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Original Research Article

MicroRNA-33b inhibits liver cancer cell proliferation, migration and invasion via down-regulation of Fli-1 and MMP-2 protein expressions

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Abstract

Purpose: *To study the influence of microRNA-33b (miR-33b) on liver cancer cell proliferation, migration and invasiveness, and the mechanism involved.*
Methods: *MicroRNA-33b or Fli-1 overexpression plasmid was transfected into liver cancer (SMMC7721) cells. Cell proliferation, migration, and invasiveness were determined using cell counting kit 8 (CCK-8), scratch test, and Transwell invasion assay, respectively. The amounts of miR-33b and Fli-1 in liver cancer tissues, paracancerous normal tissues, and miR-33b overexpression and control groups were measured using qRT-PCR, while protein concentration of matrix metalloproteinase 2 (MMP-2) was assayed using Western blotting.*
Results: *Fli-1 protein was markedly upregulated in liver cancerous cells, relative to paracancerous normal tissues ($p < 0.05$). MicroRNA-33b protein expression was also significantly upregulated in miR-33b overexpression group, but the corresponding Fli-1 expression was downregulated in miR-33b overexpression group, relative to control ($p < 0.05$). MicroRNA-33b overexpression significantly and time-dependently inhibited SMMC7721 cell proliferation and migration, but it reduced the degree of apoptosis ($p < 0.05$). Liver cancer (SMMC7721) cells in miR-33b overexpression group were less invasive than the control group ($p < 0.05$). Similarly, miR-33b overexpression significantly downregulated MMP-2 protein expression in SMMC7721cells ($p < 0.05$).*
Conclusion: *Overexpression of miR-33b suppresses the proliferation, migratory and invasive potential of hepatic cancer cells via down-regulation of Fli-1 and MMP-2 protein expression. This finding may be useful in the identification of new liver cancer drugs.*

Keywords: *Liver cancer, Proliferation, Matrix metalloproteinase-2, Fli-1, MicroRNA-33b, Over-expression plasmid*

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INTRODUCTION

Primary hepatocellular carcinoma (PHCC), the most common form of liver cancer, is prevalent among people with liver diseases, particularly in individuals with chronic hepatitis B and C. It is an insidious disease. The disease is treated with surgery, tissue transplant, cryotherapy, and chemotherapy [1]. In the hierarchy of malignant tumors, liver cancer is ranked fifth, with more

than 800,000 new cases occurring annually worldwide. In terms of mortality, it is ranked second after lung cancer. China has the highest incidence of liver cancer in the world [1].

At the time of diagnosis, most patients with liver cancer are either in the middle or late stages of the disease, leading to death within 1 year after diagnosis [1]. Liver cancer is characterized by high invasiveness and recurrence. Its pathogenesis is not well-understood, but is thought to be caused by various factors. Inflammatory factors also increase the risk of cell cancerization by promoting their conversion to malignant tumors [2]. At present, hepatectomy appears to be the most effective therapeutic method for hepatic carcinoma. In the early stage of the disease, postoperative survival is about 15 %, but survival in the long-term is drastically reduced due to metastasis and recurrence.

The role of E26 transformation-specific (ETS) family of transcription factors in various physiological and pathological processes such as cell proliferation, differentiation, migration, angiogenesis, and cell cycle was only recently discovered [3]. During the initiation and progression of various tumors, ETS-1 binds to the promoter regions of MMPs and urokinase-type plasminogen activator (uPA) genes, thereby regulating their protein expressions. Thus, it plays a key role in tumor angiogenesis and invasion.

Although Fli-1 has been reported to be involved in hepatic carcinogenesis, its specific physiological function and underlying mechanism are yet to be fully elucidated [4].

MicroRNAs (miRNAs) are endogenous non-coding RNA molecules involved in a number of important physiological and pathological processes such as growth and development of eukaryotes, cell differentiation and metabolism, and inflammation [5]. Studies have shown that miR-33b is associated with the pathogenesis of various malignant tumors such as esophageal, lung and liver cancers. However, the precise molecular mechanism involved remains unknown [6]. The purpose of this study was to unravel the influence of miR-33b on liver cancerous cell viability, migration and invasiveness, and the underlying mechanisms.

EXPERIMENTAL

Materials

Liver cancer cell line SMMC7721 was supplied by Shanghai Hongshun Biotechnology Co. Ltd. Ultra-clean workbench (YJ-VS-1) was purchased from Wuxi Yijing Purification Equipment Co. Ltd. Cell incubator (BPH-9042) was product of Shanghai Yiheng Scientific Instrument Co. Ltd. Inverted biological microscope (VMB2200A) was obtained from Jingtong Life Science Instruments Company. Low-temperature, high-speed centrifuge (TGL-16.5M) was purchased from Shanghai Luxiangyi Centrifuge Instrument Co. Ltd. Real-time fluorescence quantitative PCR analyzer (FQD-33A) was purchased from Beijing Xinxing Johnson Biotechnology Co. Ltd. Electronic balance (FA2104B) was product of Shanghai Precision Instrument Co. Ltd. Constant temperature water bath (HH-600) was obtained from Changzhou Jintan Youlian Instrument Research Institute, while FBS was product of Shanghai Yubo Biotech. Phosphate-buffered saline (PBS) was product of Jiangsu Enmoase Biotechnology Co. Ltd. Penicillin/streptomycin double antibody was obtained from Beijing Biolab Technology Co. Ltd, while DMEM was supplied by Shanghai Jiake Biotech.

Cell culture

The SMMC7721 cells were maintained in DMEM containing 10 % FBS and antibiotics (1 % streptomycin/penicillin for 24 h in a 5 % CO_2 incubator at 37 °C until the cells attained 80 % confluency. The medium was refreshed every two days. After 1 week of incubation, the adherent confluent cells were trypsinized with 0.25 % trypsin-EDTA (2 mL), and then cultured again and passaged for later use.

Cell transfection

Logarithmic growth cells were cultured in plates until they attained 80 % fusion. Then, the cells were cultured in serum-free medium with equal volume of miR-33b or Fli-1 overexpression plasmid, each at 10 a concentration of μmol/L. Incubation was carried out at room temperature for 6 h. Lipofectamine 2000 was dissolved in serum-free medium and incubated at room temperature for 10 min to form a mixture. The mixture was then added to cells in each group, and cultured at 37 ºC in a 5 % CO_2 incubator for 48 h. Normal cell culture without miR-33b or Fli-1 plasmid served as control group. The transfection efficiency was determined using qRT-PCR.

Measurement of cell proliferative potential

This was carried out using CCK-8 assay kit, with cells at a concentration of 6 x 10^3/well (100 μL/well) cultured under the same conditions as indicated earlier, for 24 h. Then, the cells were

cultured with 20 µL of CCK-8 for 72 h, after which the absorbance of each well was read in a microplate reader at 570 nm. The assay was performed in triplicate.

Apoptotic assay

The SMMC7721 cells were cultured at 37 ºC for 72 h. They were thereafter washed with PBS, and thoroughly mixed with 500 µL binding buffer. This was followed by staining with 10 µL each of Annexin V-fluorescein isothiocyanate and PI within 10 min at room temperature in darkness. Cell apoptosis was determined at 488 nm in a flow cytometer fitted with argon laser.

Cell migration measurement

The migratory potential of SMMC7721 cell line was measured using scratch test. The cells were cultured until they attained 80 % confluency, and scratches were made on the cell monolayers. After washing thrice with serum-free medium, the cells were further cultured for 72 h, and then observed and analyzed using Image Pro Plus (6.0).

Cell invasion assay

The degree of invasion of SMMC7721 cells was measured with Transwell invasion method. Cells (1×10^5/mL) were placed in Transwell chamber coated with substrate and cultured in serum-free medium, while the lower chamber contained 10 % FBS. After 24 h, cells that passed the Matrix gel membrane were subjected to staining with crystal violet after fixation, photographed and counted under an inverted microscope.

Determination of levels of expression of miR-33b and Fli-1 in liver cancer tissues and SMMC7721 cells

The expression levels of miR-33b and Fli-1 in liver cancer tissues, paracancerous normal tissues, and miR-33b-overexpression and control groups were measured using qRT-PCR. The tissues were first homogenized and thereafter trypsinized to obtain cell suspensions. Extraction of RNA was done with Trizol Total RNA extraction kit, and reverse-transcribed to cDNA. The mRNA expressions were determined using Light Cycler 1536 RT-PCR.

Variation in the cDNA content was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. The qRT-PCR reaction conditions were: pre-denaturation at 95 ºC for 5 min; PCR reaction at 95 ºC for 5 sec and 60 ºC for 30 sec, and a total of 40 cycles. The PCR reaction mixture (20 µL) consisted of 6.4 µL of dH_2O, 1.6 µL of primer (10 µM), 2 µL of cDNA and 10 µL of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as internal parameter, and $2^{-\Delta\Delta Ct}$ was used to calculate the relative protein expressions.

Western blotting

The protein expression levels of Fli-1, miR-33b and MMP-2 in each group of cells were determined using Western blotting. The cells were rinsed in PBS and lysed with chilled RIPA buffer containing protease inhibitor. The resultant lysate was clarified via centrifugation, and the protein content of the supernatant was measured with BCA kits. Then, 40 µg of each sample was subjected to electrophoretic separation on 12 % Na dodecyl sulphate-polyacrylamide, followed by transfer to PVDF membrane for 120 min. Non-specific binding was blocked by incubating the membrane with 3 % non-fat milk solution at 37 ºC. Incubation of the blots was performed overnight at 4 ºC with primary antibodies for miR-33b, Fli-1, MMP-2 and GAPDH, each at a dilution of 1 to 1000. Following rinsing with TBS-T, the membrane was incubated with HRP-linked 2^0 antibody for 90 min at laboratory temperature. Following blot development with X-ray film, the bands were subjected to grayscale analysis with Bio-rad gel imaging protocol, with GAPDH expression as standard.

Statistical analysis

Results are presented as mean ± SEM. Statistical analysis was carried out with SPSS (22.0). Groups were compared using Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

RESULTS

Levels of miR-33b and Fli-1 proteins in liver cancer and paracancerous normal tissues

As shown in Table 1, the protein level of miR-33b was significantly downregulated in liver cancer cells, when compared to paracancerous normal tissues. However, the corresponding Fli-1 protein expression was markedly upregulated in liver cancer cells, when compared to paracancerous normal tissues ($p < 0.05$).

MicroRNA-33b and Fli-1 expression levels in miR-33b-overexpression and control groups

MicroRNA-33b protein expression was significantly upregulated in transfected cells, relative to control ($p < 0.05$), but the

corresponding Fli-1 was downregulated significantly in miR-33b overexpression group, relative to control ($p < 0.05$; Figure 1 and Table 2).

Table 1: Expression levels of miR-33b and Fli-1 proteins in liver cancer and paracancerous normal tissues (n = 42)

Group	MiR-33b	Fli-1
Liver cancer tissues	0.61 ± 0.06	2.13 ± 0.16
Paracancerous normal tissue	1.23 ± 0.12	0.87 ± 0.11
t	29.949	42.056
P-value	< 0.001	< 0.001

Table 2: MicroRNA-33b and Fli-1 expressions in miR-33b overexpression and control groups

Group	MiR-33b	Fli-1
MiR-33b overexpression	9.97 ± 0.19	0.53 ± 0.01
Control	1.01 ± 0.04	1.00 ± 0.03
t	92.293	29.725
p	< 0.001	< 0.001

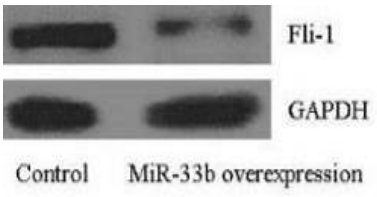

Figure 1: Levels of expression of Fli-1 protein in miR-33b-overexpression and control groups

Effect of miR-33b overexpression on proliferative ability of SMMC7721 cells

MicroRNA-33b overexpression significantly and time-dependently inhibited SMMC7721 cell proliferation (Table 3).

Table 3: Influence of miR-33b overexpression on proliferative capacity of SMMC7721 cells

Group	Cell proliferation			
	0 h	*24 h*	*48 h*	*72 h*
MiR-33b overexpression	0.26 ± 0.01	0.55 ± 0.07	1.01 ± 0.04	1.21 ± 0.09
Control	0.25 ± 0.01	0.63 ± 0.06	1.36 ± 0.09	1.65 ± 0.06
t	1.414	1.735	7.107	8.136
P-value	0.207	0.133	< 0.001	< 0.001

Effect of miR-33b overexpression on SMMC7721 cell apoptosis

As shown in Figure 2, miR-33b overexpression significantly reduced the number of SMMC7721 cell clones ($p < 0.05$). There were more apoptotic cells in miR-33b overexpression group than in the control group ($p < 0.05$).

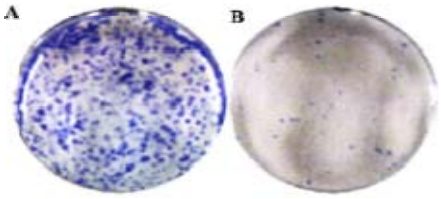

Figure 2: Effect of miR-33b overexpression on SMMC7721 cell apoptosis. A: control group; B: miR-33b overexpression group

Effect of miR-33b overexpression on SMMC7721 cell migration

When miR-33b was overexpressed, there was marked reduction in the migratory capacity of SMMC7721 cells, as shown in Table 4 and Figure 3.

Table 4: Effect of miR-33b overexpression on SMMC7721 cell migration

Group	Cell migration (%)
MiR-33b overexpression	21.47 ± 11.55
Control	53.69 ± 8.33
t	4.525
p	0.004

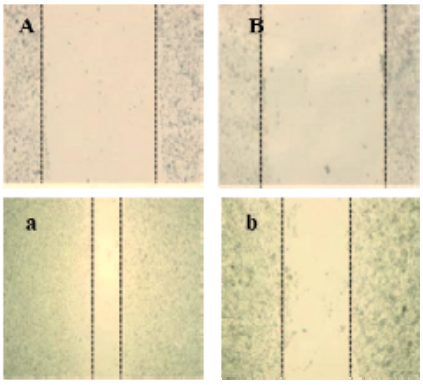

Figure 3: Effect of miR-33b overexpression on SMMC7721 cell migration. (A): Control group at 0 h; (a): Control group at 24 h; (B): miR-33b overexpression group at 0 h; and (b): miR-33b overexpression group at 24 h

Effect of miR-33b overexpression on SMMC7721 cell invasive ability

As shown in Table 5 and Figure 4, SMMC7721 cells in miR-33b overexpression group were less invasive than cells in the control group ($p < 0.05$).

Table 5: Effect of miR-33b overexpression on SMMC7721cell invasive ability

Group	Number of cell invasions (piece)
MiR-33b overexpression	123.74 ± 51.61
Control	397.83 ± 36.82
t	8.647
P-value	< 0.001

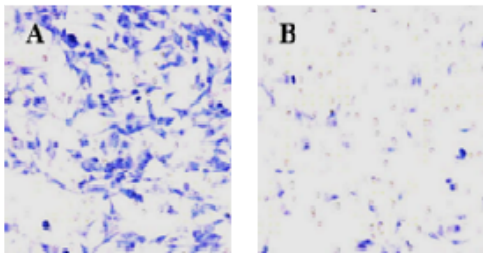

Figure 4: Influence of miR-33b overexpression on invasive ability of SMMC7721 cells. A: control group; B: miR-33b overexpression group

Effect of miR-33b overexpression on MMP-2 expression levels in SMMC7721cells

Figure 5 shows that microRNA-33b overexpression significantly downregulated MMP-2 protein expression in SMMC7721 cells.

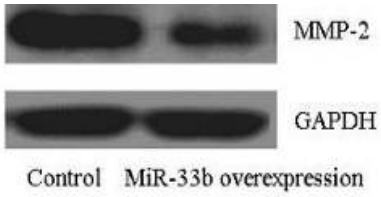

Figure 5: Influence of miR-33b overexpression on MMP-2 expression levels in SMMC7721 cells

DISCUSSION

Hepatocellular carcinoma is mostly associated with chronic liver diseases such as cirrhosis due to hepatitis B or hepatitis C. Liver cancer is a common malignant tumor and one of the main causes of cancer-related deaths worldwide. Mortality caused by liver cancer is the highest among gastrointestinal tract malignancies. At present, the most effective treatment for liver cancer is hepatectomy. The optimization of surgical equipment and improvements in surgical procedures contribute significantly to successes achieved with hepatectomy. Targeted therapy has also contributed greatly to the treatment of liver cancer. Multidisciplinary approach to treatment offers great promise for patients with this disease. Liver cancer is characterized by poor prognosis because of its insidious onset, and the 5-year survival of patients is very low [7]. Therefore, a proper understanding of the pathogenesis of this disease is key to development of a reliable therapeutic strategy.

The miRNAs are single-stranded small molecules with a nucleotide length of about 22. They function through base-pairing with complementary sequences within mRNA molecules. They are involved in post-transcriptional regulation in cells [8]. MicroRNAs have been implicated in cellular events such as programmed cell death, growth and differentiation, autophagy, metabolism and metastasis [9]. Abnormal expressions of miRNAs have been reported to contribute to the initiation and progression of tumors [10]. More than a dozen abnormally-expressed miRNAs (such as miR-15a) are reported to participate in the pathogenesis of liver cancer, thereby making them potential biomarkers for the diagnosis of the disease in its early stage [11]. Factors such as tumor type and progression, and individual differences determine the type of miRNA expressed at a particular time. This study investigated the influence of miR-33b on liver cancerous cell viability, migration and invasiveness. The miR-33b protein was downregulated in liver cancer tissues, an indication that it may be implicated in etiology of hepatic cancer.

It is known that Fli-1 is involved in the regulation of hematopoietic and vascular endothelial systems. Its involvement in malignant transformation of cells and maintenance of malignant phenotypes has been reported. The Fli-1 gene has been shown to be abnormally expressed in leukemia, breast, colon, lung and liver cancer [12]. Studies have also shown that Fli-1 overexpression in breast cancer cells influences the level of MMP-1 protein expression [13-15]. Data from the present investigation indicate that the overexpression of miR-33b may significantly downregulate Fli-1 protein expression.

Metastasis, a multi-step, multi-pathway and cytokine-regulated process, involves intercellular skeletal changes, integrin-based adhesion, and cell-extracellular matrix interactions [14]. The principal processes involved are active angiogenesis and extracellular matrix (ECM) degradation [16]. Matrix metalloproteinases (MMPs) belong to the ECM protease family which regulate tumor invasion and metastasis. These proteins catalyze the degradation of the ECM via reconstruction, maintenance of tumor microenvironment and release of relevant growth factors. It is known

that MMP-2 catalyzes the degradation of the ECM and cellular barriers to tumor cell invasion and metastasis. Thus, MMP-2 is vital in angiogenesis and migration of malignant tumors [17,18]. In this study, miR-33b overexpression significantly downregulated MMP-2 protein expression in SMMC7721 cells.

CONCLUSION

The findings from this investigation indicate that miR-33b overexpression suppresses the proliferative, migratory and invasive potential of hepatic carcinoma cells via the down-regulation of Fli-1 and MMP-2 protein expressions. This research provides new ideas which may beneficial in the identification of new liver cancer drugs.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Authors' contributions

This study was done by the authors named in this article, and they accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Yunquan Guo. Yunquan Guo, Yanzhen Cao, Huan Zhang, Cuicui Wang and Wenli Ji collected and analyzed the data, while Yunquan Guo wrote the text. All authors read and approved the manuscript for publication.

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