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ORIGINAL ARTICLE

Long Non-Coding RNA IGF2AS in Serum may be a Biomarker for Diagnosis of Hepatitis B Virus-Related Hepatocellular Carcinoma

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SUMMARY

Background: **The current study aims to evaluate whether serum IGF2AS can be used as a marker for early diagnosis of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC).**
Methods: **First, the expression of IGF2AS in serum and cancer tissues of HBV-related HCC patients was detected using RT-PCR. Then, according to the Barcelona Staging Method (BCLC), HBV-related HCC patients were further divided into group A, B, C, and D. The serum IGF2AS levels of different groups were further detected. Additionally, HBV-related HCC patients were divided into alpha-fetoprotein (AFP) negative cases and AFP positive cases. The serum level of IGF2AS was also evaluated.**
Results: **Our data showed that the level of IGF2AS in liver cancer tissues of HBV-related HCC was significantly higher than that in the adjacent non-cancerous tissues. Meanwhile, the serum level of IGF2AS in the HBV-related HCC group was higher than that in healthy control group, chronic HBV group, and HBV-related cirrhosis. Moreover, serum IGF2AS in patients with HBV-related HCC gradually increased in groups A, B, C, and D of HBV-related HCC patients according to BCLC classification. Further analysis showed that serum IGF2AS levels were increased in both AFP-negative HCC patients and AFP-positive HCC patients compared to that in HBV-related cirrhosis patients, suggesting that serum IGF2AS can be used in early identification and diagnosis of HBV-related HCC irrespective of AFP levels.**
Conclusions: **In summary, for the first time, the current study demonstrated that serum IGF2AS may be a potential biomarker for the diagnosis of HBV-related HCC.**
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KEY WORDS

long non-coding RNA, IGF2AS, hepatitis B virus, hepatocellular carcinoma

INTRODUCTION

Primary hepatocellular carcinoma (HCC) is the fifth most common malignant tumor in the world [1]. It is also one of the tumors with the fastest rising incidence and the highest mortality in recent years [2]. At present, surgical resection is one of the most commonly used clinical treatment methods, but due to the lack of early diagnostic measures, most patients miss the best opportunity for surgery, resulting in poor prognosis [3,4]. It is reported that 10.25% of chronic hepatitis B virus (HBV)

infections develop into HCC in their lifetime, and HBV infection is the main cause of HCC [5,6]. Therefore, early diagnosis and detection is important prevent development from HBV infection to final HCC.

At present, the diagnosis of HCC mainly depends on non-invasive examination and pathological examination [7]. For the differential diagnosis between cirrhosis and HCC, the commonly used clinical examinations include the detection of serum alpha fetoprotein (AFP) and imaging examinations, including computed tomography (CT) and magnetic resonance imaging (MRI) [8,9]. However, for patients with early HCC, detection by examination is difficult and the rate of misdiagnosis is high since 30% of the patients are clinically negative for AFP [10]. Therefore, it is very important to find a detection method with strong predictive ability, which can identify early HCC patients and provide timely targeted treatment.

In recent years, some new diagnostic indicators have been found, such as circulating nucleic acid (cfCNA) [11,12]. cfCNA is a kind of RNA and DNA that exists in the circulatory system, including blood, urine, and cerebrospinal fluid [11,13]. Currently, studies have confirmed that cfCNA are closely related to the occurrence and development of diseases [14]. Detection of cfCNA can achieve the purpose of diagnosing diseases [15]. Long noncoding RNA (lncRNA) exists widely in the body and can play a role in transcription, translation, and at other levels [16]. Panzitt et al. first detected the expression of lncRNA HULC in serum of patients with gastric cancer [16]. However, up to now, there are few reports on whether serum lncRNA can be used as an early diagnostic indicator for HCC patients.

Insulin-like growth factor 2 antisense RNA (IGF2AS) is shown to be significantly upregulated in the tissues and cells of HCC [17]. However, whether IGF2AS in serum can be used as a marker for early diagnosis of HBV-related HCC has not been reported.

MATERIALS AND METHODS

Patient samples

Sixty patients with chronic hepatitis B virus (chronic HBV group), 70 patients with hepatitis B cirrhosis (HBV-related cirrhosis), 34 patients with hepatitis B cirrhosis complicated with primary HCC (HBV-related HCC group) were selected from January 2017 to October 2017 in the infectious outpatient and in-patient department of Jinan Infectious Diseases Hospital. All patients were aged between 21 and 68 years. Inclusion criteria: Patients with chronic hepatitis B virus and hepatitis B cirrhosis should meet the following diagnostic criteria [18]. Patients with hepatitis B cirrhosis should conform to the imaging, hematological or biochemical examination of hepatocyte synthesis dysfunction, or portal hypertension evidence, or histological basis for the diagnosis of liver cirrhosis. The diagnostic criteria of patients with HBV-related HCC were in accordance with the criteria for diagnosis and treatment of HCC, and serum HBsAg was positive [19].

In addition, from January 2017 to October 2017, 25 patients aged 24 to 62 were selected during their health examination in the physical examination center of Jinan Infectious Diseases Hospital. All patients did not have diabetes, hypertension, fatty liver, hyperlipidemia and other acute and chronic diseases.

All patients underwent abdominal ultrasonography or abdominal CT examination on an empty stomach and were diagnosed as liver cirrhosis or HCC by ultrasound or CT. No significant abnormalities were found in the control group. The above subjects signed informed consent forms. This study was approved by the ethics committee of Jinan Infectious Diseases Hospital.

All subjects gave fasting venous blood at 7:00 a.m. the next day, and it was centrifuged 10 minutes at 4,000 r/minute. The blood was stored -80°C. All the remaining venous blood samples were sent for detection of blood routine, blood biochemistry, blood sugar, blood lipid, liver and fibrography, and serum protein. At the same time, 34 cases of primary hepatitis B cancer specimens and adjacent tissues were collected and stored in liquid nitrogen for reserve.

RNA isolation and real time PCR

Total RNA was isolated from serum samples or tissue samples using RNAVzol LS or RNAVzol (Vigorous Biotechnology Beijing Co., Ltd, Beijing, China) according to the manufacturer's protocol. The concentration and the purity of RNA samples was determined by measuring the optical density (OD) 260/OD280. Reverse transcription was carried out according to the instructions of PrimeScript RT Reagent Kit (Takara, Japan). The reaction conditions were 37°C 15 minutes, 85°C 5 seconds, 4°C 10 minutes. Then, qPCR was carried out according to the instructions of SYBR Premix Ex Taq (Takara, Japan). GAPDH was used as the internal reference gene. Reaction procedure was listed as follows: 95°C for 5 minutes; 40 cycles of 95°C 10 seconds, 60°C 10 seconds, 72°C 10 seconds. Relative mRNA expression was normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ method [20]. The primers used in the current study were as follows:

IGF2AS-F: CTGCCTAGAGCTCCCTCTTTC;
IGF-2AS-R: ATATTCCGTGCCATGACCCC;
GAPDH-F: GACCACAGTCCATGCCATCA;
GAPDH-R: GTCAAAGGTGGAGGAGTGGG.

Statistical analysis

The data are represented as the mean ± standard deviation (SD). The two-tailed unpaired Student's *t*-tests were used for comparisons of two groups. The one-way ANOVA multiple comparison test (SPSS 20.0) followed by the Tukey post hoc test were used for comparisons of two or more groups. Receiver operating characteristic (ROC) curves were used to assess IGF2AS as a biomarker, and the area under the curve (AUC) was reported (version 20.0, SPSS, IBM Corp. Armonk, NY,

Table 1. Comparison of baseline data among different groups.

	HBV	HBV-related cirrhosis	HBV-related HCC	Healthy controls
N	60	70	34	25
Male:Female	28:31	42:28	22:12	15:10
Age (years)	41.2 ± 13.1	46.8 ± 13.5	51.3 ± 9.2	42.5 ± 10.2
BMI (kg/m^2)	22.5 ± 3.9	22.4 ± 3.6	21.8 ± 3.8	20.8 ± 5.4
ALT (U/L)	57.2 ± 75.6 **	91.2 ± 183.5 **	48.9 ± 38.5 **	24.8 ± 14.5
AST (U/L)	47.6 ± 45.8 **	100.3 ± 146.8 **	89.45 ± 81.32 **	22.1 ± 4.8
TBIL (μmol/L)	22.1 ± 27.6 *	89.5 ± 113.4 **	68.2 ± 75.3 **	12.5 ± 4.6
Albumin	56.2 ± 5.8	93.2 ± 115.8 **	68.3 ± 73.8 **	13.4 ± 4.5
AFP	7.89 ± 6.92	11.34 ± 7.68	235.3 ± 27.5 **	6.24 ± 9.12

* - $p < 0.05$, ** - $p < 0.01$ vs. healthy controls.

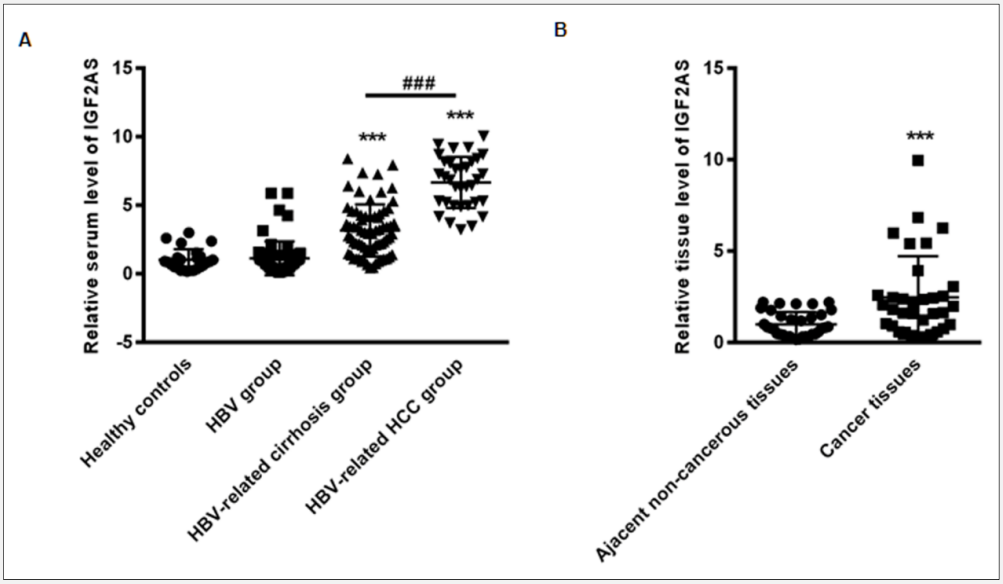

Figure 1. The level of IGF2AS was determined in serum and cancer tissues of HBV-related HCC patients.

(A) The serum level of IGF2AS in the HBV-related HCC group was significantly higher than that in the healthy control group, chronic HBV group, and HBV-related cirrhosis. (B) Compared with the adjacent tissues, the level of IGF2AS in liver cancer tissues of HBV-related HCC patients also increased significantly. *** **- $p < 0.001$ vs. Controls,** ### **- $p < 0.001$ vs. HBV-related cirrhosis group.**

USA). $p < 0.05$ was considered significant.

RESULTS

Baseline characteristics

This study included 60 cases of chronic HBV patients, 70 cases of HBV-related cirrhosis, 34 cases of HBV-related HCC patients, and 25 healthy controls. There was no significant difference in the basic information of height, weight, gender, and age between the experimental group and the control group ($p > 0.05$). Alanine ami-

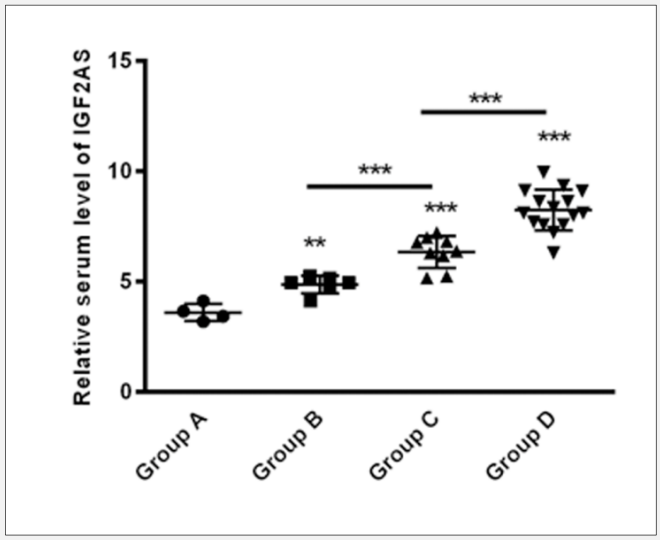

Figure 2. The level of serum IGF2AS was gradually increased in A, B, C, and D groups of HBV-related HCC patients, grouped according to BCLC classification.

** - p < 0.01, *** - p < 0.001 vs. as indicated.

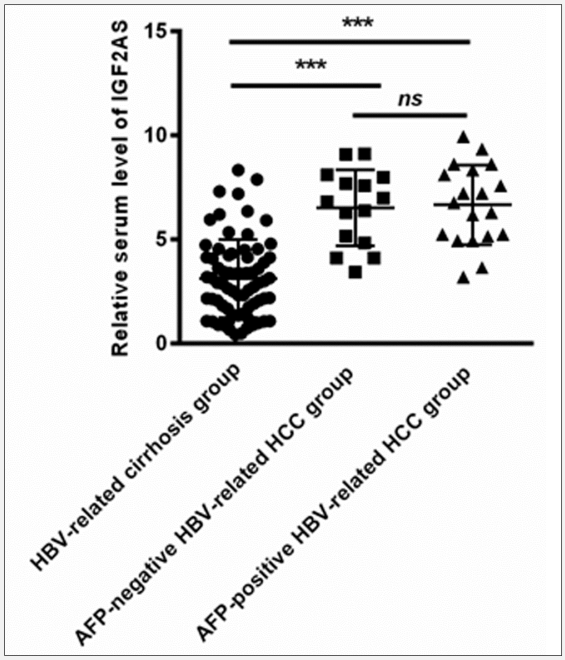

Figure 3. Compared with the HBV-related cirrhosis group, the serum IGF2AS levels of both AFP-negative and AFP-positive patients in the HBV-related HCC group were higher than those in the cirrhosis group.

ns - non-sense, *** - p < 0.001 vs. HBV-related cirrhosis group.

notransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL) and albumin in the HBV-related cirrhosis group and HBV-related HCC group were significantly different from those in healthy control group ($p < 0.01$). There was no significant difference in albumin between the chronic HBV group and healthy control group ($p > 0.05$), but there was significant difference in ALT, AST, and TBIL between the chronic HBV group and healthy control group ($p < 0.05$). Moreover, the level of alpha-fetoprotein (AFP) in the HBV-related HCC group was higher than that in the HBV-related cirrhosis group, chronic HBV group, and healthy control group, but no difference was found among HBV-related cirrhosis group, chronic HBV group, and healthy control group (Table 1).

Expression of IGF2AS in serum and cancer tissues of HBV-related HCC patients

First, we detected the level of serum IGF2AS in 60 cases of chronic HBV patients, 70 cases of HBV-related cirrhosis, 34 cases of HBV-related HCC patients, and 25 healthy controls. As shown in Figure 1A, the serum level of IGF2AS in HBV-related HCC group (6.65 ± 1.86) was significantly higher than that in the healthy control group (1.00 ± 0.79), chronic HBV group (1.11 ± 1.24), and HBV-related cirrhosis (3.15 ± 1.89). Furthermore, we also detected the level of IGF2AS in cancer tissues and the adjacent non-cancerous tissues of HBV-related HCC patients. Compared with the adjacent tissues (1.00 ± 0.69), the level of IGF2AS in liver cancer tissues of HBV-related HCC patients also increased significantly (2.48 ± 2.24) (Figure 1B). These data indicated that IGF2AS was an important regulator in the progression of HBV-related HCC patients.

Serum IGF2AS level increased sharply with the aggravation of HBV-related HCC

According to the Barcelona Staging Method (BCLC), we divided HBV-related HCC patients into four groups, including A ($n = 4$), B ($n = 6$), C ($n = 9$), and D ($n = 15$). The serum IGF2AS levels of different groups were detected and the results were as shown in Figure 2. Our data showed that serum IGF2AS levels were low in patients with better prognosis (group A: 3.60 ± 0.40) and increased sharply with the aggravation of the disease (group B: 4.87 ± 0.40; group C: 6.34 ± 0.73; group D: 8.24 ± 0.93) (Figure 2).

Higher serum IGF2AS levels of both AFP-negative and AFP-positive patients in HBV-related HCC group than those in HBV-related cirrhosis

According to the critical value of AFP (AFP = 8 ng/mL), there were 15 AFP negative cases and 19 AFP positive cases in the 34 HCC patients. We first compared the level of serum IGF2AS between 15 AFP negative cases (6.51 ± 1.83) and 19 AFP positive cases (6.66 ± 1.91). As shown in Figure 3, there was no significant difference in serum IGF2AS level between the two groups. Interestingly, compared with HBV-related cirrhosis group (3.12 ± 1.88), the serum IGF2AS levels of both AFP-negative and AFP-positive patients in HBV-related HCC group were higher than those in the cirrhosis group (Figure 3).

DISCUSSION

At present, the commonly used diagnostic methods of HCC include B-mode ultrasonography, CT, MRI, and laboratory tests such as AFP [21]. However, imaging examination is still not accurate enough for the diagnosis of HCC [22]. The sensitivity and specificity of AFP in the diagnosis of HCC are also not ideal [10,21]. Therefore, it is necessary to find new diagnostic markers for HCC.

In HCC cell lines and tissue samples, IGF2AS is found to be increased, indicating that IGF2AS may affect the formation and deterioration of HCC tumors [17]. Consistent with previous studies, we also found that the level of IGF2AS in the liver cancer tissues of HBV-related HCC was significantly higher than that in the adjacent non-cancerous tissues. Meanwhile, serum IGF2AS levels in patients with HBV-related HCC were also measured. The results showed that the serum level of IGF2AS in the HBV-related HCC group was higher than in healthy control group, chronic HBV group, and HBV-related cirrhosis, indicating that serum IGF2AS has an increasing trend in the development from chronic HBV-related cirrhosis to HCC, and can be used to monitor the course of chronic HBV-related disease. At the same time, according to BCLC classification, patients with HBV-related HCC were further divided into four groups: A, B, C and D. Our data showed that the enhancement of serum IGF2AS was consistent with the severity of HCC, validating that serum IGF2AS may be of diagnostic value for primary HCC.

Since AFP was reported to have low sensitivity and specificity in the diagnosis of HCC [23,24], we further analyzed the level of serum IGF2AS based on the critical value of serum AFP levels. Further analysis showed that no significant difference in serum IGF2AS was found between AFP-negative and AFP-positive HCC patients. More importantly, we also compared the level of serum IGF2AS among HBV-related cirrhosis patients, AFP-negative HCC patients, and AFP-positive HCC patients. It was found that serum IGF2AS levels were increased in both groups of HCC patients compared to HBV-related cirrhosis patients, suggesting that serum IGF2AS can be used in early identification and diagnosis of HBV-related HCC irrespective of AFP levels. However, due to the relatively small sample size included in this study, we have not conducted in-depth diagnostic value analysis. In future studies, we will collect more patient samples including both AFP-negative and AFP-positive HCC patients, thereby further evaluating the diagnostic value of serum IGF2AS in HBV-related HCC patients.

CONCLUSION

In summary, this study found for the first time that serum IGF2AS may be a potential biomarker for the diagnosis of HBV-related HCC, which provides a clinical basis for the role of serum IGF2AS in the disease course from chronic HBV infection, HBV-related cirrhosis, and HBV-related HCC.

Declaration of Interest:
We declare no conflicts of interest.

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