MiR-223 modulates hepatocellular carcinoma cell proliferation through promoting apoptosis via the Rab1-mediated mTOR activation

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Abstract

Hepatocellular carcinoma (HCC) is a common digestive malignancy. MiR-223, a well-identified miRNA, exhibits diverse properties in different cancers. In this study, we demonstrated that miR-223 could suppress cell growth and promote apoptosis in HepG2 and Bel-7402 HCC cell lines. We screened and identified a novel miR-223 target, Ras-related protein Rab-1(Rab1). Upregulation of miR-223 would specifically and markedly down-regulate Rab1 expression. In addition, miR-223-overexpressing subclones showed significant cell growth inhibition by increasing cell apoptosis in HepG2 and Bel-7402 cells. To identify the mechanisms, we firstly investigated the mTOR pathway and found that pmTOR, p70S6K and Bcl-2 were dramatically down-regulated after miR-223 transfection, while no changes in the level of Bax was visualized. Furthermore, our data showed that the anti-tumor effects arising from miR-223 transfection in HCC cells may be due to the deactivation of mTOR pathway caused by the suppression of Rab1 expression when miR-223 is overexpressed. In summary, our results indicate that miR-223 functions as a tumor suppressor and plays a critical role in inhibiting the tumorigenesis and promoting the apoptosis of HCC through the mTOR signaling pathway in vitro. By targeting Rab1, miR-223 efficiently mediates the mTOR pathway. Given these, miR-223 may be a potential therapeutic target for treating HCC.

Key words: Rab1; Hepatocellular carcinoma; miR-223; Apoptosis; mTOR

Introduction

Hepatocellular carcinoma (HCC) is one of the most malignant and recalcitrant form of liver cancers and remains the third leading cause of all cancer-related death [1]. It has been reported that HCC may causes 662,000 deaths worldwide per year [2]. The majority of HCC patients with advanced stages are treated with surgery, radiotherapy and systemic chemotherapy. Even with those aggressive intervention, the median survival period varies from 4 to 15 months in different countries [3, 4]. Over the last years, focusing on known genes for HCC has already unveiled new treatment possibilities. However, previously unknown noncoding RNAs, such as microRNAs (miRNAs), may also lend insight into the biology of HCC.

miR-223 is known as a short RNA molecule with important functions in promoting granulocytic differentiation while also being related with the suppression of erythrocytic differentiation [5]. Despite their crucial functions in healthy individuals, they have also been implicated in several cancers. Recent studies have shown that miR-223 could participate in cellular transformation, angiogenesis and tumor growth and cause sensitive to radiotherapy [6-8]. Specifically, miR-223 is repressed in some malignant cancers, including hepatocellular carcinoma, recurrent ovarian cancer and several types of leukemia[6, 9-11]. Furthermore, it has been demonstrated that serum miR-223 expression may prognosticate disease outcome in HCC patients and could be used as a novel potential biomarker for hepatocellular carcinoma detection[12, 13]. However, its biological functions in HCC have remained largely unclarified.

Rab1, a founding member of Rab GTPase family, has been reported to regulate membrane trafficking between the endoplasmic reticulum (ER) and Golgi apparatus [14, 15]. Several key regulatory proteins such as mitogen receptors and transcription factors are synthesized, modified and transported to the cell surface or nucleus by ER-Golgi membrane system [14]. Importantly, recent studies have identified that Rab1 is a mediator of some critical signal transduction pathways, particularly mTOR

pathway [16]. In this study, we firstly explored the association between miR-223 and Rab1 expression in HCC cells.

Here, we examined miR-223 expression in HCC patients, and explored its functions in HepG2 and Bel-7402 cells. We up-regulated the expression of miR-223 in HepG2 and Bel-7402 cells and showed that miR-223 stimulation inhibited cell proliferation and enhanced apoptosis. Moreover, we identified that Rab1 is a novel miR-223 target and demonstrated that miR-223-Rab1-mTOR axis plays a key role in mediating proliferation and apoptosis of HCC cells.

Materials and Methods

Cell Culture

Huh7, Hep3B, Bel-7402 and the HepG2 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). All these cell lines were cultured according to the instruction provided by ATCC. Briefly, all cell lines were grown in DMEM supplemented with 8% fetal bovine serum (FBS), penicillin G (100 U/mL), and streptomycin (100 g/ml), and maintained in monolayer culture at 37 °C in humidified air with 5% CO₂. Viability of the cells was monitored trypan blue staining.

Cell proliferation assay

Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. All cells were plated in 96-well plates at 4×10^3 cells/well and incubated with 0.2 mg/ml MTT for 4 h at 37°C. The optical density of each sample was read using a microplate reader (BioRad) at 570 nm.

Western blotting

Proteins were extracted from subconfluent cultures and then subjected to Western blot analysis as described previously. Briefly, after blocking with 5% nonfat milk in PBS-T for 1h at room temperature, the membranes were blotted with primary

antibodies, followed by incubation with a peroxidase-conjugated secondary antibody. Bound antibodies were visualized using enhanced chemiluminescence (Bio-Rad).

Real-time RT-PCR

Total cellular RNA was isolated using the Trizol reagent (Invitrogen). First-strand cDNA was synthesized using a ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Real-time PCR was performed using the ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). The primer sequences for has-miR-223 were 5'-GCCCAAACAUAUACAACGCUGUC -3' (sense) and 5'-CAGTGCAGGGTCCGAGGT-3' (anti-sense). The PCR was run at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 55°C for 45 s. Melting curve analysis was used to check the specificity of amplification. Parallel reactions were performed using primers to U6 as an internal control. Relative gene expression was calculated by using the comparative Ct method.

MiRNAs and transfection

MiR-223 mimics, negative controls (miR-NCs), miR-223 inhibitor (anti-miR-223), GFP siRNA, Lenti-Rab1 were designed and performed by RiboBio Co. Ltd. (China). Rab1 inhibition constructs (Rab1 siRNA) were purchased from Life-technologies (California, USA) and transfected using the manufacturer's reagents and protocol. The malignant HCC cells were stably transfected with GFP-siRNA, or Rab1-siRNA according to the manufacturer's protocol (Invitrogen). The synthetic interfering RNA (siRNA) sequences and the full length of Rab1 were carried out using pLVTHM vector and purity was confirmed by Western blot analyses. Target gene expression was evaluated 3 d after transfection and did not decrease significantly by 9 d after transfection as determined by Western blot analysis.

Apoptosis assay

ApoScreen Annexin V Apoptosis Kit (Bender Med System, CA) were used for labeling of apoptotic cells. For each experiment, 20,000 cells were analyzed using

FACSCalibur flow cytometer (BD Biosciences). Experiments were performed in triplicate.

Statistical analysis

All data are expressed as mean $\pm$ SD for triplicate determination, and analyzed using Student's t test. All statistics were performed with the SPSS 16.0 software (SPSS Inc, Chicago, IL). Statistical significance was set at $p < 0.05$.

Results

Expression of miR-223 in HCC cell lines

To choose HCC cell lines with different miR-223 expression levels for exploring the biological function of miR-223, the expression of miR-223 was detected using RT-qPCR analysis in four cell lines: Huh7, Hep3B, Bel-7402, and HepG2. As shown in Fig.1A, Bel-7402 and HepG2 cells revealed significantly lower level of miR-223 expression than that of LO2, Huh7, and Hep3B cells. Therefore, Bel-7402 and HepG2 HCC cell lines were selected to ascertain the role of miR-223 in HCC cancer.

Apoptosis is induced by miR-223 in HCC cells in vitro

Transfection of HepG2 and Bel-7402 cells with miR-223 mimics resulted in an approximate 5.9-fold increase in miR-223 expression in HepG2 cells and a 6.8-fold increase in Bel-7402 cells compared to the parent cell lines (Fig. 1B). The results of MTT assays at 24, 48, 72, 96, and 120 hours after miR-223 and scramble transfection (miR-NC) are shown in Fig. 1C. Compared with scramble transfection, miR-223 transfection significantly suppressed the proliferation of HepG2 and Bel-7402 cells. We next explored the effect of miR-223 on the apoptosis of HepG2 and Bel-7402 cells after miR-223 transfection. Scramble transfection induced the apoptosis of 10.1% HepG2 cells and 11.8% Bel-7402 cells; meanwhile, miR-223 transfection significantly induced the apoptosis of 24.6% HepG2 cells and 19.4% Bel-7402 cells (Fig. 1D). Furthermore, we used TEM analysis to confirm in evaluation that annexin V stain showed the apoptotic changes in HepG2 and Bel-7402 cells transfected with miR-223 mimics. The miR-223-transfected HepG2 and Bel-7402 cells showed classical apoptosis features, such as membrane blebbing, nuclear fragmentation, and chromatin condensation compared with control HepG and Bel-7402 cells (Fig. 1E). These results strongly indicate that miR-223 can suppress HCC cell survival by inducing apoptosis.

miR-223-induced apoptosis is associated with mTOR pathway

To determine the underlying mechanism of cell death induced by miR-223, we initially measured expression of signaling molecules involved in the mTOR and cellular apoptosis pathways by western blot verification. Compared with miR-223 NC, p-mTOR and p-p70s6k protein expressions were significantly downregulated in HepG2 and Bel-7402 cells transfected with miR-223 mimics, while no changes in the level of total mTOR and p70s6k were visualized (Fig. 1F). In addition, the levels of the antiapoptotic protein Bcl-2 was significantly suppressed in miR-223-transfected HepG2 and Bel-7402 cells compared to HepG2 and Bel-7402 cells expressing miR-NC, while the levels of the proapoptotic protein Bax did not appear to differ between groups (Fig. 1F). These results suggest that activating miR-223 function in HCC cells may enhance the dephosphorylation of mTOR and p70s6k, which promotes the cell apoptosis through suppressing the levels of cellular antiapoptosis protein Bcl-2.

Rab1 is a direct target gene of miR-223

As predicted by Targetscan, complementarity can be found between has-miR-223 and Rab1 3'-UTR (Fig. 2A). To explore whether Rab1 is a direct target of miR-223, we cloned the target site, or its mutant into an identical luciferase reporter vector (Fig. 2A). We found when co-transfected with Rab1-3' UTR luciferase reporter plasmid, miR-223 mimics led to a significant reduction of luciferase activity of Rab1 ($P<0.01$), while the luciferase activity was unaffected by the miR-223-MT (Fig. 2B). Moreover, qRT-PCR and western blot analysis showed that ectopic expression of miR-223 markedly decreased the mRNA ($P<0.05$, Fig. 2C) and protein ($P<0.05$, Fig. 2D) expressions of Rab1 in HepG2 cells, whereas inhibition of miR-223 increased the Rab1 mRNA ($P<0.05$, Fig. 2C) and protein ($P<0.05$, Fig. 2D) levels in Bel-7402 cells. To further validate that Rab1 was a direct downstream target of miR-223, we investigated the association between miR-223 level and Rab1 expression in HCC tissues. qRT-PCR analysis showed that the mRNA expression of Rab1 was obviously

decreased in patients with high level of miR-223 ($P<0.05$, Fig. 2E). Furthermore, statistical analyses of mRNA expression demonstrated that the level of Rab1 mRNA associated inversely with miR-223 level ($r=-0.28$, $P=0.038$, Fig. 2F). Consistently, we found Rab1 protein level in the miR-223 high-expressing tumors was significantly lower than those in the miR-223 low-expressing tumors by immunohistochemical staining ($P<0.05$, Fig. 2G). Thus, these results strongly indicated that Rab1 is a novel target of miR-223 in HCC.

Knockdown of Rab1 enhances apoptosis in HCC cells

We first measured Rab1 expression in four HCC cancer cell lines (Huh7, Hep3B, Bel-7402, and HepG2) by using qRT-PCR and Western blot (Figure 3A, B). Bel-7402, and HepG2 cells showed significantly higher Rab1 expression than Huh7 and Hep3B cells on both mRNA (Fig. 3A) and protein levels (Fig. 3B). So Bel-7402, and HepG2 cells were selected to perform the next experiments in vitro. Western-blot analysis revealed that Rab1 protein expression was significantly suppressed by siRNA targeting Rab1 (Fig. 3C). We then measured the contribution of Rab1 expression to the HepG2 and Bel-7402 cell proliferation by MTT assay. We found HepG2 and Bel-7402 cells exhibited reduced survival when Rab1 was specifically knocked down by Rab1 siRNA (Fig. 3D). Furthermore, flow cytometry of annexin V-stained HCC cells showed that the rate of apoptosis was significantly higher in HepG2 and Bel-7402 cells transfected with the Rab1-targeted siRNA compared with cells transfected with the GFP-siRNA (Fig. 3E). These observations clearly suggested that Rab1 expression was associated with HCC cell survival and apoptosis.

Apoptosis induction by miR-223 is Rab1-dependent

To exam whether Rab1 is a regulator in miR-223-induced apoptosis, full-length Rab1 was transfected into HepG2 and Bel-7402 cells treated with miR-223. MTT assay and apoptosis assay showed that overexpression of Rab1 in HepG2 and Bel-7402 cells expressing miR-223 reversed the lower growth (Fig. 4A) and enhanced apoptosis

(Fig. 4B, C) observed in cells expressing only the miR-223. These results imply that miR-223 induces apoptosis through an Rab1–dependent mechanism.

Suppression of mTOR pathway activation by miR-223 is mediated by Rab1

Considering the fact that Rab1 plays a critical role in cell proliferation and apoptosis induction mediated by miR-223 in HCC, we next evaluated the effects of Rab1 expression inhibition by miR-223 in the mTOR signaling pathway. We found upregulation of Rab1 expression by using Lenti-Rab1 dramatically increased the levels of the phosphorylated mTOR and p70S6K (Fig. 4D), leading to increased Bcl-2 expression in HepG2 and Bel-7402 cells (Fig. 4E). These observations imply that Rab1 may be participated in the mediation of mTOR and p70S6K phosphorylation in HCC cells. Importantly, the effect of miR-223 transfection on dephosphorylation of mTOR and dephosphorylation of p70S6K was abrogated by Rab1 transfection (Fig. 4D). Furthermore, when Rab1 was transfected into both cells to increase Rab1 expression, suppression of Bcl-2 protein expression by miR-223 was blocked (Fig. 4E). These results indicate that miR-223 down-regulation of Rab1 results in the suppression of phosphorylation of mTOR and p70S6K followed by an induction of apoptosis.

Dicussion

Over the past decade or so, progress has been made in the understanding of miR-223 functions beyond its physiological role in granulocytic differentiation, showing that miR-223 is also involved in a myriad of cell signaling and regulatory functions. Moreover, miR-223 expressions have been implicated in human diseases such as cancer, sclerosis and systemic lupus erythematosus [17, 18]. These new findings provide new insights into their physiological and pathological functions. Importantly, modulation of miR-223 expression has been shown to have therapeutic benefits toward leukemia, pancreatic and non-small cell lung cancer, suggesting that miR-223 is a potentially useful therapeutical target [11, 19, 20]. Previous reports revealed that miR-223 significantly decreased in HCC, but its biological functions were unclear and few relevant studies on miR-223 in HCC were reported. In this investigation, we explored the functions of miR-223 and the mechanism of its transcriptional regulation. MTT assays showed that miR-223 significantly suppressed proliferation of HepG2 and Bel-7402 cells. In addition, we evaluated the effect of miR-223 on the cell apoptosis. Our observations elaborated that miR-223 could promote apoptosis of HCC cells and might be involved in the dephosphorylation of mTOR and p70s6k. In summary, we considered that miR-223 might be an important target gene for anti-HCC therapy in the future.

MiRNAs mediate various cellular activities through negatively regulating special target genes. Identification of the candidate target genes of specific miRNAs has led to the elucidation of the diverse functions of miRNAs [21]. Our results revealed that miR-223 directly regulates the expression of Rab1. Rab1 is a well-known oncogene which are found predominantly at the membrane of ER and Golgi apparatus [22]. The ER-Golgi membrane system serves as a major hub for cell signaling and regulatory functions in response to extracellular and intracellular cues such as nutrients and ER stress [23]. Recent studies demonstrated that Nutrients such as amino acid (AA) is a

key activating signal for mTOR [24]. Notably, genetic screens identifies Rab1 as an essential factor for activation of mTOR by AA [25]. In the present study, we showed that Rab1 is a notable downstream target of miR-223. Furthermore, our results indicated that miR-223-mediated apoptosis in HCC might be associated with the dephosphorylation of mTOR and p70S6K, which is regulated by Rab1. These results are consistent with earlier studies that found ectopically expressed Rab1, a key mediator of ER-to Golgi transport, activates mTOR and promotes tumor growth, invasion and progression [16].

mTOR, a serine/threonine protein kinase and ‘target of rapamycin’, acts as a primary regulator of protein synthesis and cell growth, and functions as a key integrator of the receptor tyrosine kinases, which in turn is the most frequently hyperactivated pathway in HCC [26]. Previous studies demonstrated that mTOR pathways, including pRPS6, p-AKT, IGF-1R and RICTOR are up-regulated in 40-50% of HCCs, highlighting its importance in HCC [26]. Additionally, inhibition of mTOR signaling has been reported to sensitize tumor cells to apoptosis induced by several anticancer agents [27, 28]. Here we find that the upregulation of miR-223 could suppress the expression of its target gene Rab1, which can inhibit mTOR pathway activation, and leads to enhancement of miR-223-regulated cell death and apoptosis in HCC cells. Several mTOR inhibitors, such as sirolimus and everolimus, are currently undergoing clinical evaluation in patients with HCC [29, 30]. Considering the importance of miR-223 in mTOR signaling pathway, it is reasonable to speculate that agents specifically targeting miR-223, particularly in HCCs with low expression of miR-223, could be beneficial in HCC patients

Deregulated apoptosis is a hallmark of HCC. Apoptosis is an endogenous cellular process whereby an external signal activates a metabolic pathway that results in cell death [31]. This form of cell death appears to be a common feature in many biological processes where cell deletion is a mechanism for altering tissue structure and function

[32]. In cancer, suppression of apoptotic signaling contributes significantly to carcinogenesis and tumor progression [31]. An important component of the apoptotic pathway is the family of proteins commonly known as the B cell lymphoma-2 (Bcl-2). A number of studies have demonstrated that anti-apoptotic Bcl-2 protein can enhance the survival of HCC cells and facilitate tumorigenesis by inhibiting apoptosis [33, 34]. Moreover, Zhao et al. found that HCC patients with nuclear expression of Bcl-2 had worse clinical outcomes than patients exhibiting cytoplasmic expression [35]. To explore the molecular mechanisms of miR-223-mediated apoptosis in HepG2 and Bel-7402 cells, we investigated Bcl-2 and Bax expression and demonstrated that increased miR-223 expression was associated with lower antiapoptotic Bcl-2 expression, whereas no changes in the level of Bax was visualized, indicating a functional interaction between miR-223 and the Bcl-2-mediated apoptotic pathways. However, this issue remains interesting and requires further studies in the future. In summary, we demonstrate that miR-223 can inhibit HCC cells proliferation and tumorigenesis by inducing cell apoptosis. We also identified a novel target gene Rab1 for miR-223. Our findings reveal that the anti-tumor effect of miR-223 is associated with the depression of Rab1 expression, leading to the promotion of the cell apoptosis by regulating the mTOR pathway. We believed that exogenous overexpression of miR-223 may prove to be a promising strategy for targeted HCC therapies.

Disclosure Statement

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Figure Legends

Fig1 (A) MiR-223 expression was measured by qRT-PCR in Huh7, Hep3B, Bel-7402, and HepG2 cell lines. All expression was normalized to the level of U6 small nuclear RNA expression. * $P < 0.05$. (B) The expression of miR-223 was measured in Bel-7402 and HepG2 cells transfected with miR-223 mimics or scramble vector (miR-NC). * $P < 0.05$. (C) miR-223 overexpression inhibits IHCC cell proliferation in Bel-7402 and HepG2. The growth index was assessed after 1, 2, 3, 4 and 5 days. The bars represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$. (D) The number of apoptotic cells was significantly higher in Bel-7402 and HepG2 cells transfected with miR-223 mimics as measured by PI staining and flow cytometry. * $P < 0.05$. (E) Morphological analysis of Bel-7402 and HepG2 cells apoptosis induced by miR-223 mimics. The morphology of HCC cells were imaged by TEM. Magnification in TEM analysis was $\times 5000$. (F) Altered mTOR signaling and apoptosis are associated with overexpression of miR-223. Detection of mTOR, p70s6k and apoptosis-associated proteins in Bel-7402 and HepG2 cells by Western blotting.

Fig2 (A) The putative binding sequence of miR-223 in the 3'-UTR of Rab1. (B) Luciferase reporter assay in HepG2 HCC cells. * $P < 0.05$. (C) qRT-PCR analysis of Rab1 mRNA expression in HepG2 cells transfected with miR-223 mimics or control vector (miR-NC) and in Bel-7402 cells transfected with miR-223 inhibitor or negative control vector (anti-miR-NC). * $P < 0.05$. (D) Overexpression of miR-223 reduced the expression of Rab1 protein in HepG2 cells and suppression of miR-223 increases the level of Rab1 protein in Bel-7402 cells. * $P < 0.05$. (E) The expression of Rab1 mRNA in miR-223 high-expressing tumors was significantly lower than that in miR-223 low-expressing tumors. ** $P < 0.01$. (F) A significant inverse correlation between the mRNA levels of miR-223 and Rab1 was observed in HCC tissues. $P = 0.038$. (G) Representative immunostaining showed negative expression of Rab1 in miR-223 high-expressing HCC tissue and positive expression of Rab1 in miR-223

low-expressing tumor. The expression of Rab1 protein in miR-223 high-expressing tumors was significantly lower than that in miR-223 low-expressing tumors. * $P < 0.05$.

Fig3 (A) mRNA levels of Rab1 measured in four HCC cell lines by qRT-PCR. * $P < 0.05$ (B) Expression of Rab1 protein in four HCC cell lines measured by Western blot. (C) The protein levels of Rab1 expression were elevated in Bel-7402 and HepG2 cells after transfection of Rab1 siRNA for 72 h by western-blot analysis. (D) Effect of Rab1 knockdown on Bel-7402 and HepG2 cell proliferation as measured by the MTT assay. * $P < 0.05$. (E) Effect of Rab1 knockdown on Bel-7402 and HepG2 cell apoptosis as measured by PI staining and flow cytometry. * $P < 0.05$.

Fig4 (A) MTT assay of Bel-7402 and HepG2 cells co-transfected with miR-223 mimics and Rab1 expression vector or the control. * $P < 0.05$. (B) Apoptosis assay of Bel-7402 and HepG2 cells co-transfected with miR-223 mimics and Rab1 expression vector or the control. (C) Quantification of scatter plots from (B) showing apoptotic cells as a percent of the total cell number examined by flow cytometry. * $P < 0.05$. (D) Rab1 overexpression blocks the effect of miR-223 on dephosphorylations of mTOR signaling without affecting total mTOR and p70s6k /2 expression. Values representing the mean $\pm$ SD ($n = 3$) with different letters are significantly different. * $P < 0.05$. (E) miR-223 suppresses Bcl-2 protein expression through Rab1. Values representing the mean $\pm$ SD ($n = 3$) with different letters are significantly different. * $P < 0.05$.

Figure 1

Figure 2

Figure 3

Figure 4