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Diagnostic value of LncRNA-MEG3 as a serum biomarker in patients with hepatitis B complicated with liver fibrosis

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Abstract. – OBJECTIVE: The aim of this work was to explore whether lncRNA-MEG3 could serve as a serum biomarker for diagnosing chronic hepatitis B (CHB) and improve the early diagnostic and treatment efficacies.

PATIENTS AND METHODS: Serum level of lncRNA-MEG3 in CHB patients and healthy controls was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Subsequently, CHB patients were divided into HBeAg-positive group and HBeAg-negative group based on the infection condition of hepatitis B virus. Correlation analyses were conducted to investigate the relationship between lncRNA-MEG3 level and HBV. Correlation between serum level of lncRNA-MEG3 and liver fibrosis was also analyzed. Survival analyses were performed to elucidate whether lncRNA-MEG3 could be served as a serum biomarker for diagnosing CHB combined with liver fibrosis. Expression levels of lncRNA-MEG3, α-SMA, and COL1A1 in mouse hepatic stellate cells (HSCs) were detected.

RESULTS: Serum level of lncRNA-MEG3 was lower in CHB patients compared with that of healthy controls, which was negatively correlated to liver fibrotic degree. Survival analyses showed that serum level of lncRNA-MEG3 exerts significant diagnostic value on the liver fibrotic degree in CHB patients. ROC (receiver operating curve) results showed the AUC was 0.9395, the sensitivity was 100%, and the specificity was 78.13% in comparing the serum level of lncRNA-MEG3 between CHB patients with liver fibrosis and healthy controls. Further analyses showed that serum level of lncRNA-MEG3 was negatively correlated to levels of α-SMA and COL1A1. However, no significant correlations were found among the serum level of lncRNA-MEG3, HBV, hepatic inflammation and liver function. *In vitro* experiments showed that lncRNA-MEG3 expression was gradually decreased, whereas expression levels of α-SMA and COL1A1 in HSCs were gradually increased in a time-dependent manner.

CONCLUSIONS: Serum level of lncRNA-MEG3 is lowly expressed in CHB patients, which is negatively correlated to the liver fibrotic degree. LncRNA-MEG3 may serve as a diagnostic biomarker for CHB.

Key Words

HBV, LncRNA-MEG3, Liver fibrosis, Liver function.

Introduction

Hepatitis B is a serious infectious disease caused by infection of hepatitis B virus (HBV). Globally, about 350 million people are infected with HBV. Due to repeated and persistent inflammation, patients with chronic hepatitis B (CHB) have a greater risk for developing cirrhosis, liver decompensation and/or hepatocellular carcinoma[1]. At present, liver biopsy is commonly applied to assess the severity of liver damage caused by HBV and/or to monitor the progress of CHB[2]. However, liver biopsy is an invasive examination. Hence, searching for serum biomarkers for CHB is of great significance to improve early diagnosis and early treatment.

Long non-coding RNA (lncRNA) is a type of non-coding RNA with a transcript of more than 200 nucleotides in length. It was originally considered as a by-product of RNA polymerase II transcription without any biological functions[3,4]. However, with the in-depth researches, we found that lncRNA exerts extremely complex and important biological functions, including gene reg-

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ulation[5], cell cycle checkpoints[6], and cell migration[7]. It also plays an important role in the occurrence and progression of various diseases[8,9]. It is worth noting that lncRNA could not be degraded by nucleases, which is stably present in the circulatory system and is easily detected in serum[10]. Therefore, lncRNA has high diagnostic value in different diseases, especially in tumors[11]. For example, lncRNA H19 is highly expressed in peripheral blood of gastric cancer patients than that of healthy controls, which is closely related to the survival rate[12]. MALAT1 was previously identified as a prognostic marker for patients with non-small cell lung cancer (NSCLC), particularly in patients with early-stage lung adenocarcinoma[13]. Also, HULC is an upregulated lncRNA found in hepatocytes and can be routinely detected in blood sample by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)[14]. However, few studies have been reported on the relationship between lncRNA and liver fibrosis.

LncRNA-MEG3 is a tumor-suppressor gene mainly regulated by epigenetics. It is located on human chromosome 14q32.2 and widely expressed in normal tissues[15]. In many tumors, lncRNA-MEG3 is downregulated in cancerous tissues compared to that of adjacent tissues[16,17]. In this research, we found that lncRNA-MEG3 is lowly expressed in CHB patients. We aim to explore whether lncRNA-MEG3 could be served as a potential biomarker for diagnosing CHB.

Patients and Methods

Sample Collection

100 CHB patients and 80 healthy controls without a history of liver diseases and hepatitis virus infection in Muping District Hospital of traditional Chinese Medicine from 2015 year to 2017 year were collected. Serum samples and tissues samples of enrolled subjects were harvested. Basic characteristics of enrolled subjects were recorded and they were all followed up. The informed consent was obtained from all the participating. This study was approved by the Muping District Hospital of Traditional Chinese Medicine Ethics Committee.

Liver Histological Analyses

Liver biopsy was performed using the Menghini needle for collecting 2-cm liver tissues. Samples were fixed and paraffin embedded for HE (hematoxylin-eosin) staining (Boster, Wuhan, China). Liver biopsy examination was pathologically confirmed. Liver histologic activity index (HAI) and liver fibrotic degree were evaluated using ISHAK method (F0=no fibrosis, F6= cirrhosis).

Serum Sample Collection

The blood sample was harvested when performing the liver biopsy. After centrifugation at 3400 g/min for 7 min, the supernatant was collected and centrifuged at 1200 g/min, 4°C for 10 min. The serum sample was finally harvested and preserved at -80°C.

HBV Detection

HBV DNA replication level was accessed using Artus HBV QS-RGQ kit (Qiagen, Hilden, Germany). HBsAg, HBeAg, anti-HBsAg, anti-HBeAg, and anti-HBc were detected using Modular E170 Immunoanalyzer.

Cell Culture

Primary mouse hepatic stellate cells (HSCs) were extracted as previously described[18]. HSCs were cultured in DMEM (Dulbecco's Modified Eagle's Medium) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA). Cells were incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed until 85% of cell density.

RNA Extraction and qRT-PCR

Total RNA in treated cells was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using a spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). Primers used in the study were as follows: LncRNA-MEG3: forward, 5'-CCTGCTGCCCATCTACACCTC-3', reverse, 5'-CCTCTTCATCCTTTGCCATCCTGG-3'; GAPDH (glyceraldehyde 3-phosphate dehydrogenase): forward, 5'-CTGGGCTACACTGAGCACC-3', reverse, 5'-AAGTGGTCGTTGAGGGCAATG-3'.

Western Blot

Cells and peripheral blood samples were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bi-

cinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Millipore, Billerica, MA, USA). After incubation with primary and secondary antibodies (Cell Signaling Technology, Danvers, MA, USA), immunoreactive bands were exposed by enhanced chemiluminescence method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Measurement data were compared using the Mann Whitney or Kruskal Wallis test. AUC (area under the curve), sensitivity and specificity under ROC (receiver operating curve) were calculated. The correlation coefficient (r) was calculated using the Spearman rank correlation. The $p<0.05$ considered the difference was statistically significant.

Results

Serum Level of LncRNA-MEG3 Was Lower in CHB Patients and Was Correlated to Liver Fibrosis

We first recorded the basic characteristics of enrolled CHB patients and healthy controls (Table I). No significant differences in age and sex were found between the two groups ($p>0.05$). QRT-PCR data showed that the serum level of lncRNA-MEG3 was lower in CHB patients compared with that of healthy controls (Figure 1A). The correlation between lncRNA-MEG3 expression and liver fibrotic degree was further analyzed. Enrolled CHB patients were assigned into low-level fibrosis group (F0-F1), middle-level fibrosis group (F2-F4), and high-level fibrosis group (F5-F6). Our results found the highest serum level of lncRNA-MEG3 in low-level fibrosis group, indicating that lncRNA-MEG3 expression was negatively correlated to the liver fibrotic degree (Figure 1B).

Table I. Basic characteristics of CHB patients (n=100) and healthy controls (n=80).

Patient characteristics		
Parameter	**CHB patients**	**Healthy subjects**
Epidemiology		
Gender, m/f (%)	51/49 (51.0/49.0)	42/38 (52.5/47.5)
Age, years, median (range)	45.5 (31-59)	46.8 (30-60)
Virology		
HBe antigen-positive, n (%)	48 (48.0)	
HBe antigen-negative, n (%)	52 (52.0)	
ALT		
Elevated ALTa	72 (72.0)	
Normal ALT	28 (28.0)	
Fibrosis stage (Ishak)		
F0, n (%)	9 (9.0)	
F1, n (%)	10 (10.0)	
F2, n (%)	19 (19.0)	
F3, n (%)	18 (18.0)	
F4, n (%)	23 (23.0)	
F5, n (%)	11 (11.0)	
F6, n (%)	10 (10.0)	
HAI		
2, n (%)	7 (7.0)	
3, n (%)	14 (14.0)	
4, n (%)	11 (11.0)	
5, n (%)	16 (16.0)	
6, n (%)	13 (13.0)	
7, n (%)	9 (9.0)	
8, n (%)	5 (5.0)	
9, n (%)	15 (15.0)	
≥11, n (%)	10 (10.0)	
a>40U/L		

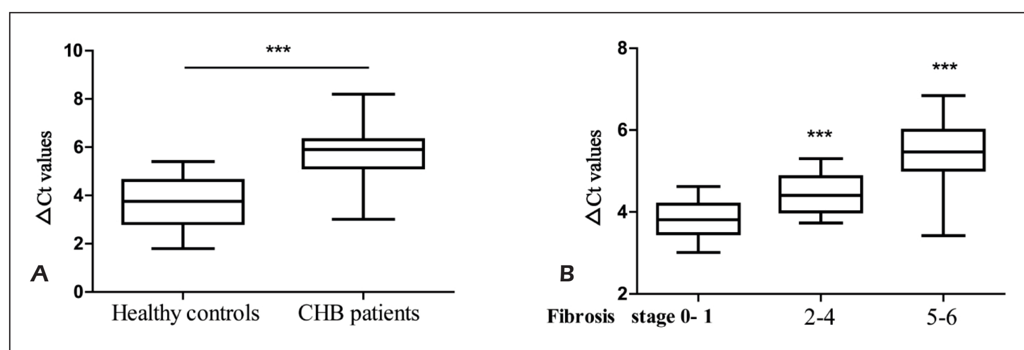

Figure 1. Serum level of lncRNA-MEG3 was lower in CHB patients and was correlated to liver fibrosis. **A**, Serum level of lncRNA-MEG3 in CHB patients and healthy controls. **B**, Serum level of lncRNA-MEG3 in low-level fibrosis group (F0-F1), middle-level fibrosis group (F2-F4), and high-level fibrosis group (F5-F6).

Diagnostic Value of Serum LncRNA-MEG3 in Liver Fibrosis of CHB Patients

To verify whether serum lncRNA-MEG3 could be served as a diagnostic biomarker for CHB patients combined with liver fibrosis, survival analyses were conducted. By analyzing CHB patients and healthy controls, the data showed that AUC was 0.8844 and cut-off value was 5.112 (Figure 2A). The AUC and cut-off value between low-level fibrosis group and control group were 0.5237 and 2.988, respectively (Figure 2B). The AUC and cut-off value between middle-level fibrosis group and control group were 0.7085 and 3.812, respectively (Figure 2C), which were 0.9395 and 4.689 between high-level fibrosis group and control group (Figure 2D).

Subsequently, we speculated whether serum level of lncRNA-MEG3 could distinguish from CHB patients with different liver fibrotic degrees. The AUC and cut-off value between low-level fibrosis group and middle-level fibrosis group were 0.8281 and 3.963 (Figure 2E). The AUC and cut-off value between high-level fibrosis group and low-level fibrosis group were 0.8857 and 4.818 (Figure 2F). On the contrary, the AUC and cut-off value between high-level fibrosis group and middle-level fibrosis group were 0.7861 and 5.312 (Figure 2G). The above data all detected that serum lncRNA-MEG3 exerts diagnostic value in CHB patients combined with liver fibrosis.

Serum LncRNA-MEG3 and HBV DNA Replication

CHB patients were divided into HBeAg-positive group and HBeAg-negative group based on the infection condition of HBV. We did not observe a significant difference in serum level of lncRNA-MEG3 between the two groups (Figure 3A). Correlation analyses further suggested that serum level of lncRNA-MEG3 does not correlate to HBV DNA replication level (r^2=0.01383, *p*=0.5216, Figure 3B).

Serum LncRNA-MEG3 and Liver Injury Markers

Since lncRNA-MEG3 was lowly expressed in CHB patients, we speculated whether serum level of lncRNA-MEG3 was related to liver inflammation and injury. The data showed no obvious correlation between HAI and serum level of lncRNA-MEG3 in CHB patients (Figure 3C). Similarly, we did not observe a remarkable correlation between ALT (alanine aminotransferase) level and serum level of lncRNA-MEG3 in CHB patients (Figure 3D).

Serum LncRNA-MEG3 and Liver Function

No remarkable correlation was found between serum level of lncRNA-MEG3 and international normalized ratio (INR) (r^2=0.03276, *p*=0.3215, Figure 3E). Levels of bilirubin and albumin in CHB patients were detected. We did not observe correlation between serum level of lncRNA-MEG3 and bilirubin level (r^2=0.09244, *p*=0.0907, Figure 3F) or albumin level (r^2=0.04173, *p*=0.2621, Figure 3G).

Serum LncRNA-MEG3 and Liver Fibrosis

α-SMA and COL1A1 are the indicators of liver fibrosis. Our results found that the serum level of lncRNA-MEG3 was negatively correlated to α-SMA level (r^2=0.4478, $p<0.001$, Figure 3H) and COL1A1 level (r^2=0.1687, *p*=0.0195, Figure 3I). HSCs are involved in the occurrence and progression of liver fibrosis. In the present work, primary mouse HSCs were used for *in vitro* experiments.

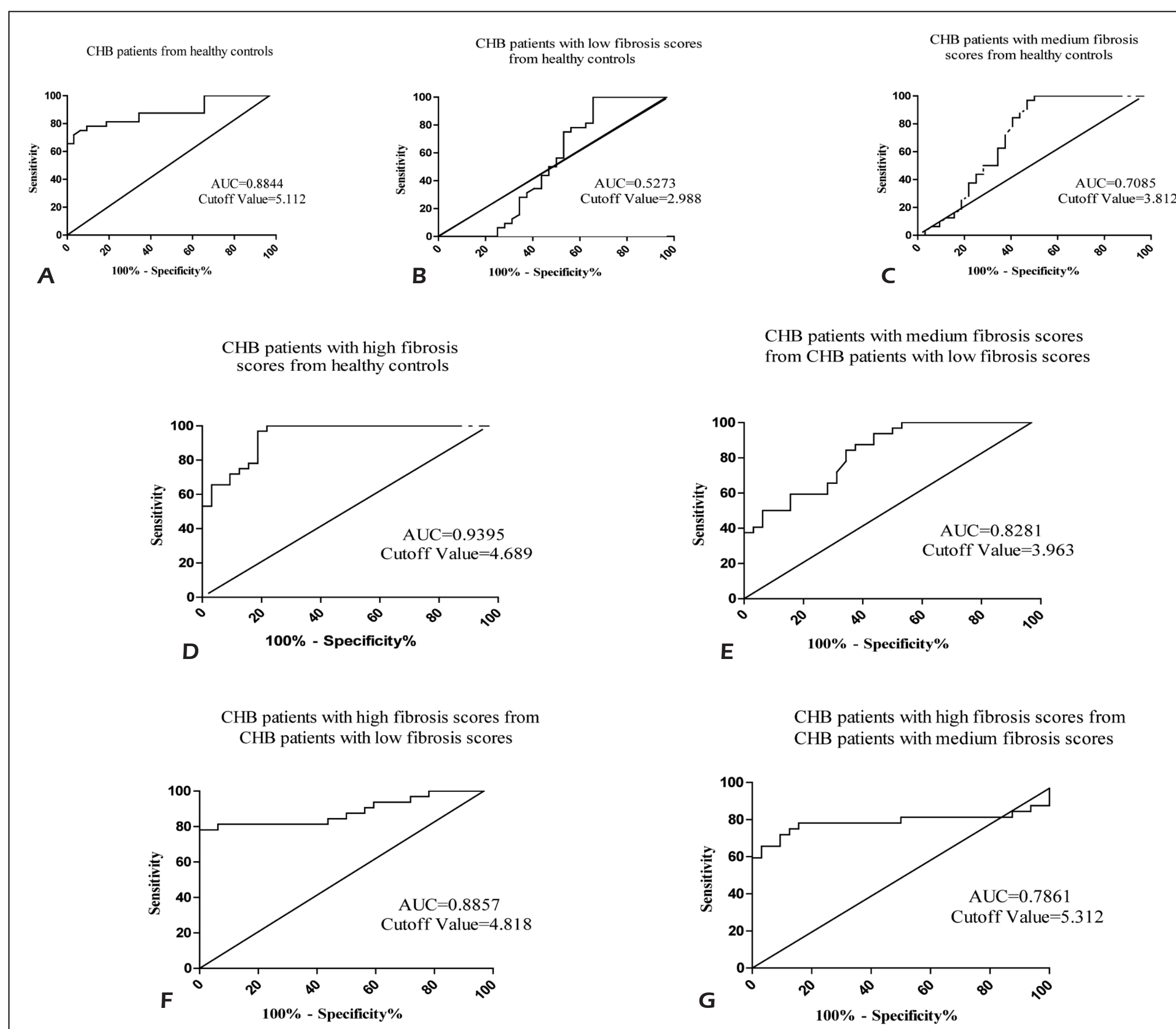

Figure 2. Correlation between serum level of lncRNA-MEG3 and liver fibrosis. ROC curve between **A**, CHB group and control group; **B**, low-level fibrosis group and control group; **C**, middle-level fibrosis group and control group; **D**, high-level fibrosis group and control group; (**E**, middle-level fibrosis group and low-level fibrosis group; F, high-level fibrosis group and low-level fibrosis group; **G**, high-level fibrosis group and middle-level fibrosis group.

Western blot results found that protein levels of α-SMA and COL1A1 in HSCs were gradually upregulated (Figure 4A and 4B). The mRNA levels of α-SMA and COL1A1 were elevated as well (Figure 4C). However, the lncRNA-MEG3 level was downregulated in HSCs (Figure 4D).

Discussion

Tissue biopsy is commonly used in disease diagnosis. However, a tissue biopsy is an invasive examination that is difficult to operate. In recent years, studies have focused on serum biomarker due to the simple availability and less trauma of serum collection. Among them, microRNAs and lncRNAs have been well recognized. Serum level of miR-155 has been found to be closely related to the development of breast cancer[19]. In HBV-positive patients, the serum level of miR-122 can be used as a biomarker for disease progression[20].

In addition to miRNAs, many studies have demonstrated that lncRNA can also function as a biomarker in a variety of diseases. For example, lncRNA-uc003wbd and lncRNA-AF085935 are differentially expressed in patients with hepatocellular carcinoma and are served as potential biomarkers[21]. CCAT1 and HOTAIR are highly

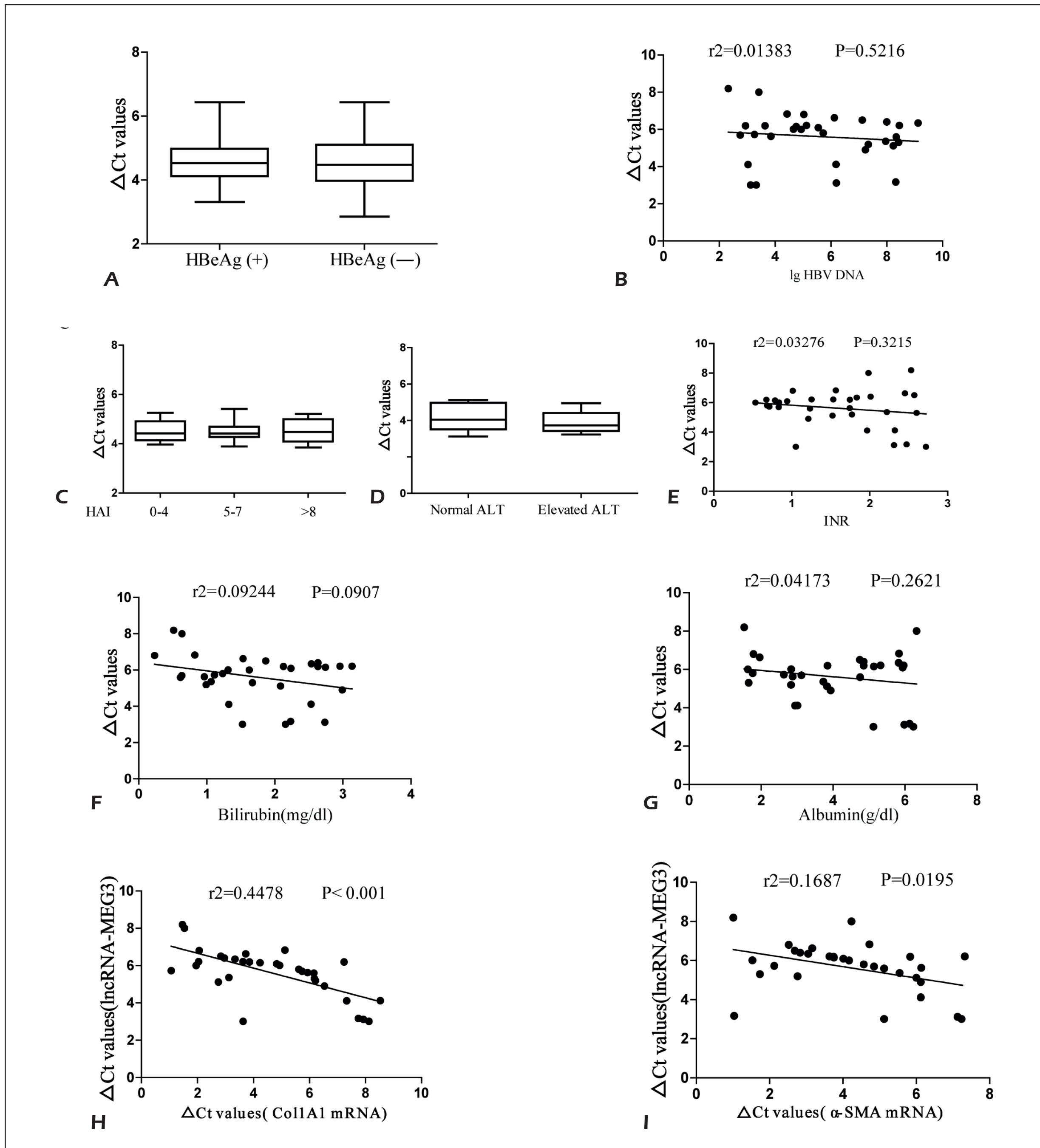

Figure 3. Serum lncRNA-MEG3 and liver indicators. **A**, Correlation between lncRNA-MEG3 and HBeAg. **B**, Correlation between lncRNA-MEG3 and HBV DNA replication level. **C**, Correlation between lncRNA-MEG3 and HAI. **D**, Correlation between lncRNA-MEG3 and ALT level. **E**, Correlation between lncRNA-MEG3 and INR. **F**, Correlation between lncRNA-MEG3 and bilirubin level. **G**, Correlation between lncRNA-MEG3 and albumin level. **H**, Correlation between lncRNA-MEG3 and α-SMA level. **I**, Correlation between lncRNA-MEG3 and COL1A1 level.

expressed in serum samples of patients with colorectal cancer. Combination of CCAT1 and HOTAIR improves the diagnostic specificity and sensitivity in colorectal cancer than those of individual detection[22]. In patients with esophageal squamous cell carcinoma, POU3F3, HNF1A-AS1, and SPRY4-IT1 are highly expressed. Among them, POU3F3 exerts the highest diagnostic value (AUC=0.842, $p<0.001$, sensitivity = 72.8%, specificity = 89.4%)[23].

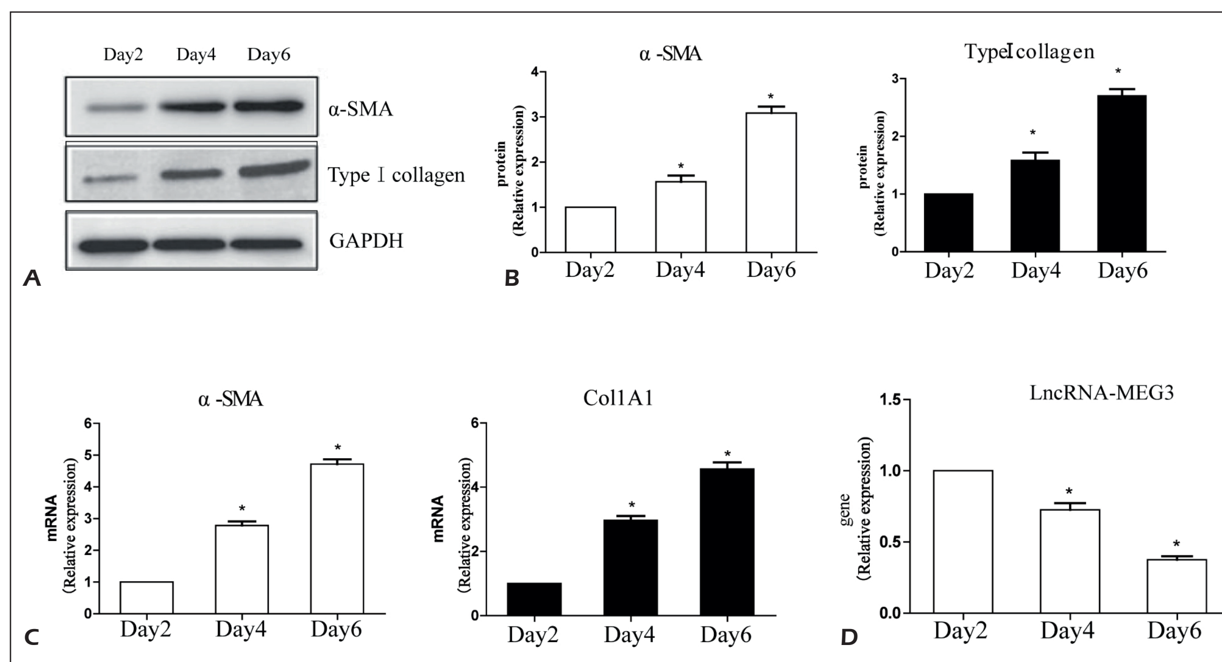

Figure 4. Serum lncRNA-MEG3 and liver fibrosis. **A-B**, Western blot results of α-SMA and COL1A1 in HSCs. **C**, QRT-PCR result of α-SMA in HSCs. **D**, Expression level of lncRNA-MEG3 in HSCs.

We found that serum level of lncRNA-MEG3 was lower in CHB patients compared with that of healthy controls, which was gradually decreased accompanied by the elevated degree of liver fibrosis. LncRNA-MEG3 has been shown to perform a variety of biological functions in different diseases. In gastric cancer, lncRNA-MEG3 can act as a miRNA "sponge" to regulate cell proliferation, migration, invasion, etc.[24]. Liu et al[25] found that in gallbladder carcinoma, lncRNA-MEG3 expression was remarkably higher than that of adjacent normal tissues. Ectopic overexpression of MEG3 can effectively inhibit the growth of gallbladder cancer cells. In liver cancer, the expression level of lncRNA-MEG3 was lower in hepatocarcinoma tissues compared with that of adjacent normal tissues, which was closely related to tumor size and Edmondson classification. Overexpression of MEG3 induces apoptosis and inhibits proliferation of liver cells[26].

In the present investigation, we explored the relationship between lncRNA-MEG3 and indicators of liver injury, liver fibrosis, liver function, and hepatitis virus replication. Serum level of lncRNA-MEG3 was negatively correlated with the liver fibrosis, but not correlated with hepatitis virus replication and liver function. Meanwhile, the serum level of lncRNA-MEG3 could not reflect the liver inflammation degree *via* detecting ALT level and HAI score. It is suggested that the serum level of lncRNA-MEG3 is correlated to CHB patients combined with liver fibrosis. Furthermore, expressions of α-SMA and COL1A1 in HSCs were gradually increased in a time-dependent manner, whereas lncRNA-MEG3 expression was downregulated. *In vitro* results further verified that lncRNA-MEG3 expression is correlated to the liver fibrotic degree of CHB patients.

Conclusions

We observed that the serum level of lncRNA-MEG3 is lowly expressed in CHB patients, which is negatively correlated to the liver fibrotic degree. LncRNA-MEG3 may serve as a diagnostic biomarker for CHB.

Competing interests

The authors declare that they have no competing interests.

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