Exosomal microRNA-122 from HCC inhibits hepatic stellate cell fibrosis activation via AMPK signaling.

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Research Article

Keywords: hepatic fibrosis, microRNA-122, small extracellular vesicles, human stellate cells

Posted Date: July 7th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3128342/v1

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Abstract

sEVs are extracellular vesicles with nanoscale bilayer membranes that deliver cell-specific proteins and nucleic acids (including mRNA and miRNA) to regulate intracellular signaling pathways. The development of hepatic fibrosis is closely related to sEV and its miRNA, which regulate the activation, proliferation, apoptosis, and migration of hepatic stellate cells. In this study, we report on the regulation of human hepatic stellate cell (HSC) LX-2 cell line by sEVs derived from serum of liver cancer patients through miR-122 and its potential signaling pathway. The effect of miR-122 on mRNA and protein expression of fibrosis markers was evaluated in human hepatic stellate cell LX-2 cell line transfected with miR-122 mimics or added serum-derived sEVs from liver cancer patients using QRT-PCR and western blot analysis. The effect of AMPK on LX-2 cell activation was validated using metformin or AMPK inhibitor. Results showed that miRNA-122 was expressed at low levels in activated LX-2 cells, but serum-derived sEVs and miR-122 mimics from liver cancer patients up-regulated miR-122 levels in activated LX-2 cells and reduced the expression of fibrosis marker proteins. The phosphorylation of AMPK decreased after activation of LX-2 cells, and the level of miR-122 was positively correlated with the phosphorylation of AMPK upon verification, suggesting that sEVs derived from serum of liver cancer patients can up-regulate miR-122 levels in LX-2 cells, change the energy status of cells, and inhibit the activation of HSC. This finding may provide an explanation for the reduced degree of fibrosis observed in HCC patients.

1 Introduction

Liver cancer, the sixth most common malignant tumor in human beings and the fourth one in China, and seriously threatens the life and health of our people. The liver undergoes reactive repair processes when stimulated by chronic hepatitis B, hepatitis C virus (HCV) infection, alcoholic liver disease, and non-alcoholic fatty liver disease (NAFLD). This leads to the activation of hepatic stellate cells (HSCs), resulting in the excessive accumulation and deposition of extracellular matrix. This ultimately leads to fibrosis, and may even progress to cirrhosis over time. Hepatic fibrosis and end-stage cirrhosis are strongly associated with the risk of developing hepatocellular carcinoma (HCC). In fact, approximately one-third of patients with liver cirrhosis will develop hepatocellular carcinoma, and that a significant majority (80–90%) of those diagnosed with HCC also have underlying fibrosis. Interestingly, while the reasons are not entirely clear, the degree of fibrosis often decreases following the hepatocarcinogenesis. In our clinical practice, we have observed that the levels of fibrosis markers in patients with hepatocellular carcinoma resulting from cirrhosis are significantly lower than those in patients with HLD complicated by cirrhosis. Additionally, imaging analysis has revealed that the degree of fibrosis in patients with hepatocellular carcinoma is significantly attenuated when compared to patients with cirrhosis. Therefore, we speculate that hepatocarcinogenesis inhibits the fibrosis process. The tumor microenvironment is believed to play a crucial role in the progression of liver cancer. Liver cancer cells can influence surrounding cells via the microenvironment and interact with normal cells.
sEV, an extracellular vesicle with lipid bilayer and with a diameter of about 30 ~ 150 nm, is secreted by a variety of cells and deliver specific proteins and nucleic acids of cell types, including mRNA and miRNA, to regulate intracellular signal pathways. It is directly related to the progression of various liver diseases. MicroRNA is encoded by genes and exerts its intracellular effects by targeting post transcriptional events on genes. Many studies have shown that sEVs and their miRNAs are closely related to the development of liver fibrosis, and regulate the process of liver fibrosis by acting on hepatic stellate cells to change their activation, proliferation, apoptosis and migration. For example, microRNA-378 restricts hepatic stellate cell activation and hepatic fibrosis by inhibiting Gli3 expression. Overexpression of microRNA-25-3p inhibits Notch1 signal transduction and TGF induced collagen expression in hepatic stellate cells. MicroRNA-145 promotes activation of hepatic stellate cell by targeting Krüppel like factor 4. In brief, microRNA can regulate the transcription and expression of liver fibrosis related genes in target cells through signal transduction and substance transduction. Hsa-microRNA-122-5p (miR-122), a specifically enriched microRNA in liver, is highly expressed in normal liver and decreases in advanced liver diseases such as liver cirrhosis and hepatocellular carcinoma, which has been proved to be an important marker of liver disease. Studies have suggested that miR-122 is closely related to the process of liver fibrosis, and it plays a role by participating in activation of HSC, chronic liver inflammation and lipid metabolism.

In our previous studies, miR-122 was abnormally elevated in exosomes of HCC. In this paper, we found that the activation state of HCC derived SEVs was inhibited when co-cultured with human stellate cells. It has been confirmed that HCC derived sEV can up-regulate the level of miR-122, and then change the energy state of cells through AMPK signaling pathway, inhibit the activation of HSC, and thus inhibit the progression of fibrosis. This finding may explain the decreased degree of fibrosis in HCC patients.

2 Materials and methods

2.1 Cell line and TGFβ-1 induced HSC activation

Human hepatic stellate cell line Lieming Xu-2 (LX-2) cell was purchased from cellcook Biotechnology company (Guangzhou, China; CC4023), and was cultured in DMEM containing 1% Penicillin-Streptomycin Solution (PS) and 10% Fetal Bovine Serum (FBS, Gibco) at 37°C and 5% CO2.

TGF-β1 (R&D Systems, Shanghai, China) was used as a stimulator of HSC activation. Cells were divided into two groups: TGF-β1 group (treated with 5 ng/mL TGF-β1 for 48h) and control group (treated with the same amount of PBS for 48h).

2.2 sEVs isolation from hepatocellular carcinoma (HCC) and human liver cancer cell lines

2mL serum was collected from patients with HCC and sEVs were separated by differential centrifugation. The samples were centrifuged at 3,000×g twice for 15 minutes to ensure that no platelets remained, then
the supernatant was diluted with ice PBS at 1:20 and was filtered with 0.22µm, and finally was concentrated with 100k ultrafiltration tube. The resulting sample was washed twice with PBS and concentrated, and then was subjected to ultracentrifugation twice at 120,000 ×g for 90mins at high speed. The sediment in the collecting tube is sEVs from human hepatoma cell line and was resuspended with 100ul PBS for reserve.

Human hepatoma cell line Huh7 cells and normal liver cell line WRL68 cells were subcultured in DMEM complete medium, and then the supernatants of the above human hepatoma cell lines were collected after 12h starvation. SEVs was isolated by supernatant centrifugation at 3,000×g twice for 15 mins to remove live and dead cells and cell fragments. Then, microvesicles and sEVs were separated by centrifugation at 12,000×g for 30min. The supernatant was concentrated in an 100k ultrafiltration tube, and was ultracentrifuged at 120,000 ×g twice for 90min. The sediment in the collecting tube is the sEVs from human hepatoma cell line, which was resuspended with 100ul PBS for standby.

2.3 LX-2 cells co-cultured with sEVs from HCC

LX-2 cells at the logarithmic growth stage of the third generation were taken and inoculated in a 10cm dish after routine digestion. LX-2 cells were co-treated with sEVs from HCC and 5ng/mL TGFβ-1 for 48h after cell adhesion overnight. Then, cell lysates were collected and used for further study.

2.4 Cell transfection

LX-2 cells at the third generation of logarithmic growth stage were routinely digested and inoculated in a 10cm dish for overnight culture. 5ng/mL TGFβ-1 was added to stimulate LX-2 cells for 24h after cell adherence, and followed transfection was carried out when cell fusion reached 70%~80%. First, 5ng/mL TGFβ-1 was added to High glucose-DMEM culture medium without FBS and PS for culture of LX-2 cells. MiR-122 mimics were diluted in DMEM to 50nM and same amount of Lipofectamine 2000 transfection reagent were diluted in DMEM, respectively. Then these two fluids were gently mixed and incubated for 10 mins. Next, the mixture was transfected into LX-2 cells and cultured in an incubator at 37℃ and 5%CO2. The NC group was also processed in the same way. DMEM was replaced with DMEM containing FBS after 6h. And cell lysates were collected and used for further study after 24h.

2.5 RT-PCR and QRT-PCR

Total RNA was extracted from the experimental group and control group using Trizol reagent (Ambion, 10296028). According to the requirements of the protocol, the first chain cDNA synthesis system (Vazyme, R312-02) was used to synthesize the first chain. Quantitative real-time PCR analysis was performed using “2×AceQ qPCR SYBR Green Master Mix” (Vazyme, Q111-02-AA) to calculate the relative expression level, and the mRNA level of GAPDH was used as internal control. Data were analyzed by 2-ΔΔCt method. Primer sequences of four fibrosis markers were shown in Table 1. miRNA-122 upstream and downstream primers were purchased from Ribobio Inc.
Table 1
Primer sequences

<table>
<thead>
<tr>
<th>Name of primer</th>
<th>Sequences</th>
</tr>
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<tbody>
<tr>
<td>α-SMA-F</td>
<td>AAAAGACAGCTACGTGGGTGA</td>
</tr>
<tr>
<td>α-SMA-R</td>
<td>GCCATGTTCTATCGGTACTTC</td>
</tr>
<tr>
<td>Collagen I-F</td>
<td>GAGGGCCAAGACGAAAGCATC</td>
</tr>
<tr>
<td>Collagen I-R</td>
<td>CAGATCACGTCATCGCACAAAC</td>
</tr>
<tr>
<td>TIMP-1-F</td>
<td>CTTCTGGAATTCCGACCTCGT</td>
</tr>
<tr>
<td>TIMP-1-R</td>
<td>ACGCTGGTATAAGGTGGTCTG</td>
</tr>
<tr>
<td>Desmin-F</td>
<td>TCGGCTCTAAGGGCTCCTC</td>
</tr>
<tr>
<td>Desmin-R</td>
<td>CGTGGTAGAAACTCCTGGT</td>
</tr>
</tbody>
</table>

2.6 Immunofluorescent staining (IF)

LX-2 cells at the third generation of logarithmic growth stage were taken and inoculated into 12-well plates after routine digestion. The cells were treated with PBS, TGFβ-1 or TGFβ-1 + sEVs respectively. After 48 hours, the LX-2 cells growing on round cover glass were fixed in 4% paraformaldehyde. The cytoskeleton was stained with “Actin-Tracker Green” (microfilaments green fluorescent probe, Beyotime, C1033). The nuclei were stained with “Antifade-Mounting Medium with DAPI” (Beyotime, P0131). The staining results were observed under a fluorescence microscope, and the green fluorescence representing the cytoskeleton and the blue fluorescence representing the nucleus were observed and photographed.

2.7 Western blot


According to instructions, total protein samples were extracted using NP40 and PMSF protease inhibitors. The protein samples (10ug-20ug) were separated by SDS-PAGE (5% concentrated gel and 10% separated gel) and transferred to 0.45 NC membrane. The membrane was blocked in TBS/Tween-20 containing 5% non-fat milk powder for 1 hour. The designated primary antibody was incubated overnight at 4°C for detection. The signal was visualized using enhanced chemiluminescence (ECL) system, and quantitative intensity analysis was performed using ImageJ software. The “Prestained Protein Ladder” was loaded in parallel to indicate the molecular weight of the Protein.

3 Results
3.1 The progression of liver fibrosis in HCC patients is inhibited in clinical practice

As shown in Figure 1, we have studied four markers of liver fibrosis in 32 patients with HLD combined with cirrhosis and 26 patients with liver cancer caused by liver cirrhosis (Table 2), and there was no significant difference in the baseline characteristics between the two groups (Table 3). Statistical analysis using T-test showed that the levels of fibrosis markers IV-C, HA, and PCIII in patients with liver cancer caused by liver cirrhosis were significantly lower than those in patients with HLD combined with cirrhosis (P<0.01), excluding LN (Figure 1A). Imaging analysis of the two groups of patients showed that the degree of liver fibrosis in HCC patients was significantly inhibited compared with that in HLD patients (Figure 1B). It indicated that the occurrence of liver cancer inhibited the progression of liver fibrosis.

Table 2. Comparison of the rate of indicators between two groups of patients

<table>
<thead>
<tr>
<th>Fibrosis indicators</th>
<th>HLD n=32</th>
<th>HCC n=26</th>
<th>P value</th>
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<tbody>
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<td>IV-C</td>
<td></td>
<td></td>
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<tr>
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<td>0.01</td>
</tr>
<tr>
<td>Abnormal</td>
<td>21</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>HA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>8</td>
<td>20</td>
<td>0.01</td>
</tr>
<tr>
<td>Abnormal</td>
<td>24</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>LN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>21</td>
<td>17</td>
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</tr>
<tr>
<td>Abnormal</td>
<td>11</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>PCIII</td>
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<tr>
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<td>0.01</td>
</tr>
<tr>
<td>Abnormal</td>
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Table 3. Baseline characteristics of patients

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<thead>
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<th>HCC n=26</th>
<th>P value</th>
</tr>
</thead>
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<td>Age</td>
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<td>54.88±7.92</td>
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<td>Gender</td>
<td></td>
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<td>16</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>10</td>
<td>0.05</td>
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<tr>
<td>Child-Pugh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>14</td>
<td>16</td>
<td>0.05</td>
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<tr>
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<td>0.05</td>
</tr>
<tr>
<td>No</td>
<td>10</td>
<td>7</td>
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</tbody>
</table>

3.2 Isolation and identification of serum-derived sEVs from HCC
Blood samples from different liver cancer patients before tumor resection were collected and sEVs were isolated from blood for RNA sequencing, and the results are shown in Figure 2A. The total amount of hsa-miR-122-5p (miR-122) in the blood of different liver cancer patients is the highest. Then we searched the expression level and expression amount distribution of miR-122 in different kinds of tissues through YM500v2 database. As shown in Figure 2B, it is indicated that miR-122 is a kind of miRNA specifically expressed in the liver.

Next, we identified the vesicles extracted from the serum of patients with liver cancer. Western blot was used to detect sEV marker proteins, in which TSG101, Alix, CD63 and Flotillin proteins were positive, while GM130 was not expressed (Figure 2C). Transmission electron microscope (TEM) observation showed that clear vesicle structure with disc shape was visible (Figure 2D). NTA results showed that the diameter of the detected vesicles was about 150 nm (Figure 2E). The isolated vesicles were detected by nanowow cytometry, and the results showed that their diameters were between 80nm and 180nm (Figure 2F). The above results indicated that the vesicles isolated from the serum of liver cancer patients were sEVs.

3.3 sEVs from HCC inhibit HSC fibrosis activation induced by TGFβ-1

We first used 5ng/mL TGFβ-1(resuspended with 4mM HCl containing 1mg/mL human or bovine serum albumin) to treat LX-2 cells for 48h, and PBS treatment as the control group. The successful construction of HSC activated cell model was verified by RT-QPCR (Figure 3A) and Western blot (Figure 3B, 3C). The results showed that after treatment with TGFβ-1, the mRNA levels of the fibrosis markers α-SMA, collagen I and TIMP-1 were all up-regulated (Figure 3A). Compared with the control group (Figure 3B, Lane 2), the mRNA (Figure 3A) and protein (Figure 3B, 3C) expression levels of α-SMA, desmin, collagen I and TIMP-1 were significantly up-regulated after TGFβ-1 treatment (Figure 2B, Lane 1). Phalloidin which carrying fluorescent probe were combined with actin filament and observed under fluorescence microscope, the results showed that LX-2 cells were fusiform or polygonal under normal conditions (Figure 3D, Line 1), and that actin was evenly distributed in the cells. And yet after TGFβ-1 treatment (Figure 3D, line 2), the proportion of actin filaments on the cell membrane increased. sEVs isolated from HCC were added to the cell model for 48h, comparing to TGFβ-1 Group (Figure 3E, Lane 2), the three fibrosis markers α-SMA, collagen I and TIMP-1 proteins were significantly down regulated (Figure 3F) in TGFβ-1+sEVs group (Figure 3E, Lanes 3-7). These results show that sEVs isolated from HCC can inhibit the activation of HSC.

3.4 Negative correlation between hsa-miR-122-5p and HSC fibrosis activation

Next, we detected the content of miR-122 in activated LX-2 cells. The results are represented in Figure 4A below. MiR-122 is low expressed in activated LX-2 cells, but its expression level was significantly up-regulated after sEVs isolated from HCC were added. The results were significantly different. The expression level of miR-122 in six hepatoma cell lines was detected by RT-QPCR (Figure 4E). It was found that the expression level of miR-122 in six hepatoma cell lines was higher than that in normal hepatocytes. According to the above results, we selected hepatic cell line WRL68 cells to overexpress miR-122 and liver cancer cell line Huh7 cell. The culture supernatant were collected after starvation for 12 hours to isolated sEVs, and these were co-cultured with activated LX-2 cells. The results showed that
(Figure 4B), compared with TGFβ-1 group (Figure 4B, Lane 2), the fibrosis markers α-SMA, collagen I and TIMP-1 were down regulated in activated LX-2 cells treated with the above cell-derived sEVs (Figure 4B, lanes 3 and 4), indicating that cell-derived sEVs that carried rich miR-122 could also inhibit the activation of LX-2 cells. Then, the activated LX-2 cell was transfected with miR-122 mimics for overexpression treatment. NC group indicated that the LX-2 cell was transfected with miR-122 empty load as a negative control. The results of RT-QPCR are shown in Figure 4C. The miR-122 expression level of LX-2 cell was significantly up-regulated after transfection of miR-122 mimics compared with TGFβ-1 group, and there was no statistical difference between NC group and TGFβ-1 group. Western blot results (Figure 4D) showed that the two fibrosis markers collagen I and TIMP-1 protein were down-regulated (Figure 4D, Lane 3) in the TGFβ-1+miR-122 mimics group compared with TGFβ-1 group (Figure 4D, Lane 2), while the protein level in the NC group did not decrease (Figure 4D, Lane 4). It indicates that up-regulating the expression level of miR-122 can inhibit the fibrosis activation of HSCs.

3.5 AMPK pathway is negatively correlated with HSCs activation

AMPK, a cellular energy sensor, is closely associated with fibrosis process. AMPK deactivation can promote liver fibrosis, and its activation can effectively improve the process of fibrosis. We found that the phosphorylation of AMPK was inhibited and the expression of P-AMPK protein was significantly down regulated in TGFβ-1 group (Figure 5B, Lane 2; Figure 5C, Lane 2) compared with PBS group (Figure 5B, Lane 1; Figure 5C, Lane 1). AMPK was activated after the activated LX-2 cells treated with sEVs from HCC and the expression level of P-AMPK protein was significantly up-regulated (Figure 5B, Lane 3-7). The same results were also obtained in TGFβ-1+mimics group (Figure 5C, Lane 3), while there was no statistical change in NC group (Figure 5C, Lane 4). These results showed that the activation of AMPK was negatively correlated with the fibrotic activity of HSCs.

We activated AMPK with metformin and inhibited AMPK with AMPK inhibitor for further verification. As results shown in figures 5D and 5E, four fibrosis markers α-SMA, collagen I, TIMP-1 and desmin were down-regulated in the TGFβ-1+metformin group (Figure 5D, Lane 2) compared with the TGFβ-1 group (Figure 5D, Lane 3). On the contrary, three fibrosis markers were significantly up-regulated in AMPK inhibitor group (Figure 5E, Lane 3) compared with PBS group (Figure 5E, Lane 1), while there was no statistical difference compared with TGFβ-1 group (Figure 5E, Lane 2). The proteins of α-SMA, collagen I and desmin, three markers of fibrosis, were significantly down-regulated after AMPK inhibitor and sEVs from HCC co-treated in activated LX-2 cells. This indicates that sEVs from HCC affect the activation of HSC at least partly through AMPK pathway, and thus affect the fibrosis process.

3.6 "Jianpi Huoxue Recipe" affects HSC activation by regulating AMPK signaling

Next, we treated LX-2 cells with the traditional Chinese medicine “Jianpi Huoxue recipe” (hereinafter referred to as “drug”) compared with TGFβ-1 group (Figure 6A, Lane 2). The results showed that P-AMPK was significantly up-regulated in the TGFβ-1+drug group (Figure 6A, Lane 3), and that the proteins of collagen I, TIMP-1 and desmin, three fibrosis markers, were significantly down-regulated (Figure 6B, Lane...
These results indicated that “Jianpi Huoxue Recipe” could activate AMPK signaling, thereby inhibiting the activation of LX-2 cells. Then we verified the relationship between “Jianpi Huoxue Recipe” and miR-122. MiR-122 was significantly up-regulated after LX-2 cells were steated with TGFβ-1+drug compared with the TGFβ-1 group, indicating that “Jianpi Huoxue recipe” can activate AMPK and inhibit the activation of HSCs by up regulating miR-122.

4 Discussion

Liver cancer is a very common cancer and the second leading cause of cancer death in China. Hepatocellular carcinoma (HCC) accounts for 90% of liver cancer and is the deadliest form of liver cancer. About 80% of patients with HCC in China have cirrhosis or some degree of liver fibrosis. Liver fibrosis is the only way from chronic liver disease to cirrhosis, and fibrosis caused by mild or transient injury is generally considered to be a reversible process. However, excessive accumulation of extracellular matrix (ECM) will form scar tissue and lead to liver decompensation, eventually resulting to cirrhosis and hepatocellular carcinoma when severe or chronic stimulation continues to progress. The structure and function of liver can be irreversibly damaged, even threatening the life of the patient. MicroRNAs have been found to play a key role in fibrosis and cirrhosis, including in HSC activation and proliferation and extracellular matrix protein production. Hsa-microRNA-122-5p (miR-122) accounts for about 70% of the total microRNAs in liver cells, and it is one of the most abundant microRNAs in the liver, and miR-122 expression is negatively correlated with the development of liver fibrosis in patients with chronic hepatitis. In this study, LX-2 cells were activated under TGFβ-1 stimulation, miR-122 was down-regulated and the proteins of α-SMA, collagen I, desmin and TIMP-1 were up-regulated. RNA sequencing of sEVs isolated from HCC revealed that they carried abundant miR-122. These sEVs co-cultured with activated LX-2 cells can inhibit the proliferation of α-SMA, TIMP-1 and collagen I in hepatic stellate cells. The activated LX-2 cells treated with miR-122 mimics revealed that both α-SMA and collagen I were inhibited, and it indicates that sEVs from HCC could inhibit fibrosis through up-regulation of miR-122 (Fig. 6).

AMPK is a member of the Serine / Threonine (Ser / Thr) kinase family and consists of α, β, and γ subunits as heterotrimer complex. AMPK, distributed in multiple organs including the liver, is a cellular energy sensor that maintains cell energy homeostasis. Experimental or clinical studies have shown that AMPK is closely related to liver fibrosis or cirrhosis, and AMPK activation can effectively improve the process of fibrosis. AMPK activity in patients with advanced fibrosis and cirrhosis is lower compared with that in healthy patients. In this study, we found that phosphorylation of AMPK was low in activated LX-2 cells, but AMPK was activated while activation of HSC was inhibited after activated LX-2 cells treated with sEVs from HCC or miR-122 mimics. Activation of AMPK by metformin can inhibits HSCs activation, whereas AMPK inhibitors promote this process. These results indicated that AMPK activation is negatively correlated with HSC activation, and sEVs from HCC affects fibrosis at least partly through AMPK pathway. Taken together, these data indicate that AMPK can actively inhibit activation of HSC to inhibit liver fibrosis. Warburg-like effect occurs during HSC activation and transdifferentiation into
myofibroblasts, ensuring energy supply by up-regulating glycolysis\textsuperscript{30,31}. AMPK inhibits aerobic glycolysis and HSC activation in a concentration-dependent manner, which is associated with a reduction in lactic acid and ATP production, thereby blocking the normal energy supply of HSC and attenuating the process of fibrosis\textsuperscript{32}. Therefore, the study of these metabolic changes and their related mechanisms is conducive to the discovery of metabolism-related anti-fibrosis therapeutic targets, thereby inhibiting the activation of HSC to prevent the occurrence and development of fibrosis.

In conclusion, this study demonstrates that sEVs from HCC patients can up-regulate the expression level of miR-122 in LX-2 cells (Fig. 7), and inhibit activation of HSCs by regulating AMPK phosphorylation (Fig. 7) to play an anti-fibrosis role (Fig. 7). This finding may explain the decreased degree of fibrosis observed in HCC patients. However, this study only reveals the effect of sEVs on HSC activation through regulation of the AMPK pathway via miRNA-122. The specific mechanism underlying the inhibition of liver fibrosis has not yet been elucidated, and further studies are needed to determine the inhibitory effect of HCC-derived sEVs on liver fibrosis and other factors affecting fibrosis in the microenvironment of HCC patients.

**Declarations**

**Acknowledgement**

We acknowledge the support from Cancer Research Center in USTC.

**7.1 Funding information**

This work was supported by grants from National Natural Science foundation of China (NSFC: 81773112 and 82073186 for Q.F.).

**7.2 Competing Interests**

The authors have no relevant financial or non-financial interests to disclose.

**7.3 Author Contributions**

All authors contributed to the study conception and design. Hongyang Wang and Qiyu Feng conceived the study. Material preparation, data collection and analysis were performed by Ziyu Wang. The first draft of the manuscript was written by Ziyu Wang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

**7.4 Data Availability**

The datasets generated during and/or analysed during the current study are not publicly available due to individual privacy could be compromised but are available from the corresponding author on reasonable request.
7.5 Ethics approval

This study was approved by the Medical Ethics Committee of the First Affiliated Hospital of Anhui University of traditional Chinese Medicine and written informed consent was obtained 2020AH-13.

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Figures
Figure 1

A: Comparison of liver fibrosis indicators between patients with HLD cirrhosis and patients with liver cirrhosis leading to liver cancer. B: Imaging comparison of the degree of liver fibrosis between HLD patients with liver cirrhosis and Patients with liver cirrhosis resulting in liver cancer, the degree of liver fibrosis in patients with liver cancer is significantly inhibited compared to patients with HLD liver fibrosis.
Figure 2

A: Quantitative analysis of miRNA in the sEVs from HCC. B: Quantitative analysis of miR-122 in various organs (from YM500v2 Database). C-F: Extracellular vesicles isolated from HCC were analyzed by
Western blot (C), transmission electron microscope (TEM, D), nanoparticle tracking analysis (NTA, E), and nanoflow cytometry (F).

**Figure 3**

A. The mRNA levels of α-SMA, collagen I and TIMP-1 in LX-2 cells before and after TGFβ-1 treatment were quantitatively analyzed by RT-QPCR. B and C: LX-2 cells lysates were collected after PBS (lane 2) and
TGFβ-1 treatment (lane 1) for 48h, western blot was performed with antibodies against α-SMA, TIMP-1, collagen I and desmin (B), and conduct quantitative analysis (C). The results showed that there were significant differences (p<0.05). D: LX-2 cells treated with PBS, TGFβ-1 and TGFβ-1+sEV for 48h were detected by fluorescent staining using Phalloidin. E and F: LX-2 cells were treated with PBS (lane 1), TGFβ-1 (lane 2) and TGFβ-1+sEV (lane 3) for 48h. Western blot was performed with anti-α-SMA, anti-TIMP-1, anti-collagen I and anti-desmin antibodies (E), and conduct quantitative analysis (F). The results were significantly different (P <0.05).
A: The expression level of miR-122 in LX-2 cells which were treated with PBS, TGFβ-1 or TGFβ-1+sEVs for 48h was quantitatively detected. B: LX-2 cells were treated with PBS (lane 1), TGFβ-1 (lane 2), TGFβ-1+Huh7 cells-derived sEVs (lane 3) and TGFβ-1+WRL68 cells-derived sEVs (lane 4) for 48h, respectively, and western blot was performed with antibodies against α-SMA, TIMP-1 and collagen I. C and D: The activated LX-2 cells treated with miR-122 mimics, and DMEM was replaced with DMEM containing FBS, PS after 6h, and then the overexpression effect was detected after 48h by RT-QPCR (C). Western blot was performed with anti-α-SMA, anti-TIMP-1 and anti-collagen I antibodies (D). e: One normal liver cell line WRL68 cells and six liver cancer cell lines were selected for quantitative detection of miRNA-122.
Figure 5

A: The expression level of P-AMPK in LX-2 cells which were treated with PBS, TGFβ-1 or TGFβ-1+sEVs for 48h was quantitatively detected. B: LX-2 cells were respectively treated with PBS (lane 1), TGFβ-1 (lane 2) and TGFβ-1+sEVs (lane 3-7) for 48h, and western blot was performed with anti-AMPK and anti-P-AMPK antibodies (B). LX-2 cells were treated with PBS (lane 1), TGFβ-1 (lane 2), TGFβ-1+miR-122 mimics (lane 3) and TGFβ-1+NC (lane 4) respectively for 48h, and western blots were performed with anti-AMPK and
anti-P-AMPK antibodies (C). LX-2 cells were respectively treated with PBS (lane 1), TGFβ-1 (lane 2), metformin + TGFβ-1 (lane 3) for 48h, and western blot was performed with anti-α-SMA, anti-TIMP-1, anti-collagen I, anti-desmin, anti-AMPK and anti-P-AMPK antibodies. e: LX-2 cells was respectively treated with PBS (Lane 1), TGFβ-1 (lane 2), AMPK inhibitor (lane 3) and AMPK inhibitor +sEVs for 2h, and western blot was performed with anti-α-SMA, anti-collagen I and anti-desmin antibodies.

Figure 6

A. LX-2 Cell Lysate

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B. LX-2 Cell Lysate

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</table>

C.

- Relative bands: ![Image](image22.png)

Figure 6
A and b: LX-2 cells were respectively treated with PBS (Lane 1), TGFβ-1 (lane 2), AMPK inhibitor (lane 3) and AMPK inhibitor + sEVs for 2h, and western blot was performed with anti-AMPK and anti-P-AMPK antibodies (A), and western blot was performed with anti-collagen I and anti-desmin antibodies (B). C: LX-2 cells were respectively treated with PBS, TGFβ-1 or TGFβ-1 + “jianpi huoxue recipe” for 48h, and the expression level of miR-122 in LX-2 cells was quantitatively detected.

![Figure 7 Diagram depicting sEVs from HCC inhibiting hepatic stellate cells activation via miR-122. sEVs from HCC can up-regulate the level of miR-122 in activated LX-2 cells ( ), and change the energy status of cells by regulating the phosphorylation of AMPK ( ), thereby inhibit the activation of HSCs ( ).]