High Rate of Detection of Human ESPL1-HBV S Fusion Gene in Patients With HBV-related Liver Cancer: A Chinese Case–Control Study

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Abstract. Aim: It has been shown that the integration of hepatitis B virus (HBV) gene into the host genome is a high-risk factor for development of hepatocellular carcinoma (HCC). However, the relationship between HBV S-integrated human extra spindle pole bodies-like 1 (ESPL1) gene and HCC is unknown. This study was designed to detect HBV S-integrated human ESPL1 fusion gene in patients with HCC for potentially using this fusion gene as a biomarker for HCC diagnosis. Patients and Methods: Nineteen and 70 patients with chronic hepatitis B (CHB) were recruited to the experimental and control groups, respectively, and both groups underwent an effective nucleoside/nucleotide analog therapy and follow-up for HCC occurrence for up to 11 years. HCC tissues were obtained by surgical resection from the experimental group, while liver tissues were collected by liver biopsy in the control group prior to treatment with nucleoside/nucleotide analogs. Alu polymerase chain reaction was used to assess HBV S gene integration in the liver tissues from both groups. HBV S-integrated human ESPL1 fusion gene was then detected in patients with HBV S gene integration using a gene database. Results: All patients in the

experimental group developed HCC, whereas no HCC was diagnosed in the control group. HBV S gene integration was identified in 12 out of 19 HCC tissues in the experimental group, giving a detection rate of 63.2%, which was significantly greater than that of 15.7% (11/70) in the control group ($p < 0.001$). We further showed that HBV S-integrated human ESPL1 fusion gene was detected in eight patients (rate of 66.7%) among the 12 patients with HCC with HBV S gene integration in the experimental group, whereas the fusion gene was not detectable in any of the patients in the control group ($p = 0.001$). Conclusion: This research demonstrates a high detection rate of HBV S-integrated human ESPL1 fusion gene in patients with HBV-related HCC and shows that this fusion gene appears to be associated with HCC development in patients with CHB. These findings suggest that HBV S-integrated human ESPL1 fusion gene may potentially serve as a biomarker for early detection of HCC in HBV-infected populations.

This article is freely accessible online.

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Key Words: Integration, fusion gene, HBV S, human ESPL1, hepatocellular carcinoma, biomarker.

Hepatitis B virus (HBV) infection is a major hygiene and health problem worldwide as well as the main cause of hepatic inflammation, liver cirrhosis, and hepatocellular carcinoma (HCC). It is estimated that there are 2.4 hundred million HBV-infected patients in the world, and 60 million of them may eventually die from HCC, an aggressive form of liver cancer. It has been shown that there is a strong relationship between HBV infection and HCC and that intrahepatic HBV replication is significantly correlated with HCC occurrence (1-3). Therefore, prevention of HBV infection has been proposed as an effective way of reducing HBV-related HCC incidence, and anti-HBV treatment is given more and more attention in regard to reduction of HCC occurrence (4-9). Nucleoside/nucleotide analog (NUC) therapy of HBV infection is widely accepted because of its oral convenience, strong antiviral effect, and high safety.

Figure 1. Schematic diagram of the experimental design of this study. Patients with chronic hepatitis B (CHB) in both the experimental group and the control group underwent an effective nucleoside/nucleotide analog (NUC) therapy and follow-up observation for hepatocellular carcinoma (HCC) development for up to 11 years. In the experimental group, HCC was diagnosed in all patients with CHB at a time frame of between 2 and 11 years after receiving NUC therapy, whereas no HCC was observed in the control group during 11 years of treatment and follow-up. HCC tissues were obtained by surgical resection in the experimental group when HCC was diagnosed, while liver tissues were collected by liver biopsy in patients of the control group prior to NUC treatment.

Despite the antiviral effectiveness of NUC treatment in HBV infection, a small proportion of HBV-infected patients still develop HCC after a long-term effective NUC therapy, especially those with occult HBV infection or with lower HBV DNA expression levels which are beyond assay detection sensitivity and efficiency (10).

Clinically, 80% of patients with HCC have HBV-related HCC (11), suggesting an important role of HBV infection in the development of HCC. Increasing research has confirmed that HBV infection and HBV gene integration are involved in the pathogenesis of HCC. Although the mechanism is not completely understood, evidence shows that induction of genetic instability during HBV DNA integration into the host cell chromosomes, generation of insertional mutagenesis in cancer-associated cellular genes, and activation of proto-oncogenes due to HBV gene integration to the specific sites and regions of the nearby host genes may account for the carcinogenesis through HBV gene integration in HCC development (12-17). Currently, most studies have focused on the relationship between HBV X gene integration and HCC development (12, 18). While HBV S gene integration is regarded as a small probability event, there is a lack of extensive and profound investigations exploring the role of HBV S gene integration in the oncogenesis and tumorigenicity of human liver cancer. We recently showed that HBV S gene integration is a crucial factor leading to a sustained high level of hepatitis B surface antigen (HBsAg) in patients with chronic hepatitis B (CHB) following antiviral therapy (19). Accumulating evidence also shows that persistent expression of integrated HBV DNA and sustained higher level of HBsAg are risk factors for HCC occurrence in patients with CHB (20-22). However, NUC treatment of HBV infection cannot completely block HCC occurrence in the clinic because

these drugs do not act on the HBV genes integrated into the genome of the host cell.

The role of HBV S gene integration in the development of HCC is unknown. In the current study, we conducted a case-control clinical study to investigate the relationship between HBV S integration and HCC.

Patients and Methods

Patient selection. This project was a prospective study. The patients for the study were selected from those with CHB who then underwent NUC therapy and a long-term follow-up cohort. During the follow-up period, all patients with CHB underwent abdominal ultrasound or computed tomography every 6 months. When a liver mass was found, the patient underwent contrast-enhanced ultrasound or enhanced magnetic resonance imaging examination in order to facilitate early detection of liver tumors. The inclusion criteria of the patients were as follows: for the experimental group, patients with CHB were provided with effective NUC therapy for 2-11 years; during this period of time, when HCC occurred and was confirmed by histopathological diagnosis, the patients underwent surgical excision and HCC tissues were cryopreserved. For the control group, prior to NUC treatment, patients underwent liver puncture biopsy and the liver tissues were conserved. The patients then received 7-11 years of NUC therapy and follow-up as above. The exclusion criteria were as follows: (i) Co-infection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus; (ii) a history of interferon therapy; (iii) diagnosis of HCC at baseline; and (iv) lost during the follow-up period. After excluding 256 patients who did not meet the inclusion criteria, one patient who not have surgical excision, and 93 patients who were lost during the follow-up time, 19 patients were eligible and enrolled in the experimental group and 70 patients were selected for the control group (Figure 1). The use of human specimens from patients in the present study was approved by the Ethics Committee of the First Affiliated Hospital of Guangxi Medical University (approval number: 2019-K-Y-E-193), and all experiments were performed in accordance with the ethical standards of the 1995 Declaration of

Table I. Primers for Alu-polymerase chain reaction analysis of hepatitis virus B S gene integration used in this study.

Primer*	Sequence (5'→3')	Location	
UP5	CAGUGCCAAGUGUUUGCUGACG		Forward sequence
T3-515	CCAAAGUGCUGGGGAUUA		Reverse sequence
UP6	AUUAACCCUCACUAAAGCCUCGAU		Forward sequence (Tag)
Mid-T3	AGAUYYRRCAYUGCAC		
52821UF	CAAGTGTGTTGCTGACGCCAAAG		Reverse sequence (Tag)
	ATTAACCCCTCACTAAAGCCTCG		HBV S1
	CACCAUAUUCUUGGGAACAAG	2818-2838	
3031F	GGAGCATTCGGGCCAGGGTTC	3028-2048	HBV S2
3132F	TGCCTCCACCAATCGGCAGT	282-301	HBV S3

*Primers design reference PubMed Nucleotide GenBank: V00866. The tag sequences of the primers UP5 and T3-515 are underlined.

Helsinki, as revised in Tokyo in 2004. Written informed consent was obtained from all patients.

Sample collection and storage. HCC tissue specimens were collected by surgical resection in the experimental group (5 mg), and liver puncture biopsy specimens were collected from the control group prior to NUC therapy (5 mg). All the samples from both groups were kept frozen at -80°C.

Histopathology and immunohistochemistry. For histopathological examination, paraffin-embedded sections were stained with hematoxylin and eosin (HE) and were sealed with neutral gum.

Immunohistochemical assay was performed using a *Streptomyces* antibiotic protein-peroxidase ligation method. In brief, 5-μm tissue sections were cut from formalin-fixed, paraffin-embedded specimens. The expression of HBsAg protein was measured with UltraSensitive™ S-P Kit (Maixin, Fuzhou, Fujian, PR China). Before labeling with primary antibody, the sections were deparaffinized, antigens were restored, and the sections were incubated with hydrogen peroxide to block endogenous peroxidase. The specimen sections were incubated overnight at 4°C with primary antibody to HBsAg (1:2000; Maixin) and then with a secondary antibody (1:2000; Maixin) conjugated to streptavidin-peroxidase. After color development with diaminobenzidine peroxidase substrate solution, the sections were counterstained with hematoxylin. For the negative control, phosphate-buffered saline was used instead of primary antibody. The immunohistochemical results were examined independently by two pathologists. When all of the cytoplasm exhibited a brown reaction product, sections were considered positive. The sections were examined under a microscope at low power to identify evenly labeled areas. Images were acquired using a Leica DMRD optical microscope (Leica, Wetzlar, Germany) at 100-200× magnification.

Detection of HBV S gene integration. DNA was isolated from the excisional hepatocellular carcinoma tissues and liver biopsy specimens using DNeasy Tissue Kit (Qiagen Bio-engineering Co., Ltd., Dusseldorf, Germany). The Alu polymerase chain reaction (PCR) assay was used to verify HBV S gene integration into the hepatocyte genome (see Table I for the primers used in this study). The amplified products were electrophoresed on 1% agarose gel, recycled, and checked by sequencing (Guangzhou Biological Engineering Co., Ltd., Guangzhou, PR China).

Bioinformatic and statistical analysis. Statistical analyses for comparisons among groups were performed with SPSS software version 22.0 (IBM, Armonk, NY, USA). The continuous variables are represented by the mean±standard deviation (SD). Categorical variables are summarized as counts and percentage. The rates of HBV S gene integration were compared between the experimental group and the control group by chi-square test of a four-fold table. The detection rate of human ESPL1-HBV S fusion gene of the patients with HBV S gene integration was compared between the experimental and the control groups by Fisher's exact probability. The expression of human ESPL1 gene in normal liver tissues and liver cancer tissues was searched from the GeneCloud Biotechnology Information (GCBI) gene database, and then compared by independent samples *t*-test. Statistical significance was defined as a *p*-value of less than 0.05. All statistical tests were two-sided. The human ESPL1-HBV S fusion gene was analyzed by using the NCBI, Ensembl, and GeneCards gene databases.

Results

Patients. All the patients entered in the study were Chinese. Nineteen patients in the experimental group underwent 2 to 11 years of effective NUC therapy and HCC developed in these patients at an average of 4.8 years. Seventy patients in the control group underwent 7 to 11 years of effective NUC therapy and had no HCC during an average of 7.8 years. The general information of the patients in the experimental and the control groups is given in Table II.

Histopathological and immunohistochemical diagnosis of HBV infection and HCC. The liver tissue samples from surgical resection in the experimental group were confirmed to be HCC by HE staining (Figure 2A). In the control group, chronic inflammation and fibrosis degree in the liver biopsy tissues from patients with CHB was shown to be at least G2S2 (Figure 2B). Immunohistochemical staining of the liver tissues from the control group showed HBsAg positivity (Figure 2C).

Rate of HBV S gene integration is higher in patients with HCC than in those with CHB alone. The detection rate of HBV S gene integration was compared by chi-square test of four-fold table. As can be seen in Table II, 63.2% (12/19) patients in the experimental group had HBV S gene integration in the HCC tissues, while only 15.7% (11/70) patients in the control group had HBV S gene integration in the liver puncture biopsy tissues at baseline prior to NUC treatment. The integration rate of HBV S gene in the experimental group was significantly higher than that in the control group ($\chi^2=17.5$, $p<0.001$).

Human *ESPL1*-HBV S fusion gene is detected in HCC patients but not in CHB patients. We used gene sequence analysis software to identify the fusion gene at the sites of HBV S gene integration, and applied Fisher's exact probability to compare the detection rate of human *ESPL1*-HBV S fusion gene between the experimental group and the control group. The detection rate of human *ESPL1*-HBV S fusion gene was 66.7% (8/12) in the experimental group, but 0 in the control group, which was a statistically significant difference ($\chi^2=10.7$, $p=0.001$).

Bioinformatic analysis of human *ESPL1*-HBV S fusion gene. We searched NCBI, Ensembl, and GeneCards gene databases for *ESPL1* gene. We then analyzed the sequence of the human *ESPL1*-HBV S fusion gene in eight patients with HCC in the experimental group by gene database and identified a consensus sequence of CTGGGAACCCCGAATG in both the human *ESPL1* gene and HBV S gene. This sequence is a part of the human *ESPL1* coding sequence and is connected to the HBV S gene SP II promoter, as illustrated in Figure 3.

Differential expression of human *ESPL1* gene between HCC tissues and normal tissues. We searched and compared the expressive abundance data of human *ESPL1* gene between different normal tissues and different tumor tissues from the GCB database and found that the expression of human *ESPL1* gene was significantly higher in most of the tumor tissues assessed, including HCC tissues, than in normal tissues (301.2 vs. 104, $p<0.01$), as shown in Figure 4.

Human *ESPL1*-HBV S fusion gene in patients with CHB is associated with HCC development. To understand the role of human *ESPL1*-HBV S fusion gene in HCC development, we carried out a clinical study in the experimental and control groups. Both HBV S gene integration and human *ESPL1*-HBV S fusion gene were detected in eight patients with CHB in the experimental group, whereas only HBV S gene integration, but not human *ESPL1*-HBV S fusion gene was detected in 11 patients with CHB in the control group. All patients in both groups received an effective NUC therapy

Table II. Selected patient characteristics. Continuous variables are expressed as mean±standard deviation (SD).

Factor	Experimental group	Control group
Total number of patients	19	70
Age, years	48.7±11.3	45.5±9.2
Male, n (%)	17 (89.5)	50 (71.4)
Effective therapy time, years	4.8±1.6	7.8±1.1
History positive, n (%)	16 (84.2)	39 (55.7)
Rate of S gene integration, n (%)	12 (63.2)	11 (15.7)
Detection rate of fusion gene, n (%)	8 (66.7)	0 (0)

and follow-up in the study. Interestingly, all patients in the experimental group developed HCC starting from 2 to 11 years after effective NUC treatment. In contrast, none of the patients in the control group developed HCC following 7-11 years of effective NUC therapy. These results indicate that human *ESPL1*-HBV S fusion gene is strongly associated with HCC in HBV-infected patients.

Discussion

It is well known that HBV X gene integrates into the host genome and fuses with some human genes at certain sites and forms human-HBV X fusion genes leading to HCC development (13, 23). However, little is known about the role of HBV S gene integration and fusion of human and HBV S genes in the pathogenesis of HCC. We carried out a case-control study to investigate the relationship between HBV S gene integration and HCC, and we found for the first time a high rate of the human *ESPL1*-HBV S fusion in patients with HBV-related HCC but not in those with CHB alone. We further showed that the human *ESPL1*-HBV S fusion gene is significantly associated with HCC occurrence in patients with CHB following long-term effective NUC therapy.

Human *ESPL1* gene, also known as *SEPA* gene, is a protein coding gene for separase protein, whose main function is to catalyze fibronectin decomposition at mitosis anaphase in order to separate sister chromatids during cell division (24). Studies have reported that mutation of human *ESPL1* gene and fusion of the human *ESPL1* gene with itself or with viral genes, as well as the expression products of the fusion genes are highly relevant to the development of different tumor types (12, 16, 25-27). Consistent with the literature, we showed that the rate of HBV S gene integration and the detection rate of human *ESPL1*-HBV S fusion gene were significantly higher in patients with HCC than in patients with CHB alone ($p<0.01$), suggesting the involvement of HBV S gene integration and human *ESPL1*-HBV S fusion gene in HCC development.

Figure 2. Histopathological and immunohistochemical assessment of the liver tissues from the experimental and control groups. The liver cancer tissues in the experimental group were obtained from tumor resection of patients with hepatocellular carcinoma; the liver tissues in the control group were obtained from liver biopsy of patients with chronic hepatitis B (CHB). Both the HCC (A) and CHB (B) tissues were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin for histopathological examination. Immunohistochemical staining with antibody to HBsAg was positive in cytoplasm in tissues from a patient with CHB (C). Representative sections of the liver tissues from both groups are shown (original magnification, $\times 200$).

Figure 3. Human extra spindle pole bodies-like 1 (*ESPL1*)-hepatitis B virus (HBV) S fusion gene. A: The diagram of fusion gene formation by human *ESPL1* gene and HBV S gene. B: Analysis of the human *ESPL1*-HBV S fusion gene sequence. The genomic location of the fusion fragment of human *ESPL1* gene is at 1253287647-53288141, which is the coding sequence, including relevant exons and introns. HBV S gene fragment (nt3148-nt2845) is chimeric reservedly into (3'-5') human *ESPL1* gene and fuses with it. Furthermore, this sequence is a part of the promoter SP II of the HBV S gene, which is connected to the nearby initiator codon ATG and controls human *ESPL1*-HBV S fusion gene expression.

Figure 4. Abundance of expression of human extra spindle pole bodies-like 1 (*ESPL1*) gene in different tumor and normal tissues. A: Comparison of the abundance of human *ESPL1* gene expression in various tumor tissues and normal tissues. Student's t-test was used to analyze the statistical significance of differences between different cancer tissues and different normal tissues. As seen, the level of human *ESPL1* gene expression was higher in the liver hepatocellular carcinoma (LIHC) tissue than in the normal liver tissue ($p < 0.01$). B: Comparing human *ESPL1* gene expression between normal hepatocytes and hepatocellular carcinoma cells by independent samples t-test; the level of human *ESPL1* gene expression in normal hepatocytes was significantly lower than that in hepatocellular carcinoma cells ($**p < 0.01$). CESC: Cervical squamous cell carcinoma and endocervical adenocarcinoma; GBM: glioblastoma multiforme; UCEC: uterine corpus endometrial carcinoma; LUSC: lung squamous cell carcinoma; LUAD: lung adenocarcinoma; BRCA: breast invasive carcinoma; CHOL: cholangiocarcinoma; SARC: sarcoma; READ: rectum adenocarcinoma; LIHC: liver hepatocellular carcinoma; PRAD: prostate adenocarcinoma; COAD: colon adenocarcinoma; PCPG: pheochromocytoma and paraganglioma; BLCA: bladder urothelial carcinoma; PAAD: pancreatic adenocarcinoma; ESCA: esophageal carcinoma; KIRC: renal clear cell carcinoma; SKCM: skin cutaneous melanoma; THCA: thyroid carcinoma; STAD: stomach adenocarcinoma; KICH: kidney chromophobe; HNSC: head and neck squamous cell carcinoma; THYM: thymoma; KIRP: papillary cell renal carcinoma; TGCT: testicular germ cell tumor; DLBC: diffuse large B-cell lymphoma; OV: ovarian serous cystadenocarcinoma; UCS: uterine carcinosarcoma; LAML: acute myeloid leukemia; ACC: adrenocortical carcinoma; MESO: mesothelioma; UVM: uveal melanoma; LGG: low-grade glioma.

To further understand the role of HBV infection and human *ESPL1*–HBV S fusion gene in this process, we analyzed the sequence and structure of the human *ESPL1*–HBV S fusion gene in eight patients with HCC in our experimental group by gene database. As illustrated in Figure 3, the human *ESPL1* sequence fragment at the fusion site is the coding sequence, including exons and introns. HBV S gene (the base sequence from 3' to 5') backward inserts into the human *ESPL1* gene sequence and shares the CTGGGAACCCCGAATG sequence of with human *ESPL1* gene. HBV S gene sequence at the fusion site is a part of SP II promoter of HBV virus. Therefore, the expression of the human *ESPL1*–HBV S fusion gene is under the control of the initiation codon ATG and SP II promoter, which lead to the expression of human *ESPL1* gene. This may account for our finding (Figure 4) that the levels of human *ESPL1* gene expression were higher in HCC cells and in different tumor tissues as compared with the normal tissues (28, 29).

In addition to the increased expression of the gene, the structure and function of the mutated human *ESPL1* gene product may also be changed. As a result, altered human *ESPL1* protein may cause an error of sister chromatid segregation, production of heteroploidy, instability of the gene, uncontrollability of apoptosis (30, 31), and finally tumor occurrence. These mechanisms may underly our study results in which only those patients with CHB with HBV S gene integration and detection of human *ESPL1*–HBV S gene fusion gene developed HCC, even under 2-11 years of effective NUC treatment, whereas patients with CHB without fusion gene did not develop HCC. Moreover, the patients with CHB and HBV S gene integration alone in our control group had no HCC occurrence during an up to 11-year period of effective NUC therapy and follow-up, indicating that both HBV S gene integration and human *ESPL1*–HBV S fusion gene are required in the process of HCC development in HBV-infected patients. It is possible that HBV S gene integration and human *ESPL1*–HBV S fusion gene, together with other genes, may act coordinately and collaboratively to cause tumor development and to promote tumor progression of HCC in patients with CHB. However, the relationship between HBV S gene integration and HCC occurrence as well as the mechanism for human *ESPL1*–HBV S fusion gene in causing HCC development remains to be experimentally determined.

Collectively, these findings indicate that patients with CHB with human *ESPL1*–HBV S fusion gene are a high-risk population for HCC development and further suggest that this fusion gene may potentially serve as a biomarker for early detection and diagnosis of HCC in HBV-infected populations.

Funding

This study was supported by grants from the National Natural Science Foundation (81960115), Natural Science Foundation of

Guangxi Key Laboratory for the Prevention and Control of Viral Hepatitis (no. GXCDCKL201802), Science and Technology Major Project of China (no. 2017ZX10202201), and the Key Laboratory of High-Incidence-Tumor Prevention & Treatment (Guangxi Medical University), Ministry of Education (GKE2019-04).

Conflicts of Interest

The Authors declare that they do not have any commercial or associative interests that represent a conflict of interests in connection with this study.

Authors' Contributions

BH and WH conducted the studies, performed the experiments, drafted the article, and are the primary Authors of this article. MS performed the experiments. RW, WZ, HL, HW, BC, and DD collected specimens and analyzed the data. JJ and QQL conceived the project, interpreted data, and revised the article. JJ obtained the funding and designed the research. All Authors met the authorship criteria and read and approved the final article.

Acknowledgements

The Authors sincerely express their thanks for the financial support of the funds for our research (see under Funding) and gratefully acknowledge our colleagues and many individuals for their help during the study processes. We would also like to thank Brigit Sullivan, NIH Library, for reviewing the article.

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Received November 13, 2019

Revised November 19, 2019

Accepted November 22, 2019