Original Article

miR-181a negatively regulates Kras to inhibit the proliferation and migration of cholangiocarcinoma cell line QBC939

Ningfu Peng1, Jingrong He1, Jindu Li1, Hao Huang1, Yingyang Liao2, Lequn Li1, Shaoliang Zhu1

Departments of 1Hepatobiliary Surgery, 2Nutrition, Affiliated Tumor Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region, China

Received October 2, 2019; Accepted December 10, 2019; Epub February 15, 2020; Published February 28, 2020

Abstract: Objective: To investigate whether miR-181a can regulate the expression of Kras to affect the proliferation and migration of cholangiocarcinoma cell line QBC939. Methods: QBC939 cells were divided into D group (cells without treatment), Dz group (cells transfected with negative control), Dm group (cells transfected with miR-181a inhibitor), and Dy group Dy (cells transfected with miR-181a mimics). The morphology of cells was observed by Hoechst staining, and the expression of Kras mRNA and miR-181a was determined by RT-PCR. The expression of Kras protein was detected by Western blot, and the invasion ability of QBC939 cells was detected by transwell assay. Clone formation assay and MTT assay were used to detect cell cloning ability and viability, and the dual luciferase reporter gene assay was performed. Results: Cells in Dz group and D group were arranged in a regular manner, while cells in Dm group were tightly arranged with increased quantity. Besides, cells in Dy group were sparsely arranged and fractured with increased loss of nuclear membrane. The expression of Kras mRNA was the lowest in Dy group and the highest in Dm group, while the expression of miR-181a was the opposite. The transfection of miR-181a could reduce Kras activity (P<0.05), but the effect on the mutated gene was not significant (P>0.05). Western blot showed that Kras protein expression in Dm group was higher than that in other groups (P<0.05), and the expression in Dz and D group was higher than that in Dy group (P<0.05). Transwell assay showed that the overall invasion ability of cells in Dy group was enhanced compared with other three groups (all P<0.05). Compared with other groups, the invasion ability of cancer cells was the weakest and the number of cells under microscope was the lowest in Dy group (all P<0.05). The clone formation assay and MTT assay showed that the number of monoclonal populations and cell viability in Dy group were the lowest. The monoclonal formation rate of QBC939 cells in Dm group, Dz group and D group showed an uptrend, which was significantly higher than that in Dy group (all P<0.05). Conclusion: Overexpression of miR-181a could inhibit proliferation and migration of cholangiocarcinoma cell line QBC939, which may be mediated by targeting and inhibiting the expression of Kras.

Keywords: QBC939 cells, miR-181a, Kras gene, cholangiocarcinoma

Introduction

Cholangiocarcinoma is a malignant tumor derived from biliary epithelial cells. Early onset of cholangiocarcinoma is not easy to detect, and it is difficult to control when the disease enters the development stage, resulting in poor prognosis and high mortality. In recent years, the prevalence of cholangiocarcinoma presents a rising trend, which has a great impact on the health of people in China [1]. At present, the pathogenesis of cholangiocarcinoma is still unclear, and the early diagnostic criteria are vague. Therefore, most patients have missed the optimal time for surgical treatment when they were diagnosed. At this stage, chemotherapy is the main clinical treatment for cholangiocarcinoma, but the effect is not ideal [2]. Most patients with cholangiocarcinoma are already in the advanced stage after diagnosis, and there is still a high recurrence rate after surgery, which is related to the metastasis or infiltration of cancer cells caused by delayed treatment at the initial stage of the disease [3].

Existing studies have shown that microRNAs play an important role in tumor progression, acting as proto-oncogenes or anti-oncogenes.
miR-181a regulates Kras expression and the proliferation and migration of QBC939

MicroRNAs have various biological characteristics and endogenous regulatory functions, which regulate about half of protein expressions in human body and inhibit the mRNA transcription of target genes while promoting the mRNA degradation. Among them, miR-181a is one of the anti-oncogenes that have been well studied [5, 6]. MiR-181a is in a low expression state in various cancers, such as liver cancer, colorectal cancer, gastric cancer, breast cancer, and the inhibition of miR-181a expression significantly promotes tumor proliferation, invasion and metastasis [7, 8].

Kras, one of the carcinogenic genes of the RAS family, exists in human chromosomes 11, 12 and 1. The stereoscopic structure and function of Kras protein change after activation, and the activated Kras protein continuously activates downstream signaling molecules, thus causing massive proliferation and malignant transformation of cancer cells [9, 10]. The relationship between miR-181a and Kras and the mechanism of action on cholangiocarcinoma cells are currently inconclusive. Therefore, this study aims to explore the effect of miR-181a on the proliferation and migration of cholangiocarcinoma cell line QBC939 and its role in regulating the expression of Kras gene.

Materials and methods

Main reagents and instrument

Reverse transcription-polymerase chain reaction (RT-PCR) kit was produced by Shanghai Superchip Biotechnology Co., Ltd. Trizol reagent was purchased from Shanghai Lianshuo Biotechnology Co., Ltd., and reverse transcription kit was purchased from Wuhan Servicebio Technology Co., Ltd. MiR-181a mimics, miR-181a inhibitor and miR-181a negative control were synthesized by Guangzhou Ribobio Technology Co., Ltd. Methyl thiazolyl tetrazolium (MTT) cell proliferation assay kit was purchased from Wuhan Amyjet Scientific Incorporation. Fluorescence microscope was produced by Beijing Keyu Technology Co., Ltd.

Cell transfection and grouping

The cholangiocarcinoma cell line QBC939 was purchased from American Type Culture Collection (ATCC). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 10% fetal bovine serum (FBS) and 1% cyano streptomycin at 37°C in 5% CO2 incubator. The cultured cells (80%~90% cell density) were prepared to be transfected after centrifugation and passage. MiR-181a mimics, miR-181a inhibitor and miR-181a negative control were prepared into compounds of 30 pmol each, then the compounds were transfected into QBC939 cells according to the lipofectamine 2000 instructions. After 48 h, the cells were collected and divided into Dz group (cells transfected with negative control), Dm group (cells transfected with miR-181a inhibitor), Dy group (cells transfected with miR-181a mimics), and D group (cells without treatment).

Hoechst staining

QBC939 cells in four groups were collected and centrifuged for 5 min, then the supernatant was removed, and the cells were washed with PBS. The cell precipitation was resuspended in 1 mL of 70% ethanol and then washed twice with PBS. After that, the cells were stained with 15 μL of Hoechst33258 at a final concentration of 16 μL/mL and incubated for 15 min. Then 10 μL of final cell suspension was placed on a glass slide and the cell morphology was observed under a fluorescence microscope.

RT-PCR

The total RNA of QBC939 cells was extracted using Trizol solution, and cDNA was reverse transcribed according to the manufacturer’s instructions. SYBR Green I was used as fluorescent dye and the miRNA U6 was used as the internal reference. PCR amplification reaction system was performed under the following conditions: initial denaturation at 94°C for 4 min, 40 cycles consisting of denaturation at 94°C for 40 s, annealing at 55°C for 30 s and extension at 65°C for 10 min. The experiment was conducted at least 3 times, and the expression of Kras mRNA and miR-181a was calculated by relative quantitative 2-ΔΔCT. Primers are listed in Table 1.

Dual luciferase reporter gene assay

QBC939 cells with high transfection efficiency were used for the dual luciferase reporter gene assay, and placed in a 12-well plate after digestion. Kras 3'-UTR plasmid was co-transfected with negative control (NC) or miR-181a into...
miR-181a regulates Kras expression and the proliferation and migration of QBC939

Table 1. The PCR primer sequence

<table>
<thead>
<tr>
<th>Primers</th>
<th>RT</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-181a</td>
<td>F</td>
<td>5'-GCCGAAACATTCAACCGCTTC-3'</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-CAGTGCAGGGTGTTGCTGGT-3'</td>
</tr>
<tr>
<td>Kras</td>
<td>F</td>
<td>5'-TCATATTTTATTGAGGTCTGCAAA-3'</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-AAAGACTGGTCCTGCACCAGTA-3'</td>
</tr>
<tr>
<td>U6</td>
<td>F</td>
<td>5'-CTCGCTCGGAGCGCACA-3'</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>5'-AACGCTTCGAGAATTGCGT-3'</td>
</tr>
</tbody>
</table>

Human embryonic kidney-293T cells (HEK-293T cells), while Kras 3'-UTR mutant plasmid was co-transfected with NC or miR-181a into cholangiocarcinoma cell line QBC939. After 6 h of transfection, cells were cultured in complete medium, and were lysed for dual luciferase reporter gene detection after 60 h. The fluorescence value of renilla plasmid was used as the internal reference.

Western blot

The transfected QBC939 cells were lysed and the total protein concentration was determined using the Bradford method. Total proteins were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were sealed in blocking solution and placed in a horizontal shaker for 2 h. After that, membranes were incubated with the primary antibodies (Abcam company, UK) in a self-made hybrid bag at 4°C overnight (anti-GAPDH was diluted with hybrid solution at 1:1000), and then incubated with the secondary antibodies for 1.5 h after washing 3 times with 1×TBST solution for 10 min/time at room temperature. After incubation, PVDF membranes were washed with TBST again for 3 times (10 min/time). Then the electrochemiluminescence (ECL) chromogenic solution A and B were mixed into the working solution at the ratio of 1:1, and the working solution was added to the membrane. About 1 min later, PVDF membranes were visualized using automatic chemiluminescence imaging analysis. The Quantity one 4.0 software was used to analyze Kras protein expression.

Transwell assay

Transfected cells were diluted to 1×10^5 cells/mL and cultured for 12 h. Transwell chambers were prepared as follows: serum culture medium was added in the lower chamber, and ECM (extracellular matrix) gel was applied in the top chamber. Then liquid in the top chamber was sucked out after 4 h of standing. A total of 200 µL cultured QBC939 cells were seeded in the migration chamber, and the cells were stained with the crystal violet hydrate solution after 24 h and observed under a microscope (100×).

Plate clone formation

The transfected QBC939 cells were cultured in a petri dish containing 10 mL of culture medium at 37°C. After incubation for 14 days, the cloned cells were collected and fixed with 4% paraformaldehyde for 15 min, and then stained with crystal violet solution for 20 min. The number of cell cloning was counted, and the survival rate of cells in each group were calculated (clone formation rate (%) = (number of formed cell cloning/number of seeded cells) x 100%).

MTT

The transfected QBC939 cells were seeded into a 96-well plate with 1×10^6 cells/well, and placed in an incubator at 37°C and 5% CO₂. After cells were cultured for 24 h, 48 h and 72 h, respectively, 20 µL of MTT solution with a concentration of 5 g/L was added to each well. After further incubation for 4 h, the supernatant was carefully discarded, and 150 µL of DMSO solution was added to each hole. The 96-well plate was placed on a shaker and slowly shaken for 15 min in the dark, and then absorbance values of each well were detected at the wavelength of 490 nm in the microplate reader. The experiment was repeated 3 times, and the results were averaged and plotted into a chart.

Statistical analysis

SPSS 19.0 software was used to perform statistics and analysis of experimental data. The count data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to detect differences among three groups, and least significant difference-t (LSD-t) test or Bonferonni correction test was used for intragroup pairwise comparison. P<0.05 was considered to be statistically significant.
miR-181a regulates Kras expression and the proliferation and migration of QBC939

Results

Hoechst staining

Hoechst staining showed that the cells in Dz and D groups were arranged regularly accompanied by the disappearance of a small amount of cell nuclear membranes, and most cell nuclear staining presented the central aggregation. In the Dm group, the cells were closely arranged with increased quantity, and the nuclear staining showed the central aggregation. In the Dy group, cells were sparsely arranged and fractured accompanied by different sizes of nucleosomes, and the loss of nuclear membranes increased. See Figure 1.

Detection of Kras mRNA and miR-181a expression by RT-PCR

RT-PCR was used to detect the expression of Kras mRNA and miR-181a in QBC939 cells of four groups. The results showed that Kras mRNA expression was significantly decreased and miR-181a expression was significantly increased in Dy group compared with Dz and Dm groups. Compared with the Dz group, Kras mRNA expression increased significantly while miR-181a expression decreased significantly in the Dm group. Besides, Kras mRNA expression was significantly decreased while miR-181a expression was significantly increased in the D group compared with the Dm group. Moreover, compared with the Dy group, the expression of Kras mRNA was significantly increased while the expression of miR-181a was significantly decreased in the D group (all P<0.05). The results of Kras mRNA expression in group Dz, Dy, Dm and D were 1.02±0.65, 1.56±0.78, 0.51±0.12, 1.03±0.64, and miR-181a expression was 1.21±0.41, 0.61±0.11, 1.67±0.65, 1.22±0.39, respectively, as shown in Figure 2.

The result of dual luciferase reporter gene assay

After constructing the vector, the dual luciferase reporter gene assay was conducted, and the results showed that the transfection of miR-181a could reduce the Kras activity (P<0.05), but the effect on the mutated gene was not significant (P>0.05). See Figure 3.

Detection of Kras protein expression by western blot

Western blot analysis showed that Kras protein expression in Dy group was lower than that in Dz group and Dm group, and the expression in

Figure 1. The result of Hoechst staining. A: Dz group (cells transfected with negative control); B: Dm group (cells transfected with miR-181a inhibitor); C: Dy group (cells transfected with miR-181a mimics); D: D group (cells without treatment).

Figure 2. Expression of Kras mRNA and miR-181a by RT-PCR. A: The expression of Kras mRNA in four groups; B: The expression of miR-181a in four groups. Dz group (cells transfected with negative control); Dm group (cells transfected with miR-181a inhibitor); Dy group (cells transfected with miR-181a mimics); D group (cells without treatment). Compared with Dz group, 1P<0.05; compared with Dm group, 4P<0.05; compared with Dy group, 4P<0.05.
miR-181a regulates Kras expression and the proliferation and migration of QBC939

cancer cells was stronger and the number of cells under microscope was higher in Dm group. Compared with Dz and Dm groups, the invasion ability of cancer cells was weaker and the number of cells under microscope was lower in Dy group. Besides, compared with Dy group, the number of cancer cells in D group was significantly increased, and the overall invasion ability was significantly enhanced (all P<0.05). See Figure 5.

Discussion

Cholangiocarcinoma is a relatively severe malignant tumor, which is generated by malignant transformation of biliary epithelial cells. It is difficult to be treated by surgical resection as the risk of recurrence is high. Besides, it is less sensitive to radiotherapy and chemotherapy, so the prognosis of such diseases is not ideal [10]. Most patients with cholangiocarcinoma have been diagnosed in advanced stage. The tissue limitations of cholangiocarcinoma are relatively high, so the relevant pathogenesis research cannot be carried out thoroughly [11]. In recent years, the incidence of global cholangiocarcinoma has been increasing year by year. Despite the rapid progress of medical technology, the 5-year survival rate of this disease has not been improved, which is only 10% [12].

MiR-181a is one of the most studied miRNAs for its multiple functions. It exists on chromosomes prone to abnormalities in hepatobiliary malignancies, and its expression is inhibited in most tumors [13]. Kras gene is one of the common oncogenes, which can be mutated in 30% to 50% of cancer patients. Kras mutations could predict patients’ characteristics such as increased age, low tumor differentiation, and slow clinical stage, which are important indica-
miR-181a regulates Kras expression and the proliferation and migration of QBC939

Figure 5. Invasion ability of cells in four groups by transwell assay. A: The results of cell invasion; B: Comparison of cell invasion. Dz group (cells transfected with negative control); Dm group (cells transfected with miR-181a inhibitor); Dy group (cells transfected with miR-181a mimics); D group (cells without treatment). Compared with Dz group, *P<0.05; compared with Dm group, #P<0.05; compared with Dy group, &P<0.05.

Figure 6. Cell proliferation in four groups. A: The result of cell cloning; B: The result of cell viability. Dz group (cells transfected with negative control); Dm group (cells transfected with miR-181a inhibitor); Dy group (cells transfected with miR-181a mimics); D group (cells without treatment). Compared with Dm group, *P<0.05; compared with Dy group, #P<0.05.
miR-181a regulates Kras expression and the proliferation and migration of QBC939
tors for understanding the occurrence, prognosis and therapeutic effectiveness of tumors [14].

This study found that Kras mRNA and protein expressions in the miR-181a overexpressed group were significantly lower than those in the other three groups, which was highest in the miR-181a inhibiting group. As an oncogene, Kras is a signaling gene in the downstream of multiple signal pathways and plays an important role in the occurrence and development of various malignant tumors [15]. Studies have confirmed that the gene most closely related to malignant tumors is Kras gene, which can act as a molecular switch that causes a series of feedbacks from malignant cells by transmitting cell signals [16]. In addition, studies have shown that Kras protein can regulate the activity of multiple signal transduction pathways after transformation, and it plays a crucial role in cell proliferation and differentiation [17]. Related proteins are also activated after Kras gene mutation, which in turn affects the stereoscopic structure and function of Kras protein. When Kras protein was activated, the activities of downstream signaling molecules would also increase, leading to migration and differentiation of cancer cells. Then the condition began to change dramatically, and the difficulty of disease control also began to increase [18], which was consistent with the result of this study.

Transwell assay showed that the invasion ability of cancer cells in the Dm group was the strongest, which in the Dy group was the weakest. Cell cloning formation assay and MTT assay showed that the monoclonal population number and cell viability of QBC939 cells in the Dy group were significantly reduced, which in the Dm and D groups showed a rising trend. Studies have shown that the expression of miR-181a is abnormal in various malignant tumors, and overexpression of miR-181a can also exert an inhibitory effect on the proliferation and differentiation of tumor cells [19]. What’s more, a related study found that miR-181a level in cholangiocarcinoma tissues and cell line QBC939 was lower than that of normal tissues, indicating that miR-181a may act as an anti-oncogene in cholangiocarcinoma, so the increase of miR-181a level indicated accelerated apoptosis of tumor cells [20]. Another study found that nos2 deficiency may reduce miR-181a expression and activate Kras expression, leading to an inflammatory response aggravating the condition, suggesting that low expression of miR-181a may have a synergistic effect on the promotion of tumorigenesis and inflammation by Kras [21]. Besides, studies have confirmed that in malignant tumors with low incidence, the tumor progression can be controlled by regulating the level of miR-181a in patients, suggesting that miR-181a can be a new target for multiple tumor therapies [22]. All these findings in literature were consist with ours.

In conclusion, overexpression of miR-181a can inhibit migration and proliferation of QBC939 cells, and its mechanism may be related to the inhibition of Kras gene expression. However, this experiment has certain shortcomings in the research process. Due to time and other problems, apoptosis and migration of cells were not detected. Therefore, more experimental methods should be added in future studies to explore the mechanism of miR-181a and Kras on QBC939 cells, so as to provide more favorable experimental basis for the treatment of cholangiocarcinoma.

Acknowledgements

This work was supported by the National Nature Science Foundation of China (81560460/H1602) and the Self-Raised Scientific Research Fund of the Ministry of Health in Guangxi Zhuang Autonomous Region (Z2016479).

Disclosure of conflict of interest

None.

Address correspondence to: Lequn Li and Shaoliang Zhu, Department of Hepatobiliary Surgery, Affiliated Tumor Hospital of Guangxi Medical University, No. 71 Hedi Road, Qingxiu District, Nanning 530021, Guangxi Zhuang Autonomous Region, China. Tel: +86-0771-5330855; Fax: +86-0771-5312000; E-mail: lilequn67uy@163.com (LQL); zhushaoliang2oyh@163.com (SLZ)

References

miR-181a regulates Kras expression and the proliferation and migration of QBC939


