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Antiviral therapy may decrease HBx, affecting cccDNA and MSL2 in hepatocarcinogenesis

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Abstract. Chronic hepatitis B virus (HBV) is the leading cause of hepatocellular carcinoma (HCC). Covalently closed circular DNA (cccDNA) is an intermediate in the life cycle of HBV. HBV-encoded X protein (HBx), a key viral oncoprotein, can be specifically ubiquitylated by male specific lethal 2 (MSL2), which causes upregulation of HBx activity and promotes transcription, cell proliferation and tumor growth. The present study compared the levels of cccDNA, MSL2 mRNA and HBx mRNA in tumor and peri-tumor tissues, and clarified the effect of antiviral therapy on these indicators. Levels of intrahepatic cccDNA, MSL2 mRNA and HBx mRNA were determined using quantitative PCR in patients with HBV-associated HCC who had undergone liver surgery. A total of 50 patients were included in the present study. Prior to surgery, 31 patients had undergone antiviral treatment. Intrahepatic cccDNA levels were significantly higher in the tumor tissues compared with the peri-tumor tissues (P=0.001), particularly in the hepatitis B e antigen-positive (P=0.008), tumor recurrence (P=0.002) and <3 cm tumor size (P=0.003) groups. Furthermore, in patients with preoperative cirrhosis, levels of cccDNA and MSL2 mRNA were significantly higher in tumor tissues compared with that in peri-tumor tissues (P<0.001 and P=0.023, respectively). The expression levels of HBx mRNA in antiviral-treated tumors and peri-tumor tissues were significantly lower compared with those in untreated tissues (P=0.026 and P=0.035). The levels of cccDNA and MSL2 mRNA in the HBx-positive group were significantly higher in tumor tissues compared with those in peri-tumor tissues (P=0.026 and P=0.013). In conclusion, cccDNA participated in the tumorigenesis of HBV-associated HCC, and antiviral therapy was found to modulate hepatocarcinogenesis by decreasing the levels of HBx to inhibit the tumorigenic effect of MSL2 and cccDNA.

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Abbreviations: cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus total DNA; HBx, HBV-encoded X protein; HCC, hepatocellular carcinoma; MSL2, male specific lethal 2

Key words: covalently closed circular DNA, male specific lethal 2, hepatitis B virus-encoded X protein, hepatitis B, hepatocellular carcinoma

Introduction

Hepatitis B virus (HBV) infection is a global health concern that causes >1,000,000 deaths worldwide per year (1). HBV-infected patients face the risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC) (1,2). HCC is the fifth most common type of cancer and the third highest cause of cancer-associated mortality worldwide, next to lung and stomach cancer (3). The high mortality associated with HCC is due to its unresponsiveness to treatment and a delay in recognizing symptoms (2,4). Chronic hepatitis B infection is a leading precursor of HCC; however, little is currently known about the pathogenesis of HCC.

Covalently closed circular DNA (cccDNA) is an important intermediate in the life cycle of HBV. It does not directly participate in HBV replication, but maintains a stable pool within the hepatocyte nucleus (5). A previous study demonstrated that HCC often develops as HBV replication intensifies during the late stage of hepatitis B (6), a period in which cccDNA becomes predominant in quantity (7). Therefore, it may be possible to predict the involvement of cccDNA in HBV-associated HCC by measuring the levels of intrahepatic cccDNA in paired tumor and peri-tumor tissues.

HBV-encoded X protein (HBx) is a key viral oncoprotein produced during the development of HBV-associated HCC (8,9). It regulates signaling pathways by interacting with a variety of proteins (10). For example, HBx can be specifically modified by E3 ubiquitin ligases to upregulate its expression and promote transcription, cell proliferation and tumor growth (11).

The human male specific lethal 2 (MSL2) ortholog is an E3 ubiquitin ligase that ubiquitylates the tumor suppressor p53, as well as histone H2B to mediate transcriptional control (12). Gao *et al* (13) reported that HBx-mediated upregulation of MSL2 activates HBV cccDNA in hepatoma cells to promote

hepatocarcinogenesis, forming a HBx/MSL2/cccDNA/HBV positive feedback loop. The present study aimed to compare the cccDNA, MSL2 mRNA and HBx mRNA levels in tumor and peri-tumor tissues, and to compare differences in cccDNA and MSL2 mRNA levels between HBx-positive and HBx-negative patients. Furthermore, the present study investigated the effect of antiviral therapy on these indicators.

Materials and methods

Patients. The present study included a total of 53 patients, including three patients without hepatitis B as a control. The 50 HBV-associated patients with HCC were divided into three groups according to hepatitis B envelope antigen (HBeAg) status, tumor size and HCC recurrence for comparison of cccDNA contents. The contents of cccDNA, MSL2 mRNA or HBx mRNA in the control patients were determined separately and one patient was included as a control in each analysis. The extraction procedures for cccDNA, MSL2 mRNA and HBx mRNA were based on the sample to be assayed. cccDNA, MSL2 mRNA, HBx mRNA were stored at -20˚C from a single HBV-negative sample, based on a fixed control patient, and were added to each PCR analysis; fold-changes were determined based on the relative levels of cccDNA, MSL2 mRNA and HBx mRNA. A total of 50 patients with HBV-associated HCC and 3 patients with HCC without hepatitis B who had undergone curative liver resection or liver transplantation at the Department of Surgery, Seoul National University Hospital (Seoul, South Korea) between October 2016 and March 2018 were included in the present study. Samples were prepared with tumor and peri-tumor liver tissues collected from these patients. The history of antiviral therapy before surgery and tumor recurrence following surgery were obtained via each patient's medical records, follow-up until September 2018. The diagnosis of HCC was evaluated by two experienced pathologists at Seoul National University Hospital and analysis was conducted in a blinded manner. Patients with HCC with evidence of HBV-infected history were enrolled in this study, and patients who had consumed alcohol or had been infected with hepatitis C or hepatitis D viruses were excluded from this study. In addition, patients with cholangiocarcinoma were excluded from this study. Tumor and peri-tumor tissues were resected, and peri-tumor tissue were obtained >1 cm from the edge of the tumor, and rapidly frozen and stored at -80˚C. Written consent, approving the use of tissues for research purpose following surgery, was obtained from each patient included in the present study. The experimental protocol was approved by the Institutional Review Board of Seoul National University Hospital (IRB no. H-1809-001-967).

Detection of HBsAg, HBeAg, and HBV-DNA. The hepatitis B surface antigen (HBsAg) and HBeAg levels were assessed by the chemiluminescence enzyme immunoassay (CLEIA) method using a commercially available enzyme immunoassay kit (Lumipulse, Fuji Rebio, Inc.), according to the manufacturer's protocols. Serum concentrations of HBV-DNA were determined using a PCR HBV monitoring kit (Roche Diagnostics K.K.), which had a quantitative range between 2.6 and 7.6 log copies/ml.

Isolation of intrahepatic total DNA and cccDNA. Total genomic DNA was extracted from ~20-30 mg of liver tissue using the QIAamp DNA Mini kit (Qiagen GmbH) according to the manufacturer's protocol. The concentration of total DNA was determined at 260 nm with a spectrophotometer (Eppendorf). Plasmid-safe™ ATP-dependent DNase (Epicentre; Illumina, Inc.) incubation with Ambion™ RNase A (Thermo Fisher Scientific, Inc.) for 30 min at 70˚C was used to hydrolyze linear double-stranded DNA, linear and closed circular single-stranded DNA, for the isolation of double-stranded closed circular DNA, including HBV cccDNA (14).

cccDNA detection using quantitative PCR (qPCR). Intrahepatic levels of cccDNA in tumor and peri-tumor tissues were compared. cccDNA was isolated by digestion from 300 mg of total DNA with Plasmid-safe™ ATP-dependent DNase and diluted with 20 ml of diethyl pyrocarbonate water. A total of 1 ml of cccDNA was then used for qPCR amplification (7500 Real-time PCR Instrument system; Applied Biosystems; Thermo Fisher Scientific, Inc.), which was performed using TOPreal™ qPCR PreMIX SYBR Green (Enzynomics). β-actin amplicons were used as the internal reference for subsequent PCR analysis. Each sample was assayed three times to determine the mean cycle threshold (Ct) values for HBV cccDNA and β-actin. Relative transcriptional fold-changes were calculated as $2^{-\Delta\Delta Ct}$ (15). The detected cccDNA levels in patients with HBV-associated HCC were presented as a fold-change relative to that in the one HBV-negative control patient. The thermocycling was performed as follows: 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec, 63˚C for 30 sec, 72˚C for 25 sec and 95˚C for 15 sec (16). Optimal sensitivity was obtained by combining two forward primers with a reverse primer. The sequences of the PCR primers are listed in Table I (17).

MSL2 and HBx mRNA detection using reverse-transcription qPCR (RT-qPCR). Total RNA was extracted from tumor and peri-tumor tissues using TRIzol® reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) was used to reverse transcribe total RNA into cDNA. Synthesis conditions were as follows: 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min. cDNA was used as the template for qPCR using a TOPreal™ qPCR PreMIX SYBR Green. β-actin was used as an internal control for normalization. Relative transcriptional fold-changes were calculated using the $2^{-\Delta\Delta Ct}$ method (15). Thermocycling was performed as follows: 95˚C for 10 min, followed by 45 cycles of 95˚C for 15 sec, 63˚C for 30 sec, 72˚C for 25 sec and 95˚C for 15 sec. All primers used are listed in Table I. MSL2 and HBx mRNA levels in patients with HBV-associated HCC were presented as fold-changes relative to that in the fixed one HBV negative control patient.

Statistical analysis. All statistical analyses were performed using SPSS software (version 23; IBM Corp.). Variables with normal and skewed distribution of paired samples were analyzed using paired Student's t-tests and Wilcoxon signed ranks tests, respectively. The Mann-Whitney U test was used to analyze continuous variables. Correlation analysis was performed using Spearman's correlation test. $P<0.05$ was

Table I. Primers used for qPCR and reverse transcription-qPCR for the detection of hepatitis B virus cccDNA, MSL2, HBx and β-actin.

Gene	Primer sequence
cccDNA	Forward, 5'-GCGGWCTCCCCGTCTGTG CC-3'; 5'-GTCTGTGCCTTCTCATCTGC-3' Reverse, 5'-GTCCATGCCCCAAAGCCA ACC-3'
MSL2	Forward, 5'-ACAGTGAGAAAGTTCAG CCA-3' Reverse, 5'-AGCACGCCCACATTTACT-3'
HBx	Forward, 5'-ATGGCTGCTAGGCTGTGC-3' Reverse, 5'-TTAGGCAGAGGGGAAAAAGT TG-3'
β-actin	Forward, 5'-GTGCACCTGACTCCTGAGGA GA-3' Reverse, 5'-CCTTGATACCAACCTGCC CAG-3'

qPCR, quantitative PCR; cccDNA, covalently closed circular DNA; MSL2, male-specific lethal 2; HBx, hepatitis B virus-encoded X protein.

considered to indicate a statistically significant difference. Data are expressed as the mean ± standard deviation, with bars in the graphs representing standard deviation.

Results

Clinical characteristics of patients. A total of 50 patients with HBV-associated HCC were included in the present study (40 males and 10 females; mean age, 58±10 years; age range, 34-80). Of these patients, 49 were HBsAg-positive, and one exhibited seroconversion following antiviral treatment; 31 patients had received different degrees of antiviral therapy and 19 had not; 14 patients were HBeAg-positive (28%) and 36 were HBeAg-negative (72%). Postoperative HCC recurrence occurred in 17 patients (34%), and 33 (66%) did not present with recurrence; 30 patients (60%) had a tumor size ≥3 cm, whereas 20 patients (40%) had a tumor size <3 cm (Table II).

Comparison of cccDNA in tumor and peri-tumor tissues. For 50 patients with HBV, intrahepatic cccDNA levels were significantly higher in tumor tissues compared with those in peri-tumor tissues (44.68±65.14 vs. 11.47±23.03, P=0.001; Fig. 1A). Furthermore, the difference in intrahepatic cccDNA levels was more apparent in the tumor tissues compared with the peri-tumor tissues in the HBeAg-positive (90.07±93.94 vs. 10.05±10.48, respectively; P=0.008; Fig. 1B), tumor recurrence (65.78±79.85 vs. 8.55±7.16; P=0.002; Fig. 1C) and <3 cm tumor size (59.65±72.67 vs. 7.02±9.02; P=0.003; Fig. 1D) groups.

Comparison of cccDNA/MSL2/HBx levels in tumor and peri-tumor tissues of patients with HCC exhibiting liver cirrhosis. From the cohort of 50 patients with HBV-associated HCC in the present study, 25 with preoperative cirrhosis were included in this analysis. Of these 25 patients with cirrhosis, six samples could not be paired due to lack of tumor tissue after analysis of cccDNA when analyzing MSL2 mRNA and HBx mRNA. Therefore, HBx and MSL2 mRNA levels were measured in 19 paired samples. Of these, 14 samples were HBx-positive. In the HBx-positive patients, the cccDNA and MSL2 mRNA levels were compared in tumor and peri-tumor tissues. The results revealed that the level of cccDNA was significantly higher in tumor tissue compared with that in peri-tumor tissue, in patients with HCC exhibiting liver cirrhosis (70.42±78.56 vs. 6.36±8.47; P<0.001; Fig. 2A). Similarly, the level of MSL2 mRNA was also higher in tumor tissue compared with that in peri-tumor tissue (191.78±566.32 vs. 9.77±18.56; P=0.023; Fig. 2B). However, the level of HBx mRNA was not significantly different between the two tissue types (8.24±14.38 vs. 14.26±27.40; P=0.638; Fig. 2C).

Comparison of cccDNA/MSL2/HBx levels in tumor and peri-tumor tissues of patients receiving or not receiving antiviral therapy. Of the 50 patients, 31 had undergone antiviral treatment prior to surgery. The results of the present study demonstrated that in tumor and peri-tumor tissues, antiviral therapy did not significantly change the levels of cccDNA (43.54±69.09 vs. 45.38±61.49; P=0.624 and 11.19±10.47

Table II. Clinical characteristics of patients with HCC (n=50).

Characteristic	n (%)
Sex	
Male	40 (80)
Female	10 (20)
Pre-op serum HBV DNA	
Positive	42 (84)
Negative	8 (16)
Pre-op HBeAg status	
Positive	14 (28)
Negative	36 (72)
Pre-op antiviral treatment	
No antiviral treatment	19 (38)
Antiviral treatment	31 (62)
Operation method	
Curative resection	45 (90)
Liver transplantation	5 (10)
HBV recurrence	2 (40[a])
HCC recurrence	3 (60[a])
Post-op HCC recurrence	17 (34)
Lung or lymphatic metastasis	5 (10)
Tumor size, cm	
≥3	30 (60)
<3	20 (40)

[a]Percentage of patients who underwent liver transplantation. HCC, hepatocellular carcinoma; pre-op, pre-operative; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; post-op, post-operative.

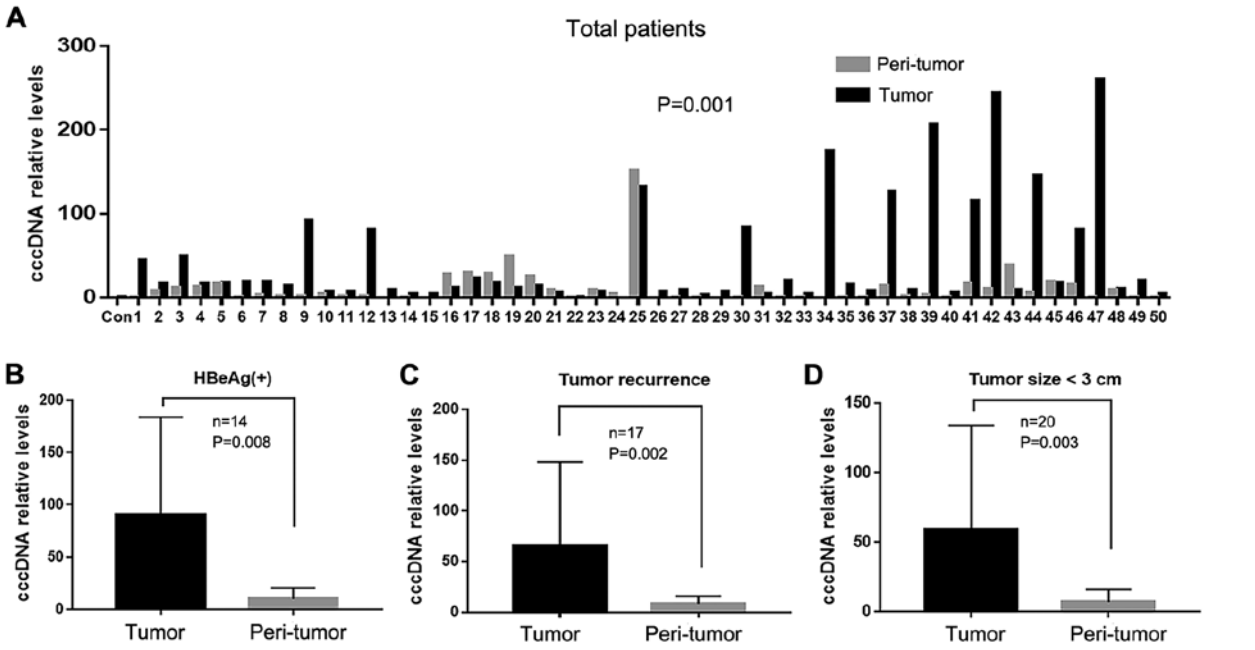

Figure 1. Comparison of intrahepatic cccDNA in tumor and peri-tumor tissues of patients with HBV-associated HCC. (A) Comparison of intrahepatic cccDNA levels in tumor and peri-tumor tissues of 50 patients (44.68±65.14 vs. 11.47±23.03; P=0.001). Liver tissue from a patient not infected with HBV was used as a control and the level was set to 1. (B) Comparison of intrahepatic cccDNA levels in tumor and peri-tumor tissues of 14 HBeAg-positive patients (90.07±93.94 vs. 10.05±10.48; P=0.008). (C) Comparison of intrahepatic cccDNA levels in tumor and peri-tumor tissues of 17 patients with HCC recurrence (65.78±79.85 vs. 8.55±7.16; P=0.002). (D) Comparison of intrahepatic cccDNA levels in tumor and peri-tumor tissues of 20 patients with a tumor size <3 cm (59.65±72.67 vs. 7.02±9.02; P=0.003). The detected cccDNA levels in patients with HBV-associated HCC were presented as fold-changes relative to those in the control patient. cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen.

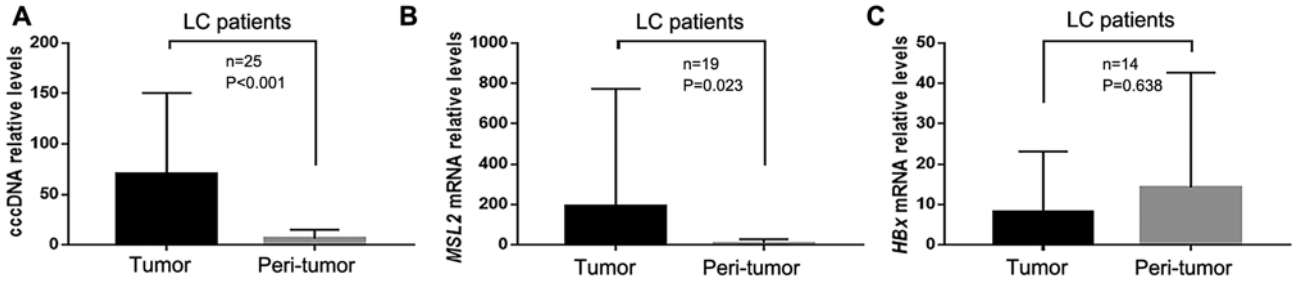

Figure 2. Comparison of cccDNA/MSL2/HBx levels in tumor and peri-tumor tissues of patients with HCC exhibiting LC. (A) Comparison of cccDNA levels in tumor and peri-tumor tissues of patients with HCC exhibiting LC (70.42±78.56 vs. 6.36±8.47; P<0.001). (B) Comparison of MSL2 mRNA levels in tumor and peri-tumor tissues of patients with HCC exhibiting LC (191.78±566.32 vs. 9.77±18.56; P=0.023). (C) Comparison of HBx mRNA levels in tumor and peri-tumor tissues of patients with HCC exhibiting LC (8.24±14.38 vs. 14.26±27.40; P=0.638). cccDNA, covalently closed circular DNA; MSL2, male-specific lethal 2; HBx, hepatitis B virus-encoded X protein; HCC, hepatocellular carcinoma; LC, liver cirrhosis.

vs. 11.64±27.77; P=0.095; Fig. 3A and B, respectively) or MSL2 mRNA (159.79±502.34 vs. 46.28±85.56; P=0.187 and 4.36±5.81 vs. 5.34±13.96; P=0.244; Fig. 3C and D, respectively). The levels of HBx mRNA in tumor and peri-tumor tissues from patients treated with antivirals were significantly lower compared with those in untreated patients (26.05±50.05 vs. 5.36±11.59; P=0.026 and 19.65±31.10 vs. 9.51±25.74; P=0.035; Fig. 3E and F, respectively).

Comparison between HBx, cccDNA and MSL2 in tumor and peri-tumor tissues of HBx-positive patients. When analyzing MSL2 mRNA and HBx mRNA, 8 of the total 50 patients could not be paired due to the lack of tumor tissues after analysis of cccDNA. Therefore, HBx mRNA and MSL2 mRNA levels were measured in 42 paired samples. Of these, HBx mRNA was only detected in 23 pairs. In these 23 HBx-positive patients, there was no significant difference in HBx mRNA levels observed between the tumor and peri-tumor tissues (23.98±42.70 vs. 24.24±34.70; P=0.527; Fig. 4A). However, HBx mRNA levels in tumor and peri-tumor tissues were positively correlated (r=0.587; P=0.003; Fig. 4B). Furthermore, the levels of cccDNA and MSL2 mRNA in the HBx-positive group were significantly higher in tumor tissues compared with those in peri-tumor tissues (38.76±56.85 vs. 10.49±12.60; P=0.026 and 55.17±87.50 vs. 8.09±14.79; P=0.013; Fig. 4C and D, respectively). However, in the HBx-negative group, these two parameters were not significantly different between tumor and peri-tumor tissues (31.90±46.27 vs. 13.82±33.23; P=0.064 and 20.95±55.00 vs. 1.19±2.19; P=0.077; Fig. 4E and F, respectively).

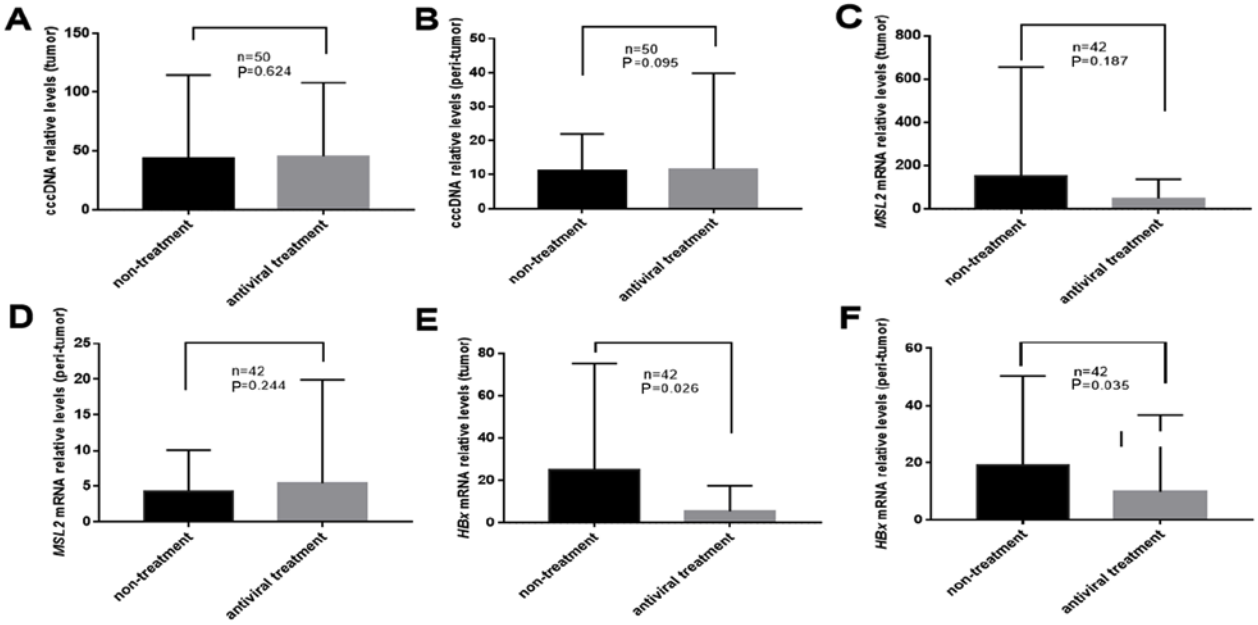

Figure 3. Comparison of cccDNA/MSL2/HBx levels in tumor and peri-tumor tissues from patients with HCC who received antiviral therapy or were untreated. (A) Comparison of cccDNA levels in tumor tissues of patients with or without antiviral therapy (43.54±69.09 vs. 45.38±61.49; P=0.624). (B) Comparison of cccDNA levels in peri-tumor tissues of patients with or without antiviral therapy (11.19±10.47 vs. 11.64±27.77; P=0.095). (C) Comparison of MSL2 mRNA levels in tumor tissues of patients with or without antiviral therapy (159.79±502.34 vs. 46.28±85.56; P=0.187). (D) Comparison of MSL2 mRNA levels in peri-tumor tissues of patients with or without antiviral therapy (4.36±5.81 vs. 5.34±13.96; P=0.244). (E) Comparison of HBx mRNA levels in tumor tissues of patients with or without antiviral therapy (26.05±50.05 vs. 5.36±11.59; P=0.026). (F) Comparison of HBx mRNA levels in peri-tumor tissues of patients with or without antiviral therapy (19.65±31.10 vs. 9.51±25.74; P=0.035). cccDNA, covalently closed circular DNA; MSL2, male-specific lethal 2; HBx, hepatitis B virus-encoded X protein.

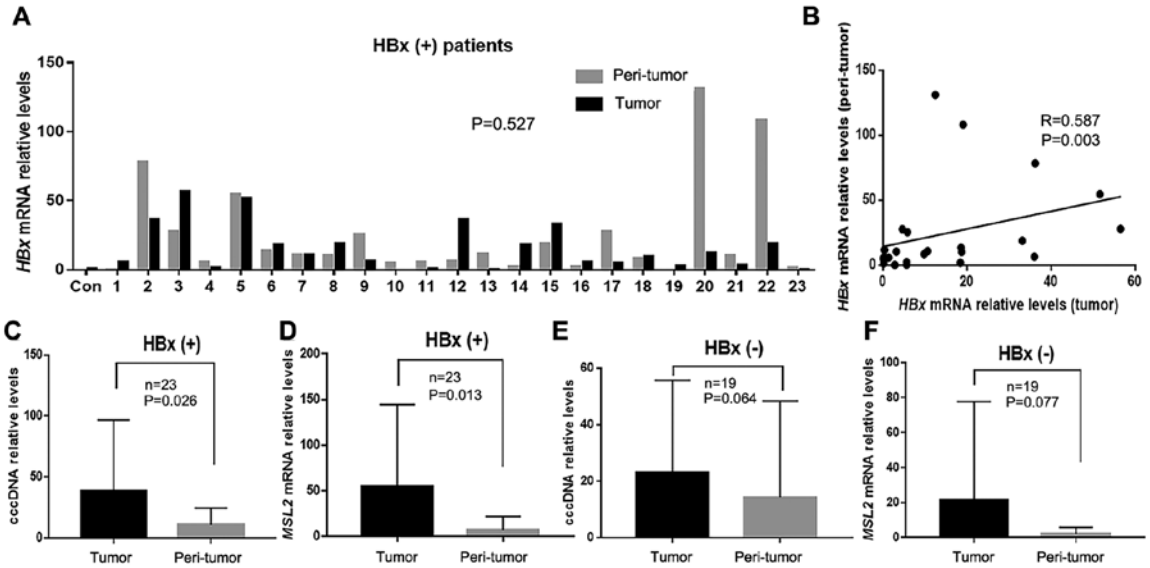

Figure 4. Relative levels of HBx mRNA in tumor and peri-tumor tissues of patients with HCC. (A) Comparison of HBx mRNA levels in tumor and peri-tumor tissues of 23 patients with HBx-positive HCC (23.98±42.70 vs. 24.24±34.70; P=0.527). The liver tissue from a patient not infected with HBV was used as a control and the level was set to 1. (B) Correlation between tumor and peri-tumor tissues based on HBx mRNA levels (r=0.587; P=0.003). Comparison of (C) cccDNA and (D) MSL2 mRNA in tumor and peri-tumor tissues of HBx-positive patients (38.76±56.85 vs. 10.49±12.60 and 55.17±87.50 vs. 8.09±14.79; P=0.026 and 0.013, respectively). Comparison of (E) cccDNA and (F) MSL2 mRNA in tumor and peri-tumor tissues of HBx-negative patients (31.90±46.27 vs. 13.82±33.23 and 20.95±55.00 vs. 1.19±2.19; P=0.064 and 0.077, respectively). The detected HBx and MSL2 mRNA levels in patients with HBV-associated HCC were presented as fold-change relative to those in the control patient. HBx, HBV-encoded X protein; HCC, hepatocellular carcinoma; cccDNA, covalently closed circular DNA; MSL4, male-specific lethal 4; HBV, hepatitis B virus.

Discussion

HBV is a small enveloped DNA virus that replicates via an RNA intermediate (18). Following infection, which is hepatocyte-specific, the capsid is transported to the nucleus and the relaxed circular (rc) DNA is released and converted into the persistent form, cccDNA. This cccDNA serves as a template for the transcription of different viral RNAs (18,19).

The 3.5-kb pregenomic RNA is encapsulated and reverse-transcribed into new rcDNA. Then, capsid-containing rcDNA is enveloped and released as newly formed virions or redirected toward the nucleus to establish a cccDNA pool. The long half-life of the cccDNA ensures the persistence of HBV in infected cells (18,20).

As the majority of patients receive different degrees of antiviral treatment following the diagnosis of hepatitis B, viral replication activity is inhibited in certain patients (21). Furthermore, new antiviral technology can detect low levels of HBV DNA and HBsAg at follow-up, even in those patients with negative HBsAg and HBV DNA loads (20,22). A previous study has reported that HBsAg, HBeAg and HBV DNA are negative in patients with seroclearance, but that cccDNA is positive in the liver tissues of all patients (23). As cccDNA maintains a stable pool in the hepatocyte nucleus, antiviral therapy cannot completely remove HBV (18). In addition, studies on liver transplantation for HBV-associated HCC have reported that high levels of cccDNA in tissues can lead to post-operative HBV recurrence despite the use of high-dose hepatitis B immunoglobulin prophylaxis during liver transplantation (24). Furthermore, univariate analyses have previously revealed significant associations between HCC recurrence and HBV reinfection (25). Therefore, the present study hypothesized that cccDNA may be involved in the development of HBV-associated HCC.

To investigate this hypothesis, the present study compared cccDNA content in tumor and peri-tumor tissues by dividing patients with HCC into three groups based on HBeAg status, tumor size and post-operative HCC recurrence. The results revealed that HBV cccDNA was present in both tumor and peri-tumor tissues, and that levels were higher in the former. It was also observed that cccDNA levels were significantly higher in tumor tissues compared with levels in peri-tumor tissues of the HBeAg-positive, <3 cm tumor size and HCC recurrence groups. HCC recurred in 8 of the 14 (57%) HBeAg-positive patients within 18 months of surgery. Therefore, it was speculated that HBV activity is associated with the formation of early tumors (tumor size, <3 cm) and tumor recurrence, and that this association may be due to the presence of cccDNA levels in liver tissues. Levels of HBeAg as a serological marker can indicate the state of viral replication activity in patients with chronic hepatitis B (26). A number of studies have reported that viral replication is more robust in HBeAg-positive patients, which leads to inflammatory liver injury (27-29) and a higher risk of tumorigenesis compared with HBeAg-negative patients (29). It has also been suggested that HBeAg-positive patients are at risk of early post-operative HCC recurrence (30-32). Therefore, the present study hypothesized that HBV cccDNA may exert a tumorigenic effect on HBV-associated HCC, particularly in HBeAg-positive patients with strong viral activity. Furthermore, cccDNA, MSL2 mRNA and HBx mRNA levels were compared in tumor and peri-tumor tissues in patients with HBV-associated HCC who exhibited preoperative cirrhosis. It was revealed that, in patients with cirrhosis, the levels of cccDNA and MSL2 mRNA were also significantly higher in tumor tissues compared with levels in peri-tumor tissues. However, HBx mRNA was not significantly different between the two tissue types. These results further indicate that cccDNA and MSL2 are oncogenic during the development of HBV-associated HCC.

The present study assessed whether the cccDNA, MSL2 and HBx levels were changed by antiviral therapy. The results revealed no significant difference in cccDNA and MSL2 mRNA levels between the antiviral treatment and untreated groups. However, HBx mRNA in the tumor and peri-tumor tissues of the antiviral treatment group was significantly lower compared with that in the untreated group. These results suggest that antiviral therapy can affect the tumorigenic effect of cccDNA and MSL2 by decreasing the amount of HBx. Therefore, the present study further verified the association between levels of cccDNA and MSL2 mRNA in the HBx-positive and HBx-negative groups.

The present study detected the levels of HBx and MSL2 mRNA in 42 pairs of tumor and peri-tumor tissue. The results revealed that HBx mRNA was detectable in 23 of these tissue pairs, but not in the other 19. The HBx mRNA levels in 23 pairs of tumor and peri-tumor tissues were not significantly different; however, in terms of expression, there was a positive correlation between tumor and peri-tumor tissues. Furthermore, in the HBx-positive group, the levels of cccDNA and MSL2 mRNA were significantly higher in tumor tissues compared with those in peri-tumor tissues. However, in the HBx-negative group, the levels of these markers were not significantly different between these two types of tissue. These results suggest that in the presence of HBx, cccDNA and MSL2 may regulate the formation of HBV-associated HCC. Gao *et al* (13) reported that HBx-elevated MSL2 mRNA led to the activation of HBV cccDNA in hepatoma cells, which promoted hepatocarcinogenesis and formed a positive feedback loop of HBx/MSL2/cccDNA/HBV. However, the molecular mechanism underlying the involvement of cccDNA in patients with HBV-associated HCC receiving antiviral therapy remains unclear. Furthermore, regional differences are also an influencing factor for liver cancer. Therefore, the molecular mechanism underlying the effects of antiviral therapy on tumorigenesis were analyzed from the perspective of cccDNA, MSL2 and HBx in patients from the Seoul National University College of Medicine. The results from the present study are also consistent with those in previous studies, as cccDNA was significantly higher in the tumor tissues compared with that in peri-tumor tissues. Furthermore, it was demonstrated that antiviral therapy can inhibit HBx mRNA expression, but does not directly affect tumor cccDNA and MSL2 levels. These findings provide a guide for future research on antiviral therapy for HBV-associated HCC.

One limitation of the present study was that the specimens collected were taken only once from explanted liver samples during surgery. Since biopsy specimens were not obtained during pre- and postoperative follow-up, changes in cccDNA levels in the liver tissue during tumorigenesis could not be determined, and levels of cccDNA, MSL2 mRNA and HBx mRNA during tumorigenesis could only be inferred from intraoperative specimens. In addition, due to insufficient samples in the present study, we were unable to observe expression of MSL2 and HBx at the protein level. Thus, further research is required in order to address this issue. Another limitation of the present study is that the association between cccDNA and MSL2 mRNA was not analyzed at the sequence level. Certain key mutations in HBV cccDNA have been reported to have prognostic value for patients with HBV-associated

HCC (33-35). Therefore, further research should include a genetic analysis of HBV cccDNA mutations to fully elucidate the association between HBV cccDNA and HBV-associated HCC.

In summary, cccDNA may promote the development of HBV-associated HCC, particularly in HBeAg-positive patients with high levels of viral replication. This phenomenon may occur as a result of crosstalk between HBx, MSL2 and cccDNA. Decreasing HBx levels via antiviral therapy may inhibit this process. However, this should be investigated by performing additional experiments, such as studying the interaction between cccDNA and MSL2, and performing genetic analyses on HBV cccDNA mutations.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XJ, NY, KL and KS designed the study. XJ and SL performed the experiments. XJ, SH, HK, NY, KL and KS analyzed the data. XJ, SH and KS wrote the article. All authors approved the final published version of this article.

Ethics approval and consent to participate

The experimental protocol was approved by the Institutional Review Board of Seoul National University Hospital (IRB no. H-1809-001-967). Written consent, approving the use of tissues for research following surgery, was obtained from each patient included in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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