의학박사 학위논문

Antiviral therapy reduces MSL2 by downregulation of HBx, without direct effect on cccDNA level in patients with hepatocellular carcinoma

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Abstract

Antiviral therapy reduces MSL2 by downregulation of HBx, without direct effect on cccDNA level in patients with hepatocellular carcinoma

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Introduction

Hepatitis B virus (HBV) infection is a global health problem and chronic hepatitis B is the leading cause of hepatocellular carcinoma. The covalently closed circular DNA (cccDNA) is an intermediate in the life cycle of HBV virus. HBV-encoded X protein (HBx), a key viral oncoprotein, can be specifically modified by E3 ubiquitin ligase, such as Male Specific Lethal 2 (MSL2), a process called ubiquitination that up-regulates HBx activity to promote transcription, cell proliferation and tumor growth. This 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. Moreover, we clarified the impact of antiviral therapy on these indicators.

Methods

The levels of intrahepatic cccDNA, *MSL2* mRNA and *HBx* mRNA in 50 patients with HBV-related HCC who had undergone liver surgery were compared. Real-Time PCR assay was performed to quantify these indicators.

Results

A total of 50 patients were included in this study (49 HBsAg positive and 1 showing seroconversion after antivirus treatment). Before surgery, 31 of them had undergone antiviral treatment and 19 had not. Intrahepatic

cccDNA levels ware significantly higher in the tumor tissues than in peri-tumor tissues (44.68 ± 65.14 vs. 11.47 ± 23.03; p = 0.001). The cccDNA level in the tumor and peri-tumor tissues was significantly different, especially in the HBeAg positive group (90.07 ± 93.94 vs. 10.05 ± 10.48; p = 0.008), tumor recurrence group (p = 0.002) and tumor size less than 3cm group (p = 0.003). Moreover, in patients with preoperative cirrhosis, levels of cccDNA and *MSL2* mRNA were significantly higher in tumor tissue than in peri-tumor tissue ($p < 0.001$ and p = 0.023). The levels of *HBx* mRNA in tumor and peri-tumor tissues were significantly lower in the antiviral treated group than in untreated group (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 than in peri-tumor tissues (p = 0.026 and 0.013). Moreover, *MSL2* mRNA levels were significantly higher in HBx-positive tumor tissues than in HBx-negative tumor tissues (p = 0.002). However, there was no difference in cccDNA levels between HBx-positive tumor tissues and HBx-negative tumor tissues (p = 0.609).

Conclusion

cccDNA level was higher in tumor tissue and antiviral therapy can modulate hepatocarcinogenesis by reducing levels of HBx to inhibit the tumorigenic effect of MSL2 and cccDNA.

Keywords: Hepatitis B, cccDNA, HBV DNA, HCC, MSL2, HBx, HCC recurrence

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Contents

Legends of Table and Figures

1. Introduction

Hepatitis B virus (HBV) infection is a global health concern that causes more than one million deaths 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 cancer and the third highest cause of cancer-associated mortality, 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). HBV-infected chronic hepatitis B is a leading precursor of HCC; however, little is 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 showed that HCC often arises as HBV replication intensifies during the late stage of hepatitis B (6), a period in which cccDNA becomes predominant in quantity (7). Therefore, it might be possible to predict the pathogenesis of HBV-related HCC by measuring 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-related 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 up-regulate 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). Yuen Gao et al. reported that HBx-mediated up-regulation of MSL2 activates HBV cccDNA in hepatoma cells to promote hepatocarcinogenesis, forming a HBx/MSL2/cccDNA/HBV positive feedback loop (13). This 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. Moreover, we clarified the effect of antiviral therapy on these indicators.

2. Methods

2.1 Patients

The study included a total of 53 patient specimens, including three patients without hepatitis B as a negative control group, The contents of cccDNA, *MSL2* mRNA, *HBx* mRNA in the negative control group were determined. 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 negative sample, based on a fixed control group, and added to each PCR analysis; fold changes were determined based on relative levels of cccDNA, *MSL2* mRNA, and *HBx* mRNA. 50 HBV-related HCC patients who had undergone curative liver resection or liver transplantation at the Department of Surgery, Seoul National University Hospital, between October 2016 and March 2018. Samples were prepared with tumor and peri-tumor liver tissues collected from these patients. Patient HBsAg, HBV DNA levels, and antiviral therapy history were obtained through medical records. The diagnosis of HCC was confirmed by histological examination of the tissues. No patient had consumed alcohol or had been infected with hepatitis C or hepatitis D viruses. Tumor and peri-tumor tissues were resected and rapidly frozen and stored at−80 °C. Written consent, approving the use of tissues for research purpose after the operation, was obtained from each patient. The experimental protocol was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. H-1809-001-967).

2.2 Isolation of intrahepatic total DNA and cccDNA

Total genomic DNA was extracted from approximately 20–30 mg of liver tissue using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The concentration of total DNA was determined at 260 nm with a spectrophotometer (Eppendorf, Hamburg, Germany). Total DNA was digested using Plasmid-safe^{TM} ATP-dependent DNase (Epicentre Technologies, Madison, WI), which hydrolyzes linear double-stranded DNA as well as linear and closed circular single-stranded DNA for the detection of HBV cccDNA (14). The remaining extracted total DNA was used for the detection of β-actin.

2.3 cccDNA detection by quantitative real-time polymerase chain reaction (RT-qPCR)

Intrahepatic levels of cccDNA in tumor and peri-tumor tissues were compared. cccDNA was extracted from 300 mg of total DNA and diluted with 20 ml of DEPC water. Then, 1 ml of cccDNA was added for RT-qPCR amplification (7500 Real-time PCR Instrument system, Applied Biosystems, Foster City, Calif, USA), which were performed with TOPreal^{TM} qPCR PreMIX SYBR Green (Enzynomics, Cheongju, Korea). β-actin amplicons were used as the internal reference for subsequent PCR analysis. Each sample was assayed for three times to determine the mean cycle threshold (Ct) values for HBV cccDNA and β-actin. Relative transcriptional fold-changes were calculated as $2^{-\triangle\triangle Ct}$ (15). The detected cccDNA levels in patients with HBV-related HCC was presented as a fold-change relative to that in the control group (patient without hepatitis B). Thermal cycling was performed as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C (15 s), 63 °C (30 s), 72 °C (25 s), and 95 °C (15s) (16). The sequence of the PCR primers is listed in Table 1 (17).

2.4 MSL2 mRNA and HBx mRNA detection by reverse-transcription polymerase chain reaction (RT-PCR) and RT-qPCR

Total RNA was extracted from tumor and peri-tumor tissues using TRIzol reagent (Thermo, Fisher, MA) according to the manufacturer's instructions. A first-strand cDNA synthesis kit (Thermo, Fisher, MA) was used to reverse transcribe total RNA into cDNA, served as the templates for RT-qPCR, which were performed with TOPreal^{TM} qPCR PreMIX SYBR Green (Enzynomics, Cheongju, Korea). β-actin was used as an internal control for normalization. Relative transcriptional fold-changes were calculated as $2^{-\triangle\triangle Ct}$. Thermal cycling was performed as follows: 95 °C for 10 min, followed by 45 cycles of 95 °C (15 s), 63 °C (30 s), 72 °C (25 s), and 95 °C (15s). All primers are listed in Table 1. Liver tissue from patient not infected with HBV were used as controls. *MSL2* and *HBx* mRNA levels in patients with HBV-related HCC were presented as fold-change relative to levels in the control group.

2.5 Statistical analysis

All statistical analyses were performed using SPSS 23. (SPSS Inc. Chicago, IL). Variables with normal and skewed distribution of paired samples were analyzed with paired t-tests and Wilcoxon signed ranks tests, respectively. The Mann-Whitney U test was used to analyze continuous variables. Correlation analyses was performed by Spearman correlation analysis. $P < 0.05$ was considered statistically significant. Data are expressed as the mean ± standard deviation (SD), and bars in the graph represent standard deviation.

3. Results

3.1 Clinical characteristics of patients

Fifty HBV-related HCC patients were included in our study (40 males and 10 females; mean age, 58 ± 10 years). Of them, 49 were HBsAg-positive and one showed seroconversion after antiviral treatment; 31 patients had received different degrees of antiviral therapy, whereas 19 had not; 14 patients were HBeAg-positive (28%) and 36 were HBeAg-negative (72%). Post-operative HCC recurrence was 30% (n = 17), and 33 (70%) 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 2).

3.2 Comparison of cccDNA in tumor and peri-tumor tissues

We divided the 50 patients into three groups according to HBeAg status, tumor size, and HCC recurrence. HBeAg status and tumor size were obtained through medical records and tumor recurrence after surgery was obtained by follow-up records until September 2018. For all specimens, intrahepatic cccDNA levels were significantly higher in tumor tissues than in peri-tumor tissues (44.68 ± 65.14 vs. 11.47 ± 23.03, respectively; p = 0.001; **Fig. 1A**). Further, this difference was more obvious in the HBeAg-positive (90.07 ± 93.94 vs. 10.05 ± 10.48, respectively; p = 0.008), tumor recurrence (p = 0.002, Wilcoxon signed rank test) and < 3cm tumor size (p = 0.003, Wilcoxon signed rank test; **Fig. 1B–D**) groups. Moreover, in all patients, the level of cccDNA was significantly higher than that in control group tissues, and even 100-fold higher in some samples.

3.3 Comparison of cccDNA/MSL2/HBx levels in tumor and peri-tumor tisues of HCC patients with liver cirrhosis

From our cohort of 50 patients with HBV-related HCC, 25 with preoperative cirrhosis were included in this analysis. Of these, six samples could not be paired due to lack of tumor tissue. Therefore, *HBx* mRNA and *MSL2* mRNA levels were measured in 19 paired samples. Of these 14 samples were HBx- positive. In these patients, we compared cccDNA and *MSL2* mRNA levels in tumor and peri-tumor tissues. The results showed that the level of cccDNA was significantly higher in tumor tissue than in peri-tumor tissue ($p < 0.001$, Wilcoxon signed rank test; **Fig. 2A**). Similarly, the level of *MSL2* mRNA was also higher in tumor tissue than in peri-tumor tissue ($p = 0.023$, Wilcoxon signed rank test; **Fig. 2B**). However, the level of *HBx* mRNA was not significantly different between the two tissue types ($p = 0.638$, Wilcoxon signed rank test; **Fig. 2C**)

3.4 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 before surgery. Our results showed that in tumor and peri-tumor tissues, antiviral therapy did not significantly change the levels of cccDNA ($p = 0.624$ and 0.095, respectively, Mann-Whitney U test; **Fig. 3A, B**) and *MSL2* mRNA ($p = 0.187$ and 0.244, respectively,Mann-Whitney U test) (**Fig. 3C, D**). The levels of *HBx* mRNA in tumor and peri-tumor tissues from patients treated with antivirals were significantly lower than those in untreated patients ($p = 0.026$ and 0.035, respectively, Mann-Whitney U test; **Fig. 3E, F**).

3.5 Comparison of HBx, cccDNA, and MSL2 in tumor and peri-tumor tissues of HBx-positive patients

Because of the lack of tumor tissues, eight samples could not be paired. Therefore, *HBx* mRNA and *MSL2* mRNA levels were measured in 42 paired samples. Of these, *HBx* mRNA was only detected in 23 pairs (23/42). In HBx-positive patients, no difference in *HBx* mRNA levels were found

between tumor and peri-tumor tissues (p = 0.527, Wilcoxon signed rank test; **Fig. 4A**). However, *HBx* mRNA levels in tumor and peri-tumor tissues were positively correlated (r = 0.587; p = 0.003, Spearman's rank correlation; **Fig. 4B**). Moreover, the levels of cccDNA and *MSL2* mRNA in the HBx-positive group were significantly higher in tumor tissues than in peri-tumor tissues (p = 0.026 and 0.013, respectively, Wilcoxon signed rank test; **Fig. 4C, D**). However, in the HBx-negative group, these two parameters were not different between tumor and peri-tumor tissues (p = 0.064 and 0.077, respectively, Wilcoxon signed rank test; **Fig. 4E, F**).

3.6 Comparison of cccDNA and MSL2 levels in tumor tissues of HBx positive patients and negative patients

In 42 pairs tumor and peri-tumor tissues, *HBx* mRNA was detected in 23 pairs samples. The *MSL2* mRNA levels were significantly higher in HBx-positive tumor tissues than in HBx-negative tumor tissues (p = 0.002, Mann-Whitney U test; **Fig. 5A**). However, there was no difference in cccDNA levels between HBx-positive tumor tissues and HBx-negative tumor tissues (p = 0.609, Mann-Whitney U test; **Fig. 5B**).

4. Discussion

HBV is a small enveloped DNA virus that replicates via an RNA intermediate. After infection, which is hepatocyte-specific, the capsid is transported to the nucleus and the relaxed circular DNA is released and converted into the persistent form, cccDNA. This cccDNA serves as a template for the transcription of different viral RNAs. The 3.5-kb pregenomic RNA is encapsidated 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).

Since most patients receive different degrees of antiviral treatment after being diagnosed with hepatitis B, viral replication activity is inhibited in some patients. Moreover, new antiviral technology can detect low levels of HBV DNA and HBsAg at follow-up, even in some patients with negative HBsAg and HBV DNA loads (20, 21). Studies have 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 (22). Because cccDNA maintains a stable pool in the hepatocyte nucleus, antiviral therapy cannot completely remove HBV (18). In addition, studies on liver transplantation for HBV-related 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 (HBIG) prophylaxis during liver transplantation (23). Further, univariate analysis previously showed a significant correlation between HCC recurrence and HBV reinfection (24). Therefore, we speculated that cccDNA might be involved in the development of HBV-related HCC. To verify this hypothesis, we compared cccDNA content in tumor and peri-tumor tissues by dividing patients into three groups based on HBeAg status, tumor size, and post-operative HCC recurrence. The results showed that HBV cccDNA was present in both tumor and peri-tumor tissues, and that levels were higher in the former. We further discovered that cccDNA levels were significantly higher in tumor tissues than in peri-tumor tissues of the HBeAg-positive, < 3cm tumor size, and HCC recurrence groups. HCC recurred in eight of 14(57%) HBeAg-positive patients with in 18 months after surgery. Therefore, we speculated that HBV activity is related to the formation of early tumors (size, < 3cm) and tumor recurrence, and that this relationship might be reflected by 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. Some studies have reported that viral replication is more robust in HBeAg-positive patients, which leads to inflammatory liver injury (25-27) and a higher risk of tumorigenesis

compared to that in HBeAg-negative patients (27); it was also suggested that HBeAg-positive patients are at risk of early post-operative HCC recurrence (28-30). Therefore, we hypothesized that HBV cccDNA might have a tumorigenic effect on HBV-related HCC, especially in HBeAg-positive patients with strong viral activity. Further, we compared cccDNA, *MSL2* mRNA and *HBx* mRNA levels in tumor and peri-tumor tissues in patients with HBV-associated HCC with preoperative cirrhosis. We found that the levels of cccDNA and *MSL2* mRNA were also significantly higher in tumor tissue than in peri-tumor tissue. 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-related HCC.

Next, we analyzed whether the cccDNA, MSL2, and HBx levels were changed by antiviral therapy. The results showed no significant difference in cccDNA and *MSL2* mRNA levels between the antiviral treatment and untreated groups. However, *HBx* mRNA levels in the tumor and peri-tumor tissues of the antiviral treatment group were significantly lower than those in the untreated group. Regarding this phenomenon, we can assume that antiviral therapy can affect the tumorigenic effect of cccDNA and MSL2 by reducing the amount of HBx. Therefore, we further verified the relationship between levels of cccDNA and MSL2 mRNA in the HBx-positive and HBx-negative groups.

We detected levels of HBx and MSL2 mRNA in 42 pairs of tumor and peri-tumor tissue. The results showed 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 different; however, in terms of expression, there was a positive correlation between tumor and peri-tumor tissues. Moreover, in the HBx-positive group, the levels of cccDNA and MSL2 mRNA were significantly higher in tumor tissues than in peri-tumor tissues. However, in the HBx-negative group, the levels of these markers were not different between these two tissue types. These findings suggest that in the presence of HBx, cccDNA and MSL2 might regulate the

formation of HBV-related HCC. Yuen Gao et al. reported that HBx-elevated MSL2 activated HBV cccDNA in hepatoma cells to promote hepatocarcinogenesis, forming a positive feedback loop of HBx/MSL2/ cccDNA/HBV. However, the mechanism of cccDNA on HBV-related HCC patients with receiving antiviral therapy was still unclear. Moreover, regional differences are also an influencing factor for liver cancer. Therefore, we analyzed the mechanism underlying the effects of antiviral therapy on tumorigenesis from the perspective of cccDNA, MSL2 and HBx in our local patients. Our results are also consistent with previous studyies, as the cccDNA levels were significantly higher in tumor tissue than in peri-tumor tissue. Further, we found that antiviral therapy can inhibit HBx mRNA expression, but it does not directly affect the tumorigenic cccDNA and MSL2. In addition, *MSL2* mRNA levels were significantly higher in HBx-positive tumor tissues than in HBx-negative tumor tissues. However, there was no difference in cccDNA levels between HBx-positive tumor tissues and HBx-negative tumor tissues. This finding will provide a powerful guide for future research on antiviral therapy for HBV-related HCC.

One limitation of this study was that the specimens collected in this study were taken only once from explant liver samples during surgery. Since biopsy specimens were not obtained during pre-operative and post-operative 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 this study, we were unable to observe MSL2 and HBx levels at the protein expression level. Thus, further research is required to address this issue. Another limitation of this study is that we did not analyze the relationship between cccDNA and *MSL2* mRNA at the sequence level. Some key mutations in HBV cccDNA have been reported to have prognostic value for patients with HBV-related HCC (31-33). Therefore, further research needs to include a genetic analysis of HBV cccDNA mutations to fully elucidate the relationship between HBV cccDNA and HBV-related HCC.

In summary, cccDNA might promote the development of HBV-related HCC, particularly in HBeAg-positive patients with high viral replication activity. This phenomenon might occur as a result of a crosstalk between HBx, MSL2, and cccDNA. Reducing HBx levels through antiviral therapy might inhibit this process. However, this should be investigates by performing additional experiments like studying the interaction between cccDNA and MSL2 and the performing genetic analysis of HBV cccDNA mutations.

Reference

1. Aljarbou AN. The Emergent Concern of Hepatitis B globally with special attention to Kingdom of Saudi Arabia. International journal of health sciences. 2013;7(3):333-40.
2. Allweiss L, Dandri M. The Role of cccDNA in HBV Maintenance. Viruses. 2017;9(6).
3. Joachim Lupberger EH. Hepatitis B virus-induced oncogenesis. World Journal of Gastroenterology. 2007.
4. Blum HE. Hepatocellular carcinoma: therapy and prevention. World J Gastroenterol. 2005;11(47):7391-400.
5. Wu M, Li J, Yue L, Bai L, Li Y, Chen J, Zhang X, Yuan Z. Establishment of Cre-mediated HBV recombinant cccDNA (rcccDNA) cell line for cccDNA biology and antiviral screening assays. Antiviral Res. 2018;152:45-52.
6. Mazet-Wagner AA, Baclet MC, Loustaud-Ratti V, Denis F, Alain S. Real-time PCR quantitation of hepatitis B virus total DNA and covalently closed circular DNA in peripheral blood mononuclear cells from hepatitis B virus-infected patients. J Virol Methods. 2006; 138(1-2):70-9.
7. Wong DK, Yuen MF, Yuan H, Sum SS, Hui CK, Hall J, Lai CL. Quantitation of covalently closed circular hepatitis B virus DNA in

chronic hepatitis B patients. Hepatology. 2004;40(3):727-37.
8. Ng SA, Lee C. Hepatitis B virus X gene and hepatocarcinogenesis. J Gastroenterol. 2011;46(8):974-90.
9. Zhang X, Zhang H, Ye L. Effects of hepatitis B virus X protein on the development of liver cancer. J Lab Clin Med. 2006;147(2):58-66.
10. Ding R, Han S, Lu Y, Guo C, Xie H, Zhang N, Song Z, Cai L, Liu J, Dou K. Overexpressed Id-1 is associated with patient prognosis and HBx expression in hepatitis B virus-related hepatocellular carcinoma. Cancer Biology & Therapy. 2014;10(3):299-307.
11. Rabut G, Peter M. Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep. 2008;9(10):969-76.
12. Villa R, Forne I, Muller M, Imhof A, Straub T, Becker PB. MSL2 combines sensor and effector functions in homeostatic control of the Drosophila dosage compensation machinery. Mol Cell. 2012;48(4):647-54.
13. Gao Y, Feng J, Yang G, Zhang S, Liu Y, Bu Y, Sun M, Zhao M, Chen F, Zhang W, et al. Hepatitis B virus X protein-elevated MSL2 modulates hepatitis B virus covalently closed circular DNA by inducing degradation of APOBEC3B to enhance hepatocarcinogenesis. Hepatology. 2017;66(5):1413-29.
14. Scott Bowden Kj, Margaret Littlejohn, and Stephen Locarnini. Quantification Of HBV Covalently Closed Circular DNA from Liver Tissue by Real-Time PCR. Methods in Molecular Medicine. 2004;95:41-50.
15. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
16. Duan BW, Lu SC, Lai W, Liu XE, Liu Y. The detection of (total and ccc) HBV DNA in liver transplant recipients with hepatitis B vaccine against HBV reinfection. Hum Vaccin Immunother. 2015;11(10):2490-4.
17. Kim JW, Lee SH, Park YS, Hwang JH, Jeong SH, Kim N, Lee DH. Replicative activity of hepatitis B virus is negatively associated with methylation of covalently closed circular DNA in advanced hepatitis B

virus infection. Intervirology. 2011;54(6):316-25.
18. Schreiner S, Nassal M. A Role for the Host DNA Damage Response in Hepatitis B Virus cccDNA Formation-and Beyond? Viruses. 2017;9(5).
19. Fu S, Li N, Zhou PC, Huang Y, Zhou RR, Fan XG. Detection of HBV DNA and antigens in HBsAg-positive patients with primary hepatocellular carcinoma. Clin Res Hepatol Gastroenterol. 2017;41(4): 415-23.
20. Chuaypen N, Sriprapun M, Praianantathavorn K, Payungporn S, Wisedopas N, Poovorawan Y, Tangkijvanich P. Kinetics of serum HBsAg and intrahepatic cccDNA during pegylated interferon therapy in patients with HBeAg-positive and HBeAg-negative chronic hepatitis B. J Med Virol. 2017;89(1):130-8.
21. Roche B, Feray C, Gigou M, Roque-Afonso AM, Arulnaden JL, Delvart V, Dussaix E, Guettier C, Bismuth H, Samuel D. HBV DNA persistence 10 years after liver transplantation despite successful anti-HBS passive immunoprophylaxis. Hepatology. 2003;38(1):86-95.
22. Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J Med Virol. 2009;81(1):27-33.
23. Chauhan R, Lingala S, Gadiparthi C, Lahiri N, Mohanty SR, Wu J, Michalak TI, Satapathy SK. Reactivation of hepatitis B after liver transplantation: Current knowledge, molecular mechanisms and implications in management. World J Hepatol. 2018;10(3):352-70.
24. Song GW, Ahn CS, Lee SG, Hwang S, Kim KH, Moon DB, Ha TY, Jung DH, Park GC, Kang SH, et al. Correlation between risk of hepatitis B virus recurrence and tissue expression of covalently closed circular DNA in living donor liver transplant recipients treated with high-dose hepatitis B immunoglobulin. Transplant Proc. 2014;46(10): 3548-53.
25. Ganem D. hepatitis B virus infection-natural history and clinical consequences. The New England Journal of Medicine. 2004 sep:16; 351(12):.

26. Wang Q, Lin L, Yoo S, Wang W, Blank S, Fiel MI, Kadri H, Luan W, Warren L, Zhu J, et al. Impact of non-neoplastic vs intratumoural hepatitis B viral DNA and replication on hepatocellular carcinoma recurrence. Br J Cancer. 2016;115(7):841-7.
27. YANG H-I. HEPATITIS B e ANTIGEN AND THE RISK OF HEPATOCELLULAR CARCINOMA. The New England Journal of Medicine. 2002.
28. Sun HC, Zhang W, Qin LX, Zhang BH, Ye QH, Wang L, Ren N, Zhuang PY, Zhu XD, Fan J, et al. Positive serum hepatitis B e antigen is associated with higher risk of early recurrence and poorer survival in patients after curative resection of hepatitis B-related hepatocellular carcinoma. J Hepatol. 2007;47(5):684-90.
29. Kubo S, Hirohashi K, Yamazaki O, Matsuyama M, Tanaka H, Horii K, Shuto T, Yamamoto T, Kawai S, Wakasa K, et al. Effect of the presence of hepatitis B e antigen on prognosis after liver resection for hepatocellular carcinoma in patients with chronic hepatitis B. World J Surg. 2002;26(5):555-60.
30. K s. factors linked to early recurrence of small hepatocellular carcinima after hepatectomy; univariate and multivarate analysis. hepatology. 1991:802-5.
31. Jiao F, Long L, Ding S, Xie X, Jia L, Lu F. Complete genome sequencing and clinical analysis of intrahepatic hepatitis B virus cccDNA from HCC. Microb Pathog. 2017;109:49-55.
32. Yang G, Han M, Chen F, Xu Y, Chen E, Wang X, Liu Y, Sun J, Hou J, Ning Q, et al. Hepatitis B virus genotype B and mutations in basal core promoter and pre-core/core genes associated with acute-on-chronic liver failure: a multicenter cross-sectional study in China. Hepatol Int. 2014;8(4):508-16.
33. Xu H, Zhao M, Lou G, Zheng M, Cao Q, Chen Z. New point mutations in surface and core genes of hepatitis B virus associated with acute on chronic liver failure identified by complete genomic sequencing. PLoS One. 2015;10(4):e0123139.

Table 1. Primers used in quantitative real–time PCR for the detection of HBV cccDNA, MSL2, HBx, and β–actin

Gene		Primer sequence
cccDNA	Forward:	5’-GCG GWC TCC CCG TCT GTG CC-3'
		5’-GTC TGT GCC TTC TCA TCT GC-3'
	Reverse:	5’-GTC CAT GCC CCA AAG CCA ACC-3'
MSL2	Forward:	5’-ACA GTG AGA AAG TTC AGC CA-3'
	Reverse:	5’-AGC ACG CCC ACA TTT ACT-3'
HBx	Forward:	5’-ATG GCT GCT AGG CTG TGC-3'
	Reverse:	5’-TTA GGC AGA GGG GAA AAA GTT G-3'
β–actin	Forward:	5’-GTG CAC CTG ACT CCT GAG GAG A-3'
	Reverse:	5’-CCT TGA TAC CAA CCT GCC CAG-3'

Table 2. Clinical characteristics of Hepatocellular carcinoma patients (50)

Variable	n (%)
Age	34–80 (5810)
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 anti-viral treatment	19 (38%)
Anti-viral treatment	31 (62%)
Operation method	
Curative resection	45/50 (90%)
Liver transplantation	5/50 (10%)
HBV recurrence	2/5
HCC recurrence	3/5
Post-op HCC recurrence	17/50 (30%)
Lung or lymphatic metastasis	5/50 (10%)
Tumor size	
≥ 3cm	30 (60%)
< 3cm	20 (40%)

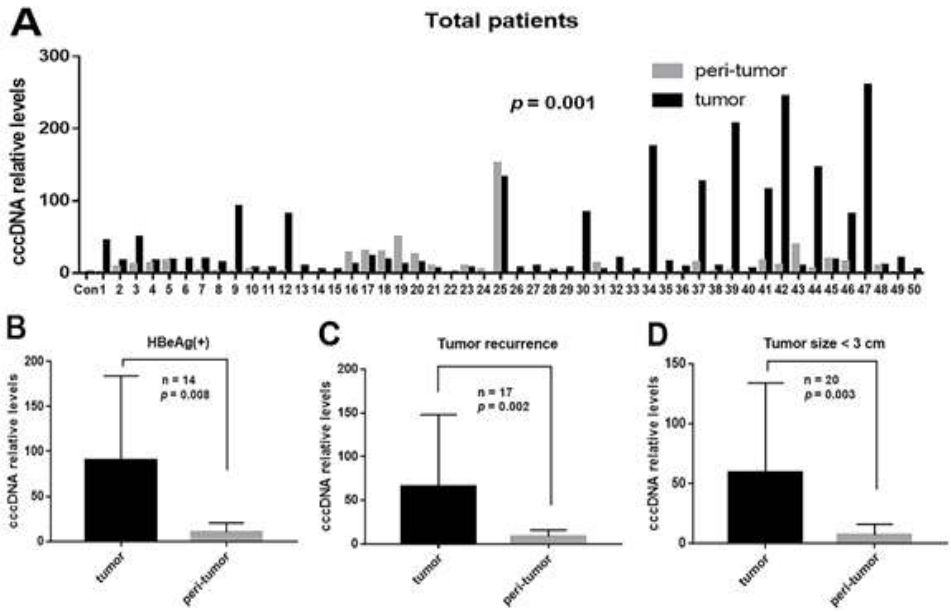

Fig. 1. Comparison of intrahepatic covalently closed circular (cccDNA) in tumor and peri-tumor tissues of patients with hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). (A) Comparison of intrahepatic cccDNA levels in tumor and peri-tumor tissues of 50 patients (44.68 ± 65.14 vs. 11.47 ± 23.03; p = 0.001, paired t-test). 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, paired t-test). (C) Comparison of intrahepatic cccDNA levels in tumor and peri-tumor tissues of 17 patients with HCC recurrence (z = −3.101, p = 0.002, Wilcoxon signed rank test). (D) Comparison of intrahepatic cccDNA levels in tumor and peri-tumor tissues of 20 patients with a tumor size < 3cm (z = −2.987, p = 0.003, Wilcoxon signed rank test). The detected cccDNA levels in patients with HBV-related HCC were presented as fold-changes relative to those in the control patient.

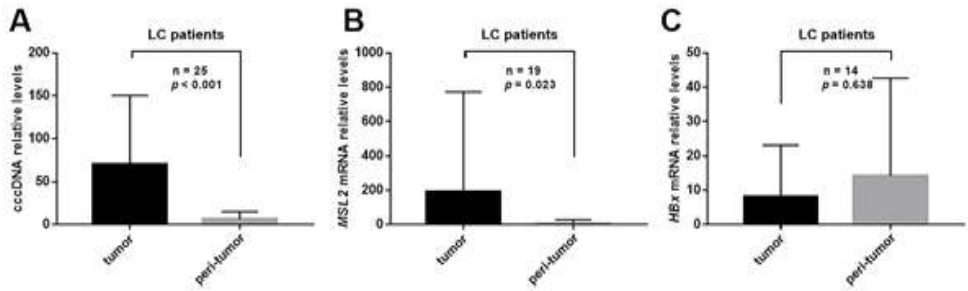

Fig. 2. Comparison of cccDNA/MSL2/HBx levels in tumor and peri-tumor tissues of hepatocellular carcinoma patients with liver cirrhosis. (A) Comparison of cccDNA levels in tumor and peri-tumor tissues of hepatocellular carcinoma patients with liver cirrhosis ($p < 0.01$, Wilcoxon signed rank test). (B) Comparison of MSL2 mRNA levels in tumor and peri-tumor tissues of hepatocellular carcinoma patients with liver cirrhosis ($p < 0.023$, Wilcoxon signed rank test) (C) Comparison of HBx mRNA levels in tumor and peri-tumor tissues of hepatocellular carcinoma patients with liver cirrhosis ($p < 0.638$, Wilcoxon signed rank test). LC, liver cirrhosis.

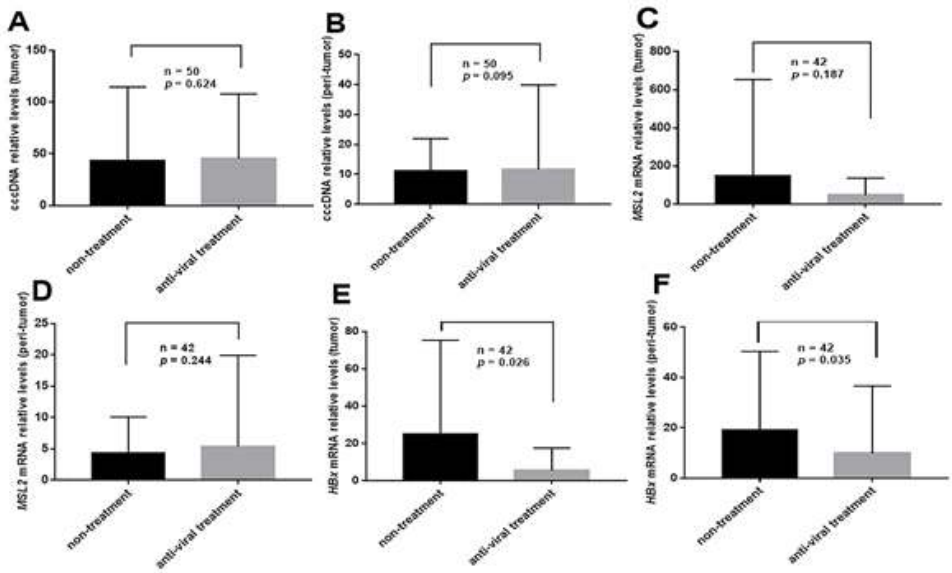

Fig. 3. Comparison of cccDNA/MSL2/HBx levels in tumor and peri-tumor tissues of hepatocellular carcinoma patients receiving or not receiving antiviral therapy. (A) Comparison of cccDNA levels in tumor tissues of patients with or without antiviral therapy ($z = -0.490$, $p = 0.624$, Mann-Whitney test). (B) Comparison of cccDNA levels in peri-tumor tissues of patients with or without antiviral therapy ($z = -1.669$, $p = 0.095$, Mann-Whitney test). (C) Comparison of MSL2 mRNA levels in tumor tissues of patients with or without antiviral therapy ($z = -1.320$, $p = 0.187$, Mann-Whitney test). (D) Comparison of MSL2 mRNA levels in peri-tumor tissues of patients with or without antiviral therapy ($z = -1.166$, $p = 0.244$, Mann-Whitney test). (E) Comparison of HBx mRNA levels in tumor tissues of patients with or without antiviral therapy ($z = -2.232$, $p = 0.026$, Mann-Whitney test). (F) Comparison of HBx mRNA levels in peri-tumor tissues of patients with or without antiviral therapy ($z = -2.114$, $p = 0.035$, Mann-Whitney test).

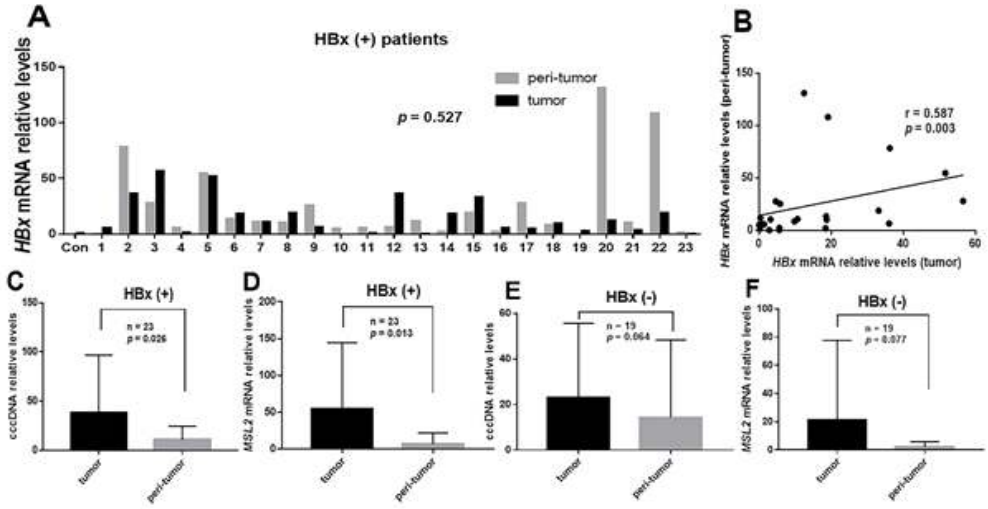

Fig. 4. Relative levels of HBx mRNA were examined in tumor and peri-tumor tissues of hepatocellular carcinoma patients by qRT-PCR. (A) Comparison of HBx mRNA levels in tumor and peri-tumor tissues of 23 patients with HBx-positive hepatocellular carcinoma (z = −0.633, p = 0.527, Wilcoxon signed rank test). 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, Spearman's rank correlation). (C, D) Comparison of cccDNA and MSL2 mRNA in tumor and peri-tumor tissues of HBx-positive patients (z = −2.220 and −2.484, p = 0.026 and 0.013, respectively, Wilcoxon signed rank test). (E, F) Comparison of cccDNA and MSL2 mRNA in tumor and peri-tumor tissues of HBx-negative patients (z = −1.851 and −1.771, p = 0.064 and 0.077, respectively, Wilcoxon signed rank test). The detected HBx mRNA, MSL2 mRNA levels in patients with HBV-related HCC were presented as fold-change relative to those in the control patient.

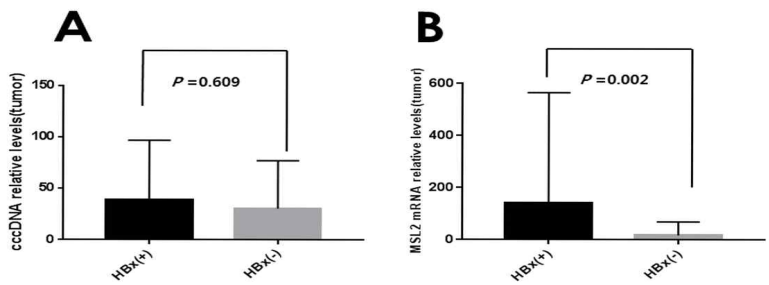

Fig. 5. Comparison of cccDNA and MSL2 levels in tumor tissues of HBx positive patients and negative patients. (A) Comparison of cccDNA level in tumor tissues of HBx positive patients and negative patients(p = 0.609, Mann-Whitney U test). (B) Comparison of *MSL2* mRNA level in tumor tissues of HBx positive patients and negative patients(p = 0.002, Mann-Whitney U test).

국문 초록

간세포암 환자에서 항바이러스 요법이 cccDNA에 직접 영향 없이 HBx를 하향 조절하여 MSL2 레벨을 저하시키는 기전에 대한 연구

배경

B형 간염바이러스(HBV)의 감염은 세계적인 건강 문제로서 만성 B형 간염은 간세포암의 주요 원인이기도 하다. B형 간염 바이러스의 covalently closed circular DNA (cccDNA) 는 B형 간염 복제에 필요한 주형으로서 HBV 복제에 직접 참여하지는 않지만, 간세포 핵 내에서 안정적인 폴을 유지한다. HBV-encoded X protein (HBx)는 종양 발생 기능을 지니고 있는 주요한 oncoprotein으로서 Male Specific Lethal 2 (MSL2)와 같은 E3 ubiquitin ligase에 의하여 특이적으로 조정되어 HBx의 활성을 상향 조절하는 ubiquitination화 되어 전사과정, 세포 증식 및 종양의 성장을 촉진하는 역할을 한다. 이 연구에서는 종양 및 종양 주변 간 조직에서 cccDNA, *MSL2* mRNA 및 *HBx* mRNA양을 비교하였고 또한 HBx 양성인 환자와 HBx 음성인 환자에서 cccDNA및 *MSL2* mRNA양의 차이를 비교하는 것을 목표로 하였다. 또한 항바이러스 치료요법이 이러한 지표에 미치는 영향을 관찰 하였다.

방법

간 절제 수술을 받은 B형 간염을 동반한 간세포암 환자 50명을 대상으로 Real-time PCR 분석법을 통해 간 내 cccDNA, *MSL2* mRNA 및 *HBx* mRNA의 발현 양상을 비교 하였다.

결과

본 연구에 총 50명의 환자가 포함되었다 (49명의 HBsAg 양성, 1명은 항바이러스 치료 후 혈청 전환 되였음). 수술 전 31명은 항바이러스 치료를 받았고 19명은 치료받지 못했다. 간 내 cccDNA양을 비교하여 볼 때 종양 조직에서 종양 주변조직에 비하여 유의하게 높았다 (44.68 ± 65.14 vs. 11.47 ± 23.03; p = 0.001). 이러한 종양 조직과 종양 주변조직에서의 cccDNA 수준은 특히 HBeAg 양성 군 (90.07 ± 93.94 vs. 10.05 ±

10.48; p = 0.008), 수술 후 종양 재발 군 (p = 0.002)과 종양 크기가 3cm 미만인 군 (p = 0.003)에서 현저하게 차이가 있었다. 더욱이 수술 전 간경변증 환자의 경우 cccDNA와 *MSL2* mRNA양은 종양 조직에서 종양 주변조직에 비하여 유의미하게 높았다 (p < 0.001, p = 0.023). 항바이러스 치료를 받은 환자 종양 조직과 종양 주변조직에서의 *HBx* mRNA양은 치료 받지 않는 군에 비하여 유의미하게 낮았다 (p = 0.026, p = 0.035). HBx 양성 그룹의 cccDNA와 *MSL2* mRNA 수준은 종양 주변조직에서 보다 종양 조직에서 유의하게 높았다 (p = 0.026, 0.013). 또한 *MSL2* mRNA 수준은 HBx 음성 종양 조직보다 HBx 양성 종양 조직에서 유의하게 높았다 (p = 0.002). 그러나 HBx 양성 종양 조직과 HBx 음성 종양 조직 사이에는 cccDNA 수준의 차이가 없었다 (p = 0.609).

결론

cccDNA는 HBV 관련 간세포암 조직에서 높게 관찰되며 항바이러스 치료를 통하여 만성 B형간염에 의한 HCC 형성과정에서 HBx의 형성을 억제함으로서 cccDNA와 MSL2의 간세포암 형성 과정에서의 영향을 조절할 수 있을 것이다.

주요어: B형 간염, cccDNA, HBV DNA, HCC, MSL2, HBx, HCC 재발
학번: 2016-30780

감사의 글

서울대학교 의과대학으로부터 박사과정 입학 통지서를 받으면서부터 저는 기쁘고 긴장한 마음으로 처음으로 되는 유학생활을 꿈꾸게 되었습니다.

2016년 3월에 입학하여서부터 유학생활을 하면서 많은 어려운 점은 있었지만 그 어려움을 알고계신 지도교수님이신 서경석 교수님께서 무한한 관심과 배려 속에서 저의 박사과정 학업을 마칠 수 있게 되여 충심으로 되는 감사와 존경을 보내드립니다. 이 논문이 완성되기까지 서경석 교수님께서는 부족한 저를 격려해 주시고 지도해 주시면서 실험에서의 많은 곡절 속에서도 열정이 식지 않도록 인도해 주셨고 또한 외과 의사로서 수술과정에서의 책임 감, 침착성을 키우게끔 완벽하고 뛰어난 수술기법을 배워 주신데 대하여 감사드립니다.

학위 과정에서 많은 조언과 도움을 주신 이광웅 교수님, 이남준 교수님, 홍석균 교수님에께도 진심으로 감사드립니다. 의학 박사로서의 질병에 대한 연구능력 질병에 대한 치료방법들을 배우는데 주변 교수님들의 도움을 떠날 수 없었습니다. 그리고 논문 연구과정에서 도움을 주신 김화정 박사님, 이선경 연구원, 서수인 연구원, 박민영 연구원에께 진심으로 감사드립니다.

그리고 바쁘신 와중에도 심사위원으로 맡아 주시고 많은 조언을 해주신 류지곤 교수님, 장진영 교수님, 김윤준 교수님, 최기홍 교수님께도 감사드립니다.

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