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The increase of miR-27a affects the role of cisplatin on proliferation and migration capacities of liver cancer cells

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Abstract. – OBJECTIVE: To study the effects of chronic virus-mediated micro ribonucleic acid miR-27a on proliferation and migration capacities of liver cancer cells.

PATIENTS AND METHODS: A total of 60 patients with primary liver cancer from January 2015 to December 2016 were selected as observation group, 60 patients with chronic liver disease were selected as control group, and another 60 healthy subjects who received physical examination during the same period were selected as healthy group. All patients received serum miRNA detection. The different expressions of serum miRNAs in healthy people, patients with chronic liver disease, and patients with liver cancer were analyzed. The correlations of miR-27a with growth and proliferation of liver cancer MCC-7721 cells were studied.

RESULTS: The levels of miR-27a and miR-664b in subjects in control group and healthy group were significantly lower than those in observation group, but the expression levels of miR-30a were statistically elevated ($p<0.05$). The expression of serum miR-27a in liver cancer patients with higher tumor-node-metastasis (TNM) staging (stage III-IV, number of tumors >1, tumor size >5 cm, and vascular invasion) was significantly higher than those in patients with lower TNM staging (stage I-II, number of tumors =1, tumor size ≤5 cm, and no vascular invasion) ($p<0.05$). At 48 h and 72 h after transfection, the proliferation of liver cancer MCC-7721 cells was significantly enhanced compared to that in non-transfection group ($p<0.05$). The level of miR-27a was significantly upregulated in cisplatin-resistant liver cancer A549/CDDP cells compared with that in parental A549 cells. MiR-27a regulated epithelial-mesenchymal transition (EMT) and cisplatin resistance *in vitro*, while modulated the in vivo response of liver cancer cells to cisplatin. Further studies identified the Raf kinase inhibitor protein (RKIP) as a direct and functional target of miR-27a. The knockdown of RKIP by RNAi showed a similar effect to ectopic miR-27a expression, whereas the over-expression of RKIP weakened the function of miR-27a in liver cancer cells.

CONCLUSIONS: MiR-27a participated in the proliferation and migration capacities of liver cancer cells and influenced the effect of cisplatin via targeting RKIP. The high expression of miR-27a is closely related to the malignant degree of liver cancer, which provides guidance for the diagnosis, targeted therapy, and prognostic evaluation of liver cancer.

Key Words
Liver cancer, miR-27a, Cancer cells, Proliferation, Migration.

Introduction

Liver cancer is a kind of malignant tumor that occurs in the liver. Primary liver cancer mainly occurs in the liver mesenchymal or epithelial tissues, which is a type of frequently-occurring malignant tumor in China[1]. Early symptoms of primary liver cancer are hidden without evident characteristics. But the disease progression is rapid, and it has been often in the middle and advanced stage when significant symptoms were observed. Therefore, the survival time of patients is not more than 6 months after liver cancer. Liver cancer is also known as "the king of cancer". According to the research data of liver etiology, tumor invasion and metastasis are the most important causes of death in patients with liver cancer[2,3]. At present, the pathogenesis of liver cancer remains unclear in clinical medicine, but it was found in relevant research that many factors are involved in the progression from normal liver tissues to liver cancer, including aflatoxins, steroid compounds, cirrhosis, chemical carcinogens, viral hepatitis, etc.[4]. However, molecular and cellular mechanisms of liver cancer need to be further studied. In recent years, evidence showed that cancers result from accumu-

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lation of normal gene mutations, such as oncogene activation and inactivation, and loss of tumor suppressor genes[5]. Micro-ribonucleic acid (miR)-27a gene, as a protooncogene of intracellular signal transducer protein, plays an important regulatory role in cell proliferation and angiogenesis-related signaling pathways. Moreover, it can be regarded as a molecular switch involved in the transduction process of extracellular proliferation, growth and differentiation signals into cells[6]. Therefore, it is needed to strengthen the monitoring of proliferation and migration of liver cancer cells in clinic, so as to provide guidance for the clinical treatment of liver cancer. To this end, the correlations of miR-27a with proliferation and migration capacities of liver cancer cells were analyzed in this study.

Patients and Methods

Patients

A total of 60 patients with primary liver cancer admitted into our hospital from January 2015 to December 2016 were selected as observation group, 60 patients with chronic liver disease were selected as control group, and another 60 healthy subjects who received a physical examination in our hospital during the same period were selected as healthy group. In the observation group, there were 36 males and 19 females aged 52-75 years old with an average of (63.39±3.82) years old. In the healthy group, there were 33 males and 22 females aged 53-74 years with an average of (63.22±4.05) years old. In the control group, there were 32 males and 23 females aged 53-74 years old with an average of (63.45±4.11) years old. Inclusion criteria: 1) subjects who were diagnosed via imaging examination, hematological and histopathological examination, and grouped[7], 2) liver cancer patients not complicated with liver failure, 3) patients with independent thinking ability. Exclusion criteria: 1) subjects with liver metastasis caused by other malignant tumors[8], 2) subjects complicated with severe liver failure, 3) subjects complicated with serious internal medicine diseases, organ dysfunction or mental disorders[9], 4) subjects with immune dysfunction, or 5) patients who refused to participate in the study, or were not informed of the study content. Basic data had no statistically significant differences among the three groups of patients ($p>0.05$), and they were comparable. The study was approved by the hospital Ethical Committee and all patients signed the informed consent.

RNA Isolation

All patients received serum miRNA detection. 5 mL fasting venous blood was collected from them, placed at room temperature for 30-60 min, and centrifuged at 3000 g for 10 min after coagulation. The supernatant was taken into an Eppendorf (EP) tube (RNase-free), and centrifuged at 2500 g and 4°C for 15 min. Then, the supernatant was taken again and stored in a refrigerator (at -80°C). The specimen was taken and thawed via water bath. The blood sample was mixed with TRIzol at a ratio of 1:3. Then, the solution was moved to an Ep tube and added with 200 μL chloroform. After vibrated for 15 s, the upper aqueous phase was added with 500 μL isopropanol for 10 min. After centrifuged at 12000 g for 10 min, the precipitation was added with 1 mL ethanol (75%). After centrifuged at 4°C and 7500 g for 5 min, the supernatant was removed and the tube was dried for 10 min. Next, the RNA was solved in DEPC water and qualified by 0.8% agarose gel electrophoresis (Figure 1). RNA content and purity were determined by ultraviolet spectrophotometer.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

The different expressions of serum miRNAs in healthy people, patients with chronic liver disease, and patients with liver cancer were analyzed by qRT-PCR. After RNA concentration was determined, reverse transcription was performed in a 20 μL system including 4 μL 25 mM $MgCl_2$, 2 μL Reverse Transcription 10X Buffer, 2 μL 10 mM dNTP Mixture, 0.5 μL Ribonuclease Inhibitor, 0.5 μL AMV Reverse Transcriptase, 1 μL Random Primers, 1 μg total RNA and Nuclease-Free Water to a final volume of 20 μL under 42°C for 15 min and 85°C denature (Promega, Madison, WI, USA). Real-Time PCR was then performed by using SYBR Premix Ex Taq GC kit (TaKaRa, Otsu, Shiga, Japan) (7.5 μL 2×premix, 10 mM forward and reverse primers, dH_2O to a final volume of 15 μL) in the following condition: 94°C denature for 30 sec, followed by 40 cycles each containing 94°C denature for 5 s, and 60°C annealing for 30 s with LightCycler 480 (Roche Diagnostics, Basel, Switzerland). Primer sequence and amplification length were shown in Table I. GAPDH was selected as internal reference. Relative gene expression was semiquantitatively analyzed by $2^{-\Delta\Delta t}$ method. $2^{-\Delta\Delta Ct}$ = gene copy number in test group/gene copy number in control. Experiments were carried out in triplicates.

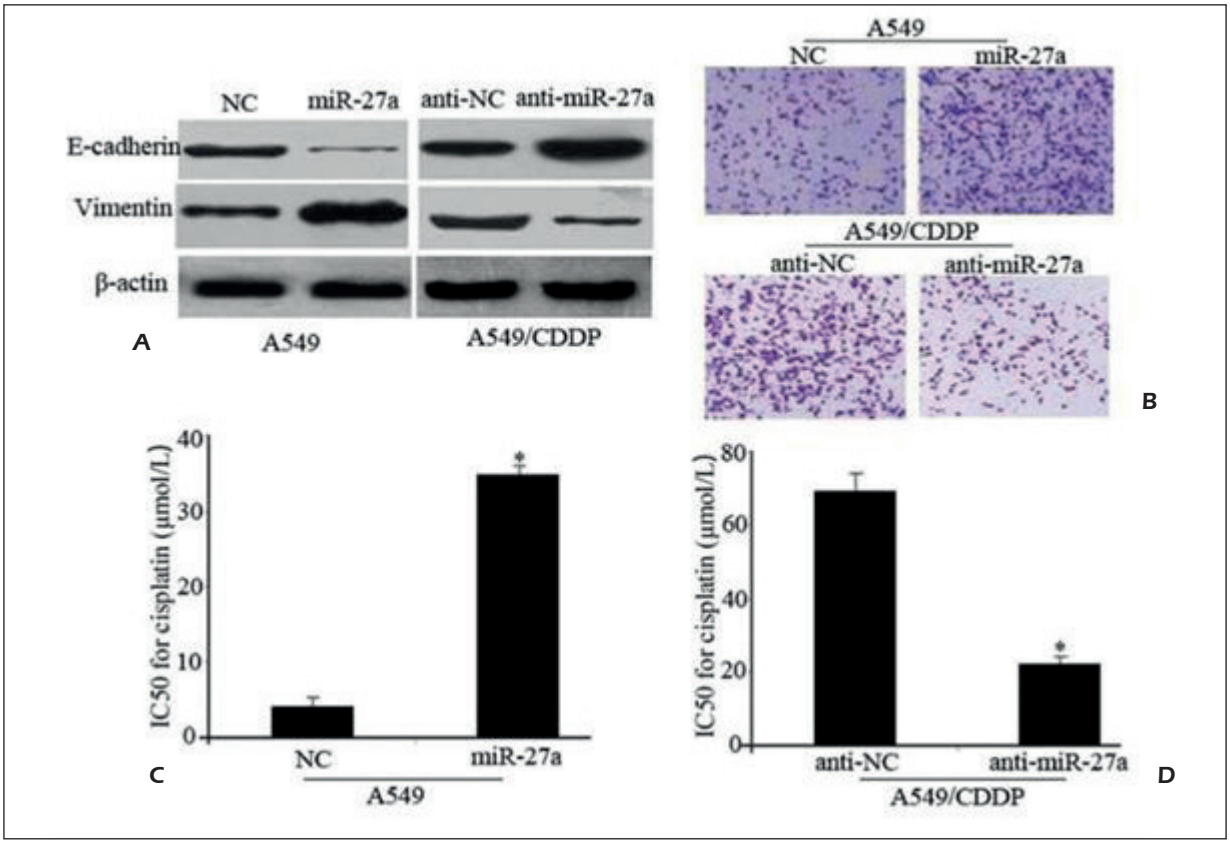

Figure 1. MiR-27a promotes EMT and cisplatin resistance *in vitro*. Expressions of E-cadherin and vimentin were detected via Western blotting with β-actin as an internal reference (**A**). Transwell invasion assay (**B**) and methyl thiazolyl tetrazolium (MTT) assay (**C** and **D**) were performed to detect invasion and cisplatin sensitivity. Data were presented as mean ± standard deviation in three independent experiments (*p<0.05).

Cell Transfection

MCC-7721 cells were bought from ATCC (Manassas, VA, USA). Cells were cultured in RMPI-1640 medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). The culture condition was 37°C, 5% CO_2. Cells were passaged once every 2 to 3 days and cells at logarithmic growth were used in the experiment. Control RNA and miR-27a mimics were provided by RiboBio Co., Ltd., (Guangzhou, Guangdong, China) and Lipofectamine 2000 was obtained from Thermo Fisher Scientific (Waltham, MA, USA) for transfection, according to the kit instructions. Subsequent experiments were performed 24-72 h after transfection. MCC-7721 cells were transfected with miR-27a mimics; growth, and proliferation of MCC-7721 cells were observed, and cell proliferation was monitored using the cell counting kit. After the treatment, cells were incubated at 37°C for 2 h; the specific absorbance value was detected at a wavelength of 450 nm for 3 times, and the average was taken. The higher the value was, the larger the number of proliferating cells would be.

Table I. Expressions of different miRNAs in different subjects ($\bar{x}$±s).

Group	miR-27a	miR-30a	miR-664b
Healthy group (n=55)	1.06±0.07	0.99±0.06	0.55±0.05
Control group (n=55)	1.24±0.07	0.89±0.07	0.71±0.06
Observation group (n=55)	1.45±0.12	0.77±0.05	0.83±0.04

Note: In the comparison between healthy group and observation group, ^{a}p<0.05 (t=20.819, 20.890 and 32.430, p=0.000, 0.000 and 0.000). In the comparison between control group and observation group, ^{b}p<0.05 (t=11.210, 10.345 and 12.341, p=0.000, 0.000 and 0.000). In the comparison between healthy group and control group, ^{c}p<0.05 (t=13.485, 8.044 and 15.193, p=0.000, 0.000 and 0.000).

Cell Proliferation by Methyl Thiazolyl Tetrazolium (MTT) Assay

Cell activity at 12 h, 24 h, 48 h, and 72 h was detected. In brief, 5 mg/mL MTT solution was added into each well (20 μL) for 4 h incubation. 0.15 mL dimethyl sulfoxide (DMSO) was thus added into each well, followed by 10 min slow vortex to dissolve crystals. Absorbance values at 570 nm wavelength were measured in each well.

Generation of A549/Cisplatin (CDDP) Cell Model

A549/CDDP cell model was generated by challenging with gradient concentrations of CDDP for induction. In brief, cells at log-growth phase with satisfactory growth status were firstly treated with 0.1 μM CDDP for 24 h. PBS was then used to remove fresh medium containing CDDP for continuous incubation. When cells reached a stable growth and had been passed for 3 generations, induction concentration of CDDP was gradually elevated 0.2 μM, 0.5 μM, 1.0 μM, and 2.0 μM, until A549 cells could maintain stable growth at 2.0 μM CDDP and repeated passage, thus generating drug-resistant cell strain A549/CDDP.

Luciferase Reporter Gene Assay

HEK293T cells were lysed by TRIzol for extracting mRNA. Using HEK293T mRNA as the template, 3'-UTR of RKIP gene containing targeted binding sites or its mutant form was amplified. PCR products were extracted from agarose gel, and were digested by enzymes to ligate into pUC19 plasmid for transforming DH5α competent cells. Positive clones of bacteria colony were picked for sequencing those plasmids with correct insertion. Plasmids were renamed as pUC19-RKIP-wt or pUC19-RKIP-mut. Lipo 2000 was used to co-transfection 100 ng pUC19-wt (or pUC19-mut), 50 pmol miR-29a mimic (or miR-NC, miR-29a inhibitor), and 50 ng pRL-null renilla luciferase into HEK293T cells. After 48 h incubation, Dual-Glo Luciferase Assay System kit (Promega, Madison, WI, USA) was used to measure relative luciferase activity.

Western Blot Detection

After transfection the cells were collected and tested for cell lysate concentration. Western blot was performed starting with 60 V electrophoresis for 30 min, followed by 120 V electrophoresis for 120 minutes. After electrophoresis, proteins were transferred to NC membrane under 300 mA for 30 minutes. The membrane was blocked with 5% defatted milk powder for 60 min at room temperature. Mouse anti-human E-cadherin, Vimentin, RKIP, and β-actin antibody (all diluted at 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added for 4°C room temperature incubation overnight. The membrane was then washed with phosphate-buffered solution Tween (PBST) for 30 min, followed with incubation with secondary antibody for 60 min (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washed three times with phosphate-buffered saline Tween (PBST), a chemiluminescence detection reagent was used to develop and fix. Gel image system was used to analyze the band density (Bio-Rad, Hercules, CA, USA).

Histological Examination

The obtained specimens, the cells were fixed in 4% paraformaldehyde solution (pH7.2) and were prepared for paraffin-based slices (5 μm). After hematoxylin-eosin (HE) staining, slices were observed under a light field microscope.

Statistical Analysis

The whole group of data was proofread using Statistical Product and Service Solutions (SPSS) 22.0 software package (IBM Corp., Released 2012, IBM SPSS Statistics for Windows, Armonk, NY, USA). Measurement data were presented as ($\overline{x}\pm s$), and *t*-test was used. Enumeration data were presented as case (n) and rate (%), and chi-square test was used. Two-tailed Pearson correlation was performed to determine the relations between level of miR-27a and RKIP. $\alpha=0.05$ was set as the test level; $p<0.05$ suggested that the difference was statistically significant.

Results

Expression Levels

The levels of miR-27a and miR-664b in the healthy group and the control group were significantly lower than those in the observation group, but the expression of miR-30a was statistically higher than that in the observation group ($p<0.05$) (Table I).

Correlations of MiR-27a With Pathological Features of Liver Cancer

The level of serum miR-27a in liver cancer patients was related with the tumor-node-metastasis (TNM) staging. In patients with higher

Table II. Correlations of miR-27a with pathological features of liver cancer [n (%)].

Type		Low expression	High expression	χ^2	*p*
TNM staging	I-II (n=36) III-IV (n=19)	23 (63.89) 4 (21.05)	13 (36.11) 15 (78.95)	9.131	0.003
Number of tumor	1 (n=33) >1 (n=22)	23 (69.70) 6 (27.27)	10 (30.30) 16 (72.73)	7.639	0.006
Tumor size	≤5 cm (n=23) >5 cm (n=32)	15 (65.22) 11 (34.38)	8 (34.78) 21 (65.62)	5.107	0.024
Vascular invasion	Yes (n=20)	6 (30.00)	14 (70.00)	5.498	0.019

tumor-node-metastasis (TNM) staging (stage III-IV, number of tumors > 1, tumor size > 5 cm, and vascular invasion), significantly higher miR-27a level was found compared to that in patients with lower TNM staging (stage I-II, number of tumors =1, tumor size ≤ 5 cm, and no vascular invasion) (p<0.05) (Table II).

Correlation Between MiR-27a and Liver Cancer Cells

At 48 h and 72 h after transfection, the proliferation of liver cancer MCC-7721 cells was significantly increased than that in non-transfection group (p<0.05) (Table III).

MiR-27a Promoted Epithelial-Mesenchymal Transition (EMT) and Cisplatin Resistance In Vitro

A549 cells were transfected with NC or miR-27a mimics, while A549/CDDP cells were transfected with anti-NC or miR-27a inhibitor, respectively. Of note, the overexpression of miR-27a apparently decreased the level of E-cadherin but elevated the expression of Vimentin compared to that in NC group. However, in A549/CDDP cells, the reduction of miR-27a dramatically upregulated the level of E-cadherin whereas deterred the expression of vimentin. Moreover, miR-27a significantly enhanced the proliferation of A549 cells and resistance against the effect of CDDP as A549/CDDP cells did, compared to that in NC group (p<0.05) (Figure 1B, 1C). In contrast, the suppression of miR-27a inhibited the cell proliferation and resistance to CDDP, compared to that in anti-NC group (p<0.01) (Figure 1B, 1D).

MiR-27a Directly Targeted Raf Kinase Inhibitor Protein (RKIP)

In silico prediction by microRNA.org showed the potential binding site of miR-27a in RKIP 3'-untranslated region (UTR), and mutation of PKIP 3'UTR was also designed as shown in Figure 2A. Luciferase assay was further performed and showed that the relative luciferase activity was significantly inhibited by miR-27a compared to that of control, while the inhibitory role of miR-27a became invalid since RKIP 3'-untranslated region (UTR) was mutated, indicating the targeting effect of miR-27a on RKIP (p<0.05) (Figure 2D). Alternatively, the level of RKIP in A549/CDDP cells was found decreased compared to that in A549 cells, suggesting that the resistance to CDDP reduced the expression of RKIP in A549 cells (Figure 2B). Of note, the overexpression of miR-27a inhibited the level of RKIP, while the suppression of miR-27a elevated the expression of RKIP and counteracted the resistance to CDDP (Figure 2C), which indicated the regulatory role of miR-27a on RKIP expression.

Correlations of High Expression of MiR-27a in Liver Cancer Tissues With Decreased RKIP Expression, Chemotherapy Resistance and Poor Prognosis

We then detected and compared the relative levels of miR-27a and RKIP between cisplatin-sensitive and non-sensitive liver cancer tissues.

Table III. Correlation between miR-27a and proliferation of liver cancer MCC-7721 cells ($\bar{x}$±s).

Group	0 h	24 h	48 h	72 h
Before transfection (n=55)	0.55±0.02	0.71±0.05	0.93±0.04	1.25±0.08
After transfection (n=55)	0.55±0.01	0.72±0.04	1.03±0.07	1.61±0.07
t	0.000	1.158	9.199	25.116
p	1.000	0.076	0.000	0.000

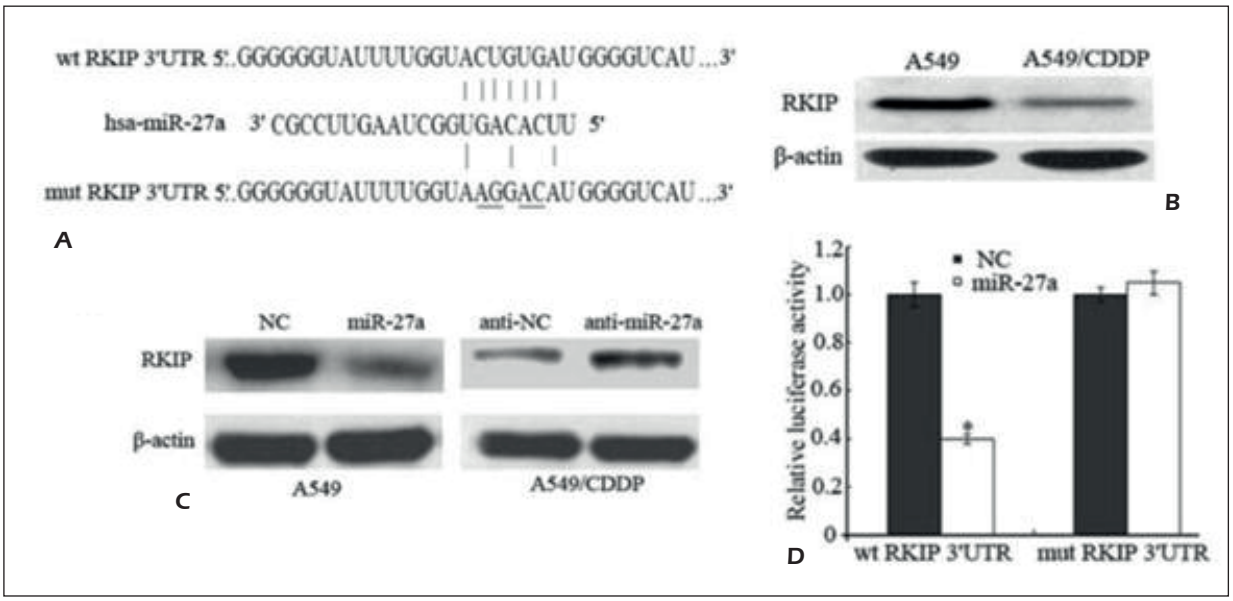

Figure 2. MiR-27a directly targets RKIP. **A**, The binding site of miR-27a predicted in RKIP 3'-untranslated region (UTR) and the type of mutation induced through the site are shown in Figure 3. **B**, Variable RKIP expressions in A549 and A549/CDDP were obtained via Western blotting. **C**, A549 cells were transfected with NC or miR-27a mimics, and A549/CDDP cells were transfected with anti-NC or miR-27a inhibitor, respectively. RKIP expression was detected via Western blotting with β-actin as an internal control. **D**, Luciferase assay was performed in A549 cells cotransfected with miRNA mimics, and the relative luciferase activity was compared between wild-type and mutant-type RKIP 3'-UTR transfected with NC and miR-27a. Data were presented as mean ± standard deviation in three independent experiments (*p<0.05).

The result of qRT-PCR showed that the level of miR-27a in non-sensitive tissues was significantly increased, with statistical reduction of RKIP expression (p<0.05) (Figure 3A, 3B). Moreover, data on two-tailed Pearson correlation analysis showed that there was a negative correlation between miR-27a and RKIP mRNA expression levels in all tissue samples (n=30) (r=-0.691, p<0.05) (Figure 3C). The result of Kaplan-Meier survival curves indicated that the overall survival time of patients with high miR-27a expression was shorter than that of patients with low miR-27a expression (log-rank test, p<0.05) (Figure 3D), manifesting the high risk of poor prognosis of liver cancer with a high level of miR-27a.

Discussion

The current treatments of liver cancer include surgery, chemotherapy, and radiotherapy, which are also primary means for all malignant tumors[10]. Surgery is a preferred treatment method for liver cancer, which can reduce the tumor volume via resection of primary and metastatic lesions. Surgery, combined with radiotherapy and chemotherapy, was applied in the therapy for the early phase of cancers[11]. At the same time, with the rapid development of minimally-invasive techniques, interventional therapy of liver cancer has been widely used, and it is recognized as the preferred treatment means of middle-advanced liver cancer because of its favorable therapeutic effect[12]. Through interventional therapy, the unresectable advanced liver cancer or liver cancer with larger primary lesions and more metastatic lesions can be reversed, which become resectable[13,14]. However, the early symptoms of liver cancer are not noticeable, so that patients are mostly in the middle-advanced stage when diagnosed. Additionally, the recurrence rate after treatment is regularly higher with unsatisfactory 5-year survival rate[15].

At present, the specific pathogenesis of liver cancer is not yet clear, but it has been found that many factors are involved in the progression from normal liver tissues to liver cancer, which are associated with aflatoxins, steroid compounds, cirrhosis, chemical carcinogens, viral hepatitis, etc.[16]. Recent evidence showed that cancers resulted from the accumulation of normal gene mutations, such as oncogene activation and inactivation, and loss of tumor suppressor genes[17]. For instance, Raf-1 kinase inhibitor protein (RKIP) is

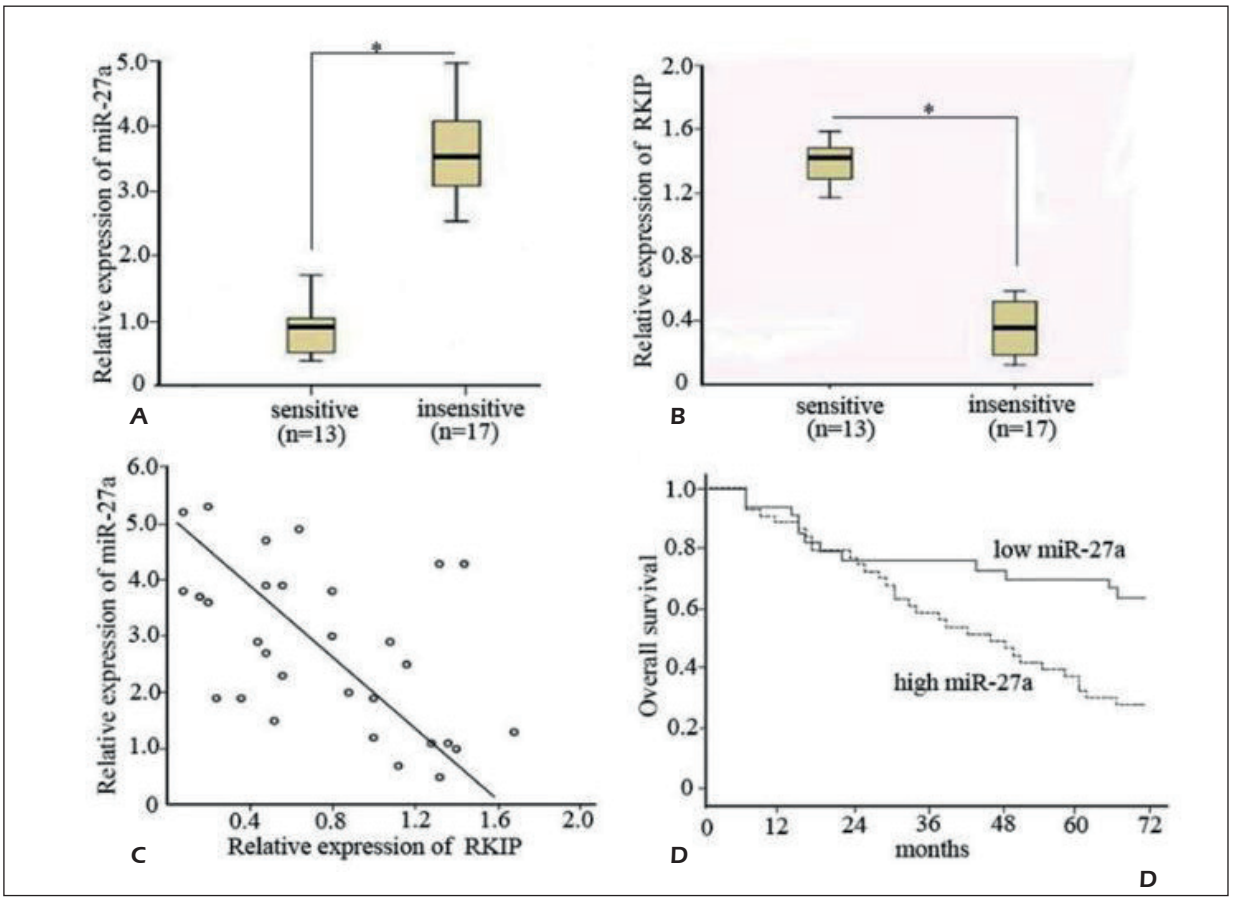

Figure 3. Correlations of high expression of miR-27a in liver cancer tissues with decreased RKIP expression, chemotherapy resistance, and poor prognosis. The relative expression levels of miR-27a (**A**) and RKIP mRNA (**B**) were detected in cisplatin-sensitive (n=13) and non-sensitive (n=17) liver cancer tissues via qRT-PCR. The abundance of miRNA and RKIP mRNA was normalized for U6 RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. **C**, Two-tailed Pearson correlation analysis showed that there was a negative correlation between miR-27a and RKIP mRNA expression levels in all tissue samples (n=30) ($r=-0.691$, $p<0.05$). **D**, Kaplan-Meier survival curves showed that the overall survival time of patients with high miR-27a expression was shorter than that of patients with low miR-27a expression (log-rank test, $p<0.05$).

a tumor and metastasis suppressor in cancer cells and suppresses the proliferation and metastasis of breast cancer cell lines through up-regulation of miR-185 targeting HMGA2[18].

MiRNA is a kind of non-coding micro RNA with only 20-24 nucleotides in length and a regulatory function, which belongs to a small fragment of RNA[19]. It has been shown that the decreased levels of two miRNAs are associated with chronic lymphocytic leukemia, suggesting that there is a clear potential relationship between miRNAs and cancer[20]. MiR-27a has been proven in clinical research to be related to various cancers, such as breast cancer, gastric cancer, and prostate cancer. It promotes tumor expansion and distant metastasis via acting on the target gene Spry2[21]. Results of this study showed that miR-27a and miR-664b expression levels were gradually increased in healthy group, control group, and observation group, but the expression levels of miR-30a were gradually decreased, indicating that there is a significant correlation between miRNAs and occurrence of liver cancer. Furthermore, the increase of miR-27a level has evident positive correlations with the formation and development of cancer, and it plays a guiding role in the diagnosis of liver cancer[22]. Our data indicated that miR-27a promoted the proliferation and migration of liver cancer cells, which was in favor of the malignant development of liver cancer, as in agreement with previous finding[23]. Ren et al[24] revealed that miRNA represented a regulatory way of gene expression and played irreplaceable roles in cell differentiation, occur-

rence and development of diseases. Consistent to the previous finding that miRNAs such as miR-126, miR-155 modulated the proliferation and apoptosis of liver cancer cells[25,26], our study found the similar regulatory role of miR-27a in liver cancer, particularly, its effect on CDDP and related mechanism towards RKIP protein. However, the exact efficacy of miR-27a in the therapy of liver cancer remains further evaluation based on a large number of clinical data.

Conclusions

We demonstrated that miR-27a was closely related to the patients with liver cancer, which was in a malignant degree dependent manner. The reduction of miR-27a elevated the expression of RKIP and counteracted the resistance to CDDP, MiR-27a proliferation and migration capacities of liver cancer cells, which provides a new drug target for the clinical treatment of liver cancer.

Conflict of Interests:

The Authors declare that they have no conflict of interests.

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