Expression of *miR-9a-5p* in cirrhosis patients with recurrent portal hypertension after treatment

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Abstract

Background. MicroRNA (miR) influences the biological activities of cirrhotic patients with recurrent portal hypertension.

Objectives. The current study was designed to investigate risk factors related to the survival of cirrhosis patients and assessed the possibility of using *miR-9a-5p* predictability to prevent post-treatment portal hypertension.

Materials and methods. Patients with portal hypertension due to liver cirrhosis treated from January 2015 to September 2016 were included in this study. Patients without relapse after treatment were selected as the success group while patients with relapse after treatment were selected as the recurrence group. Serum samples from healthy people were also collected. The blood indexes of the 2 groups of patients before and after treatment were compared and the *miR-9a-5p* serum level in each group was determined. The Kaplan–Meier method was applied to analyze three-year survival, Cox univariate regression was used to analyze the risk factors for recurrence of cirrhotic portal hypertension, and the receiver operating characteristic curve (ROC) was used to evaluate the diagnostic value of serum *miR-9a-5p*, total bilirubin (TBIL) and platelet (PLT) levels in patients with recurrence.

Results. The *miR-9a-5p* level in the recurrence group was higher than that in the success group after treatment. In patients with recurrence, the *miR-9a-5p* level was negatively correlated with red blood cell count, TBIL, white blood cell count, and PLT count, and positively correlated with albumin. The *miR-9a-5p*, TBIL and PLT are potential markers of recurrent portal hypertension in liver cirrhosis. The *miR-9a-5p* had the highest area under the curve (AUC) value in patients with relapse.

Conclusions. The *miR-9a-5p* is a risk factor for the recurrence of cirrhotic portal hypertension after treatment. It may be used as a marker of recurrence, and so has potential clinical value for the diagnosis and treatment of recurrent portal hypertension.

Key words: *miR-9a-5p*, liver cirrhosis, portal hypertension, recurrence, risk factor

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Background

Liver cirrhosis is a common and chronic liver disease that is caused by extensive fibrosis secondary to long-term liver cell inflammation and necrosis accompanied by the formation of pseudolobules and regenerative nodules, which deform and harden the liver.[1] The main causes of cirrhosis are viral hepatitis – a few cases of which are schistosomiasis cirrhosis – alcoholism, autoimmune conditions, and Budd–Chiari syndrome.[2,3] The disease progresses slowly and has no visible symptoms. Most patients have mild or no symptoms that can be relieved by rest. However, once liver function is decompensated, the symptoms become more obvious, and are mainly manifested as liver function decline and portal hypertension.[4,5] The clinical manifestation of cirrhotic portal hypertension is usually the opening of the communicating branch of the portal systemic vein, which results in a large amount of portal vein blood directly entering systemic circulation before entering the liver, resulting in venous dilatation, hypersplenism, splenomegaly, and ascites in the esophagus and abdominal wall.[6,7] Dilatation of the esophageal and gastric fundus veins can occur in severe cases. If rupture occurs, the patient will suffer from severe upper gastrointestinal hemorrhage, which endangers their life.[8] Massive hemorrhage of the digestive tract caused by rupture of collateral circulation of portal body is one of the main causes of death in patients with decompensated liver cirrhosis. Zhao et al. reported that the death rate after the first hemorrhage is about 20%, and the rebleeding rate can reach 45% within 24 h after hemorrhage, and is as high as 75% within 1 year.[7] If untreated, about 60% of patients suffer from rebleeding within 1–2 years after the first hemorrhage.[9,10] The most effective treatment for cirrhotic portal hypertension in patients complicated with esophageal and gastric varices bleeding is a transjugular intrahepatic portosystemic shunt (TIPS), which is minimally invasive and can effectively reduce the pressure of the portal vein. However, due to hemodynamic changes after TIPS and mechanical damage during the operation, complications such as liver function damage, hepatic encephalopathy and long-term stent stenosis may occur after TIPS.[11,12]

MicroRNA (miR), which is a kind of non-coding RNA containing approx. 22 nucleotides that is widely distributed in organisms, regulates the biological functions of cells by binding to target mRNA 3'UTR to inhibit translation.[13] MicroRNA has been found to play a role in many diseases including viral hepatitis,[14] liver fibrosis[15] and liver cancer.[16] It can regulate differentiation, proliferation, apoptosis, metabolism, tumorigenesis, and other biological processes, and has gradually attracted attention in the field of molecular biology.[17]

Objectives

In order to explore the role of *miR-9a-5p* in cirrhosis patients with recurrent portal hypertension after treatment, this study assessed *miR-9a-5p* levels in recurrent patients to explore three-year survival, identify risk factors related to survival, and discuss the possibility of using *miR-9a-5p* to predict the recurrence of liver cirrhosis portal hypertension after treatment.

Materials and methods

General patient data

From January 2015 to September 2016, 42 patients (28 males and 14 females, aged 49.8 ±12.1 years) who did not relapse (successful operation) after treatment and 35 patients (23 males and 12 females, aged 50.1 ±11.5 years) who suffered from relapse (operation failure) after treatment were selected for TIPS treatment due to liver cirrhosis portal hypertension in our hospital (Tai'an Central Hospital, Tai'an, China). The general characteristics of the 2 groups before treatment were comparable. The success criteria for the TIPS operation are: 1. The portal pressure should be reduced to 12 mm Hg (16 cm H_2O), or below 25% of the pressure for treatment of the anterior portal vein after the establishment of the hepatic vein (inferior vena cava of the hepatic segment) 2. Diversion between the portal veins should occur. The diagnostic criteria of shunt dysfunction in TIPS include any of the following signs indicating that shunt dysfunction may be present: 1) ultrasound indicates that the shunt blood flow velocity is >200 cm/s, <50 cm/s or no blood flow, or the shunt diameter is <50%; and 2) recurrence of portal hypertension, i.e., hemorrhage and ascites of the esophagogastric vein after treatment. In all cases, portosystemic pressure gradient (PSG) exceeded 12 mm Hg (16 cm H_2O). Suspected shunt dysfunction can only be diagnosed after the portal vein pressure exceeds 12 mm Hg, as determined using portal vein angiography. Data from 30 healthy people were collected during the same time to serve as a control group (18 males and 12 females, aged 50.3 ±10.8 years). All patients and healthy people participating in this study provided informed consent and the study was approved by the Ethics Committee of our institution.

Blood sample collection

Patients in each group received anticoagulants through the antecubital vein on an empty stomach in the morning. A blood sample was then collected and centrifuged at 4°C for 10 min at 3000 rpm. The upper serum was placed in a microcentrifuge tube sterilized with high pressure steam, centrifuged at 4°C for 15 min at 15,000 rpm, collected, divided into smaller samples, and stored in a refrigerator at 4°C.

Quantitative polymerase chain reaction

Serum samples frozen at –80°C were thawed at room temperature. Each microcentrifuge tube contained 100 µL of serum. Total RNA was extracted from serum according to the instructions of the miRNeasy Mini kit (Qiagen, Hilden, Germany). The concentration and purity of total RNA at 260–280 nm were detected using an ultraviolet spectrophotometer. An OD260/OD280 ratio of 1.8–2.0 indicated acceptable RNA purity. According to the instructions of the reverse transcription kit (Qiagen), a 20 µL reaction system was constructed and reverse transcription was carried out using a Gene AmpPCR System 9700 (37°C for 60 min and 95°C for 5 min). The following *miR-9a-5p* and U6 primers were designed and synthesized by Sangon Biotech (Shanghai, China): *miR-9a-5p*: F: 5'-GGGTCTTTGGTTATCTAGCT-3'; R: 5'- ATCCAGTGCGTGTCGTGGA-3', U6: 5'-GCTTCGGCAGCACATATACTAAAAT-3'; R:5'-CGCTTCACGAATTTGCGTGTCAT-3'. The PCR kit was purchased from Ribo Bio (Guangzhou, China), and 20 µL of the reaction system was prepared according to the manufacturer's instructions. The standard three-step method was used: 95°C for 20 s, 95°C for 10 s, 60°C for 20 s, and 70°C for 10 s, for a total of 40 cycles. The serum miR-level was calculated using $2^{-\Delta\Delta Ct}$ method.

Patient follow-up

The non-recurrent group of patients visited the hospital for routine blood, liver function, kidney function, and hemagglutination testing at 1, 3, 6, and 12 months after TIPS treatment, and then they were examined once a year. During this period, if the patients suffer from gastrointestinal hemorrhage or ascites, they should return to hospital for examination and treatment at any time. For the relapse group, patients were followed-up by nurses every 6 months through outpatient or telephone visits for 3 years.

Data analyses

IBM SPSS v. 20.0 (IBM Corp., Armonk, USA) software was used for statistical analyses and GraphPad Prism v. 6 (GraphPad Software, San Diego, USA) software was used for visualizing the data. Measurement data are reported as the mean ± standard deviation (SD). Comparisons between groups were performed using the independent sample t-test, log rank test, and one-analysis of variance (ANOVA), and post hoc pair-wise comparisons were performed using the least significant difference (LSD) test. Count data are reported as n and comparisons were performed using the χ^2 test. Receiver operating curve (ROC) analysis was applied to explore the diagnostic value of *miR-9a-5p* for patients with recurrent cirrhosis portal hypertension after treatment. Cox regression analysis was applied to test the risk factors for recurrence. All data were tested using two-tailed tests. A value of 95% was used for the confidence intervals (95% CI). A difference was considered to be statistically significant when $p < 0.05$.

Results

Comparison of general data of patients before and after treatment

There were no significant differences in the routine blood index, liver function index, renal function index, Child–Pugh score, or grading before treatment between the 2 groups. After treatment, the routine blood index, liver function index, renal function index, and Child–Pugh score of patients in the recurrence group were significantly lower than those in the success group ($p < 0.05$; Table 1,2).

Expression of *miR-9a-5p* and its influence on prognosis

Comparison of miR-9a-5p levels in patients with different Child–Pugh grades revealed higher levels in patients with liver cirrhosis than in controls, and it was expressed differentially by distinct high Child–Pugh grade ($p < 0.05$, Fig. 1A). The *miR-9a-5p* level in the recurrence group was significantly higher than that in the success group after treatment ($p < 0.05$, Fig. 1B). Combined with the expression level of *miR-9a-5p* in patients' serum samples, the Kaplan–Meier method was applied to analyze the three-year survival of the 77 patients with liver cirrhosis portal hypertension (Fig. 1C) and Cox univariate regression was used to test the risk factors of these patients. The results showed no significant difference in three-year survival between the high and low *miR-9a-5p* groups. Cox univariate analysis showed that *miR-9a-5p*, serum TBIL, PLT, Child–Pugh score before treatment, and hepatic encephalopathy before treatment were all risk factors for three-year survival in patients with recurrent cirrhotic portal hypertension.

Correlation between serum *miR-9a-5p* and various indicators in recurrent patients

The *miR-9a-5p* levels in patients with recurrent liver cirrhosis portal hypertension were negatively correlated with red blood cell count, TBIL, white blood cell count, and PLT count, and positively correlated with albumin, as shown in Fig. 2.

Evaluation of *miR-9a-5p*, TBIL, and PLT as diagnostic markers of recurrence

In order to determine whether *miR-9a-5p*, TBIL or PLT can be used as markers for the treatment of recurrent portal

Table 1. General data of patients before treatment

Variable	Success group (n = 42)	Recurrence group (n = 35)	χ^2/t	p-value
Gender, n (%)	–	–	0.0077	0.9299
Male	28	23	–	–
Female	14	12	–	–
Age [years]	49.8 ±12.1	50.1 ±11.5	0.1108	0.9121
Red blood cell count [$\times 10^{12}$/L]	4.46 ±1.86	4.39 ±1.91	0.1624	0.8713
White blood cell count [$\times 10^{9}$/L]	3.56 ±1.03	3.48 ±1.08	0.3269	0.7446
Platelet count [$\times 10^{9}$/L]	50.35 ±15.23	53.25 ±16.98	0.7896	0.4322
Albumin [g/L]	31.65 ±3.26	31.58 ±3.03	0.0969	0.9231
TBIL [µmol/L]	23.75 ±5.98	23.52 ±5.85	0.1697	0.8657
Alanine transaminase [U/L]	52.06 ±10.74	51.97 ±10.82	0.0365	0.9710
Thrombin activity [%]	60.03 ±8.26	59.56 ±8.74	0.2421	0.8093
INR	1.15 ±0.12	1.16 ±0.09	0.4067	0.6854
Blood creatinine [µmol/L]	59.8 ±2.96	58.7 ±3.12	1.5843	0.1173
Child–Pugh score	6.85 ±1.02	6.73 ±1.16	0.4829	0.6306
Child–Pugh grading (A/B/C)	32/10/0	26/7/0	0.0711	0.7897

TBIL – total bilirubin; INR – International Normalized Ratio.

Table 2. Comparison of indexes after treatment

Variable	Success group (n = 42)	Recurrence group (n = 35)	χ^2/t	p-value
Red blood cell count [$\times 10^{12}$/L]	5.23 ±1.36	4.39 ±1.91	2.2482	0.0275
White blood cell count [$\times 10^{9}$/L]	8.06 ±1.75	3.48 ±1.08	13.4827	<0.0001
Platelet count [$\times 10^{9}$/L]	305.36 ±20.36	137.98 ±12.03	42.7823	<0.0001
Albumin [g/L]	23.85 ±3.72	28.63 ±3.16	6.0061	<0.0001
TBIL [µmol/L]	30.06 ±6.32	24.36 ±4.53	4.4631	<0.0001
Alanine transaminase [U/L]	41.05 ±5.23	49.85 ±10.82	4.6619	<0.0001
Thrombin activity [%]	78.69 ±8.03	64.26 ±7.84	7.9363	<0.0001
INR	1.89 ±0.36	1.30 ±0.18	8.8144	<0.0001
Blood creatinine [µmol/L]	55.3 ±1.29	58.7 ±2.85	6.9326	<0.0001
Child–Pugh score	6.03 ±0.71	6.89 ±1.05	4.2673	<0.0001

TBIL – total bilirubin; INR – International Normalized Ratio.

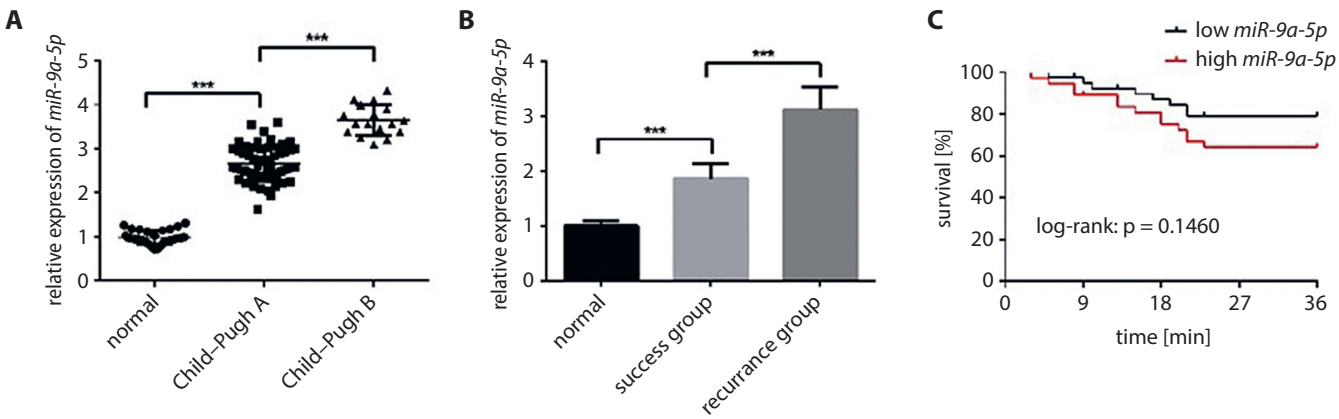

Fig. 1. Expression of *miR-9a-5p* and its relationship with prognosis. A. Expression of *miR-9a-5p* in serum of patients with different Child–Pugh grades; B. Expression of *miR-9a-5p* in serum of different groups of patients; C. Prognosis of patients with different *miR-9a-5p* expression levels

*** significant intergroup differences; p < 0.001

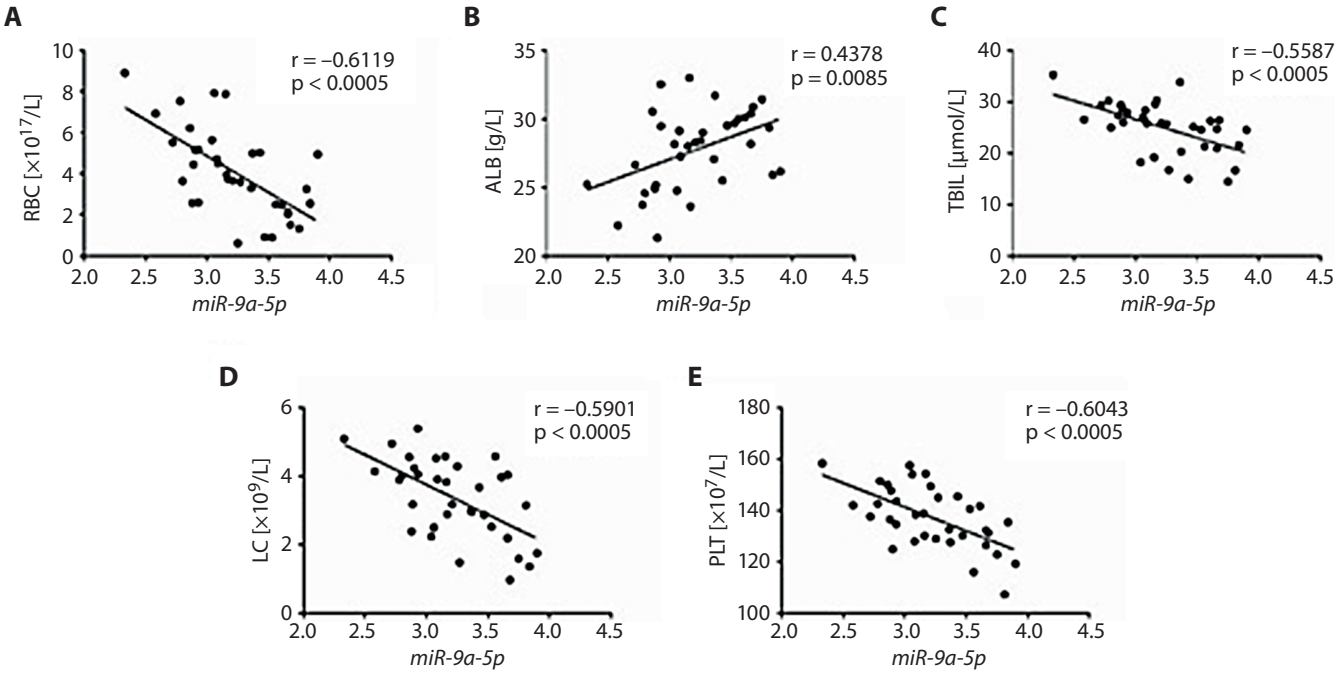

Fig. 2. Correlation between serum *miR-9a-5p* and various indexes in relapsed patients. A. Correlation between *miR-9a-5p* and red blood cell count; B. Correlation between *miR-9a-5p* and albumin; C. Correlation between *miR-9a-5p* and total bilirubin (TBIL); D. Correlation between *miR-9a-5p* and leukocyte count; E. Correlation between *miR-9a-5p* and platelet (PLT) count

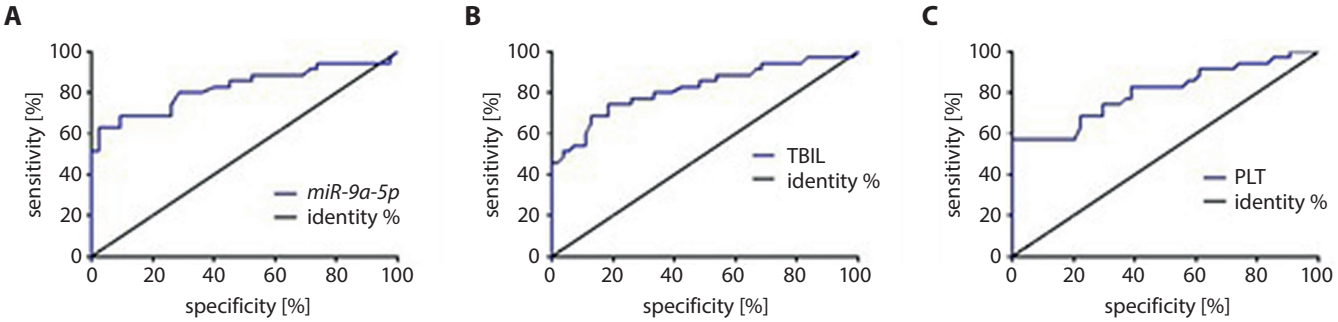

Fig. 3. Use of *miR-9a-5p*, total bilirubin (TBIL) and platelet (PLT) for diagnosis of recurrence. A. Use of *miR-9a-5p* for diagnosis of recurrent patients; B. Use of TBIL for diagnosis of recurrent patients; C. Use of PLT for diagnosis of recurrent patients

Table 3. Results of COX regression analysis

Variable	HR	95% CI	p-value
miR-9a-5p	1.032	1.001–1.062	0.034
Gender	1.385	0.921–2.052	0.192
Age	1.521	0.943–2.522	0.083
Serum TBIL	1.236	1.026–1.273	0.010
Platelet count	0.972	0.932–1.009	0.035
Child–Pugh score before treatment	1.125	1.001–1.043	0.027
HE before treatment	1.018	0.905–0.973	0.007

TBIL – total bilirubin; HE – hepatic encephalopathy; 95% CI – 95% confidence interval; HR – hazard ratio.

hypertension in cirrhosis, the ROC curves of *miR-9a-5p*, TBIL and PLT were constructed (Fig. 3). The area under the curve (AUC) values of *miR-9a-5p*, TBIL and PLT were 0.8252, 0.8209 and 0.8040, respectively. The above results revealed that *miR-9a-5p* had the best diagnostic value (Tables 3,4).

Discussion

Cirrhosis, as a common liver disease, has a rising incidence rate.[18] The liver function of patients with early cirrhosis is normal and there are no obvious clinical symptoms.

Table 4. Diagnostic value of *miR-9a-5p*, TBIL and PLT for recurrence of portal hypertension

Variable	AUC	SE	95% CI	p-value
miR-9a-5p	0.8252	0.052	0.7240–0.9263	<0.0001
TBIL	0.8209	0.049	0.7248–0.9170	<0.0001
PLT	0.8040	0.050	0.7050–0.9030	<0.0001

AUC – area under the curve, SE – standard error; 95% CI – 95% confidence interval; TBIL – total bilirubin; PLT – platelet level.

However, patients with late cirrhosis may experience a portal vein pressure increase and other phenomena that induce portal hypertensive gastropathy, gastrointestinal hemorrhage and other complications. Liver cirrhosis and portal hypertension can further induce splenomegaly and hyperfunction.[19] Although drug therapy can alleviate the disease to a certain extent, long-term medication use can lead to a drug withdrawal reaction, low hemostasis rate and other complications. Surgery is a commonly used method for clinical treatment of liver cirrhosis and portal hypertension, but the possibility of recurrence of portal hypertension is high due to the development of liver cirrhosis.[20] Studies have shown that although microRNA accounts for only 2% of human genes, it regulates the expression of more than 30% of human genes, and is closely related to the occurrence and development of diseases. Clinically, miR-130a and miR-130b are helpful for the diagnosis and prognosis of liver cirrhosis and can be applied as a joint detection method for liver cirrhosis or for a general survey of liver cirrhosis.[21]

In the present study, *miR-9a-5p* in patients with recurrent cirrhosis and portal hypertension after treatment was studied. The results revealed higher *miR-9a-5p* serum levels in patients with recurrent cirrhosis and portal hypertension than in patients in the success group after treatment. Previous research has revealed that *miR-9a-5p* is enhanced in rats with liver fibrosis portal hypertension.[22] The serum *miR-9a-5p* level in patients with recurrent liver cirrhosis portal hypertension showed a negative correlation with red blood cell count, TBIL, white blood cell count, and PLT count, and a positive correlation with albumin. About $^1/_3$ of platelets in the normal blood system are stored in the spleen, while 50–90% of platelets in patients with cirrhosis are stored there; if metabolism of the spleen is enhanced, damage to blood cells is increased and the proliferation of monocytes and macrophages is over-activated, thus leading to a decreased PLT level in peripheral blood.[23] Albumin plays a decisive role in maintaining blood volume and plasma colloid osmotic pressure. It can expand the blood volume, inhibit the aggregation of leukocyte components, reduce blood consistency, and improve blood flow, which are beneficial for tissue repair and the control of ascites formation. However, albumin also controls ascites and increases portal vein blood flow, thus increasing the risk of esophageal and gastric varices bleeding.[24] Serum bilirubin is a metabolite of heme in hemoglobin and other heme proteins in macrophages or other reticuloendothelial cells and hepatocytes. Blood detection indexes include indirect bilirubin level (IBIL) combined with albumin in blood, and direct bilirubin level (DBIL) combined with glucuronic acid in liver. The increase in bilirubin is likely caused by a related increase in bilirubin production or issues with the uptake, binding or excretion of bilirubin.[25] The *miR-9a-5p*, TBIL and PLT are potential markers for diagnosis of recurrent portal hypertension in liver cirrhosis. Previously, several investigations have found that platelet count is an independent factor affecting the survival rate of patients with refractory ascites.[26] Therefore, PLT count is related to postoperative liver function and survival rate, and a higher PLT count is beneficial to liver function.[27] In the present study, *miR-9a-5p* had the highest AUC value in patients with relapse.

Limitations

Although the most optimal care possible has been provided by the researchers during every step of this study, still some limitations exist.

This was a clinic-based study and only hematological analysis was carried out; therefore, futher investigation is required in this aspect. Due to time constraints, the collected sample size was not adequate to represent all patients' characteristics. The result might be area-specific and might not necessarily represent the community scenario. Finally, the study might lack external validity.

Conclusions

Overall, by studying the expression of *miR-9a-5p* in the serum of patients with recurrent cirrhosis portal hypertension, this study has identified factors associated with recurrence after treatment of cirrhosis portal hypertension. The *miR-9a-5p* may be used as a marker of recurrence, and so has clear potential clinical value for the diagnosis and treatment of recurrent portal hypertension.

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