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Lentivirus-Mediated Overexpression of miR-218-5p Inhibits Metastasis by Targeting CDH2 in Colorectal Cancer

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Abstract: *Introduction:* MicroRNA-218 (miR-218) acts as a tumor suppressor in various types of cancer. However, the association between miR-218-5p, the mature body of miR-218, and colorectal cancer (CRC) metastasis remains unclear. *Objective:* This study aimed to investigate the effect of miR-218-5p on CRC metastasis in vivo and the underlying molecular mechanism. *Methods:* Quantitative real-time PCR with reverse transcription (RT-qPCR) was employed to determine the expression level of miR-218-5p that trying to investigate if there is a link to clinicopathological characteristics. HCT116 cells were transfected with lentivirus for stably expressing miR-218-5p which was validated, as well as its target gene CDH2, by RT-qPCR and Western blotting. To generate xenograft models, BALB/c nude mice were treated with transfected cells for stably overexpressing miR-218-5p. Finally, the lung metastasis of CRC was observed. *Results:* The miR-218-5p expression was significantly downregulated in CRC tissues, playing the role of indicator for lymph node metastasis, advanced clinical stage, and poor prognosis. In addition, the expression level of CDH2 was negatively related to miR-218-5p in lentiviral transfected HCT116 cells. At the same time, overexpressed miR-218-5p inhibited CRC cell xenografts metastasis. *Conclusion:* Data indicates that miR-218-5p suppresses the metastasis of CRC cells, in part by inhibiting CDH2.

Keywords: Colorectal Cancer, CDH2, miR-218-5p

1. Introduction

Colorectal cancer (CRC) is one of the commonest gastrointestinal tract tumor types. Although nowadays there are many advances in systemic therapy, the prognosis of CRC patients remains poor especially those with metastasis [1]. In this setting, novel therapeutic strategies are urgently required for CRC metastasis.

MiRNA, known as small non-coding RNA molecules, can inhibit translation and also affect the stabilization through targeting the mRNA 3'-untranslated regions [2]. There is evidence that pathogeneses such as cancer can be caused by abnormal miRNA expression. Recently, researchers showed that low miR-218 expression is tightly linked to neoplasms throughout the human body, such as pulmonary [3], cervical [4], and osteosarcoma [5]. Moreover, miR-218 dysregulation will lead to a particularly poor prognosis for these cancers [3-5]. Despite this, it remains unclear whether its mature morphology as miR-218-5p exhibits a correlation with clinical outcomes.

This study is to figure out whether miR-218-5p get the ability to regulate the fate of CRC metastasis and the underlie molecular mechanism. The results can provide a potential therapeutic strategy for inhibiting CRC metastasis.

2. Material and Methods

2.1. CRC Samples

There were 42 CRC samples and corresponding normal samples (N samples) acquired from the Tongji Hospital of Tongji University from January 2008 to May 2016. Inclusion criteria were: 1) CRC patients underwent tumor resection; 2)

No preoperative anticancer treatment, including chemotherapy and radiotherapy. These CRC specimens were reviewed by two gastrointestinal pathologists. Clinicopathological characteristics including patient age, patient gender, tumor size, histological grade, lymph node metastasis (LNM), TNM stage (according to the 8th TNM system) and survival time, were collected by medical records and telephone follow-up. The Tongji Hospital Ethics Committe gave its approval to this study.

2.2. MiR-218-5p Quantification in CRC Tissues

RNA was obtained from samples by RNAprep Pure FFPE Kit (TIANGEN) following the protocol. The PrimeScript™ RT reagent Kit (TAKARA) was employed during the preparation for cDNA. The reverse transcription conditions were following the steps: 42°C, 60 min; 70°C, 10 min. Quantify miRNA using RT-qPCR on a LightCycler 1.5 real-time system (Roche) with Bulge-LoopTM miRNA RT-qPCR Primer (RIBOBIO). U6 was detected as the internal control. The RT-qPCR primer pairs were ordered from RIBOBIO. Reaction conditions included: 95°C for 20 seconds, 1 cycle; 95°C for 30 seconds, 60°C for 20 seconds, 72°C for 20 seconds, and 40 cycles respectively. Differences in miR-218-5p value were represented by -ΔCt (Ct U6 - Ct miR-218-5p) within CRC and N samples [6].

2.3. Construction of Lentiviral Vector

To construct a lentiviral vector expressing miR-218-5p, the precursor and its flanking sequences were amplified from human genomic DNA (Hanbio) using PCR. Then using EcoRI/BamHI restriction enzyme to digest. Inserted the product in the pHBLV-U6-ZsGreen-Puro plasmid (Hanbio). To generate the miR-218-5p-expression lentiviruses (LV-miR-218), the validated recombinant plasmid (pHBLV-miR-218-5p), packaged plasmid pSPAX2, and encapsulated plasmid pMD2G were co-transfected into 293T cells by LipofiterTM (Hanbio) mediating. To construct CRC cells stably expressing miR-218-5p, we infected HCT116 cells using LV-miR-218. To determine miR-218-5p expression in transduced HCT cells, we used RT-qPCR and calculated fold changes values by $2^{-\Delta\Delta Ct}$ formula [7].

2.4. Western Blotting

Radio Immunoprecipitation Assay buffer combined with Protease Inhibitor (Keygenbio) was used to lyse HCT116 cells that were stably overexpressing miR-218-5p. Following the instructions, the BCA protein assay kit (Keygenbio) was used to detect protein concentration. Proteins (35 μg per lane) were separated by 10% SDS-PAGE. Electrophoresed proteins were transferred to PVDF membranes and blocked with 5% defatted milk for 1 h at room temperature, then incubated with mouse anti-GAPDH (1:1,000; Boster) and mouse anti-CDH2 (1:400; Boster) primary antibodies at 4 ˚C overnight. An enhanced chemiluminescence substrate kit (Keygenbio) was used to examine the blots, followed by 1 h incubation of secondary anti-mouse IgG antibody (1:1,000; Beyotime) labeled with goat radish peroxidase at room temperature. FluorChem Q Imaging Systems (ProteinSimple) were used to obtain the Western blotting image, and AlphaView software (ProteinSimple) was used to assess the densitometry.

2.5. Tumor Xenografts Growth in Nude Mice

The Tongji Hospital Ethics Committe authorized all animal investigations. 20 male BALB/c nude mice (5-7 weeks) were offered by Tongji Hospital Laboratory Animal Facility. HCT116 cells which highly expressed miR-218-5p were injected subcutaneously into the right anterior side of mice, with a dosage of 1x107. After 7 weeks, the participants were euthanized, and their bilateral lungs were dissected.

2.6. Statistics Analysis

SPSS 17.0 ((IBM, Inc.) and GraphPad Prism 5.0 (GraphPad, Inc.) were used for statistical analysis. The independent samples t-test was used to see whether there were any differences between the groups. The *m*±*SE* of quantitative data is presented. Survival analysis was performed by Kaplan–Meier curve and log-rank test. Statistical significance was shown by $p<0.05$.

3. Results

3.1. The Expression of miR-218-5p in CRC Tissues

RT-qPCR showed that the expression of miR-218-5p in CRC samples (-9.99 ± 3.20) was lower than in N samples (-6.56 ± 2.39). (Figure 1a). According to expression levels that more or less than the mean value (-9.99), we separate the including cases into two groups, low expression (n=18) and high expression (n=24). (Figure 1b). Kaplan-Meier survival analyses showed that patients in the low expression group had a lower survival rate than those in the high expression group. (Figure 1c). The expression of miR-218-5p was considerably downregulated in individuals with LNM or advanced stage (III+IV) (Table 1).

Table 1. *Correlation of miR-218-5p with clinicopathological features.*

Parameter		*n*	miR-218-5p expression (*m*±*SE*)	*p* value
Age (years)	<55	8	-10.24±3.34	
	≥55	34	-9.94±3.22	0.815
Gender	Male	28	-9.70±3.40	
	Female	14	-10.59±2.79	0.403
Tumor size (cm)	<5	20	- 9.90±2.41	
	≥5	22	-10.08±3.84	0.860
Histologic grade	G1+G2	38	-9.90±3.34	

Parameter		*n*	miR-218-5p expression (*m*±*SE*)	*p* value
	G3	4	-10.90±1.05	0.559
LNM	No	19	-8.05±1.72	
	Yes	23	-11.59±3.28	＜0.001
TNM Stage	I + II	14	-8.39±1.54	
	III + IV	28	-10.80±3.52	0.004

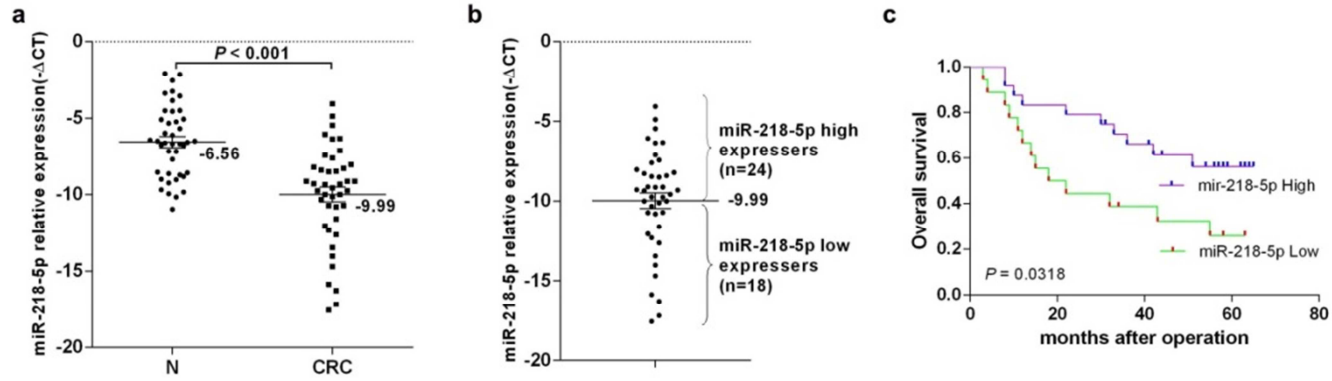

Figure 1. *The expression of miR-218-5p in CRC tissues.*

3.2. Overexpression of miR-218-5p Inhibits CDH2 Expression in HCT116 Cell Lines

The target gene of miR-218-5p was inferred according to four online databases, including miRanda, miRWalk, Pictar, and TargetScan. CDH2 was deemed as a potential target gene from intersections of the aforementioned databases. Subsequently, HCT116 cells were transfected with LV-miR-218 and the results were confirmed by RT-qPCR (Figure 2a). Overexpressed miR-128-5p in HCT116 cells reduced CDH2 protein expression, according to Western blotting data (Figure 2b).

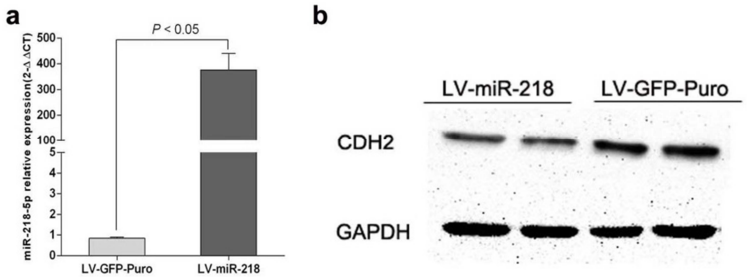

Figure 2. *Overexpression of miR-128-5p suppressed CDH2 expression in CRC cell lines.*

3.3. Lentivirus-Mediated miR-218-5p Overexpression Suppresses CRC Metastasis in Vivo

We injected the HCT116 cells which were filled with LV-miR-218 or the blank lentiviral vector (LV-GFR-Puro) subcutaneously for further studying the inhibitory effect of miR-218-5p on tumor metastasis in vivo (Figure 3a). The results showed that lung metastases were seen in four out of ten mice treated with LV-GFR-Puro cells (Figure 3b). Mice injected with LV-miR-218 cells, on the other hand, showed no signs of metastasis (Figure 3 and Table 2).

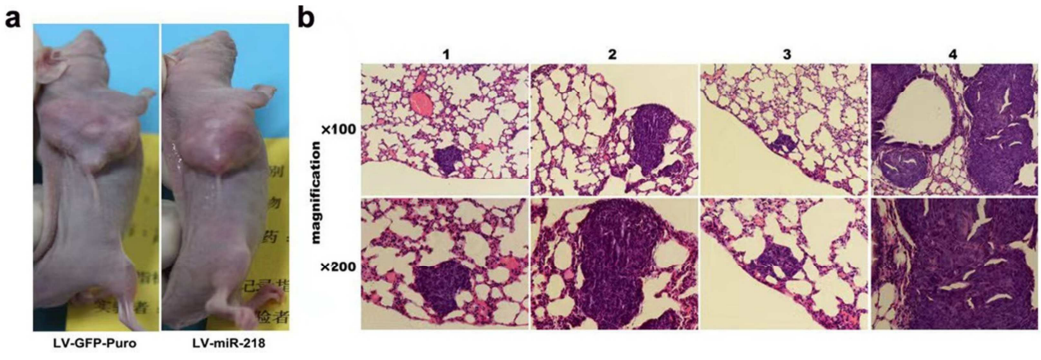

Figure 3. *Lentivirus-mediated overexpression of miR-218-5p suppresses CRC cells migration in vivo.*

Table 2. *Incidence of metastasis.*

Parameter		LV-GFP-Puro	LV-miR-218	*p* value
Lung metastasis	Yes	4	0	0.025
	No	6	10	

4. Discussion

Small non-coding RNA molecules, which are known as miRNAs, may play an important role in the development of several human diseases, including cancer [8]. In light of the strong link between miRNA expression and malignancy, numerous research studies have explored their potential application in cancer diagnostics and medical management fields [9].

The miR-218, one of the most commonly found intronic miRNAs, has been taken into account as a factor preventing the development of various cancers [5, 10]. Recently, the findings of Yang`s group showed that the serum levels of miR-218 are much lower among those patients suffering from hepatocellular carcinoma (HCC), and the lower level leads to a poorer overall survival rate for HCC patients [11]. A recent publication on prostate cancer (PCa) patients found that lower miR-218-5p levels were directly connected to the worse clinicopathological characteristics and bone metastasis-free survival [12]. This serves as a reminder that miR-218 might be regarded as a promising therapeutic strategy and prognostic indicator for those who suffered from cancers. Based on findings in this study, miR-218-5p was found steadily dropped in the CRC group which was negatively associated with the LNM and advanced clinical stage. Depending on the follow-up findings and survival analyses, patients with lower miR-218-5p expression appeared lower overall survival rate than those with higher expression. In addition, miR218-5p was also found to be capable of inhibiting the migration of CRC cells in vivo. It is suggested that miR-218-5p could be used as a potential treatment target for CRC based on these findings.

Previous research has shown that the epithelial-mesenchymal transition (EMT) played a critical role in controlling tumor invasiveness, metastasis, and treatment resistance [13]. According to previous studies, CDH2, also known as neuronal cadherin, is expressed during the EMT process [14]. EMT is accumulating evidence that CDH2 can be used as a prognostic and predictive molecular biomarker in various cancers. It was shown that CDH2 was substantially linked with glioma grade in a prior investigation, and the higher CDH2 expression represented the adverse prognostic factor in glioma cases [15]. In this study, Pictar, TargetScan, miRanda, and miRWalk were utilized to detect miR-218-5p target genes. Finally, a total of 127 target genes were identified based on these four databases. Among these, the CDH2 gene was closely linked to cell movement, according to biological function annotation. This study also discovered that CRC cells with miR-128-5p overexpression can suppress CDH2 protein production. Overall, the study fully illustrated that upregulation of miR-218-5p inhibits the migration of CRC cells by reducing CDH2 expression.

5. Conclusion

Taken together, this study demonstrates that CRC samples had a significantly low miR-218-5p level compared to healthy controls, and it was linked to clinical stage, LNM, and prognosis. Lentivirus injection led to the increase of miR-218-5p, therefore, hindering CRC cells' migration by targeting the CDH2 gene. Taking into account these findings, lentivirus-mediated overexpression of miR-218-5p could be a promising option for treating mCRC. It should be noted that the present research was not a multicenter trial, and the sample size was small due to constraints of funds. In future work, the molecular mechanisms of miR-218-5p regulating CRC metastasis is still needed to clarify in larger samples.

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