Original Article

Exosomal microRNA-155 as a biomarker for hepatic fibrosis diagnosis and progression

Lian-Jie Niu[1,2]^, Ya-Min Zhang[2]^, Tao Huang[1], Xian-Fu Sun[1], Su-Xia Luo[3]

[1]Department of Mammary Gland Department, Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, China; [2]Department of Hepatobiliary Surgery, Tianjin First Central Hospital, Tianjin, China; [3]Department of Gastroenterology, Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, China

Contributions: (I) Conception and design: LJ Niu, YM Zhang, XF Sun; (II) Administrative support: YM Zhang; (III) Provision of study materials or patients: YM Zhang, SX Luo; (IV) Collection and assembly of data: LJ Niu, T Huang; (V) Data analysis and interpretation: LJ Niu, XF Sun, SX Luo; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Su-Xia Luo; Xian-Fu Sun. Affiliated Cancer Hospital of Zhengzhou University, 127 Dongming Road, Jinshui District, Zhengzhou, China. Email: luosuxiahnszlyy@163.com; zlyysunxianfu1256@zzu.edu.cn.

Background: Pathological examination of liver biopsies remains the gold standard for evaluating the stage of hepatic fibrosis, which are a number of disadvantages associated with biopsy. The aim of the present study was to investigate the potential of exosomal microRNA (miR)-155 as a non-invasive biomarker for the diagnosis and progression of hepatic fibrosis.

Methods: Exosomal miR-155 quantity was analyzed by sampling serum exosomes of patients with hepatic fibrosis and a hepatic fibrosis rat model. A total of 94 patients were divided into three groups based on Child-Pugh rating. Additionally, 30 patients with primary liver fibrosis who underwent liver transplantation were divided into the low miR-155 expression group and the high expression group; 56 rats were divided into 7 groups (n=8, 0, 2, 4, 6, 8, 10, and 12 weeks). Rats in every group were intravenously injected with CCl4 (3% vol/vol in olive oil; 0.3 mL/100 g body weight) twice weekly to produce different degrees of liver necrosis and liver fibrosis.

Results: Exosomal miR-155 was found to be closely associated with the progression of cirrhosis and clinical prognostic indicators of cirrhosis. Exosomal miR-155 gradually increased with the severity of hepatic necrosis and fibrosis.

Conclusions: The findings of the present study indicate that exosomal miR-155 can act as a non-invasive biomarker for the diagnosis and progression of hepatic fibrosis.

Keywords: Exosome; microRNA-155; biomarker; liver fibrosis; diagnosis; progression

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Introduction

The activation of hepatic stellate cells (HSCs) results in hepatic fibrosis (1-3). Liver fibrosis is closely associated with the development of hepatocellular carcinoma (HCC); therefore, the accurate assessment of fibrosis is critical for HCC screening (4). Distinguishing patients with advanced disease, especially cirrhosis, from patients with mild or no fibrosis is important for clinical treatment decisions (5).

Exosomes are small extracellular membrane vesicles (EVs) containing nucleic acids and proteins excreted

^ ORCID ID: Lian-Jie Niu, 0000-0002-1138-1502; Ya-Min Zhang, 0000-0001-7886-2901.

by HSCs and mesenchymal stem cells (6,7). Damaged epithelial cells secrete exosomes that activate fibrocytes (8). In addition to exosomes, there are other EVs including microvesicles and apoptotic bodies, EVs enter the intercellular space or they may enter body fluids such as urine, saliva or blood, which may be taken up by neighbouring cells or potentially be delivered to target cells. Exosomes are a readily accessible and rich source of biomarkers that have potential for assessing organ disease, including that of the liver (9,10). miRNAs, 19–24 nucleotide non-coding RNAs, negatively regulate gene expression and profibrogenic signaling (11,12). miRNAs are involved in HSC activation, and regulate apoptosis, differentiation, migration, and proliferation of activated HSCs (13,14). Circulating miRNAs are associated with the progression of fibrosis, and may be promising fibrotic markers present in plasma (15,16). However, the clinical benefit of using exosomal miRNAs for assessing the stage of hepatic fibrosis is not yet clear. Several studies have shown that miRNA-155 (miR-155) is closely associated with hepatic cancer and hepatitis (17-19). miR-155 appeared in different serum components in different liver injuries. In alcohol liver injury, miR-155 was found in the EV-enriched serum fraction, whereas in APAP-induced DILI, miR-155 is mainly present in the protein rich fraction (20). Therefore, miR-155 in different fractions of serum may be a potential biomarker for identifying the aetiology of liver injury, and it will be interesting to further explore its contribution to communication between hepatic fibrosis and hepatic cancer. profiling exosomal miR-155 during the progression of fibrosis may identify new diagnostic biomarkers and therapeutic targets for the treatment of hepatic fibrosis.

Pathological examination of liver biopsies remains the gold standard for evaluating the stage of hepatic fibrosis (21,22). There are a number of disadvantages associated with biopsy; for example, sampling errors and intra- and inter-observer variations (23,24). In addition to pathological examination to stage liver fibrosis, albumin, Type IV, hydroxyproline (Hyp), aspartate aminotransferase (AST), aminotransferase (ALT), albumin, hemoglobin, bilirubin ascites and other indicators can indirectly reflect the degree of liver fibrosis. In this context, the inclusion of exosomal miRNAs in the prediction algorithms for determining hepatic fibrosis stage may be useful, although this has not yet been investigated. In the present study, the capacity of exosomal miR-155 as a non-invasive biomarker to predict the stage of hepatic fibrosis in patients was investigated.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-20-7787).

Methods

Plasma sampling

Peripheral blood serum was collected from each participant (50 healthy volunteers and 94 patients with cirrhosis) and liver fibrosis model rats and transferred to tubes. The tubes were centrifuged at 1,200 ×g for 15 min at 26 ℃. After plasma centrifugation, the samples were stored in liquid nitrogen until use. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The Institutional Review Board of Tianjin First Central Hospital authorized the study protocol. All participants provided written informed consent ahead of recruitment.

Animal model

All animal procedures followed national guidelines for animal care and use, and were authorized by the Animal Care Ethics Committee of Tianjin First Central Hospital. Adult male Sprague-Dawley rats, weighing 200–220 g and raised on standard laboratory chow with freely available drinking water, were used in the experiments. They were housed at 23 ℃ with a light/dark (12 h/12 h) cycle. A total of 56 rats were randomly assigned to 7 groups (n=8, assigned as 0, 2, 4, 6, 8, 10, and 12 weeks). Rats in all groups were intravenously injected with CCl4 (3% vol/vol in olive oil; 0.3 mL/100 g body weight) twice weekly. When the liver and blood were harvested, the rats were killed by cervical dislocation under anesthesia. Blood was collected from the vena cava at each time point, and the plasma was collected according to the procedure described above. A portion of each rat liver was soaked in formalin, and the remainder was stored in liquid nitrogen for later use.

Exosome extraction and identification

Exosomes were isolated from plasma samples using the Exoquick kit (System Biosciences, California, USA) according to the manufacturer's protocol. Briefly, 0.4 mL plasma and 0.1 mL Exoquick kit were mixed and incubated for 12 h at 4 ℃, and then centrifuged at room temperature at 10,000 ×g for 30 min. Exosomes were used for RNA extraction and characterization.

Table 1 Primer sequences used for quantitative reverse transcription polymerase chain reaction

Gene name	Forward and reverse primer sequences
MicroRNA-155	RT: 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACCCCT-3'
	Forward: 5'-TGCTAATCGsTGATAGGGG-3'
	Reverse: 5'-CAGTGCAGGGTCCGAGGTAT-3'
U6	RT: 5'-AAAATATGGAACGCTTCACGAATTTG-3'
	Forward: 5'-CTCGCTTCGGCAGCACATATACT-3'
	Reverse: 5'-ACGCTTCACGAATTTGCGTGTC-3'

RT, reverse transcription.

Transmission electron microscopy (TEM) to verify exosome morphology

TEM (JEM-2100; Jeol, Tokyo, Japan) was used to observe the extracted pellets and to validate characterization. Purified exosomes (10 μL) were fixed with 1% glutaraldehyde for 10 min, washed, and contrasted in 2% uranyl acetate.

Western blot analysis of CD63 and CD9

Proteins extracted from plasma exosomes were loaded onto 10% gels and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes (EMD Millipore, Temecula, CA, USA). The membranes were probed with primary antibodies at 4 ℃ for ~12 h and then with secondary antibodies for 1 h. The signal was developed using enhanced chemiluminescence reagent (EMD Millipore, Temecula, CA, USA), and visualized with the FluorChem FC2 Imaging System (Alpha Innotech, California, USA). Western blot analysis was performed following a standard protocol using antibodies targeting CD9 and CD63 (dilution 1:200; Wanleibio, Shenyang, China).

Quantification of miR-155 mRNA expression by quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from exosomes. To validate miRNA expression, qRT-PCR was performed using a SYBR Premix Dimereraser kit (TaKaRa Biotechnology, Kyushu, Japan) on a LightCycler 480 II detection system (Roche Diagnostics, New Naxi, USA). The primers for miR-155 and U6 were purchased from Sangon Biotech (Shanghai, China). The primers are listed in *Table 1*. The relative gene expression values for the target miRNA were calculated using the $2^{-\Delta\Delta CT}$ method.

Hematoxylin-eosin (HE) staining and histopathological observation

Sixty 3-μm sections were obtained from each paraffin block using a microtome (RM2255; Leica, Frankfurt, Germany) and stained with HE. Samples were immersed in xylene and alcohol, stained with hematoxylin for 5 min, stained with eosin for 3 min, and re-immersed in alcohol and xylene. Slides were mounted using a synthetic resin (Entellan; Merck, Darmstadt, Germany). Paraffin sections of liver tissue were prepared for histopathological observation, and the degree of hepatic necrosis and fibrosis was observed by HE staining.

Plasma measurements

Type IV collagen (CIV), hydroxyproline (Hyp), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, hemoglobin, and platelets in plasma samples were measured at the Tianjin First Center Hospital Laboratory.

Statistical analysis

Statistical analysis was performed using SPSS version 24.0 (IBM, Armonk, NY, USA). The results are presented as means ± standard deviations. Two groups were compared using a *t*-test. Independent risk factors of relationships between different groups in the *in vitro* assay data were analyzed using analysis of variance. Receiver-operating characteristic (ROC) curve analysis was conducted to

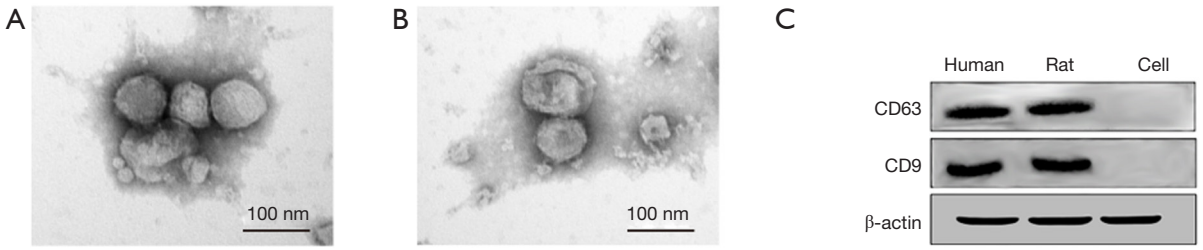

Figure 1 Characterization of isolated exosomes. (A) Human exosomes; (B) rat exosomes; (C) Western blot analysis of the protein expression of CD63 and CD9.

determine the accuracy of the diagnosis of hepatic fibrosis. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of isolated exosomes

Exosomes were characterized by western blot analysis and TEM to ensure the quality and efficiency of the exosomes (*Figure 1*). Exosome analysis by electron microscopy was consistent with previous reports (*Figure 1A,B*). Furthermore, western blot analysis of CD9 and CD63 were used to confirm the presence of exosomes (*Figure 1C*). The successful isolation of exosomes was confirmed by these results.

Clinical significance of exosomal miR-155 for liver fibrosis staging

To better understand the potential roles of plasma exosomal miR-155 in liver fibrosis development and progression, the clinical significance of miR-155 expression in the plasma exosomes of the 94 patients with liver fibrosis and 50 healthy individuals was validated by qRT-PCR. The Child-Pugh rating was used to categorize the 94 liver fibrosis cases into 3 groups. As expected, there was a significant correlation between miR-155 expression and Child-Pugh score (r2=0.728, P<0.01). Additionally, miR-155 expression in patients with a Child-Pugh C rating was substantially higher than in patients with a Child-Pugh A and B rating (*Figure 2A*). The ROC curve [area under the curve (AUC): 0.971, sensitivity: 93.62%, specificity: 94%] showed that exosomal miR-155 may have diagnostic value for liver fibrosis (*Figure 2B*). Furthermore, the association between plasma exosomal miR-155 expression and the prognosis of 30 patients with primary liver fibrosis who underwent liver transplantation was examined. Further research suggested that the low miR-155 expression group had a significantly better prognosis than the high expression group (P=0.016) (*Figure 2C*). Plasma analysis also revealed that there were differences in AST (P<0.001), albumin (P<0.001), platelet count (P<0.001), and age (P=0.028) between the Child-Pugh C group and the Child-Pugh A and B group. However, the Child-Pugh score was not associated with several other clinical features, including sex, ALT, and hemoglobin (*Table 2*). Therefore, we hypothesized that miR-155 expression can predict the stage of liver fibrosis. To validate our hypothesis, we produced rat liver fibrosis models of different stages, as described earlier.

Results of liver fibrosis rat models

As described earlier, a rat liver fibrosis model was established. The prolongation of CCl4 treatment significantly increased the degree of hepatic necrosis and cirrhosis. Hepatic fibrosis gradually worsened over time, and from week 10 onwards, a marked reduction in hepatic volume was observed. HE staining revealed that hepatic necrosis and hepatic fibrosis gradually worsened. These results demonstrated that the fibrosis model had been successfully established with different degrees of liver necrosis and cirrhosis (*Figure 3*).

Exosomal miR-155 and clinical indicators of different degrees of hepatic necrosis and fibrosis

In the present study, we found that, with the increase in hepatic necrosis and hepatic fibrosis, the expression of exosomal miR-155 significantly increased relative to the control group (P<0.05) (*Figure 4A*). The results revealed that AST, albumin, CIV, and Hyp were significantly associated with hepatic fibrosis (P<0.05); however, other

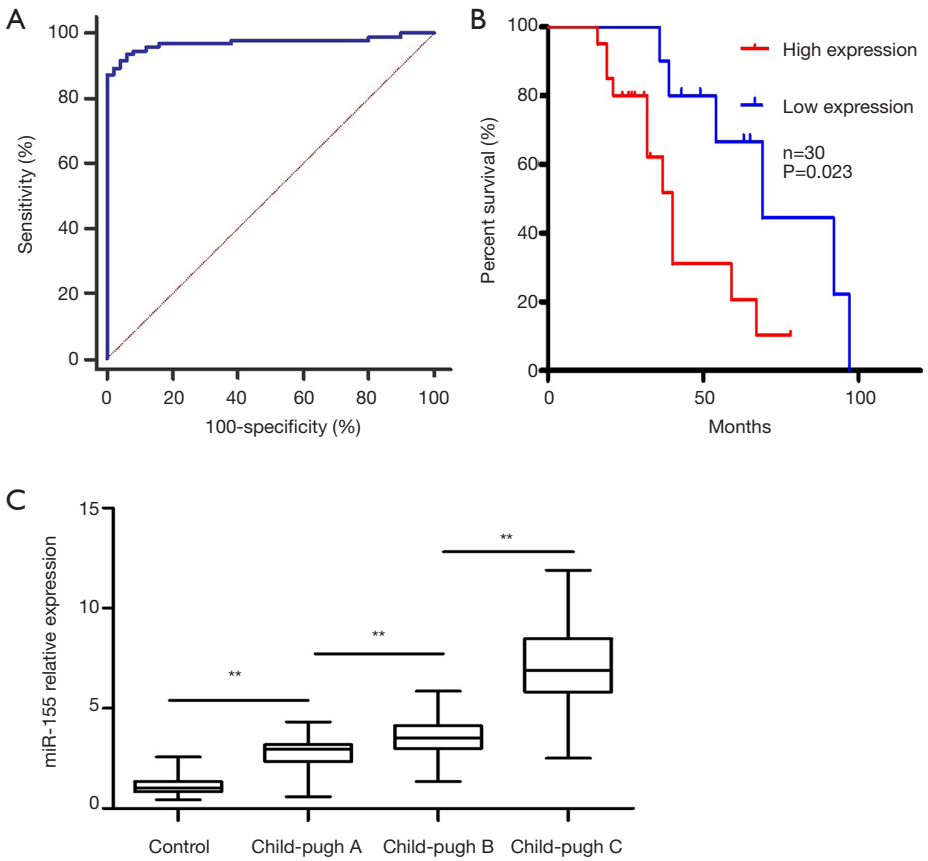

Figure 2 Clinical significance of exosomal miR-155 for liver fibrosis staging. (A) Receiver-operating characteristic curve analysis for the diagnosis of liver fibrosis; (B) high microRNA-155 (miR-155) expression group prognosis compared with the low-miR-155 expression group; (C) expression levels of exosomal miR-155 according to the Child-Pugh rating. **, P<0.01.

Table 2 Clinical data in the different patient groups

Parameter	Control (n=50)	Child A (n=39)	Child B (n=32)	Child C (n=23)	P value
Age (years)	47.2±6.78	44.51±5.65	46.78±6.32	49.65±7.57	0.028
Sex					0.612
Male	22	22	14	10	
Female	28	17	18	13	
ALT (IU/L)	51.19±24.42	55.42±21.78	57.24±22.67	60.11±18.5	0.395
AST (IU/L)	47.31±17.55	56.22±19.33	70.11±21.03	86.21±27.32	<0.001
Albumin (g/L)	41.0±0.6	35.7±0.6	30.9±0.7	26.8±0.9	<0.001
Hemoglobin (g/L)	142.2±1.87	141.3±1.42	139.8±1.67	136.9±1.52	0.624
PLT (10^9/L)	227.2±52.74	210.3±51.26	196.7±48.62	168.2±43.22	<0.001

P<0.05 indicated a statistically significant difference. Data are presented as means ± standard deviations. ALT, alanine aminotransferase; AST, aspartate transaminase; PLT, platelet.

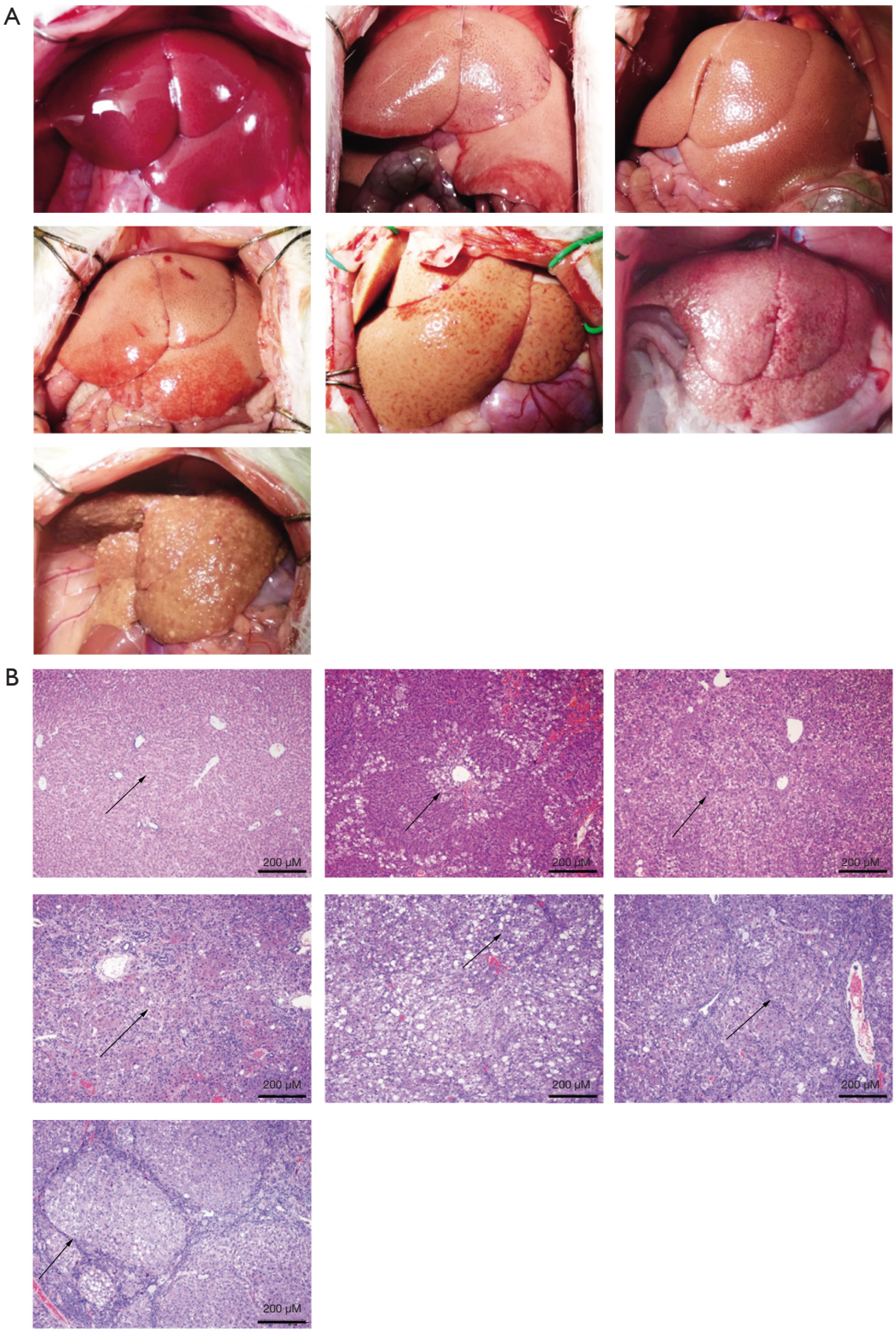

Figure 3 Results of liver fibrosis rat models. (A) Rat liver cirrhosis specimens in each group. (B) Hematoxylin-eosin staining images of rat liver cirrhosis specimens in each group (0, 2, 4, 6, 8, 10, and 12 weeks; ×100 magnification). The arrow refers to the observation position of rat liver cirrhosis.

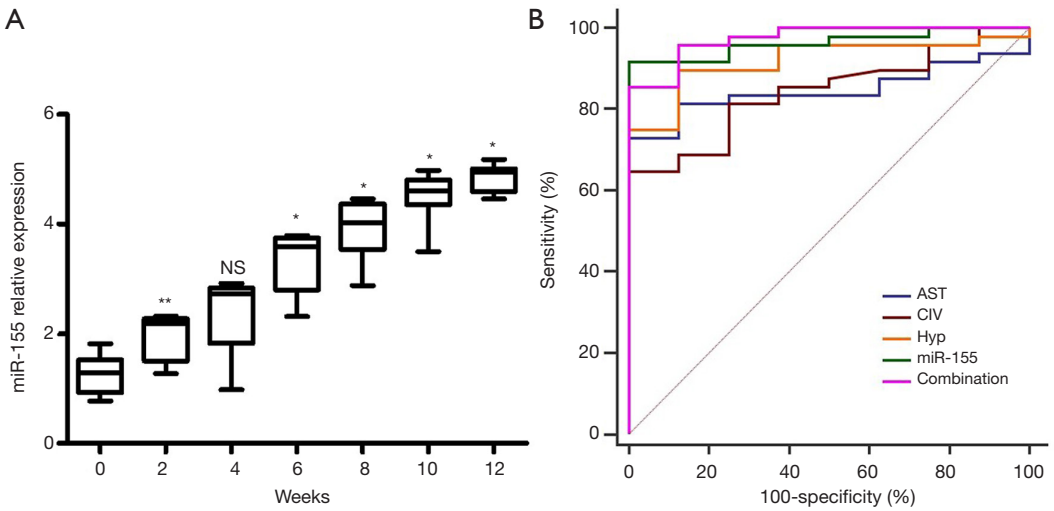

Figure 4 Exosomal miR-155 and clinical indicators of different degrees of hepatic necrosis and fibrosis. (A) Expression of exosomal microRNA-155 (miR-155) in each group; (B) receiver-operating characteristic curve analysis for the diagnosis of liver fibrosis. *, P<0.05; **, P<0.01. AST, aspartate transaminase; CIV, collagen IV; Hyp, hydroxyproline.

Table 3 Clinical data in the different animal model groups (n=8)

Parameter	0 week	2 weeks	4 weeks	6 weeks	8 weeks	10 weeks	12 weeks	P value
ALT (IU/L)	46.8±3.3	47.6±4.1	48.6±3.2	45.3±4.3	48.9±3.9	48.1±4.0	50.6±4.3	0.22
AST (IU/L)	189.6±7.8	214.8±31.4	235.0±44.4	287.9±6.3	309.9±8.2	430.3±14.1	434.8±13.5	<0.001
Albumin (g/L)	33.2±0.33	34.5±0.27	31.2±0.22	32.3±0.42	29.7±0.33	27.8±0.46	26.2±0.37	<0.001
Hemoglobin (g/L)	126.8±0.58	124.4±0.37	125.8±0.46	110.6±0.56	97.4±0.39	91.2±0.42	89.7±0.57	0.324
CIV (μg/L)	29.14±2.1	31.60±2.8	35.45±2.9	39.98±2.0	44.76±2.3	56.4±2.4	59.1±2.3	<0.001
HyP (μg/g)	214.9±69.6	302.8±88.5	309.1±87.3	449.3±88.1	492.2±58.0	516.8±58.4	552.9±79.7	<0.001

P<0.05 indicated a statistically significant difference. Data are presented as means ± standard deviations. ALT, alanine aminotransferase; AST, aspartate transaminase; CIV, collagen IV; Hyp, hydroxyproline.

clinical features, including ALT and hemoglobin, were not associated with fibrosis (*Table 3*). The ROC curve showed that exosomal miR-155 has diagnostic value for liver fibrosis, more so than CIV, Hyp, or AST. Combined diagnosis using miR-155, CIV, Hyp, and AST had the highest AUC value of 0.974 (sensitivity: 85.4% and specificity: 98.7%) (*Figure 4B*).

Discussion

The activation of hepatic stellate cells (HSCs) results in hepatic fibrosis. Liver fibrosis is closely associated with the development of HCC, so the accurate assessment of fibrosis is critical for HCC screening (4,25,26). A number of studies have found that HSC-derived exosomes promote fibrosis. The activated HSCs can release exosomes containing CCN2, which may amplify fibrogenic signalling to promote hepatic fibrosis (27). Kostallari et al. reported that the PDGF receptor-alpha (PDGFRα)-enriched exosomes released by PDGF-BB–treated HSCs promote HSC migration and liver fibrosis (28). MiRNAs have the potential to become novel, non-invasive biomarkers because they are highly stable and easily detected in circulation. Indeed, numerous studies have shown that several miRNAs have certain advantages as biomarkers for early diagnosis, prognosis and evaluation of hepatic fibrosis compared with traditional biomarkers. In addition, exosomes derived from HSCs contribute to the pathology of liver cancer.

Researchers showed that exosomes derived from HSCs deliver miR-335-5p to recipient hepatocellular carcinoma (HCC) cells, inhibiting the proliferation and *in vitro* invasion of HCC cells and inducing the shrinkage of HCC tumours *in vivo* (29). Moreover, researchers observed that the expression of miR-30a was down-regulated in exosomes derived from activated HSCs, which may prevent HSC activation by suppressing autophagy (30).

miR-155 has various liver-associated roles, including effects on hepatic cancer, hepatic injury, hepatic fibrosis, and lipid metabolism (17,31,32). It was demonstrated that miR-155, a pro-fibrotic cytokine positively regulated by hypoxia inducible factor-1α (HIF-1α), adjusted both transforming growth factor-β1 and the process of epithelial-mesenchymal transition (EMT) under hypoxia to promote fibrosis of proximal tubule cells. Another study showed that hsa-miR-155-5p was correlated with hepatic inflammatory level and fibrosis (33). In our study, miR-155 was also significantly upregulated in Child-Pugh C, and demonstrated excellent diagnostic value for advanced liver fibrosis. At the same time, the expression level of miR-155 may be used to predict the clinical outcome of liver transplantation patients; patients with high expression are likely to have reduced survival time after liver transplantation compared with patients with low expression. miR-155 is closely related to the progression of cirrhosis and clinical prognostic indicators of cirrhosis. Furthermore, exosomal miR-155 gradually increased with the degree of hepatic necrosis and liver fibrosis induced by CCl4. Combined diagnosis with miR-155, CIV, Hyp, and AST had the highest AUC, indicating that the diagnostic accuracy was improved. Exosomal microRNA-155 can be used as a non-invasive marker to avoid complications caused by liver biopsy. It has the potential to reflect these dynamic changes, which could help in identifying patients at risk for progressive fibrosis to allow for earlier intervention or maintain closer surveillance. However, there is a lack of relevant long-term longitudinal and clinical data about microRNA-155, so it is difficult to apply to clinic in the short term.

In conclusion, exosomal miR-155 was identified to be associated with liver fibrosis, according to the results obtained in the present study. It had a significant correlation with liver fibrosis, and may therefore serve as a sensitive biomarker for patients with liver fibrosis.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-7787

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-7787). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The Institutional Review Board of Tianjin First Central Hospital authorized the study protocol. All participants provided written informed consent ahead of recruitment. All animal procedures followed national guidelines for animal care and use, and were authorized by the Animal Care Ethics Committee of Tianjin First Central Hospital.

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