Original Article

MiR-206 regulates the proliferation and metastasis of HCC cells via targeting E2F5

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Abstract: Background: MicroRNA-206 (miR-206) has been identified as a tumor suppressor in many cancers. However, the molecular biological function in HCC has not been elucidated. The aim of this study was to explore miR-206 expression levels and evaluated its function in HCC cells. Methods: MiR-206 expression was examined by quantitative real-time PCR (qRT-PCR) in 4 HCC cells and a normal liver epithelium cell line. Then, the function of miR-206 expression in the progression of HCC cells were determined by molecular experiments. MTT and colony formation assay was employed to detect the function of miR-206 on proliferation. Wound healing assay, transwell assay and attachment/detachment assay was applied to exam the function of miR-206 on cell metastasis. Western blot assay was used to determine the expression level of E2F5, activated caspase-3, total caspase-3 activated PARP and total PARP. Results: MiR-206 was found almost down-regulated in HCC cells compared with normal liver epithelium cell line. Forced miR-206 could obviously inhibit cells proliferation, metastasis. E2F5 is target gene of MIR-206 and contributes to the function of miR-206 exert in HCC cells. Conclusion: These results indicated that loss miR-206 contribute to the progression of HCC cells.

Keywords: HCC, miR-206, E2F5, proliferation, migration

Introduction

HCC is the fifth most common cancer around the world with approximately 564,000 new cases diagnosed every year, and over 40 percent of all cases of HCC occur in China, which has an annual incidence of 137,000 cases [1]. It is a complex polygenetic disease ascribed to the interactions between genetic predisposition and environmental factors. Despite the fact that great research efforts have been taken in this field like significant advances in diagnostic techniques, surgical and chemotherapeutic approaches, the survival rate has shown only minor improvement. Therefore, it is still meaningful to explore the underlying detailed molecular mechanisms contributing to the progression of HCC.

MicroRNAs (miRNAs) are a class of small (18-25 nt), non-coding, endogenous RNAs that have been implicated in a wide range of cellular biological processes that involve the targeting of messenger RNAs (mRNAs) for either degradation or inhibition of translation [2]. MiR-206 was first identified as a skeletal muscle-specific miRNA that is involved in the process of skeletal muscle differentiation [3]. Deregulated expression of miR-206 has been investigated in many cancers including breast cancer, rhabdomyosarcoma, renal cell carcinoma, estrogen receptor α-positive endometrioid adenocarcinoma, colorectal cancer, gastric cancer and lung cancer [4-9].

In the present study, we identified that miR-206 could dramatically suppress HCC cells proliferation and migration. The anti-proliferation and anti-metastasis effect mediated by miR-206 was depended on the downstream signaling target E2F5. On the basis of these findings, we propose that miR-206 is a negative regulator of HCC cell growth and migration and loss of miR-206 contributes to the progression of HCC.

Materials and methods

Cell lines

Three HCC cell lines (SMMC-7721, HepG2, Hep3B), and a normal liver epithelium cell line
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(L02) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM (GIBCO-BRL) medium supplemented with 10% fetal bovine serum (10% FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin in humidified air at 37°C with 5% CO₂.

**Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)**

Total RNA from cells was isolated with Trizol reagent (Invitrogen, CA, USA) according to the manufacturer’s protocol. Reverse transcription was performed with PrimeScript RT reagent Kit (Takara, Japan) according to the manufacturer’s instructions. qRT-PCR was performed with SYBR Prime Script RT-PCR Kits (Takara, Japan) based on the manufacturer’s instructions. Results were calculated with the 2^ΔΔCt methods and normalized to the expression of GAPDH mRNA and u6. All assays were performed in triplicate. The expression levels were relative to the fold change of the corresponding controls which were defined as 1.0.

**Cell viability**

Cells were seeded into 96-well plates (3 x 10^3 cells/well) directly or 24 h after transfection. Cell viability was assessed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-trtrazolium bromide (MTT) assay. All samples were assayed in triplicate.

**Colony formation assay**

Cells (500 cells/well) were plated in 6-well plates and incubated in DMEM with 10% FBS at 37°C. Two weeks later, the cells were fixed and stained with 0.1% crystal violet. The number of visible colonies was counted manually.

**Flow cytometric analysis of apoptosis**

Apoptosis were performed using flow cytometric analyses with Annexin V: FITC Apoptosis Detection Kits (BD Biosciences, USA), according to the manufacturer’s instructions. All samples were assayed in triplicate.

**Cell migration assays**

Cell migration/invasion assays were measured by transwell chamber (8 um pore size, Corning) without or with Matrigel. 48 h after transfection, cells in serum-free media were placed into the upper chamber. Media containing 10% FBS were added into the lower chamber. Following 48 h incubation, cells remained in upper membrane were wiped, while cells migrated/invaded were fixed in methanol, stained with 0.1% crystal violet and counted under a microscope. Three independent experiments were carried out.

**Cell attachment and detachment assay**

For attachment assay, cells were seeded in 24-well plates at 5 x 10^4 cells per well. Unattached cells were removed after 1 h incubation, and the attached cells were counted after trypsinization. The data were presented as a percentage of the attached cells compared to total cells. For cell detachment assay, after 24 h incubation, the cells were incubated with 0.05% trypsin for 3 min to detach the cells. Then, the culture medium was added to inactivate the trypsin and the detached cells were collected. The remaining cells were incubated with 0.25% trypsin to detach and counted. The data were presented as a percentage of the detached cells to total cells.

**Cell transfection**

Transfections with pcDNA3.1/E2F5, si-E2F5, miR-206 mimics and inhibitor (all obtained from GenePharma, Shanghai, China) were performed using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer’s protocol.

**Western bolt analysis and antibodies**

Total protein lysates were separated in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and were electrophoretically transferred to polyvinylidene difluoride membranes (Roche). Protein loading was estimated using mouse anti-GAPDH monoclonal antibody. The membranes were blotted with 10% non-fat milk in TBST for 2 h at room temperature, washed and then probed with the rabbit anti-human E2F5 (1:2000 dilution), activated caspase-3 (1:2000 dilution), total caspase-3 (1:2000 dilution), activated PARP (1:2000 dilution), and GAPDH (1:3000 dilution) overnight at 4°C, followed by treatment with secondary antibody conjugated to horseradish peroxidase for 2 h at room temperature. The proteins were detected by the enhanced chemiluminescence system and exposed to x-ray film. All antibodies were purchased from Abcam (USA).
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Figure 1. MiR-206 is down-regulated in HCC cells and regulates cell growth. A. Differences in miR-206 expression levels between HCC cells and normal liver epithelium cell line. The expression of miR-206 was normalized to that of u6. Data are presented as mean ± standard error based on at least three independent experiments. *P<0.05,
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**P<0.01. B, C. MTT and colony formation assays on HCC cells transfected with miR-206 mimics. D. Flow cytometric analyses was employed to examine the apoptosis rate. E. Apoptosis related proteins were detected by western blot. Error bars represent the mean ± SD of at least three independent experiments. *P<0.05, **P<0.01 vs. control group.

Figure 2. Down-regulation of miR-206 represses the migration abilities of HCC cells. A-C. Wound healing, metastasis, and attachment/detachment assays were utilized to detect the migratory capability of HCC cells. Error bars represent the mean ± SD of at least three independent experiments. *P<0.05, **P<0.01 vs. control group.

Dual luciferase reporter assay

PmirGLO-E2F5-wt or pmiGLO-E2F5-mut (miR-206) was co-transfected with miR-206 mimics or miR-NC into HepG2 cells by Lipofectamine-mediated gene transfer. The relative luciferase activity was normalized to Renilla luciferase activity 48 h after transfection. The data were relative to the fold change of the corresponding control groups defined as 1.0.
Statistical analysis

Data are shown as the means ± standard error of at least three independent experiments. The SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Two group comparisons were performed with a Student t test. Multiple group comparisons were analyzed with one-way ANOVA. All tests performed were two-sided. P<0.05 was considered statistically significant.

Results

MiR-206 is down-regulated in HCC cells and regulates cell growth

To investigate the function of miR-206 in HCC cells, we first detect the expression of miR-206 both in 3 HCC cell lines (SMMC-7721, HepG2, Hep3B), and a normal liver epithelium cell line (L02). As shown in Figure 1A, obviously decreased expression of miR-206 was observed in HepG2 cell lines compared with normal liver epithelium cell line. These results indicated that miR-206 might be involved in the progression of the HCC.

To further determine the biological functions of miR-206 in HCC cells, HepG2 cells were stably transfected with miR-206 mimics using miR-NC as a negative control (NC). MTT assays revealed weakened proliferation ability of HepG2 cells transfected with miR-206 mimics compared with NC-transfected cells (P<0.01; Figure 1B). Additionally, colony formation assays revealed low proliferation of HepG2 cells transfected with miR-206 mimics compared with NC-transfected cells (P<0.01; Figure 1C). Besides, cell apoptosis analysis assays present that forced expression of miR-206 could facilitate cell apoptosis compared with the negative control (Figure 1D). As shown in Figure 1E, western blot assay demonstrated that cells transfected with miR-206 mimics showed a low level of activated caspase-3 and activated PARP, while the level of total caspase-3 and total PARP in cells transfected with miR-206 mimics show no significant compared with negative control. These data together revealed that miR-206 was associated with the HCC cells proliferation.

Down-regulation of miR-206 represses the migration abilities of HCC cells

To determine the effect of miR-206 on the migratory capability of HCC cells. Wound healing assays, and metastasis/invasion assays were utilized to detect the migratory capability of HCC cells. As shown in Figure 2A and 2B, wound healing, and transwell assays results present that forced expression miR-206 could inhibit the migratory capability of HCC cells. Additionally, in consistent with the transwell and wound healing results, attachment/detachment assays revealed that forced miR-206 suppressed HCC cells migration (Figure 2C). This indicates that miR-206 represses the migration abilities of HCC cells.

E2F5 is a target of miR-206

Overexpression of E2F5 has been found to be associated with tumor aggressiveness, metastasis, and poor prognosis in multiple cancers. To investigate whether E2F5 is involved in the function of miR-206, we first detected the level of E2F5 in three HCC cell lines and a normal liver epithelium cell line (Figure 3A). And then we utilized two bioinformatic analysis websites (http://www.targetscan.org/cgi-bin/targetscan/vert_61/view_gene.cgi?taxid=9606&rs=NM_001083588&members=&showcnc=0&shownc=0&showncf=) to forecast the binding sites between miR-206 and E2F5. As shown in Figure 3B (left), miR-206 could bind with the 3'-UTR of E3F5. Therefore, we hypothesized that the functions of miR-26a in HCC cells might be mediated by E2F5. To further validate the regulatory relationship between miR-206a and E2F5, we performed luciferase reporter assays. As shown in Figure 3B (right), miR-206 mimics reduced the luciferase activity of wild-type (WT) E2F5 reporter vector but not that of mutant reporter vector. Additionally, we examined the expression of E2F5 both in mRNA and protein level. As shown in Figure 3C, forced expression of miR-206 could inhibit the expression of E2F5. Together, these data suggest that E2F5 is a bona fide miR-206-targeting gene.

Down-regulated E2F5 suppressed HCC cells proliferation and migration

To determine the biological functions of E2F5 in HCC cells, HepG2 cells were stably transfected with si-E2F5 using siRNA as a negative control (NC). Satisfactory transfection efficiency was obtained at 48 hours post-transfection (Figure...
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Figure 3. The effects of miR-206 was depended on SDF-1. A. The level of E2F5 in three HCC cell lines and a normal liver epithelium cell line was detected both in mRNA and protein level. B. Binding sites between miR-206 and E2F5 (left), luciferase reporter assays further confirmed the regulating relationship between miR206 and E2F5 (right). C. Expression of E2F5 both in mRNA and protein level in response to the level of miR206. Error bars represent the mean ± SD of at least three independent experiments. *P<0.05, **P<0.01 vs. control group.

4A) MTT assays revealed weakened proliferation ability of HepG2 cells transfected with si-E2F5 compared with siRNA-transfected cells (P<0.01; Figure 4B). Additionally, colony formation assays revealed low proliferation of HepG2 cells transfected with si-E2F5 compared with siRNA-transfected cells (P<0.01; Figure 4C). Besides, cell apoptosis analysis assays present that forced expression of si-E2F5 could facilitate cell apoptosis compared with the negative control (Figure 4D). And western blot assay (Figure 4E) demonstrated that cells transfected with si-E2F5 shown a low level of activated caspase-3 and activated PARP, while the level of total caspase-3 and total PARP in cells transfected with si-E2F5 show no significant compared with negative control. What’s more metastasis/invasion and wound healing assays
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Figure 4. A. Cells were transfected with si-E2F5 and transfection efficiency was measured by qRT-PCR. B. MTT and colony formation assays on HCC cells transfected with miR-206 mimics. D. Flow cytometric analyses was employed to exam the apoptosis rate. E. Apoptosis related proteins were detected by western blot. Error bars represent the mean ± SD of at least three independent experiments. *P<0.05, **p<0.01 vs. control group.
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Figure 5. Down-regulated E2F5 suppressed HCC cells proliferation and migration. A-C. Wound healing, metastasis, and attachment/detachment assays were utilized to detect the migratory capability of HCC cells. Error bars represent the mean ± SD of at least three independent experiments. *P<0.05, **P<0.01 vs. control group.

were utilized to detect the function of E2F5 on migratory capability of HCC cells. As shown in Figure 5A and 5B, wound healing and transwell assays results present that si-E2F5 could inhibit the migratory capability of HCC cells. Additionally, in consistent with the transwell and wound healing results, attachment/detachment assays (Figure 5C) revealed that si-E2F5 suppressed HCC cells migration. This indicates that down-regulated E2F5 could repress the migration abilities of HCC cells.

The function of miR-206 in HCC cells was depended on E2F5

We performed rescue experiments to determine whether miR-206 influenced HCC cells proliferation, apoptosis, and the migration in
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E2F5-dependent manner. MiR-206 mimics or miR-NC were transfected into HepG2 cells co-transfected with pcDNA3.1-E2F5 or pcDNA3.1. The weakened proliferation ability caused by miR-206 in HepG2 cells was partially abolished by co-transfected with E2F5 (P<0.01; Figure 6A). The decreased colony formation capacity induced by miR-206 in HepG2 cells was abro-
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gated by the introduction of E2F5 (P<0.01; Figure 6B). Flow cytometric assays revealed that the pro-apoptotic effect of miR-206 could be partially reversed by the introduction of E2F5 (P<0.01; Figure 6C, 6D). The anti-metastasis effect of miR-206 in HepG2 could be partly abolished by co-transfection with E2F5 (P<0.01; Figure 6E-G). These results showed that miR-206 could influence cell proliferation, apoptosis, and metastasis in HepG2 cells in vitro at least in part in an E2F5-dependent manner.

Discussion

MicroRNA expression abnormal has been documented associated with various cancers contributing to tumor initiation and progression, functioning as tumour suppressors or oncogenes [10-12]. The current study showed that miR-206 expression is significantly decreased in human HCC cells. E2F5 is negatively regulated by miR-206 at the posttranscriptional level, via a specific target site within the 3'UTR. Previous studies have reported that miR-206 could inhibit cell proliferation, migration and invasion via targeting downstream genes [13-19]. Moreover, Xiao H et al. showed that miR-206 could function as a novel cell cycle regulator and tumor suppressor in clear-cell renal cell carcinoma through targeting cell cycle related gene CDK4, CDK9 and CCND1 [20]. Furthermore, Kondo N. et al. documented that miR-206 could be a novel candidate for endocrine therapy that targets only ERalpha in breast cancer [4]. Besides, in 2011, loss of miR-206 was reported to play an important role in the progress of laryngeal squamous cell carcinoma and miR-206 may function as a novel tumor suppressed miRNA [18]. However, the expression and the potential biological function of miR-206 in HCC cells have not been reported.

It was well known that miRNAs exerted its biological function by regulating target genes [21]. Therefore, identification and characterization of the targets of altered miRNAs may contribute to elucidate the molecular mechanisms involved in carcinogenesis. Two bioinformatic analysis websites (http://www.targetscan.org/cgi-bin/targetscan/vert_61/view_gene.cgi?taxid=9606&rs=NM_001083588&members=&showcnc=0&shownc=0&showncf=), and (http://mirdb.org/cgi-bin/target_detail.cgi?targetID=1804872) has been applied to forecast the binding sites between miR-206 and E2F5. E2F5 belongs to E2Fs family which is a large family of transcription factors containing one or more conserved DNA binding domains that binds to the promoters of the targeted gene involved in tumor progression [22]. Accumulating documents reported that E2F5 expression was upregulated in various solid tumors including ovarian cancer and been identified as target gene of multiple miRNAs [23-29]. In current study, we characterized E2F5 as a functional target of miR-206 by luciferase reporter gene assays, qRT-PCR and Western blot analysis, respectively. And we found that the expression of E2F5 was negative correlated with miR-206 expression in HCC cells. What’s more, deletion of E2F5 has similar inhibition effect of miR-206 overexpression on the HCC cells. These results suggested that miR-206 exerted tumor suppressor role in HCC cells by targeting E2F5.

In summary, our present results first showed that the expression of miR-206 in HCC cells was significantly low than that in normal liver epithelial cell. Moreover, overexpression of miR-206 could significantly suppress cell proliferation, promote cell apoptosis, an inhibit cell migration/invasion through targeting E2F5. This regulatory pathway provides new insight into the mechanisms underlying the progression of HCC, and targeting this signaling pathway may be a potential least therapeutic strategy for treating HCC.

Disclosure of conflict of interest

None.

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