly, although both miR-423-3p and miR-423-5p are expressed and function in HCC cells, only miR-423-3p plays a crucial role in promoting HCC cell proliferation and cell cycle progression. The possible role and significance of miR-423-5p remain to be elucidated. According to miRBase Sequences (Release 16, September 2010), there are ~1048 pre-miRNA sequences in humans, yet 1223 mature sequences are listed in total. That means that ~200 miRNA genes, roughly 20% of all human miRNA genes, might contain two mature sequences. Whether all cases behave like miR-423 remains to be explored further.

Integrating bioinformatics and experimental assays, we identified p21Cip1/Waf1 as a direct functional downstream target of miR-423 in HCC cells. Two potential target sites of miR-423 were found in the p21Cip1/Waf1 3’ UTR (Figure 3A). Mutation of site 1 only increased luciferase activity slightly. When site 2 was mutated, luciferase activity increased ~20%. However, when both site 1 and site 2 were mutated, luciferase activity increased to a level similar to that seen with the control vector. This indicates that the two sites work cooperatively and both are miR-423 targets, although site 2 is apparently a more effective target. In concordance with the luciferase reporter results, endogenous p21Cip1/Waf1 mRNA and protein levels were found to be downregulated by miR-423 in HCC cells. miR-423 decreased the p21Cip1/Waf1 mRNA by ~40–60%, which suggests that miR-423 regulates p21Cip1/Waf1 mRNA stability to some extent. However, as the amount of p21Cip1/Waf1 protein changed much more appreciably than the amount of its mRNA did, it is probably that miR-423 also regulates translation of p21Cip1/Waf1 mRNA.

In conclusion, we demonstrate for the first time that miR-423 exerts growth-promoting effects in HCC cells through downregulating the expression of the tumor suppressor p21Cip1/Waf1. These data suggest an oncogenic function and a therapeutic application for miR-423 in HCC.

Supplementary material

Supplementary Table S1 and Figures 1–3 can be found at http://carcin.oxfordjournals.org/.

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Conflict of Interest Statement: None declared.

References


Fig. 5. p21Cip1/Waf1 can block miR-423-induced liver cancer cell growth and cell cycle progression. (A–C) Western blot analysis of p21Cip1/Waf1 protein levels (A), cell proliferation assays (B) and FACS analysis (C) of SNU-449-vector and SNU-449-423 cells were performed after infection with p21Cip1/Waf1 (ORF without 3’UTR) or vector control lentivirus. (B and C) Western blot analysis of p21Cip1/Waf1

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<th>Cell Population (%)</th>
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<th>Lenti-vector+Lenti-p21</th>
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The results are presented as the mean ± standard error of the mean of triplicate experiments. Statistical analysis was performed using the Student’s t-test. ** indicates P < 0.01. NS indicates no significant change.

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