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Serum Hepatitis B Surface Antigen is Correlated With Intrahepatic Total HBV DNA and cccDNA in Treatment-Naïve Patients With Chronic Hepatitis B but not in Patients With HBV Related Hepatocellular Carcinoma

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The aim of the study was to investigate correlations between intrahepatic hepatitis B virus total DNA, covalently closed circular DNA (cccDNA), and serum HBsAg in treatment-naïve chronic hepatitis B and HBV related hepatocellular carcinoma (HCC). Liver tissues were taken from 42 HBV related HCC and 36 patients with chronic hepatitis B. A fraction of DNA extracted from liver tissue was digested with a plasmid-safe ATP-dependent DNase and used for HBV cccDNA detection. The remaining DNA was used for the detection of HBV total DNA and β-globin, the latter of which is a housekeeping gene and quantified for normalization by real-time PCR. Quantitation of serum HBsAg was performed by a chemiluminescence assay. Serum HBsAg had positive correlations with serum HBV DNA ($r = 0.636$, $P < 0.001$), intrahepatic HBV total DNA ($r = 0.519$, $P = 0.001$) and cccDNA ($r = 0.733$, $P < 0.001$) in 36 treatment-naïve chronic hepatitis B, while HBsAg correlated poorly with DNA ($r = 0.224$, $P = 0.210$), intrahepatic total DNA and cccDNA in the tumor ($r = 0.351$, $P = 0.031$; $r = 0.164$, $P = 0.324$, respectively) and non-tumor ($r = 0.237$, $P = 0.152$; $r = 0.072$, $P = 0.667$, respectively) liver tissues of 42 HCC. HBV cccDNA and total DNA were significantly higher in liver tissue from chronic hepatitis B than in tumor and non-tumor of HCC ($P < 0.001$). Serum HBsAg and HBV DNA were also higher in chronic hepatitis B than in HCC ($P < 0.001$). It was concluded that levels of serum HBsAg and intrahepatic cccDNA and total DNA were significantly higher in chronic hepatitis B than in HCC, and significant correlations among them were observed in treatment-naïve chronic hepatitis B but not in HCC. ***J. Med. Virol. 85:219–227, 2013.***

KEY WORDS: hepatitis B virus; hepatitis B surface antigen; covalently closed circular DNA; total HBV DNA; hepatocellular carcinoma; chronic hepatitis B

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INTRODUCTION

More than 400 million people worldwide are infected chronically with HBV, and every year about 1 million people die from HBV-related cirrhosis and liver cancer [Lee, 1997]. Chronic hepatitis B virus infection is generally considered as a major risk factor for the development of hepatocellular carcinoma (HCC). High baseline levels of serum HBV DNA and continued high levels of viral replication increase the incidence and mortality risk of HCC [Yu et al., 1999; Chen et al., 2006a,b; Mendy et al., 2010]. Previous studies have indicated that antiviral drugs can suppress effectively viral replication, diminish the amount of HBV DNA in peripheral blood [Turin et al., 1996; Chang et al., 2006] and delay the progression to cirrhosis of the liver and HCC. However, many studies suggest that HBV DNA could still be detected in the liver tissue of HBsAg seroclearance or patients with occult HBV, mainly in the form of cccDNA, and these patients can continue to develop HCC [Pollicino et al., 2004; Yuen et al., 2004, 2008; Tong et al., 2009]. The half-life of intrahepatic cccDNA molecules are longer compared to relaxed circular DNA (rcDNA). Antiviral agents used most commonly can lower successfully the viral load, but will not eliminate hepadnaviral cccDNA, which plays a key role in drug resistance and relapse of viral replication after discontinuation of antiviral therapy [Moraleda et al., 1997; Abdelhamed et al., 2002]. The detection and quantitation of cccDNA in liver tissues is important for monitoring antiviral treatment and regimen adjustment.

The collection of liver biopsy samples is difficult due to its invasive nature. Serum HBV markers such as HBsAg, HBV DNA, and HBeAg have been proposed as surrogates for intrahepatic HBV cccDNA. There are controversies on the correlation between serum HBV DNA and intrahepatic HBV cccDNA, particularly among HBeAg-positive patients [Werle-Lapostolle et al., 2004; Chan et al., 2007]. Some studies had shown that serum HBsAg was better than serum HBV DNA for predicting treatment response [Brunetto et al., 2009]. Clearance of serum HBsAg has been linked to a good prognosis, including improvement of liver histopathology and liver function, and prolonged survival [Arase et al., 2006; Perrillo, 2009]. HBsAg titer correlates positively with intrahepatic HBV cccDNA and HBV total DNA [Wursthorn et al., 2006]. However, this correlation differs between HBeAg-positive and HBeAg-negative patients with chronic hepatitis B. In HBeAg-positive chronic hepatitis B, HBsAg correlated positively with serum HBV DNA, and intrahepatic HBV cccDNA and HBV total DNA. However, in HBeAg-negative chronic hepatitis B, no significant correlation was observed [Thompson et al., 2010]. Some studies found serum HBV DNA, not HBsAg, reflected the amount of cccDNA and replication efficiency of HBV in HBeAg-negative patients with chronic hepatitis B [Lin et al., 2010; Guner et al., 2011].

Few studies have investigated the relationship between the titer of HBsAg and intrahepatic HBV markers in chronic hepatitis B and HCC. Intrahepatic HBV cccDNA levels and its distribution in the liver of patients with HCC and chronic hepatitis B have also been identified rarely. This study has established a sensitive and specific real time polymerase chain reaction (PCR) assay for detection and quantitation of HBV cccDNA and total DNA in liver tissues. The levels of serum HBsAg and HBV DNA were measured by commercially available methods (Abbott assays). The correlations between serum HBsAg and intrahepatic HBV total DNA and cccDNA were explored in chronic hepatitis B and HCC patients.

MATERIALS AND METHODS

Study Patients

This study was approved by the Ethics Committee of Peking University Health Science Center in accordance with the Helsinki Declaration. Informed consent was obtained from each patient. Samples of tumor and non-tumor liver tissue were taken from 42 HBsAg-positive HCC patients who had tumor resection or liver transplantation between September 2009 and May 2011. Of these patients, 20 and 22 cases were from the Third Affiliated Hospital of Peking University and Beijing Youan Hospital, respectively. Patients were aged 26–74 years (36 males and 6 females). The liver biopsies obtained from 36 patients with chronic hepatitis B without antiviral therapy (27 males and 9 females, aged 17–68 years) at the No. 88 Hospital of the People's Liberation Army (Taian, Shandong Province). The liver tissues from 10 patients not infected with HBV (two males and eight females, aged 33–77 years) were also collected from the above hospitals and used as negative controls. HCC patients were diagnosed initially by dynamic computed tomography (CT) or ultrasonography, and confirmed by pathology. Tumor tissues were obtained from HCC and non-tumor tissues from tumor adjacent cirrhosis tissues without evidence of tumor cells. All serum samples were collected before the procedure of surgery or biopsy and transported on dry ice or in liquid nitrogen and stored in the laboratory at $-70^{\circ}C$.

Real Time PCR Assays for Detecting HBV cccDNA and Total DNA

The primers and probe design were according to a previous study [Singh et al., 2004] and applied to a real-time PCR assay, replacing hybridization probes with Taqman hydrolysis probes. The primers and probes were synthesized by Shanghai GeneCore Biological Engineering Technology (China). The PCR primers for the amplification of cccDNA were designed from HBV-1,523–1,540 (c-F: 5′-GGGGCGCACCT CTCTTTA-3′) and position 1,874–1,890 (c-R: 5′-AGG-CACAGCTTGGAGGC-3′). The Taqman hydrolysis probe for the detection of the amplified 367 bp DNA fragment was selected from position 1,562–1,581 (5′-FAM-TTCTCATCTGCCGGACCGTG-BHQ-3′). The PCR

primers for the amplification of total DNA were 5′-GCCAAAATTCGCAGTCC-3′ (t-F) and 5′-AAACTGA GCCAGGAGAAA-3 (t-R), which corresponded to position 305–321 and 680–663, respectively. The Taqman hydrolysis probe for the detection of the amplified 376 bp DNA fragment was from position 403–427 (5′-FAM-TTCCTCTTCATCCTGCTGCTATGCC-BHQ-3′).

The recombinant plasmid pBB4.5HBV1.2 (constructed by Dr. Wen-peng Li [Li et al., 2008b] in the laboratory) was pBlueBac4.5-based and contained a 1.2 times sequence length of a segment of the HBV DNA genome, which belonged to the adr subtype HBV C genotype and covered the HBV genome sequence nucleotide 1,389–3,215 and 1–1,969 nt. The plasmid was extracted by Plasmid Vector Extraction Kit (Shanghai Biological Technology, China) according to the manufacturer's instructions. The concentration of the plasmid (C, μg/ml) was measured at 260 nm by spectrophotometer. The copy number of the plasmid was calculated by the formulation of copies/μl = C/M × A (M: molecular weight, A: Avogadro Constant). Tenfold serial dilutions of the plasmid stock (10^9–10^2 copies/μl) were used to establish standard curves for detecting HBV cccDNA and HBV total DNA. The original plasmid stock was stored at −20°C.

Each sample was amplified with cccDNA and total DNA primers and detected with the probes, respectively. The reaction for detecting cccDNA was carried out in a 20 μl mixture containing 2× concentration LightCycler® 480 Probe master—10 μl [Fast Start Taq DNA Polymerase, reaction buffer, dNTP mix (with dUTP instead of dTTP), 6.4 mM $MgCl_2$, Roche Diagnostics, Mannheim, Germany], 2 μl of the plasmid-safe ATP-dependent DNase enzyme treated DNA sample was used as a template, c-F (10 mM) 0.8 μl, c-R (10 mM) 0.8 μl, c-Taqman (10 mM) 0.4 μl, PCR water or double distilled water (ddH_2O) 6 μl. The cycle parameters of amplification (LightCycler 480 systems, Roche) were 95°C for 10 min, followed by 45 cycles of 95°C for 10 sec, 55°C for 20 sec, and 72°C for 20 sec. The reaction mixture for detecting total DNA and the PCR conditions were the same as the cccDNA detection except t-F, t-R, and t-Taqman were used, and the template DNA was not digested with DNase. β-globin is a housekeeping gene in the human genome, therefore the LightCycler Control Kit (containing β-globin primer and probe) and LightCycler® 480 Genotyping Master Reagent (Roche Diagnostics) were used to measure the amount of genomic DNA and to calculate the numbers of cells. The cycling conditions for β-globin detection by real-time PCR followed the manufacturer's instructions. The amount of cccDNA and total DNA in liver tissues was expressed as copies/cell with the estimation of 6.667 pg of hgDNA/cell [Newbold et al., 1995].

Quantitation of Intrahepatic HBV cccDNA and HBV Total DNA

Twenty milligram of tumor or non-tumor tissues was homogenized under liquid nitrogen and the total DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction with minor modifications (i.e., using 300 μl buffer AW1 to wash the extraction twice instead of 500 μl buffer AW1 washing once). The DNA from liver biopsies was extracted using QIAamp DNA Micro Kit (Qiagen). The concentration of total DNA was determined at 260 nm with a spectrophotometer (American GE Company GeneQuant pro).

Plasmid-safe ATP-dependent DNase (Epicentre Technologies, Madison, WI) hydrolyzes selectively linear double-strand (ds) DNA, linear, and closed-circular single-strand DNAs to deoxynucleotides at slightly alkaline pH, but does not affect the closed circular double-strand or supercoiled DNA. A total of 25 μl total DNA and 1 μl PSAD (10 U/μl) were added into the final volume of 50 μl of slightly alkaline configured solution, containing 10 mM Mg^{2+}, 1 mM ATP, 0.5 mM DTT. The solution was incubated for enzyme digestion at 37°C for 1 hr, and then heated at 70°C for 30 min for plasmid-safe ATP-dependent DNase inactivation. The digested DNA was used for cccDNA detection by real-time PCR.

The remaining fraction of extracted total DNA was used for the detection of HBV total DNA and β-globin. Each sample was repeated three times for determining the means of cycle threshold (Ct) values of HBV total DNA, HBV cccDNA and β-globin detection. The amounts (copies/cell) of HBV cccDNA and total DNA were quantified by real-time PCR, and were normalized with the β-globin housekeeping gene.

Quantitative Serum HBV DNA Assay

Serum HBV DNA levels were quantified by real-time PCR using commercial reagents in an automatic nucleic acid detection instrument (Abbott M2000 System consisting of a m2000sp/m24sp automatic nucleic acid extraction apparatus and a m2000rt real-time PCR instrument) with a dynamic range over 10–10^9 IU/ml (1 IU/ml = 3.41 copies/ml). Samples were retested after dilution if the value of initial detection was higher than 10^9 IU/ml.

Quantitative Serum HBsAg Assay

The titer of serum HBsAg was measured by a chemiluminescence assay using the ARCHITECT i2000SR platform and Abbott Architect HBsAg reagents (Abbott Laboratories, Chicago, IL). A value of HBsAg >0.05 IU/ml was defined as positive. If the initial test value was higher than the upper limit of detection (250 IU/ml), the samples were diluted (1:400) and reassessed.

Statistical Analysis

The statistical analysis was performed using the Statistical Program for Social Sciences (SPSS 13.0 for Windows, SPSS, Chicago, IL). Intrahepatic HBV total DNA and HBV cccDNA, serum HBsAg and HBV DNA

levels were logarithmic transformed for analysis. The correlations between them were analyzed using Pearson's correlation test. The statistical comparisons were analyzed by the Paired *t*-test and non-parametric test, respectively. All comparisons were two-tailed, and statistical significance was defined as a *P*-value <0.05.

RESULTS

Clinical Features

The clinical data of 42 HBsAg-positive patients with HCC and 36 patients with chronic hepatitis B is shown in Table I. The mean age was younger in patients with chronic hepatitis B (37.08 ± 12.00 years) than in patients with HCC (50.64 ± 10.01 years). The median values of serum HBV DNA, HBsAg, ALT, and AST of 42 patients with HCC were 1.39×10^4 copies/ml ($57.91–1.79 \times 10^7$), 826 IU/ml (0.64–6,508), 36 U/L (15–359.1), and 43.25 U/L (15–359.1), respectively. Of 36 patients with chronic hepatitis B without any antiviral treatment, the median values of serum HBV DNA, HBsAg, ALT, and AST were 1.66×10^6 copies/ml ($9.93–2 \times 10^8$), 5,728 IU/ml (153.76–149,248), 67 U/L (17–743 U/L) and 47 U/L (20–331), respectively. The levels of serum HBV DNA, HBsAg, ALT, and AST were significantly higher in patients with chronic hepatitis B than in patients with HCC (Table I).

Quantitation of Intrahepatic HBV cccDNA, Total DNA, and Serum HBsAg in Patients With HCC and Correlation Analysis

HBV cccDNA and total DNA were negative in the liver tissues of 10 patients with disease not infected with HBV. The levels of intrahepatic HBV cccDNA and total DNA in 42 HBsAg-positive patients are shown in Table II. The median of intrahepatic HBV cccDNA were 0.081 copies/cell (0.002–27.11) and 0.132 copies/cell (0.026–3.875) for tumor and non-tumor tissues, respectively. The log value of cccDNA was significantly lower in tumor tissues than in non-tumor tissues (-1.212 log copies/cell vs. -0.812 log -cell, $P < 0.001$). The median of intrahepatic HBV total DNA were 1.887 copies/cell (0.007–1,255.083) and 1.349 copies/cell (0.059–327.72) in tumor and non-tumor tissues, respectively. There was no significant difference between the log values of HBV total DNA in tumor and non-tumor tissues (0.236 log copies/cell vs. 0.286 log copies/cell, $P = 0.687$). The level of intrahepatic HBV cccDNA correlated positively with the intrahepatic HBV total DNA ($r = 0.568$, $P < 0.001$ in tumor tissues; $r = 0.432$, $P = 0.004$ in non-tumor tissues; see Supplementary Fig. 1A and B). The log ratio of cccDNA to total DNA was inversely related to the log total DNA levels ($r = -0.592$, $P < 0.001$ for tumor tissues; $r = -0.812$, $P < 0.001$ for non-tumor tissues; see Supplementary Fig. 2A and B). The ratio of cccDNA to total DNA was significantly higher in non-tumor liver tissues than in tumor liver tissues [11.70% (0.01–100%) vs. 5.13% (0.13–51.34%), $P = 0.002$] (Table II).

The median values of serum HBV DNA and HBsAg in HCC patients were 1.39×10^4 copies/ml ($57.91–1.79 \times 10^7$) and 826.00 IU/ml (0.64–6,508.00), respectively. The log values of serum HBsAg correlated poorly with the log values of intrahepatic HBV total DNA in tumor and non-tumor tissues ($r = 0.351$, $P = 0.031$; $r = 0.237$, $P = 0.152$; Fig. 1A and B), and did not correlate with intrahepatic HBV cccDNA and serum HBV DNA in patients with HCC ($r = 0.164$, $P = 0.324$; $r = 0.071$, $P = 0.667$ for cccDNA in tumor and non-tumor tissues; $r = 0.224$, $P = 0.210$ for serum HBV DNA; Fig. 2A–C). In addition, the log values of serum HBV DNA correlated positively with the log values of intrahepatic HBV total DNA in tumor and non-tumor tissues ($r = 0.371$, $P = 0.033$;

TABLE I. Clinical Characteristics of 42 HBsAg-Positive HCC and 36 Treatment-Naïve Patients With Chronic Hepatitis B

	HBsAg(+) HCC			Treatment-naïve CHB			
Patients clinical characteristics	HBeAg(+) (n = 13)	HBeAg(−) (n = 29)	Total (n = 42)	HBeAg(+) (n = 24)	HBeAg(−) (n = 12)	Total (n = 36)	*P*-value
Male:Female	11:2	25:4	36:6	17:7	10:2	27:9	
Age (years): Mean ± SD	48.00 ± 10.93	51.83 ± 9.52	50.64 ± 10.01	32.96 ± 9.41	45.33 ± 12.70	37.08 ± 12.00	<0.001[a]; 0.002[b]
Log ALT (U/L): Mean ± SD	1.61 ± 0.31	1.58 ± 0.29	1.59 ± 0.29	1.83 ± 0.39	1.98 ± 0.52	1.88 ± 0.44	0.001[a]; 0.350[b]
Log AST (U/L): Mean ± SD	1.63 ± 0.16	1.61 ± 0.29	1.62 ± 0.25	1.71 ± 0.28	1.87 ± 0.40	1.76 ± 0.33	0.035[a]; 0.180[b]
Log HBsAg (IU/ml): Mean ± SD	2.80 ± 0.62	2.46 ± 1.12	2.57 ± 1.00	4.23 ± 0.65	3.31 ± 0.66	3.92 ± 0.78	<0.001[a]; <0.001[b]
Log HBV DNA (copies/ml): Mean ± SD (serum)	3.54 ± 1.63	3.67 ± 1.33	3.63 ± 1.41	7.59 ± 1.84	5.78 ± 2.05	6.97 ± 2.08	<0.001[a]; 0.012[b]

HCC, hepatocellular carcinoma; CHB, chronic hepatitis B.
[a]Comparison between patients with HCC and patients with chronic hepatitis B.
[b]Comparison between HBeAg-positive and HBeAg-negative patients with chronic hepatitis B.

TABLE II. Intrahepatic HBV cccDNA and Total DNA Levels (copies/cell) in 42 HBsAg-positive HCC Patients and 36 Treatment-Naïve Patients With Chronic Hepatitis B

Patients	HBsAg(+) HCC (42)		Treatment-naïve CHB (36)			
Total DNA/cccDNA assay	Tumor	Non-tumor	HBeAg(+) (24)	HBeAg(−) (12)	Total	*P*-value[a,b,c]
Total DNA:median	1.89	1.35	343.28	4.86	94.41	0.687[a]
Range	0.01–1,255.08	0.06–327.72	2.40–1,884.30	0.20–610.03	0.20–1,884.31	<0.001[b]
Log value range	0.24 ± 0.85	0.29 ± 0.90	2.31 ± 0.78	0.86 ± 1.02	1.83 ± 1.10	<0.001[c]
cccDNA:median	0.08	0.13	3.79	0.32	1.51	<0.001[a]
Range	0.00–27.11	0.03–3.88	0.05–19.66	0.03–8.93	0.031–19.66	0.001[b]
Log value range	−1.21 ± 0.72	−0.81 ± 0.53	0.38 ± 0.67	−0.48 ± −0.68	0.09 ± 0.78	<0.001[c]
cccDNA/total DNA:median	5.13%	11.70%			1.27%	0.002[a]
Range	0.13–51.34%	0.01–100%			0.05–89.67%	0.005[c]

[a]Comparison between tumor and paired non-tumor tissues.
[b]Comparison between HBeAg-positive and HBeAg-negative patients with chronic hepatitis B.
[c]Comparison between tumor, paired non-tumor tissues, and biopsies.

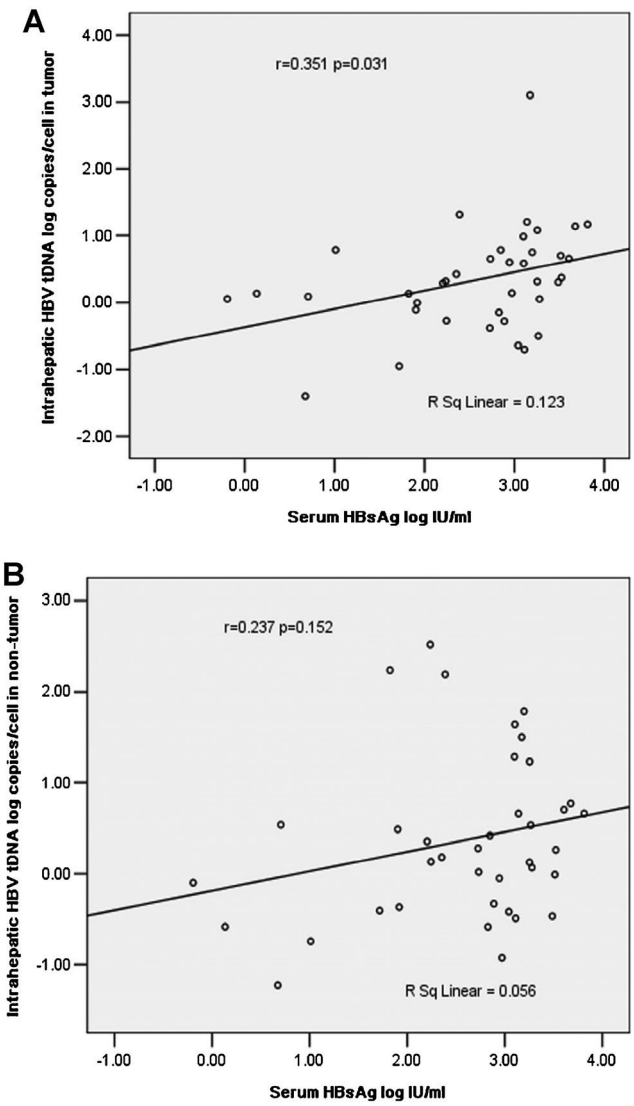

Fig. 1. **A**: Correlation between serum HBsAg and intrahepatic HBV tDNA (total DNA) in the tumor tissues of HBV-related HCC patients; (**B**) correlation between serum HBsAg and intrahepatic HBV tDNA (total DNA) in the non-tumor tissues of HBV-related HCC patients.

r = 0.617, $P < 0.001$, respectively; see Supplementary Fig. 3A and B), but had no significant correlation with intrahepatic HBV cccDNA (r = 0.092, $P = 0.609$; r = 0.014, $P = 0.939$, respectively).

Quantitation of Intrahepatic HBV cccDNA, Total DNA, and Serum HBsAg in Patients With Chronic Hepatitis B and Correlation Analysis

The median values of intrahepatic HBV cccDNA and total DNA were 1.511 copies/cell (0.031–19.655) and 94.41 copies/cell (0.195–1,884.305) in the liver biopsies of 36 patients with chronic hepatitis B, respectively (Table II). The log values of HBV cccDNA and total DNA were significantly higher in the liver tissues of patients with chronic hepatitis B than in the tumor and non-tumor tissues of HCC patients (0.093 ± 0.78 vs. −1.212 ± 0.72 and −0.812 ± 0.53 for cccDNA, $P < 0.001$; 1.828 ± 1.10 vs. 0.236 ± 0.85 and 0.286 ± 0.9 for total DNA, $P < 0.001$; Table II). In addition, the log values of intrahepatic HBV cccDNA and total DNA were significantly higher in HBeAg-positive patients with chronic hepatitis B than in HBeAg-negative patients (0.38 ± 0.67 vs. −0.48 ± −0.68, $P = 0.001$; 2.31 ± 0.78 vs. 0.86 ± 1.02, $P < 0.001$, respectively; Table II). There was a significant positive correlation between intrahepatic HBV cccDNA and total DNA in patients with chronic hepatitis B (r = 0.766, $P < 0.001$; see Supplementary Fig. 4A). The log ratio of cccDNA to total DNA was related inversely to the intrahepatic HBV total DNA level in liver biopsies (r = −0.709, $P < 0.001$; see Supplementary Fig. 4B). The ratio of cccDNA to total DNA was lower significantly in the liver biopsies of patients with chronic hepatitis B than in tumor and non-tumor tissues [1.27% (0.05–89.67%) vs. 5.13% (0.13–51.34%) and 10.70% (0.01–100%), $P = 0.005$)], as shown in Table II.

The log values of serum HBV DNA, HBsAg, and ALT were significantly higher in patients with chronic hepatitis B than in HCC patients (6.97 ± 2.08 vs. 3.63 ± 1.41, $P < 0.001$; 3.92 ± 0.78 vs. 2.57 ± 1.00, $P < 0.001$; 1.88 ± 0.44 vs. 1.59 ± 0.29, $P = 0.001$, respectively; Table I). For stratification analysis, the log

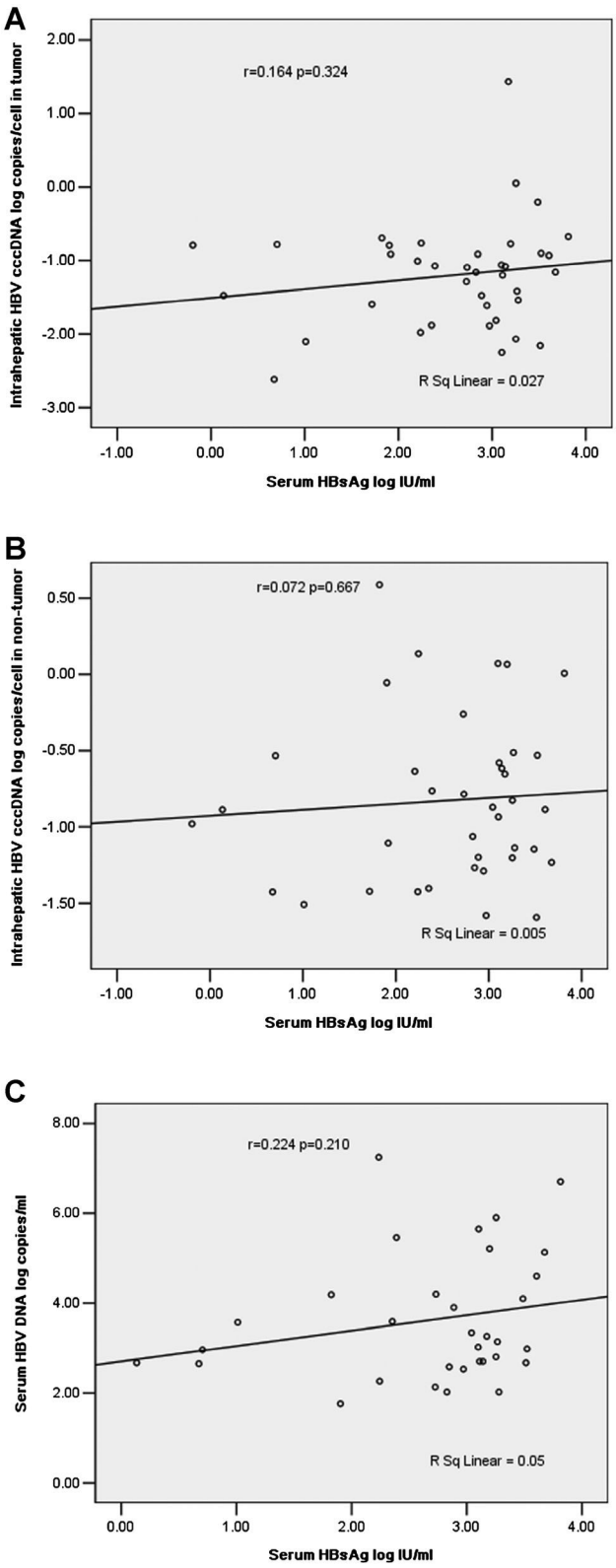

Fig. 2. **A**: Correlation between serum HBsAg and intrahepatic HBV cccDNA in the tumor tissues of HBV-related HCC patients; (**B**) correlation between serum HBsAg and intrahepatic HBV cccDNA in the non-tumor tissues of HBV-related HCC patients; (**C**) correlation between serum HBsAg and serum HBV DNA levels in HBV-related HCC patients.

values of serum HBV DNA and HBsAg were also significantly higher in HBeAg-positive patients with chronic hepatitis B than in HBeAg-negative patients (7.59 ± 1.84 vs. 5.78 ± 2.05, $P = 0.012$; 4.23 ± 0.65 vs. 3.31 ± 0.66, $P < 0.001$, respectively; Table I). For the correlation analysis, the log values of serum HBsAg titer correlated positively with the log values of intrahepatic HBV cccDNA and total DNA as well as serum HBV DNA levels ($r = 0.519$, $P = 0.001$; $r = 0.636$, $P < 0.001$; $r = 0.733$, $P < 0.001$, respectively; Fig. 3A–C). The log value of serum HBV DNA was also correlated positively with the log values of intrahepatic HBV cccDNA and total DNA ($r = 0.472$, $P = 0.004$; $r = 0.703$, $P < 0.001$, respectively; see Supplementary Fig. 5A and B).

DISCUSSION

Intrahepatic HBV cccDNA and total DNA of liver specimens in 42 patients with HBV related HCC were measured. The level of intrahepatic HBV cccDNA ranged from 0.002 to 27.110 copies/cell, which was similar to the previous report from 5 to 50 copies/cell [Addison et al., 2002]. The intrahepatic HBV cccDNA levels were significantly higher in non-tumor than in tumor tissues (0.132 log copies/cell vs. 0.081 log copies/cell, $P < 0.001$), although there was no difference in the level of intrahepatic HBV total DNA between tumor and non-tumor liver tissues ($P = 0.687$). This result was consistent with some previous studies [Zhang et al., 2007; Faria et al., 2008; Pan et al., 2009], but was different from the data reported in other studies [Wong et al., 2006; Li et al., 2008a]. The uneven distribution of HBV cccDNA [Zhang et al., 2003] was also observed in liver tissues in the present study, and the level of HBV cccDNA was lower in tumor than in non-tumor tissues. The mechanisms for this discrepancy may be complicated. One reason may be due to the long time process and persistence of hepatic damage in patients with HCC, which leads to the destruction and necrosis of many liver cells, and causes the reduction of HBV replication and the potential for gradual depletion of the HBV cccDNA pool in tumor cells. In addition, the HBV DNA sequences have been shown to be integrated into host genomic DNA in HCC and non-tumor tissues from patients with chronic HBV infection [Brechot, 2004; Kim et al., 2012]. These integrated viral DNA sequences could amplify when the hepatocyte proliferation occurs, which might be detected as total DNA. Therefore, the amounts of intrahepatic HBV total DNA in tumor and non-tumor tissues were comparable. However, other unknown mechanisms may still exist, more studies are needed.

The ratio of cccDNA to total DNA was significantly higher in patients with HCC than in patients with chronic hepatitis B. The ratio of HBV cccDNA to total DNA was significantly higher in non-tumor than in tumor tissues, which was different from a previous report [Wong et al., 2006]. The cccDNA/total DNA ratios

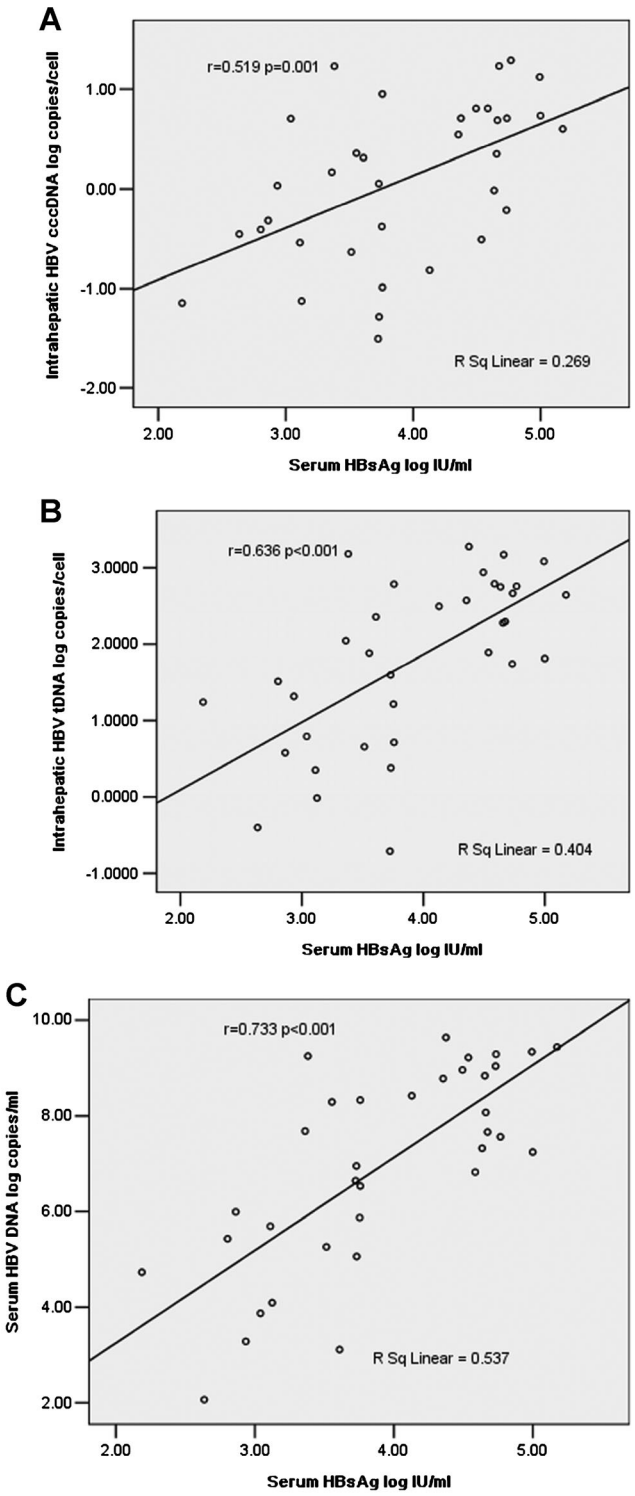

Fig. 3. **A**: Correlation between serum HBsAg and intrahepatic HBV cccDNA in patients with chronic hepatitis B; (**B**) correlation between serum HBsAg and intrahepatic HBV tDNA (total DNA) in patients with chronic hepatitis B; (**C**) Correlation between serum HBsAg and serum HBV DNA levels in patients with chronic hepatitis B.

of tumor and non-tumor tissues in patients with HCC and of liver tissues in patients with chronic hepatitis B were significantly related inversely to corresponding the intrahepatic HBV total DNA, suggesting that if intrahepatic HBV DNA was low, the cccDNA/total DNA ratio would be increasing, solely in the form of HBV cccDNA. This result was consistent with a previous study in patients with chronic hepatitis B [Wong et al., 2004], and also existed in HBV-related HCC patients.

Intrahepatic HBV cccDNA and total DNA of liver biopsies from 36 patients with chronic hepatitis B without any antiviral treatment were measured. The levels of intrahepatic HBV cccDNA and total DNA ranged from 0.031 to 19.655 copies/cell and from 0.195 to 1,884.305 copies/cell, respectively, which were similar to the results of previous studies [Wursthorn et al., 2006; Laras et al., 2006; Bourne et al., 2007]. The levels of intrahepatic HBV cccDNA and total DNA were significantly higher in HBeAg-positive patients (24 cases, median: 3.79 and 343.28 copies/cell, respectively) than in HBeAg-negative patients (12 cases, median: 0.32 and 4.86 copies/cell, respectively). The trend confirmed previous reports [Werle-Lapostolle et al., 2004; Wong et al., 2004; Laras et al., 2006; Wursthorn et al., 2006] that patients with chronic hepatitis B with different HBeAg status had different levels of intrahepatic virus. Many studies indicated that the copies and replication activities of virus in liver cells were higher in HBeAg-positive than in HBeAg-negative patients with chronic hepatitis B. This study found that serum HBV DNA and HBsAg levels were significantly higher in HBeAg-positive patients with chronic hepatitis B than in HBeAg-negative patients.

Previous studies have indicated that advanced age, male sex, elevated ALT level, higher HBV DNA level were associated with HCC development [Ganem and Prince, 2004]. In this study, patients with HCC were older than patients with chronic hepatitis B. The levels of serum HBV DNA, HBsAg, ALT, AST, intrahepatic HBV cccDNA, and total DNA were lower in patients with HCC than in patients with chronic hepatitis B. Serum HBsAg correlated well with intrahepatic HBV cccDNA, intrahepatic HBV total DNA and serum HBV DNA in treatment-naïve patients with chronic hepatitis B, and serum HBV DNA also correlated positively with intrahepatic viral level. However, serum HBsAg did not correlate with intrahepatic HBV markers in patients with HBV-related HCC. The difference might be explained by the long progressive process of HCC tumorigenesis from chronic hepatitis B to cirrhosis, and eventually to cancer, during which viral replication decreases gradually. The low levels of intrahepatic HBV DNA and HBsAg in HCC and non-tumor tissues indicated viral replication decreasing in the whole liver, not just limited to the tumor tissues. As discussed above, the integrated HBV DNA in HCC patients might play a major role in HBsAg production, thus the detected serum HBsAg might not be only

dependent of intrahepatic viral load [Brunetto, 2010]. This might explain why there was no correlation between the levels of intrahepatic viral load and serum HBsAg in HCC patients. The previous studies observed that the association between HBsAg production and HBV replication broke down in the HBeAg-negative phase of HBV infection [Thompson et al., 2010; Guner et al., 2011]. In this study, the same phenomenon occurred in HCC patients with different HBeAg status.

In addition, 14 cases of 42 HBsAg-positive HCC patients had received antiviral treatment from 9 months to 5 years before HCC was diagnosed. The stratification analysis was performed by treated and untreated groups, and correlations between HBV markers were consistent with the results of analysis without stratification (see Supplementary Tables I and II).

Unfortunately, the patient numbers were not sufficient for a correlation analysis between serum HBsAg and intrahepatic HBV markers stratified by different HBeAg status in the phase of HCC or chronic hepatitis B. A further study is needed with additional cases.

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