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HIFs-MiR-33a-Twsit1 axis can regulate invasiveness of hepatocellular cancer cells

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Abstract. – OBJECTIVE: In this study, we investigated whether miR-33a downregulation in HCC is a result of hypoxia-inducible factors (HIFs) overexpression. Then, we further studied the regulative effects of miR-33a on Twist1 and their regulation in HCC cell invasiveness.

MATERIALS AND METHODS: Human hepatocellular cancer (HCC) cell lines (HepG2 and BEL-7402) were transfected with miR-33a mimics, HIFs siRNA or Twist1 siRNA. MiR-33a level was measured using QRT-PCR. The binding between miR-33a and Twist1 3'UTR was verified using Western blot analysis and dual luciferase assay. E-cadherin and N-cadherin expression levels were detected by western blot analysis. Tumor cell invasion was assessed using transwell assay.

RESULTS: MiR-33a downregulation in HCC cells is hypoxia-induced and is a result of HIFs upregulation. HIF-1α and HIF-2α suppression partly rescued miR-33a expression under hypoxia. Both HepG2 and BEL-7402 cells with miR-33a overexpression had significantly decreased E-cadherin expression and increased N-cadherin level. Transwell analysis confirmed that miR-33a overexpression significantly suppressed the tumor cell invasion capability. Twist1 is a direct target of miR-33a in HCC. HepG2 cells with Twist1 knockdown had significantly increased E-cadherin, decreased N-cadherin and suppressed invasion capability.

CONCLUSIONS: MiR-33a downregulation in HCC cells is hypoxia-induced and is a result of HIFs upregulation. MiR-33a can modulate EMT and invasion of hepatocellular cancer cells at least partly via downregulating Twist1.

Key Words:
HIFs, miR-33a, Twsit1, Invasion, Hepatocellular cancer.

Introduction

Hypoxic mass within the neoplastic mass is one of the key features of solid malignant tumors, including hepatocellular carcinoma (HCC)[1]. The chronic hypoxia exerts strong selective pressure to tumor cells and changes cell behaviors such as extracellular matrix remodeling, increased migratory and metastatic potential[2,3]. Hypoxia-inducible factors (HIFs) are among the most important transcriptional regulators regulating the cellular response to hypoxia[4]. In human, three isoforms of HIFα, including HIF-1a, HIF-2a, and HIF-3a are involved in hypoxic responses[5].

MiRNA are conserved small noncoding RNA, which negatively regulate gene expression via base-pairing with a complementary sequence within mRNAs[6,7]. Previous studies found that hypoxia could induce changes in miRNA expression and subsequent tumor cell behaviors via HIFs mediated mechanisms. For example, miR-210 can be induced via a HIF-1 or HIF-2 dependent manner[8] and miR-210 augments the metastatic potential of HCC cells by targeting vacuole membrane protein[9] and tissue inhibitor of metalloproteinases 2 (TIMP2)[10]. Hypoxia-suppressed miR-199a can inhibit glycolysis in HCC cells by targeting hexokinase-2 (Hk2) and pyruvate kinase-M2 (Pkm2)[11]. The MiR-30c expression is inhibited by hypoxia in a HIFs-dependent manner and promotes epithelial-mesenchymal transition (EMT) in human renal cell carcinoma[12].

MiR-33a is a tumor suppressive miRNA in HCC. One recent study found that miR-33a is usually downregulated in HCC cells[13]. Functionally, miR-33a can directly target β-catenin and downregulate its expression, thereby weakening the β-catenin signaling pathway and inhibiting cell growth[13]. However, how it is downregulated in HCC and whether other downstream targets are involved in its tumor suppressive effects are not clear. In this study, we investigated whether miR-33a downregulation in HCC is a result of

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HIFs overexpression. In addition, by performing bioinformatics analysis, we found that Twist1, which is an EMT-promoting transcription factor[14,15] is a possible target of miR-33a. Therefore, we further studied the regulative effects of miR-33a on Twist1 and their regulation in HCC cell invasiveness.

Materials and Methods

Cell Culture and Treatment

HCC cell line HepG2 and BEL-7402 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Eagle's minimum essential medium supplemented with 10% fetal-bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were cultured in 37°C with 5% CO_2 in humidified atmosphere. For hypoxic treatment, oxygen supply was set to 1%. HepG2 and BEL-7402 were cultured under hypoxic condition for 12 or 24 hours and then subjected to Western blot analysis of HIF-1α and HIF-2α expression and qRT-PCR analysis of miR-33a expression.

MiR-33a mimics, HIF-1α and HIF-2α siRNA (si-HIFs), Twist1 siRNA and the corresponding negative controls were purchased from Ribobio (Guangzhou, China). HepG2 and BEL-7402 cells were transfected with 100 nM miR-33a, 100 nM Twist1 siRNA, or 50 nM HIF-1α siRNA and 50 nM HIF-2α siRNA in combination using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. HepG2 and BEL-7402 cells transfected with si-HIFs were treated under hypoxic condition for 24 hours and then subjected to qRT-PCR analysis of miR-33a expression.

QRT-PCR Analysis

Total RNA from cells samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, MiRNAs specific cDNA was synthesized using stem-loop primers and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). After the reverse transcription, mature miR-33a expression level was quantified using qRT-PCR analysis with TaqMan MicroRNA Assay Kit (Applied Biosystems). The relative expression of miR-33a was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Conventional Western blot analysis was performed. The cancer cells were collected and lyzed for protein extraction. Then, the protein samples were denatured and then 25 μg proteins were loaded in each lane for separation using SDS-PAGE with 10% acrylamide gels. After that, the proteins were transferred to nitrocellulose membrane. The primary antibodies used include anti-HIF-1α (1:1000, ab82832, Abcam, Cambridge, UK), anti-HIF-2α (1:1000, ab73895, Abcam), anti-E-cadherin (1:1000, #3195, Cell Signaling, Danvers, MA, USA), anti-N-cadherin (1:1000, #13116, Cell Signaling, Danvers, MA, USA) and anti-Twist1 (1: 2000, ab175430) and anti-β-actin (ab3280, Abcam). The signals were visualized by using the ECL Western blotting substrate (Promega, Madison, WI, USA) and the gray scale of the protein bands were quantified by using the Image-J software.

Transwell Analysis of Cell Invasion

In brief, 24 hours after transfection, 1×10^5 cells were placed into chambers coated with 150 μg of Matrigel. The chambers were then inserted into the wells of a 24-well plate and incubated for 48 hours in Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 20% fetal bovine serum. Then, The invading cells on the bottom side were fixed with 4% polyoxymethylene and stained with 0.1% crystal violet. Cell counting was performed at 100× magnification under a microscope.

Dual Luciferase Assay

Based on bioinformatics data, two short sequences of oligonucleotides containing the predicted targeting sites between miR-33a and 3'UTR of Twist1 and the corresponding mutant sequences were chemically synthesized. The sequences were, then, cloned into the downstream of the luciferase gene of pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). The recombinant vectors were named as pGLO-Twist1-WT and pGLO-Twist1-MT respectively. HepG2 cells were co-transfected with 200 ng luciferase reporter vector and 100 nM miR-33a mimics or the negative controls. Luciferase activity was examined 24 hours after the transfection using the Dual-Luciferase Assay kit (Promega) according to manufacturer's instruction.

Statistical Analysis

The statistical analysis was performed using Graphpad Prism 5.1. Data were presented in the form of means ± standard deviation based on at least three repeats. The comparison was performed using the unpaired t-test. p-value of <0.05 was considered as statistically significant.

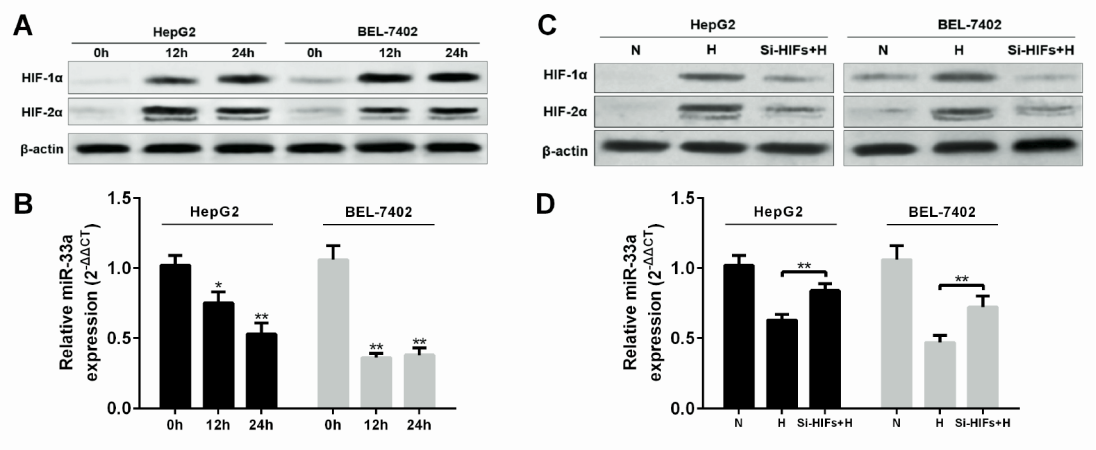

Figure 1. Hypoxia-induced significant downregulation of miR-33a in HCC cells. A-B. Western blot analysis of HIF-1α and HIF-2α levels ***(A)*** and qRT-PCR analysis of miR-33a levels (B) in HepG2 and BEL-7402 cells after 0, 12 and 24 hours hypoxic culture. C-D. Western blot analysis of HIF-1α and HIF-2α levels (C) and qRT-PCR analysis of miR-33a levels (B) in HepG2 and BEL-7402 cells with or without transfection of HIF-1α siRNA (50 nM) and HIF-2α siRNA (50 nM) in combination 24 hours after hypoxic culture. N: normoxia; H: hypoxia; $^{*}p<0.05$; $^{**}p<0.01$.

Results

Hypoxia-Induced Significant Downregulation of miR-33a in HCC Cells

Previous studies[16,17] reported that HIF-1α and HIF-2α expression is associated with poor prognosis of hepatocellular carcinoma. In this investigation, we firstly examined the level of HIF-1α and HIF-2α expression in HCC cell lines under hypoxic conditions. Western blot analysis showed that the hypoxic culture induced significant upregulation of HIF-1α and HIF-2α in both HepG2 and BEL-7402 cells (Figure 1A). Then, we analyzed the expression changes of miR-33a under hypoxic culture. QRT-PCR showed that hypoxia-induced substantial downregulation of miR-33a in both HepG2 and BEL-7402 cells (Figure 1B). Then, we decided to investigate whether there is a causative effect between HIF-1α and HIF-2α upregulation and miR-33a downregulation in HCC cells. Both HepG2 and BEL-7402 cells were transfected with si-HIF-1α and si-HIF-2α in combination. Western blot analysis confirmed that si-HIFs significantly suppressed HIF-1α and HIF-2α upregulation induced by hypoxia (Figure 1C). HepG2 and BEL-7402 cells with HIF-1α and HIF-2α suppression also had partly rescued miR-33a expression under hypoxia (Figure 1D). These findings suggest that hypoxia-induced HIF-1α and HIF-2α results in significant downregulation of miR-33a in HCC cells.

MiR-33a Can Modulate EMT and Invasion of Hepatocellular Cancer Cells

One previous study[13] reported that miR-33a has inhibitive effects on carcinogenesis of HCC via decreasing β-catenin expression and thereby weakening the Wnt/β-Catenin signaling pathway. In this work, we further investigated the role of miR-33a in HCC cells. HepG2 and BEL-7402 cells were firstly transfected for miR-33a overexpression (Figure 2A). In these two cell lines, we found that miR-33a overexpression significantly decreased E-cadherin expression, while increased N-cadherin level (Figure 2B-C). Since EMT is an important mechanism associated with enhanced tumor cell invasiveness, we then explored how miR-33a modulates invasion of HepG2 and BEL-7402 cells. Transwell assay showed that miR-33a overexpression significantly suppressed the invasion capability (Figure 2D-E). These results suggest that miR-33a can modulate EMT and invasion of hepatocellular cancer cells.

MiR-33a Modulates Invasiveness of Hepatocellular Cancer Cells Via Downregulating Twist1

Since we confirmed the role of miR-33a in EMT and invasion of hepatocellular cancer cells, we then further investigated the downstream targets. Based on our preliminary bioinformatics analysis, we found that the 3'UTR of Twist1 has a highly conserved possible target of miR-33a (Figure 3A). Twist1 is an EMT-promoting transcription factor[14,15]. Therefore, we decided to

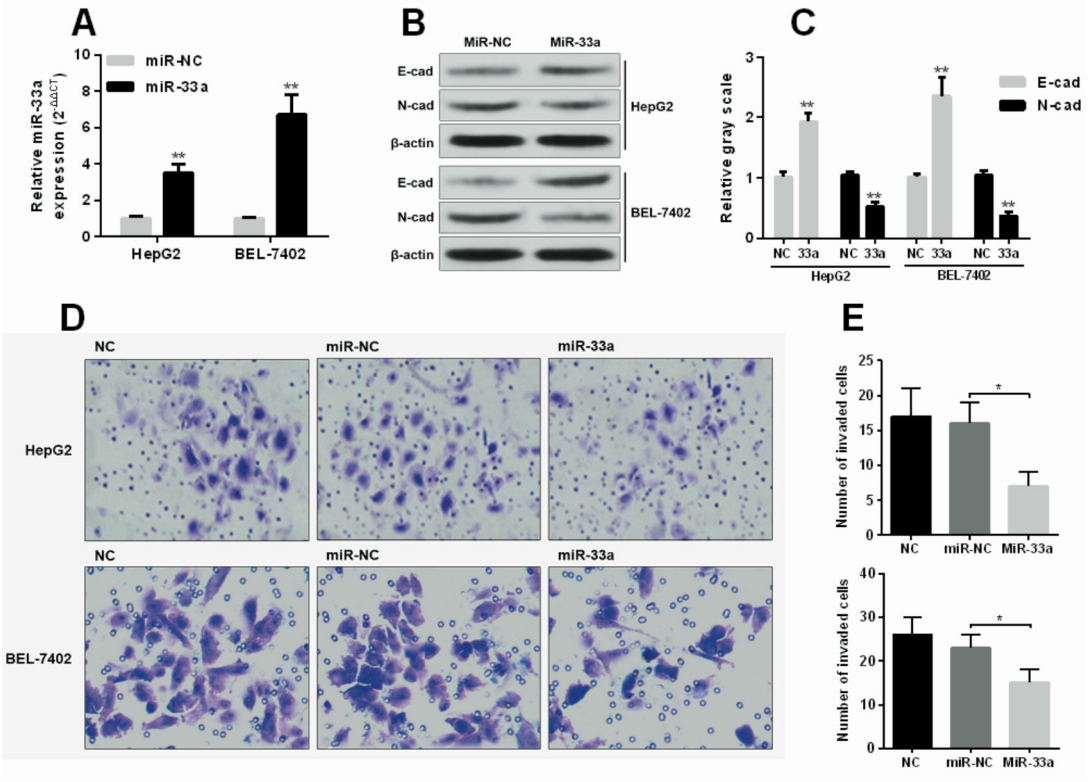

Figure 2. MiR-33a can modulate EMT and invasion of hepatocellular cancer cells. ***A.*** qRT-PCR analysis of miR-33a levels in HepG2 and BEL-7402 cells with or without transfection of miR-33a mimics (100 nM). ***B-C.*** Images (B) and quantitation (C) of Western blot analysis of E-cadherin (E-cad) and N-cadherin (N-cad) in HepG2 and BEL-7402 cells 48 hours after transfection of miR-33a mimics (33a, 100 nM). ***D-E.*** Representative images (D) and quantitation (E) of transwell assay of invading HepG2 and BEL-7402 cells with or without transfection of miR-33a mimics. $^{*}p<0.05$; $^{**}p<0.01$.

verify the regulative effect of miR-33a on Twist1. Western blot data showed that enforced miR-33a expression had a similar effect as Twist1 siRNA on suppressing Twist1 expression in HepG2 cells (Figure 3B). By performing dual luciferase assay, we observed that miR-33a mimics could significantly suppress the expression of luciferase of the reporter with wild-type sequence. However, it had no suppressive effect on the reporter carrying mutant sequence (Figure 3C). Then we explored the role of Twist1 in EMT and invasion of HepG2 cells. Knockdown of Twist1 significantly increased E-cadherin, decreased N-cadherin (Figure 3D-E), and suppressed cell invasion capability (Figure 3F-G). These results suggest that miR-33a modulates invasiveness of HCC cells at least partly via downregulating Twist1.

Discussion

Hypoxia-induced miRNA dysregulation and its involvement in pathological development are widely seen in solid tumors. For example, in prostate cancer, hypoxia-induced upregulation of miR-301a/b can increase cell autophagy and also enhance cancer cell viability by targeting NDRG2[18]. Hypoxic-induced HIF-1α can bind to the promoter region of miR-182 and increase its expression in prostate cancer cells. Also, miR-182 can decrease PHD2 and FIH1 levels, two negative regulators of HIF-1α[19]. This feedback regulation facilitates the adaption of the cancer cells to hypoxic stress during prostate tumor progression[19]. HIF-1α can also bind to the promoter region of miR-21 and enhance its transcription, thereby enhancing cell proliferation and reducing cell apoptosis in pancreatic cancer[20] and breast cancer[21]. Hypoxia-induced miRNA downregulation is also observed in some types of cancer. For example, hypoxia-induced downregulation of miR-30c can promote EMT in human renal cell carcinoma[12]. MiR-30a and miR-205 are significantly downregulated due to hypoxia in prostate cancer cells and can modulate radiosensitivity of the cancer cells by inhibiting autophagy via TP53INP1[7].

MiR-33a plays as a tumor suppressor in several types of cancer. For example, it can inhibit melanoma cell proliferation by targeting PCTAIRE1[22], suppress bone metastasis of lung cancer via targeting parathyroid hormone related protein[23] and decrease breast cancer cell proliferation and metastasis by targeting ADAM9 and ROS1[24]. In HCC, miR-33a can directly target β-catenin and downregulate its expression, thereby weakening the β-catenin signaling pathway and inhibiting tumor cell growth[13]. In these tumors, miR-33a is usually downregulated[13,22-24]. However, how it is downregulated is not quite clear. In this investigation, we observed that miR-33a downregulation in HCC cells is hypoxia-induced and is a result of HIFs upregulation. HIF-1α and HIF-2α suppression partly rescued miR-33a expression under hypoxia. One previous study[25] observed that miR-33a can inhibit EMT and metastasis of non-small cell lung cancer. Therefore, we decided to explore whether miR-33a has similar regulative effects in HCC. By performing western blot analysis, we found that both HepG2 and BEL-7402 cells with miR-33a overexpression had significantly decreased E-cadherin expression and increased N-cadherin level. Also, transwell analysis confirmed that miR-33a overexpression significantly suppressed tumor cell invasion capability. Therefore, we infer that miR-33a can modulate EMT and invasion of hepatocellular cancer cells.

One miRNA usually targets multiple genes and exerts complex regulative effects. In HCC, only β catenin is a verified target of miR-33a[13]. Therefore, we investigated further the possible downstream targets of miR-33a in HCC. Our preliminary bioinformatics analysis showed that the 3'UTR of Twistl has a highly conserved possible target of miR-33a. Twistl is an EMT-promoting transcription factor[14,15]. By performing Western blot analysis and dual luciferase assay, we confirmed that Twistl is a direct target of miR-33a in HCC. Besides, we also observed that HepG2 cells with knockdown of Twistl had significantly increased E-cadherin, decreased N-cadherin and suppressed invasion capability. Therefore, we infer that the HIFs-MiR-33a-Twsitl axis can regulate invasiveness of hepatocellular cancer cells.

Conclusions

MiR-33a downregulation in HCC cells is hypoxia-induced and is a result of HIFs upregulation. MiR-33a can modulate EMT and invasion of hepatocellular cancer cells at least partly via downregulating Twistl.

Conflicts of interest

The authors declare no conflicts of interest.

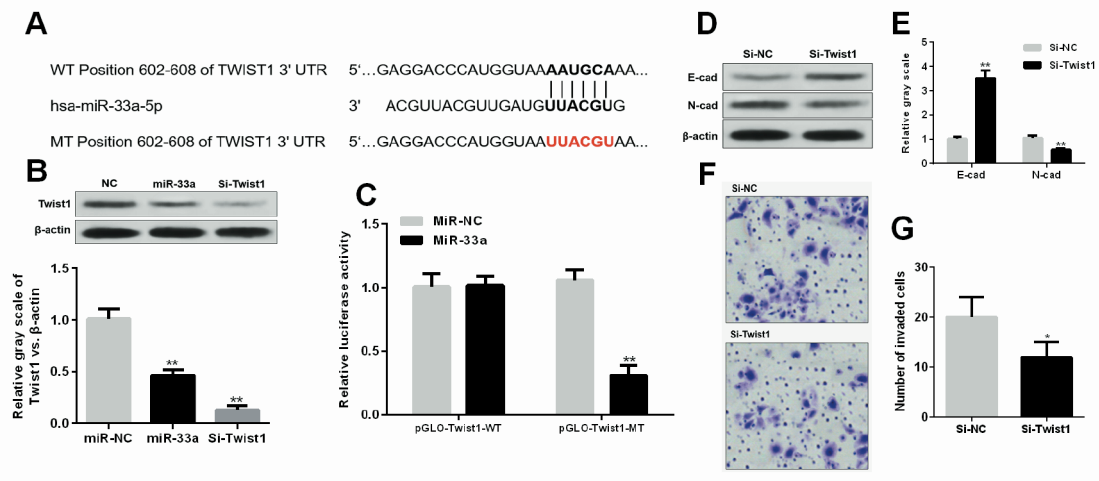

Figure 3. MiR-33a modulates invasiveness of hepatocellular cancer cells via downregulating Twistl. ***A.*** Predicted binding sites between miR-33a and 3'UTR of Twistl. ***B,*** Images and quantitation of Western blot analysis of Twistl in HepG2 cells 48hours after transfection of miR-33a mimics or Twistl siRNA. ***C.*** The relative luciferase activity in HepG2 cells with transfection of reporter carrying wild-type sequence (pGLO-Twistl-WT) or reporter carrying mutant sequence (pGLO-Twistl-MT) in combination with 100 nM miR-33a mimics. D-E. Images (D) and quantitation (E) of Western blot analysis of E-cad and N-cad in HepG2 cells 48 hours after transfection of Twistl siRNA (100 nM). F-G. Representative images (F) and quantitation (G) of transwell assay of invading HepG2 cells after transfection of Twistl siRNA. $^{*}p<0.05$; $^{**}p<0.01$.

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