Overexpression of miRNA-21Promotes the Proliferation and Invasion in Hepatocellular Carcinoma Cells via Suppressing SMAD7

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Abstract
Purpose: This study aimed to explore the molecular mechanism of microRNA-21 and smad family member 7 in hepatocellular carcinoma. Method: A total of 57 participants were divided into control group (healthy participants, n = 10) and hepatocellular carcinoma group (hepatocellular carcinoma patients, n = 37). The expression of microRNA-21 levels were first detected in these two groups. Cell transfection was performed on hepatoma cell lines, followed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Transwell assay to reveal proliferation and invasion ability. Furthermore, the relation between microRNA-21 and smad family member 7 was revealed by luciferase reporter gene and RNA immunoprecipitation assay. Finally, a transplantation tumor model of breast cancer in mice was constructed. Results: The serum indicators including \( \alpha \)-alanine aminotransferase, aspartate aminotransferase, and albumin were differentially expressed between hepatocellular carcinoma group and control group. Compared to the control group, there was a high expression of microRNA-21 in hepatocellular carcinoma group. Low expression of microRNA-21 inhibited the proliferation and invasion of HepG2.2.15 and Huh7-1.3 cells. Luciferase reporter gene and RNA immunoprecipitation assay showed that smad family member 7 was the target gene of microRNA-21. Moreover, mice model analysis showed that microRNA-21 might regulate the growth of the transplanted tumors in mice by targeting smad family member 7. Conclusion: The upregulated microRNA-21 might participate in the proliferation and migration in cells of hepatocellular carcinoma via suppression of smad family member 7. Furthermore, serum indicators such as alanine aminotransferase, aspartate aminotransferase, and albumin might be used as serum diagnostic markers for hepatocellular carcinoma.

Keywords
hepatitis B, SMAD7, miRNA-21, HBV, AFP, ALT

Abbreviations
ALB, albumin; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; mRNA, messenger RNA; miRNA, microRNA; miRNA-21, microRNA-21; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; PCR, polymerase chain reaction; qRT-PCR, quantitative real time-polymerase chain reaction; RIP, RNA immunoprecipitation; SMAD7, smad family member 7; 3'-UT, 3'-untranslated region.

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Introduction

Hepatocellular carcinoma (HCC) is a common tumor located in the human liver. Due to the complexities and reoccurrence after surgical resection, metastasis, and heterogeneity, hepatocellular carcinoma is considered one of the top 3 deadliest tumors in the world. Many therapy strategies including drug intervention, chemoembolization, conservative, and surgical treatment have been applied in the clinical treatment of HCC. However, due to the lack of deep understanding on the pathological mechanism, the clinical therapy effect of HCC is still not ideal.

Molecular alterations are proved to take part in the development of HCC. MicroRNA (miRNA) dysregulation is closely related to the inactivation of hepatocarcinoma tumor suppressor gene and oncogene activation. In patients with HCC, abnormal expression of circulating miRNA-21 can be used as a biomarker for the diagnosis of early liver cancer. A previous study shows that miRNA-21 expression is highly dependent on hepatitis B virus X protein, which can abnormally affect gene expression network, and is a unique viral carcinogenic pathway. Actually, the biological function of miR-21 in disease progression is commonly realized by targeting certain genes such as smad family member 7 (SMAD7). A previous study showed that miRNA-21 can inhibit the expression of SMAD7 via a target sequence in the 3'-untranslated region (3'-UTR) and inhibit proliferation of renal tubular epithelial cells. Meanwhile, the miR-21-SMAD7 regulatory relation is proved to participate in the process of metformin antiangiogenic activity. However, the complex regulatory mechanisms between miRNA-21 and SMAD7 during the progression of HCC is still unclear.

In this study, to reveal the role of miRNA-21 and SMAD7 in HCC, the expression levels of miRNA-21, as well as its role on HepG2.2.15 and Huh7-1.3 cells proliferation and invasion were investigated in patients with HCC. The current study hoped to provide a new understanding of miRNA-21 and SMAD7 in HCC.

Materials and Methods

Participants and Grouping

A total of 57 participants were received by our hospital from October 2016 to October 2017. All these participants were divided into control group (healthy participants, 10 males and 10 females, age: 41.9 ± 10.5) and HCC group (patients with HCC, 23 males and 14 females, age: 48.68 ± 12.7). The preoperative radiotherapy, chemotherapy, and interventional therapy were not accepted for patients with HCC. There was no significant difference in the general data of each group (P > .05). A total of 5 mL peripheral venous blood of each participants was taken on an empty stomach in the morning, followed by EDTA.[Please replace “EDTA” with its expansion, if appropriate.] treatment, centrifugation, and refrigerated at −80°C. The informed consents had been approved by the patients, and all the study protocol were approved by the Ethics Committee of our hospital.

Real Time–Polymerase Chain Reaction Analysis

Total messenger RNA (mRNA) was extracted from the serum according to the kit instructions (Molecular Research Center, Cincinnati, USA). The content of miRNA-21 and SMAD7 were detected by EzOmnics TM real-time polymerase chain reaction (PCR) detection kit (Baimax Biotech Co, Ltd, Jiangsu, China). The miRNA reverse transcription reaction and PCR quantitative reaction were carried out in 1 test tube. Each sample contained 50 ng RNA, 12.5 μL of qPCR mixture, 0.5 μL of miRNA-21, and SMAD7 reverse transcription primers (10 μmol/L). The amplification was performed based on 0.5 μL (10 μmol/L) miRNA-21 and 0.5 μL (10 μmol/L) forward/reverse primers of SMAD7. The amplification procedure was denatured at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 1 minute. Both U6 and β-actin were used as the internal reference of miRNA-21 and SMAD7, respectively. Relative expression of candidate genes was calculated by 2^−ΔΔCt method. All primers in the current study were synthesized by Sangon Biotech Co, Ltd (Shanghai, China). The amplification primer sequences of each gene and its primers were shown in Table 1.

| Name of Primer | Sequences |
|----------------|-----------|
| miRNA-21-F     | CACATCAGCTGGTAGCTTTACGACTG |
| miRNA-21-R     | TGGTGTGGAGTCTGCG |
| SMAD7-F        | CGGAAGTCAAGACCTGTG |
| SMAD7-R        | TGGACGCTAGTTGGTTGGAG |
| β-Actin-F      | TACCTCATGAAGATCACC |
| β-Actin-R      | CCTGCTTGCACGACA |
| U6-F           | AAGCTTCAGAATTGCGT |

Cell Culture and Transfection

Hepatoma cell line (HepG2.2.15 and Huh7, purchased from American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI1640 medium containing 100 μg/mL streptomycin solution, 100 U/mL penicillin, and 10% fetal bovine serum (FBS; Hyclone, Logan, UT) at 37°C (5% CO2). HepG2.2.15 cells were established by transfecting the HBV genome into these cells and screening with G418. The Huh7-1.3 cell line was transfected with pcDNA 3.0-1.3 containing the HBV genomic fragment by FuGENE HD transfection reagent (Promega, Madison, Wisconsin). Then, the HepG2.2.15 and Huh7-1.3 cells were divided into Mock group, miRNA-21 inhibitor group (transfected with miRNA-21 inhibitor) and miRNA-21 NC group (transfected with miRNA-21 negative-control inhibitor), respectively. In addition, miRNA-21 inhibitor and miRNA-21 negative-control inhibitor was obtained.
from Gene Pharma, Shanghai, China. HepG2.2.15 and Huh7-1.3 cells were inoculated into 24-well plates with $6 \times 10^5$ cells/well and cultured overnight in an incubator at 37°C and 5% CO$_2$. Cell transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, California) reagent according to the manufacturer’s instructions.

Another part of HepG2.2.15 cells were divided into four groups including NC mimics + Vector (transfected miRNA-21 negative-control mimics and SMAD7 negative control), mimics + Vector (transfected miRNA-21 mimics and SMAD7 negative), NC mimics + SMAD7 (transfected with miRNA-21 negative-control mimics and pcDNA3.1(+-SMAD7), and mimics + SMAD7 (transfected with miRNA-21 mimics and pcDNA3.1(+-SMAD7). Transfection was performed strictly in accordance with the instructions of the Lipofectamine 2000 kit. The SMAD7 expression vector pcDNA3.1(+-SMAD7 and related control were constructed by Gene Pharma.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay**

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay was used for the detection of cell proliferation. Simply, cells of each transfection group were cultured in monolayer with 0.25% trypsin digestion. A single-cell suspension was prepared from RPMI 1640 medium containing 10% FBS, seeded at a density of $3 \times 10^5$ cells/well in 96-well plates. After 24 hours of culture (50% fusion), RPMI medium containing 10% FBS was added to each well, and 10 μL MTT solution (Roche, Shanghai, China) was added to stain cells for 4 hours. Subsequently, culture medium was discarded and the cells were incubated at room temperature for 10 minutes with 150 μL dimethyl sulfoxide (TEDIA, Fairfield, Ohio). Finally, the absorbance at 490 nm was measured using an Elx-800 enzyme-linked immunosorbent assay to analyze cell proliferation.

**Transwell Assay**

Transwell assay was used to detect cell invasion. After transfection for 48 hours, approximately $1 \times 10^5$ HepG2 and Hep3B cells were added to the Transwell upper chamber with the inner membrane coated with Matrigel. Simultaneously, 500 μL complete medium containing 10% FBS was added to the Transwell lower chamber. Then, the entire Transwell chamber was cultured in a 37°C and 5% CO$_2$ incubator for 24 hours. Moreover, the Transwell lower chamber was placed under an inverted microscope and the experiment was terminated when a small amount of cell penetration was observed. Matrigel and upper chamber cells were wiped with a cotton swab, and the inferior chamber cells were fixed with 4% paraformaldehyde, stained with 0.25% Coomassie blue. Finally, 5 fields of view were randomly selected under the microscope to calculate the number of invading cells in each region.

**Western Blot Assay**

The protein expressions in cells/tissues were detected by Western blot. Briefly, total protein from different groups were extracted by RNA immunoprecipitation analysis (RIPA) lysis buffer (Beyotime Biotechnology Co., Ltd, Shanghai, China). After centrifugation, proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Roche). Then the membrane was blocked with 50 g/L skimmed milk for 12 hours at 4°C and incubated with primary antibodies (SMAD7, 1:1000, ab216428, Abcam, Cambridge, MA, USA; MMP-2, 1:1000, 40994, Cell Signaling Technology, Danvers, MA, USA; TIMP-2, 1:1000, 5738, Cell Signaling Technology) overnight at 4°C. Then, the membrane was incubated with secondary antibody (Horseradish Peroxidase-conjugated IgG, 1:2000, sc-516102, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at 37°C. Protein bands were visualized using enhanced chemiluminescence luminescence reagent (Thermo Fisher Scientific Inc., Waltham, USA). Finally, glyceraldehyde 3-phosphate dehydrogenase was used as an internal reference (1:1000, 5174, Cell Signaling Technology), and the relative gray value was analyzed using the Quantity one scanning software (Bio-Rad, Hercules, CA, USA).

**Luciferase Reporter Gene Assay**

The target sites between SMAD7 and miR-21 were determined by Target Scan, and the mutant and wild sequences were designed according to the predicted results. The miR-21 mutant sequence and the wild sequence fragment were cloned and bound to the Promega vector. HepG2.2.15 and Huh7-1.3 cells were co-transfected with mutated sequences in combination with miRNA-21 mimics or miRNA-21 negative controls, and named as MT + mimics group and MT + NC group, respectively. Meanwhile, the wild sequence was combined with miRNA-21 mimics or miRNA-21 negative control to co-transfect HepG2.2.15 and Huh7-1.3 cells, respectively, and set to WT + mimics group and WT + NC group, respectively. After transfection for 48 hours, luciferase assay was determined by dual-luciferase reporter assay kit (Yuanpinghao Biotechnology Co, Ltd, Beijing, China).

**RNA immunoprecipitation Assay**

RNA immunoprecipitation Assay analysis was performed using the Magna RPTM RNA Binding Protein Immunoprecipitation Kit (Millipore, Bedford, Massachusetts). Simply, cultured HepG2.2.15, Huh7-1.3 cells were harvested and resuspended in RIP lysis buffer (Solarbio, Beijing, China), and then the cell extract was incubated with RIP buffer containing human anti-Ago2 antibody (Millipore) magnetic beads at 4°C overnight (Input and normal IgG was considered as controls). The next day, the magnetic beads were incubated with proteinase K after 3 washes. Total RNA was subsequently isolated from the extract using TRIzol reagent. Finally, the relative enrichment of SMAD7 and miRNA-21 was determined by RT-qPCR analysis.
Table 2. Relationship Between the Expression of Serum MicroRNA-21 and Clinicopathological Features.

| Variable            | Control (n = 20) | HCC (n = 37) |
|---------------------|------------------|--------------|
| Sex (male/female)   | 10/10            | 23/14        |
| Age                 | 41.9 ± 10.5      | 48.8 ± 12.7  |
| ALT (U/L)           | 18.4 ± 10.3      | 48.3 ± 22.1a |
| AST (U/L)           | 16.4 ± 7.7       | 49.9 ± 23.4a |
| ALB (g/L)           | 44.5 ± 2.4       | 24.6 ± 8.1a  |
| TBIL (mmol/L)       | 11.7 ± 2.5       | 42.2 ± 9.3a  |
| PT (s)              | 11.8 ± 0.8       | 15.0 ± 4.9b  |

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALB, serum albumin; HCC, hepatocellular carcinoma; PT, prothrombin time; TBIL, total bilirubin.

Transplantation Tumor Model of Breast Cancer in Mice

A total of 20 BALB/c-nu mice (6 weeks old, purchased from Shanghai Pharmaceutical Research Institute, Shanghai, China) were randomly divided into 4 groups with 5 mice in each group. HepG2.2.15 cells from NC mimics + Vector, mimics + Vector, NC mimics + SMAD7, and mimics + SMAD7 groups were subcutaneously inoculated into the right axillary region of mice to establish xenotransplantation tumor model. The growth of transplanted tumors was examined every 5 days after modeling. The diameter of tumors was measured by vernier caliper. The volume of tumors was calculated by the formula of $V = \frac{1}{2}ab^2$ ($a$: the largest diameter of tumors, $b$: the smallest diameter of tumors). The volume of tumors was measured continuously for 30 days. After the last measurement, rats were sacrificed by neck region-lifting method, and the weights of the tumors were weighed. Tumor growth curve was plotted with tumor volume as ordinate and time as abscissa. All the above experiments were approved by the Animal Ethics Committee of our hospital.

Statistical Analysis

GraphPad Prism version 5.0 software was used for the statistical analysis in the current study. All results were expressed as mean ± standard deviation. The $t$ test was used for comparison between the 2 groups. Meanwhile, one-way analysis of variance (ANOVA) was used for comparison among groups. After ANOVA analysis, the Tukey multiple comparisons test was performed on the comparison between the 2 groups. A value of $P < .05$ was considered to be statistically significant.

Results

Clinicopathological Features

The characteristics of enrolled participants in the control group and HCC group were shown in Table 2. The results showed that there was no significant difference in age and sex distribution between the 2 groups ($P > .05$). Moreover, there were significant differences in $\alpha$-alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), total bilirubin, and prothrombin time between control group and HCC group (all $P < .05$).

Effects of miRNA-21 on Proliferation and Invasion of HepG2.2.15 and Huh7.1.3 cells

Polymerase chain reaction was used to detect the expression of miRNA-21. The results showed that the relative expression of microRNA-21 in HCC was significantly higher than that in control group ($P < .05$), suggesting that microRNA-21 might be involved in the occurrence of HCC (Figure 1A). To determine the trend of elevated miRNA-21 levels, the expression of miRNA-21 in Huh7, Huh7-1.3, HepG2, and HepG2.2.15 were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The results showed that the expression of miRNA-21 in Huh7-1.3 and HepG2.2.15 was significantly higher than that in Huh7 and HepG2, respectively (all $P < .001$, Figure 1B). After transfection of Huh7-1.3 and HepG2.2.15 cells with miRNA-21 for 48 hours, the expression of miRNA-21 in cells was detected by RT-PCR (Figure 1C). The result showed that compared to Mock group and NC inhibitor group, the expression level of miRNA-21 in miRNA-21 inhibitor group was significantly decreased ($P < .05$). Compared to Mock group and NC mimics group, the expression level of miRNA-21 in miRNA-21 mimics group was significantly increased ($P < .05$). This result indicated that miRNA-21 inhibitors could effectively inhibit the expression of miRNA-21 in HepG2.2.15 and Huh7-1.3 cells, and miRNA-21 mimics could effectively increase the expression of miRNA-21 in HepG2.2.15 and Huh7-1.3 cells. Moreover, the MTT assay showed that compared to the Mock group and the NC inhibitor group, the cell proliferation ability of the miRNA-21 inhibitor group was significantly decreased (all $P < .05$, Figure 1D). The proliferation of miRNA-21 mimics group was significantly higher than that of Mock group and NC mimics group (all $P < .05$). Furthermore, Transwell experiments (Figure 3D) showed that the cell degradation of miRNA-21 inhibitor group was significantly lower than that of Mock and NC inhibitor group (all $P < .05$). The cell degradation of miRNA-21 mimics group was significantly higher than that of Mock group and NC mimics group (all $P < .05$, Figure 1E). The results indicated that low expression of miRNA-21 inhibited the proliferation and invasion of human hepatoma Huh7-1.3 and HepG2.2.15 cells, while the overexpression of miRNA-21 had the opposite effect.

Smad Family Member 7 Was the Target Gene of miRNA-21

The expression level of SMAD7 mRNA in HCC was detected by qRT-PCR assay. Compared to the control group, the relative expression of SMAD7 mRNA in the HCC group was significantly decreased ($P < .05$, Figure 2A). In HCC group, there was a significant negative correlation between SMAD7 and miRNA-21 expression ($r = -0.7067$, $P < .001$, Figure 2B). Moreover, the expression of SMAD7 mRNA in Huh7, Huh7-1.3, HepG2,
and HepG2.2.15 was detected by qRT-PCR (Figure 2C). The results showed that the expression of SMAD7 in Huh7-1.3 and HepG2.2.15 was significantly lower than that in Huh7 and HepG2 (P < .01). The level of SMAD7 was detected after transfection of miRNA-21 mimics and inhibitor (Figure 2D). The results showed that the expression of SMAD7 protein was significantly decreased and increased in the miRNA-21 mimics group and miRNA-21 inhibitor group, respectively (all P < .01). Furthermore, Target Scan analysis showed that the binding site of SMAD7 to miRNA-21 was the 3′-UTR (Figure 2E). The luciferase reporter gene assay (Figure 2F) showed that there was no significant change in the intensity of luciferase activity in cells transfected with SMAD7-Mt and miRNA-21 mimics (P > .05). However, the intensity of luciferase activity decreased significantly in the cells co-transfected with SMAD7-Wt and miRNA-21 mimics (all P < .001). In addition, the results of RIP analysis (Figure 2G) showed that compared to anti-IgG group, the expression of SMAD7 and miRNA-21 was significantly increased in anti-Ago2 group (all P < .05). These results indicated that SMAD7 was a target gene of miRNA-21.

The Effect of miRNA-21-SMAD7 Regulatory Relation in HepG2.2.15 Cells

In order to determine whether miRNA-21 promoted proliferation and invasion in cancer cells mediated by SMAD7, the
HepG2.2.15 cells were divided into 4 groups including NC mimics + Vector, mimics + Vector, NC mimics + SMAD7, and mimics + SMAD7. Western blot results showed that the expression of SMAD7 in HepG2.2.15 cells was upregulated after transfection (all \( P < .05 \)). Meanwhile, the proliferation and invasion of HepG2.2.15 cells mediated by miRNA-21 were reversed in mimics + SMAD7 group (Figure 3B and C). These results suggested that miRNA-21-SMAD7 regulatory relation could regulate the proliferation and invasion of HepG2.2.15 cells.

**Figure 2.** SMAD7 was the target gene of miRNA-21. A, Quantitative real time-polymerase chain reaction was used to detect the expression level of SMAD7 mRNA HCC. B, Quantitative real time-polymerase chain reaction was used to detect the expression level of SMAD7 mRNA in liver cancer groups; \( t \)-test was used to compare the two groups; *indicated \( P < .01 \). (C) Correlation analysis between SMAD7 and miRNA-21 expression. (D) The SMAD7 levels after transfection of miRNA-21 mimics and inhibitor. (E) Target scan predicted target sites for binding of SMAD7 and miRNA-21. (F) The result of dual-luciferase reporter activity assay; \( *P < .001 \) when compared with MT + mimics group, MT + NC and WT + NC group. (G) The result of RIP analysis. One-way analysis (ANOVA) was used among the groups; Tukey’s multiple comparisons test was used for 2 groups comparison after AVOVA analysis. \( *P < .05 \) when compared with anti-IgG group. RIP indicates RNA immunoprecipitation.
The Effect of miRNA-21 on the Growth of Mice Transplanted Tumors via Regulating SMAD7

Compared with NC mimics + Vector group, the volume and weight of transplanted tumors in mimics + Vector group and NC mimics + SMAD7 group were significantly increased and decreased, respectively (all $P < .01$). The treatment in mimics + SMAD7 group reversed the effect of miRNA-21 on the volume and weight of transplanted tumors (Figure 4A-C). Western blot was used to detect the expression of MMP-2 and TIMP-2 protein in tumors (Figure 4D). The results showed that compared to NC mimics + Vector group, the expression of MMP-2 and TIMP-2 protein was significantly increased and decreased in mimics + Vector group and NC mimics + SMAD7 group, respectively (all $P < .01$). The treatment in mimics + SMAD7 reversed the expression of MMP-2 and TIMP-2 mediated by miRNA-21. These results suggested that miRNA21 might regulate the growth of transplanted tumors in mice by targeting SMAD7.

Discussion

MiRNA-21 is considered to be an onco-microRNA that inhibits the action of several tumor suppressor genes and promotes tumor cell growth, invasion, and metastasis. It is worth mentioning that serum miRNA-21 levels, a hallmark of necrotizing inflammatory activity, are upregulated in tumor tissues of patients with HCC malignancies. Moreover, the expression of miRNA-21 increased significantly with the progression of cirrhosis, increased number of lesions, and enlarged tumor volume. After transfer of HepG2 cells into a recombinant plasmid containing anti-miRNA-21, miRNA-21 expression was significantly downregulated, inhibiting cell proliferation, and promoting apoptosis, and this finding was reported by Guo et al. Interestingly, the biological function of miR-21 in disease progression is commonly realized by targeting certain genes such as SMAD7. As an inhibitory Sma-Mad family member, SMAD7 is a negative regulator of signaling by the transforming growth factor-β superfamily protein. Han et al showed that miRNA-21-induced breast cancer cell invasion and migration by suppressing SMAD7. As a target site of miRNA-21, SMAD7 play an important role in the progression of liver fibrosis. Based on the animal model, a previous study shows that miRNA-21 contribute to the myocardial fibrosis through down-regulating Smad7. Ning et al indicated that miRNA-21 mediates angiotensin II-induced liver fibrosis by targeting SMAD7. In this study, the luciferase reporter gene experiment and RIP analysis confirmed that SMAD7 was the target gene of miRNA-21. The relative expression of miRNA-21 increased with the progression HCC and decreased expression of miRNA-21 significantly inhibited the proliferation and migration of human hepatoma HepG2 and Hep3B cells. Importantly, the mice model analysis showed that...
Figure 4. The miRNA-21 contributed to the growth of mice transplanted tumors via regulating SMAD7. A, HepG2.2.15 transplanted tumor model constructed in mice. B, The weight of transplanted tumors in mice. C, Growth curve of transplanted tumors in mice. D, Western blot was used to detect the expression of MMP-2 and TIMP-2 proteins. One-way analysis of variance (ANOVA) was used among the groups; Tukey multiple comparisons test was used for 2 groups comparison after ANOVA. **P < .01 when compared to negative control (NC) mimics + vector group.
miRNA-21 take part in the growth of transplanted tumors in mice by targeting SMAD7. Thus, we speculated that miRNA-21–SMAD7 regulatory relation contributes to the proliferation and migration in cells of HCC.

The serum level of ALT is often used as a major criterion to initiate treatment in the absence of cirrhosis, though patients with lower ALT may not be free from future risk of HCC. Actual ALT levels are proved to be consistent with hepatitis activity. There is a close association between high serum ALT and more rapid recurrence in patients with HCV-associated liver cirrhosis and HCC. Moreover, the expression level of serum AST is associated with the development of liver cancer. Witjes et al showed that quantitative AST was a strong predictor for survival after HCC detection in chronic HBV patients. A previous study indicates that the AST-to-platelet ratio index can be used to predict long-term survival in patients with HCC following non-transplant therapies. Furthermore, ALB is proved to be differentially expressed between tumor cells and non-tumor cells of patients with HCC. A previous study proves that ALB is a predictor for patients with HCC. In this study, compared to control group, the expression of ALT, AST, and ALB were significantly different than those in HCC group. Thus, we hypothesized that serum ALT, AST, and ALB might be used as serum diagnostic markers for HCC. However, there were some limitations in the current study such as small samples size and lack of clinical verification. Thus, a further study based on large sample size is needed to confirm all speculation in this study.

Conclusion
In conclusion, miRNA-21–SMAD7 regulatory relation contributes to the proliferation and migration in cells of HCC. Moreover, serum ALT, AST, and ALB might be used as serum diagnostic markers for HCC.

Authors' Note
Yan Wang and Ping Zhang contributed equally to this work. This study was conducted after obtaining local ethical committee approval of Qingdao Sixth People’s Hospital and written informed consent from the patients (approval number: qddlrmyy2018006) Written/verbal consent was obtained from the patients.

Declaration of Conflicting Interests
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