RESEARCH ARTICLE

MiR-675 Promotes the Growth of Hepatocellular Carcinoma Cells Through Cdc25A Pathway

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Abstract

Background: MicroRNAs (miRNAs) have fundamental roles in tumorigenesis. MiR-675 is upregulated in hepatocellular carcinoma(HCC) cells. However, the roles of miR-675 in hepatocellular carcinogenesis are still not fully elucidated. In this study, we focus on investigating the effect and mechanism of miR-675 in proliferation of HCC cells. Materials and Methods: The cell proliferation was measured by MTT assays after transfection with miR-675 inhibitor and miR-675 mimics in HCC cells. The expression level of miR-675 was detected by real-time quantitative reverse transcription polymerase chain reaction. Protein expression of Cdc25A was measured by western blotting analysis. Results: In MTT assays, overexpression of miR-675 promoted the proliferation of HCC cells($P<0.05$. at 48 hours, $P<0.01$. at 72 hours) compared with the miR-675mimics control group. Downexpression of miR-675 inhibited the proliferation of HCC cells($P<0.05$. at 48 hours, $P<0.01$. at 72 hours) compared with the miR-675inhibitor control group. In western blotting analysis, the expression level of Cdc25A was significantly increased ($p<0.05$) after treatment with miR-675 mimics. The expression level of Cdc25A was significantly decreased ($p<0.05$) after treatment with miR-675 inhibitor. Conclusions: Our results indicate that miR-675 promotes the proliferation in human hepatocellular carcinoma cells by associating with Cdc25A signaling pathway.

Keywords: MiR-675 - hepatocellular carcinoma - proliferation - Cdc25A signaling pathway

Introduction

Hepatocellular carcinoma is the fifth most common malignancy in the world (El-Serag et al., 2007). Although the treatment of HCC has made great progress, the 5 year mortality rates of HCC have still not significantly declined over the last three decades. Therefore, it is an urgent need to investigate the underlying pathophysiological mechanisms of HCC development.

MicroRNAs (miRNAs) are short non-coding RNAs of 20-24 nucleotides and can posttranscriptionally regulate multiple target genes and thus play important roles in regulation of cellular proliferation, cell cycle control, apoptosis, differentiation (Garzon et al., 2006; Jovanovic et al., 2006; Zhang et al., 2007). Aberrant miRNAs expression in human cancer is frequently found. Dysregulation of miRNAs is associated with proliferation of cancers. The studies demonstrate that downregulation of miRNAs in human cancers is involved with inhibiting tumor cell proliferation. For example, microRNA-451 is downregulated in colorectal carcinoma (CRC) cells and has a repressive role in human colorectal carcinoma cells by inhibiting cell growth through downregulating the PI3K/AKT pathway (Li et al., 2013). MiR-497 is

downregulated in prostate carcinoma cells and inhibits growth and induces apoptosis by caspase-3 activation in prostate cancer cells (Wang et al., 2013). In contrast, some studies also suggest that upregulation of miRNAs in human cancers is associated with promoting tumor cell proliferation. For instance, the expression level of miR-590-5p is upregulated in the human hepatocellular carcinoma cell and promotes proliferation and invasion in human HCC cells by directly targeting TGF-beta RII (Jiang et al., 2012). MiR-423 is upregulated in hepatocellular carcinoma and promotes cell growth through the suppression of tumor suppressor p21Cip1/Waf1 expression (Lin et al., 2011). In these miRNAs, miR-675 has been proved to play an important role in HCC proliferation. MiR-675 may promote proliferation of HCC by regulating cell cycle through inhibition of retinoblastoma (RB) protein expression and upregulation of E2F family transcription factors (Hernandez et al., 2013). However, the underlying mechanisms of miR-675 in proliferation of HCC still remain unknown.

Previous study demonstrates that Cdc25A signaling pathway is associated with the cell cycle and proliferation in cancer cells (Kang et al., 2008). MiR-21 inhibits colon cancer cells proliferation and G1-S transition

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through downregulation of Cdc25A (Wang et al., 2009). Overexpression of miR-449a inhibits cell growth and induces G1-phase arrest in T24 and 5637 human bladder cancer cells by upregulation of Rb and p130 and downregulation of CDK6 and Cdc25A(Chen et al., 2012). In addition, overexpression of miR-503 may inhibit growth of K562 cell line by downregulation of Cdc25A at both mRNA and protein level(Roy et al., 2013). In order to determine the downstream target of miR-675 in the proliferative of HCC cells, we chose to focus on Cdc25A signaling pathway. Therefore, in this study, we investigated the role and mechanism of miR-675 in proliferation of HCC. Furthermore, we hypothesized that miR-675 affect proliferation of HCC by associating with Cdc25A signaling pathway.

Materials and Methods

Cell culture

The normal human hepatic cells (LO2) and human HCC cell line MHCC-97H was obtained from the surgical laboratory at the first affiliated hospital of Sun Yat-Sen university. These cells were incubated in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO2.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assays

MHCC-97H cells (1×10^6) were seeded into 96-well plates after transfection. Cells were incubated in 37°C and 5% CO_2 incubator for 24 hours,48 hours and 72 hours respectively. MTT reagent (30 μl; 3mg/ml) was added to each well and cells were incubated for 6h. Then the precipitate was dissolved in 100 μl DMSO and the absorbance rate was measured by a microplate reader at a wavelength of 490 nm. All experiments were repeated in triplicate.

Transfection assay

MiR-675 inhibitor, miR-675 mimics and their respective negative controls (NC) were obtained from Sagene Technology Company (Guang zhou, China). The day before transfection, MHCC-97H cells(1×106) were placed in 24-well plates. Cells were incubated overnight to obtain >70% convergence at the time of transfection. According to the protocols of the manufacturer, miR-675 mimics or miR-675 mimics negative control and miR-675 inhibitor or miR-675 inhibitor negative control were transfected into MHCC-97H cells, respectively. RNA and total cellular protein were extracted from the cells and subjected to qRT-PCR and western blotting analyses.

RNA isolation and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

MHCC-97H cells(1×10^6) were seeded in 24-well plates and cultured in DMEM overnight and then transfected. After 2 days, MHCC-97H cells

and 0.5ml Bioopure were mixed for 5 minutes. Then the solution was mixed with 100μl chloroform at room temperature for 5 minutes, and centrifuged for 15 minutes. The supernatant containing RNA ,1μl RNA precipitate, and 250μl isopropanol were mixed at room temperature for 10 minutes, and centrifuged at 4°C for 10 minutes to obtain the RNA precipitate. The RNA precipitate was dissolved by adding into 10μl DEPC. The quantitation and purity of total RNA were measured by spectrophotometer (Quawell Q5000, USA). Reverse transcription was performed by Kit (Kit Fermentas 1622,USA). Primers used were as follows:hsa-miR-675-RT,5,-CTCAACTGGTGTCGTGGAGTCGGCA ATTCAGTTGAGCACTGTGG-3,,hsa-miR-675-F,5,-ACACTCCAGCTGGGTGGTGCGGAGAGGGCCC-3,,miRNA-R,5,-TGGTGTCGTGGAGTCG-3,,U6-F,5,-CTCGCTTCGGCAGCACA-3,,U6-R,5,-AACGCTTCACGAATTTGCGT-3,. Real-time PCR was run on the ABI Stepone plus(ABI,USA). The ΔΔCt method was used to measure the relative expression quantification of miR-675. All of the reactions were repeated in triplicate.

Protein extraction and western blotting

Forty-eight hours after transfection, MHCC-97H cells were harvested with a cell lysis buffer (0.125M Tris HCl, 4%SDS, 20%glycerol, 0.004% bromphenol blue, 10% 2-mercaptoethanol). The standard protein solution was prepared by 100mg BSA and 100ml NaCl(150mmol/l). The samples were added into different amounts of protein standard solution and 5ml Coomassie Brilliant Blue G-250 solution (0.01%). Hereafter, the light absorption value was measured by spectrophotometer (Infinite F50, Tecan, Switzerland). The concentrations of protein in the samples were calculated by the standard curve of protein. Furthermore, each protein sample was separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into a polyvinylidene difluoride membrane. Then, the membrane was washed off by Tris-buffered saline with Tween(TBST) for three times and 10 minutes. Afterwards, the membrane was added into TBST containing 5% fat-free milk ,and placed in the rotary platform for 2 hours to block the first antibody. Followed, rabbit monoclonal anti-Cdc25A and rabbit monoclonal anti-GAPDH antibody(Cell Signal Technology, Boston,USA) were added and incubated overnight at 4°C. After washing with TBST for three times and 10 minutes, the membrane was added into TBST containing 5% fat-free milk and horseradish peroxidase-labeled polyclonal rabbit IgG antibody (Cell Signal Technology, Boston, USA) ,and placed in the rotary platform at 4°C for 1 hour. After washing with TBST for three times and 10 minutes, the membrane was exposed to the X-ray. The blots were scanned by Uvipro gel imaging system (Peiqing Technology, Shanghai,China). The optical density values were measured by IPP6.0 software. The expression level of target protein was indicated by the target protein and GAPDH band density ratio.

Statistical analysis

Each experiment was repeated at least three times. All data were summarized and presented as the means ±standard deviations (SDs). The differences between means were analyzed using the Student's t-test. All

statistical analyses were performed using SPSS 13.0 software. *P*<0.05 was considered to be statistically significant.

Results

Expression level of miR-675 is upregulated in HCC cells

To identify miR-675 expression in HCC cells, qRT-PCR was performed to detect expression level of miR-675 in MHCC-97H cells and normal human hepatic cells (LO2). The results revealed that miR-675 expression in MHCC-97H cells was significantly higher than that in LO2 cells (Figure 1).

MiR-675 positively regulates the proliferation of HCC cells

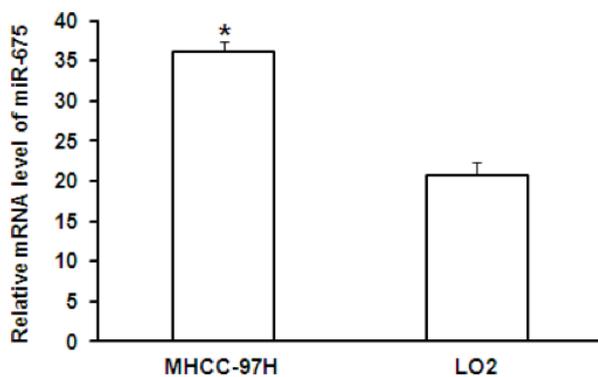To investigate the role of miR-675 in proliferation

Figure 1. Expression level of miR-675 in HCC Cells and normal Human Hepatic Cells. The expression level of miR-675 was significantly upregulated in the MHCC-97H compared with that in the normal human hepatic cells (LO2) (p<0.05)
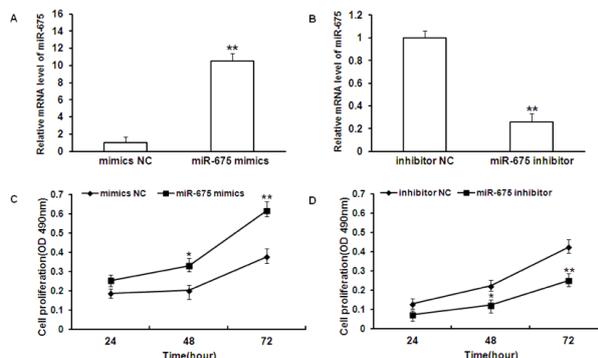

Figure 2. MiR-675 Regulates Proliferation of MHCC-97H Cells. Quantitative real-time PCR was performed to measure the expression mRNA level of miR-675. (A) The expression mRNA level of miR-675 was significantly upregulated in the miR-675 mimics group compared with the control group(*P*<0.01); (B) The expression mRNA level of miR-675 was significantly decreased in MHCC-97H cells treatment with miR-675 inhibitor compared with the control group(*P*<0.01). Cell proliferation was detected by the MTT assays at 24 hours, 48 hours and 72 hours after transfection; (C) Cell proliferation was markedly enhanced by upregulation of miR-675 compared with the control group (*P*<0.05. at 48 hours, *P*<0.01.at72 hours); (D) Cell proliferation was significantly suppressed by downregulation of miR-675 compared with the control group(*P*<0.05.at 48 hours, *P*<0.01.at 72 hours). Data are indicated by the mean of three separate experi¬ments ± SD (*P*<0.05, *P*<0.01)
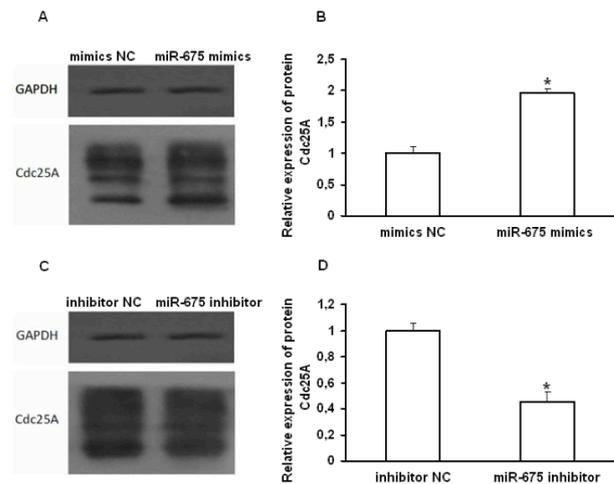

Figure 3. MiR-675 positively regulates Cdc25A. (A) Western blotting analysis was performed to detect the protein expression level of Cdc25A in MHCC-97H cells treatment with miR-675 mimics. (B) Protein expression of Cdc25A was significantly enhanced by the treatment with miR-675 mimics compared with the miR-675 mimics control group (*P*<0.05). (C) Western blotting analysis was used to detect the protein expression level of Cdc25A in MHCC-97H cells treatment with miR-675 inhibitor.(D) Protein expression of Cdc25A was significantly decreased by the treatment with miR-675 inhibitor compared with miR-675 inhibitor control group (*P*<0.05)

of HCC cells, the MTT assays were used in our study. MHCC-97H cells were treated with miR-675 mimics or miR-675 inhibitors respectively. Results revealed that the relative mRNA level of miR-675 was significantly increased by treatment with miR-675 mimics(Figure 2A) and significantly decreased by treatment with miR-675 inhibitor compared with the control group(Figure 2B). Cell proliferation was measured by the MTT assays. The results demonstrated that the upregulation of miR-675 significantly promoted cell proliferation compared with the control group(Figure 2C). Meanwhile, the downregulation of miR-675 significantly inhibited the cell proliferation of MHCC-97H cells (Figure 2D).

Cdc25A signaling pathway is modulated by miR-675

Cdc25A signaling pathway plays a crucial role in the cell cycle. We hypothesized that Cdc25A signaling pathway was modulated by miR-675.To evaluate the effect of miR-675 on the regulation of Cdc25A signaling pathway, western blotting analysis was performed to investigate the expression level of Cdc25A in the presence of miR-675 mimics and miR-675 inhibitor. In this study, our result showed that the expression level of Cdc25A was increased after treatment with miR-675 mimics in MHCC-97H cells(Figure 3B) compared with the control group. we also observed that the expression level of Cdc25A was decreased after treatment with miR-675 inhibitor in MHCC-97H cells(Figure 3D) compared with the control group. Our results suggested that miR-675

Discussion

The proliferation of HCC cells is the important factor leading to poor prognosis in HCC. However, the

molecular mechanisms of HCC proliferation is still not fully elucidated. Previous studies have discovered that miRNAs may play crucial roles in growth process of HCC. For example, microRNA-21 increases the proliferation of HCC cells by repression of mitogen-activated protein kinase-kinase3(Xu et al.,2013). miR-219-5p exerts tumor-suppressive effect in HCC by negative regulation of GPC3 expression (Huang et al., 2012). In this study, our result revealed that overexpression of miR-675 promoted the proliferation of HCC and downexpression of miR-675 led to inhibit the proliferation of HCC. Furthermore, we also found that miR-675 regulated the growth of HCC by associating with Cdc25A signaling pathway. Our results suggest that miR-675 has oncogenic function in HCC genesis and may serve as potential targets for cancer therapy.

The recent studies have revealed that miR-675 may promote the proliferation in human cancer cells. H19-derived miR-675 promotes cell growth and malignant transformation in human colon cancer cells by targeting tumor suppressor of retinoblastoma (Rb) (Tsang et al., 2010). However, the mechanisms of miR-675 in regulating the proliferation of cancer cells is still not clear. The study has showed that overexpression of miR-675 may obvious reduce E2F7 expression(Hernandez et al., 2013). E2F7 has been proved to be a repressor E2F family member, which may repress E2F1(Li etal.,2008). The repression of Rb results in upregulation of the E2F1 transcription factor (Hernandez et al., 2013). E2F1 may activate H19 and promote proliferation by the G1-S transition in breast cancer cells through E2F1 factor(Berteaux et al., 2005). Taken together, miR-675 might promote the proliferation of breast cancer cells through accelerating cell cycle division via the feedback regulation mechanism between H19/miR-675 and Rb-E2F family member. Furthermore, other microRNAs also have the feedback mechanism in regulation of cell proliferation. MiR-449a/b may inhibit the proliferation of human cancer cells by downregulation of CDK6 and Cdc25A and resulting in inactivation of Rb and cell cycle arrest at G1 phase through a negative feedback regulation mechanism between miR-449a/b and Rb-E2F1(Yang et al., 2009). In the this study, we explored the mechanisms of miR-675-induced proliferation in HCC cells. Our results revealed that Cdc25A may be a target of miR-675 in proliferation of HCC cells. In the future, we will investigate the feedback regulation mechanism between miR-675 and Cdc25A in the proliferation of HCC cells.

Cdc25A is overexpressed in many human cancers (Kristjánsdóttir etal., 2004). The cancer cell cycle division is involved with cancer cell proliferation(Tripathi et al., 2013). Cdc25A plays an important role in cancer cell cycle progression. Overexpression of Cdc25A leads to accelerate entry of cells into mitosis (Molinari et al., 2000). In addition, overexpression of Cdc25A accelerates G1/S transition by upregulating the CDK2 activity (Blomberg et al., 1999). Microinjection of Cdc25A antibodies to cells inhibits cells proliferation by inducing G1 cell cycle arrest (Hoffmann et al., 1994). Cdc25A may activate cyclinD-CDK4 complex and cyclinE-CDK2 complex, which phosphorylates Rb and results in the dissociation of Rb

from E2F1 and releases inhibition of E2F1, and promotes G1/S transition (Harbour et al., 1999). Previous study has demonstrated that expression of Cdc25A is regulated at transcriptional (Vigo et al., 1999), translational (Lin et al., 2009), and post-translational level (Busino et al., 2004). Expression of Cdc25A is regulated by PI3K signal pathway in human ovarian cancer cells (Gao et al., 2004). In addition, hypoxia inhibits the proliferation of colon cancer cells by downregulation of Cdc25A though p21Cip1 and miR-21 (de Oliveira et al., 2009). However, the role and mechanism of Cdc25A in regulation of HCC cells proliferation are still not fully known . In the present study, we detected the expression of Cdc25A and proliferation in HCC cells after overexpression and inhibition of miR-675. Our results demonstrated that Cdc25A was upregulated after overexpression of miR-675 ,which promoted the growth of HCC cells, and downregulated after inhibition of miR-675, which inhibited the growth of HCC cells. Therefore, our results suggested that Cdc25A signaling pathway played a significant role in the miR-675-mediated proliferation of HCC cells. The identification of Cdc25A signaling pathway as miR-675 downstream signaling pathway may partly demonstrate the molecular mechanism of proliferation in HCC.

In conclusion, our results demonstrate that miR-675 promotes proliferation in human hepatocellular carcinoma cells by associating with Cdc25A signaling pathway. These data suggest that miR-675 may be a diagnostic biomarker and therapeutic targets in human hepatocellular carcinoma.

Acknowledgements

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