Impact of HBx mutations in hepatocellular carcinoma due to chronic HBV infection

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Impact of HBx mutations in hepatocellular carcinoma due to chronic HBV infection

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The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

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December 2010

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Yonsei University

December 2010

ACKNOWLEDGEMENTS

I am honored to thank many grateful benefactors who contributed this thesis. In particular, I deeply appreciate supervisor, Prof. Hyon-Suk Kim for her sincere principles, thorough guidance, and honest enthusiasm. She always showed a leadership and encouraged me as “First you give something to others as best as you can.” She helped me to prepare big dreams. I hope God bless Prof. Kim and her family.

I thank many professors who helped this thesis. I especially acknowledge Prof. Jeon Han Park and Prof. Jae Myun Lee who gave many thoughtful guides for this study. In addition, I am grateful to be guided by Prof. Kwang-Hyub Han and Prof. Young Nyun Park who encouraged and supported me to complete this thesis. I thank Prof. Jeong Won Shin for her considerate advices.

I also deeply appreciate my parents who brought me up with

patience, sacrifice and eternal love. May God continue to bless my heroes.

I thank my wife who has endured a voyage with me and lovely our son “Myoung Jun”.

Finally, I trust the Lord and his words, “I can do everything through him who gives me strength.” (Philippians 4:13), “No, in all these things we are more than conquerors through him who loved us. (Romans 8:37)”

19 December 2010

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<ABSTRACT>

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Chronic hepatitis B virus (HBV) infection is known as a major etiology of hepatocellular carcinoma (HCC) pathogenesis. HBV X (HBx) protein was known as a multifunctional regulatory protein, which interacts directly or indirectly with a variety of targets and mediates many cellular functions, including cell cycle regulation, transcriptional transactivation, regulation of signaling pathways, apoptosis, angiogenesis, and DNA repair mechanism. This study aimed to elucidate the impact of HBx mutations on HCC development.

Chronically HBV infected 96 patients were enrolled in this study: 42 chronic hepatitis B (CHB) patients, 23 liver cirrhosis (LC) patients, and 31 HCC patients. Direct sequencing of the HBV X gene and *in vitro* functional study of

significant HBx mutations were performed. Genotype analysis revealed that all the patients had genotype C2 HBV. Direct sequencing showed HBx131, HBx130, HBx5, HBx94, and HBx38 amino acid mutations were relatively frequent in HCC patients in that order. Of various mutations, HBx130+HBx131 double mutations and HBx5+HBx130+HBx131 triple mutations were significantly high in HCC patients. Older HCC patients showed a trend to have more HBx130+HBx131 double mutations and HBx5+HBx130+HBx131 triple mutations than younger HCC patients. HBx130+HBx131 double and HBx5+HBx130+HBx131 triple mutations increased the risk for HCC by 3.75 fold (95% CI = 1.101-12.768, $P = 0.033$), and 5.34 fold (95% CI = 1.65-17.309, $P = 0.005$), respectively, when HCC patients were compared with CHB patients without cirrhosis.

Functionally, there were no significant differences in HBsAg and HBeAg expression in transfected cell culture supernatants with HBV construct containing wild type, HBx5 mutant, HBx130+HBx131 double mutant, and HBx5+HBx130+HBx131 triple mutant. Also the level of HBV DNA was not different among each mutant transfected cell. However, there were significantly higher levels of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activity in cells transfected with HBx5 mutant and HBx130+HBx131 double mutant than that of wild type and HBx5+HBx130+HBx131 triple mutant. HBx5 mutation and HBx130+HBx131 double mutations appear to modulate transcriptional transactivating function of the NF-κB signaling pathway.

However, HBx5+HBx130+HBx131 triple mutant did not increase NF-κB activity, there seems to be another pathway to regulate NF-κB activation.

In conclusion, specific HBx mutation may promote to HCC development by activating NF-κB transcriptional activity. HBx5 mutation in genotype C2 HBV seems to be a risk factor for the development of HCC and may be a useful marker for prediction of clinical progress of patients with chronic HBV infection.

Key words : hepatitis B virus, hepatocellular carcinoma, HBx, mutation

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I. INTRODUCTION

Hepatitis B virus (HBV) infection is a global health issue, with two billion people infected worldwide, and 350 million suffering from chronic HBV infection.[1] Chronic HBV infection is known as the most common underlying etiology of hepatocellular carcinoma (HCC) in the world.[2]

Chronic HBV infection can further progress to liver cirrhosis (LC) and HCC. Pathogenesis of these HBV-related disease progresses has not been fully clarified, because it is difficult to establish an animal model and the disease progression depends on a multi-step and complex mechanism. Demographic factors, environmental factors, and viral factors which were classified as HCC

risk factors. The 2008 National Institutes of Health (NIH) HBV management consensus report[3] suggested that male sex, age, HBV genotype, level of HBV DNA were associated with the increased risk of HCC and cirrhosis in chronic HBV infected patient.

The HBV genome is a partially double-stranded DNA and about 3.2 kb in size, which encodes four open reading frames: C, the core (nucleocapsid) protein; S, the surface protein; P, viral DNA polymerase (which contains reverse transcriptase activity); and X, the function of which has not been fully elucidated.[4] HBV is known to be associated with high frequency of mutations because inherently it lacks the proofreading function of reverse transcriptase. Mutations of HBV have been considered as an escape mechanism from the host immune system and one of the oncogenic potential of chronic HBV diseases.[5]

The associations between HBV mutations and HCC have been investigated. Recently, HBV PreS mutations, C1653T, T1753V, and A1762T/G1764A were reported as a strong risk factor of HCC in a meta-analysis.[6] On the contrary, some reports suggested that no association between Enhancer II/Basal core promoter (EnhII/BCP) mutations and HCC risk was found in the majority of patients infected with HBV genotype F or C.[7-8] Although many studies investigated specific HBV mutations and disease progression, those studies were almost non-standardized studies. Well designed case control and prospective studies are needed to further understand the impact of HBV mutations on disease progression.

HBV X is the smallest among four kinds of HBV functional genes, encodes a 154 amino acid multifunctional protein, HBx, with an N-terminal negative regulatory/antiapoptotic domain and a C-terminal transactivation/proapoptotic domain, and it is associated with modulation of a wide range of cellular functions, leading to HCC.[9] Moreover, of the four open reading frames, X gene remains enigmatic. There are conflicting suggestions about functional activity of HBx. HBx had been reported to induce HCC in certain transgenic mice *in vivo*[10-11] and *in vitro*.[12-13] Nevertheless, there are other transgenic lineages of which HBx does not lead to HCC development.[14-15] Many reports suggest that HBx is a multifunctional regulatory protein, which interacts directly or indirectly with a variety of targets and mediates many cellular functions, including cell cycle regulation[16], transcriptional transactivation[17], regulation of signaling pathways[18], apoptosis[19], angiogenesis[20], and DNA repair.[21]

Thus, the aims of this study were to elucidate (1) to analyze HBx mutations according to clinical phase of HBV infection; (2) to determine function of significant HBx mutations which appear to be related with HCC pathogenesis.

II. MATERIALS AND METHODS

1. Study subjects

Chronically HBV infected 96 patients were enrolled in this study (Table 1). There were 42 chronic hepatitis B disease (CHB) patients, 23 liver cirrhosis (LC) patients, and 31 hepatocellular carcinoma (HCC) patients. The HBV-related etiology was confirmed clinically with radiologic findings, laboratory tests and/or pathologic findings, i.e., serum ALT, HBsAg, HBeAg, HBV DNA, or liver biopsy results. All patients' serum or plasma samples were aliquoted and kept frozen at -70℃ before analysis.

This study protocol conformed to the 1975 Declaration of Helsinki and was approved by the institutional review board (IRB) of the Yonsei University Health System.

2. Sequencing of HBV DNA from the subjects

HBV DNA was extracted from serum samples using commercial QuickGene DNA Tissue Kit S (Fujifilm, Tokyo, Japan) or Qiagen DNA mini (Qiagen, Hilden, Germany). Nested PCR was performed to amplify HBV X gene. The primers for HBV X gene amplification were as follows:

sense 5'-CATGCGTGGAACCTTTGTG-3'(1233-1251),

anti-sense 5'-CTTGCCTKAGTGCTGTATGG-3'(2072-2053) for the 1st PCR amplification and

sense 5’-TCCTCTGCCGATCCATACTG-3’(1254-1263),

anti-sense 5’-CAGAAGCTCCAAATTCTTTATA-3’(1937-1916) for the 2nd PCR amplification. PCR premix (Accupower® Pfu PCR PreMix, Bioneer, Daejun, Korea) with MyGenie™ 96 Gradient Thermal Block (Bioneer) was used for 839 bp amplicon. The 683 bp amplicon of the 2nd nested PCR was identified in 1% agarose gel electrophoresis stained with ethidium bromide. PCR conditions were as follows; 1st PCR: 95℃ for 10 min, 95℃ for 20 sec, 57℃ for 20 sec, 72℃ for 1 min for 30 cycles and final extension at 72℃ for 5 min; 2nd PCR: 95℃ for 10 min, 95℃ for 20 sec, 50℃ for 20 sec, 72℃ for 1 min for 35 cycles and final extension 72℃ for 5 min. Nested PCR products were purified with Accuprep® PCR purification kit (Bioneer) then loaded with an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator v3.1 sequencing kit (Applied Biosystems). If primary PCR was not successful, supplementary primer sets of Kim et al.[22] were additionally used for direct sequencing.

First-round PCR was done with

sense XproF1: 5’-CTCTGCCAAGTGTTTGCTGA-3’(1171-1190),

anti-sense XproR1: 5’-CAAGGCACAGCTTGGAGGCT-3’(1886-1867)

for 715bp amplicon size. Second-round PCR was done with following primers;

XproF2: sense 5’-TTGCTCGCAGCCGGTCTGGA-3’ (1295-1314),

XproR2: antisense 5’-TGAACAGTAGGACATGAACA-3’ (1866-1847)

for 571 bp amplicon size. The PCR conditions were as follows; 94℃, 2 min for

initial denaturation, 30 cycles of 94℃ for 1 min, 54℃ for 45 sec, and 72℃ for 1 min. The purified PCR products were determined directly using both primers of secondary PCR, XproF2 and XproR2 primers.

All sequencing data were analyzed based on the consensus HBV sequence (GenBank access number: GQ475316) of a chronic HBV carrier patient without anti-viral therapy. Firstly, all sequence data were multiple aligned and analyzed by ClustalW and Bioedit v7.0.5 software (Ibis Therapeutics, Carlsbad, CA, USA).

3. HBV genotyping

Deduced X gene sequences were compared with several consensus sequences of HBV genotypes and a phylogenetic tree was constructed by the neighbor-joining method. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 4.0.2 (The Biodesign Institute, Tempe, AZ, USA). The genetic distances were estimated by Kimura's two-parameter method. The reliability of the phylogenetic tree analysis was assessed by the interior branch test with 1000 replicates.

4. Construction of HBx plasmid

The most frequent HBx mutations in HCC patients were HBx131, HBx130, HBx5 mutations in that order. Among them, four different plasmids were constructed: (1) wild type of HBx, (2) single amino acid mutant of HBx (HBx5;

G1386A; V5M), (3) double amino acids mutant of HBx (HBx130+HBx131; A1762T+G1764A; K130M+V131I), and (4) triple amino acids mutant of HBx (HBx5+HBx130+HBx131; G1386A+A1762T+G1764A; V5M+K130M+V131I).

All plasmids were constructed based on the HBV 1.2 mer construct within pGEM4Z vector (provided as a kind gift from Professor Kyun Hwan Kim, Department of Pharmacology, School of Medicine, Konkuk University, Seoul, Korea).

To construct G1386A mutant, site directed mutagenesis was performed at manufacturer's recommened conditions with following primers:

Mt-1386-F : 5'-GTTTCCATGGCTGCTAGGATGTGCTGCCAACTGG-3'

Mt-1386-R : 5'-CCAGTTGGCAGCACATCCTAGCAGCCATGGAAAC-3'

To construct A1762T+G1764A double mutant, following primers were used:

Mt-1762-1765-F : 5'-GAGATTAGGTTAATGATCTTTGTACTAGG-3'

Mt-1762-1765-R : 5'-CCTAGTACAAAGATCATTAACCTAATCTC-3'

PCR products were treated with restriction enzyme DpnI for 1 hour then were transformed in XL10 gold ultra competent cells (Strategene, Cedar Creek, TX, USA), according to the manufacturer's instructions.

To construct HBx5+HBx130+HBx131 triple mutant, the primary PCR was done with Mt-1386-F and Mt-1762-1765-R primers, then the secondary PCR was proceeded with Mt-1762-1765-F and M13R primers. Each PCR product was used as tertiary PCR template and was amplified with primers of Mt-1386-F and M13R. PCR products were digested with NcoI/HindIII to insert a

pGEM4Z vector. All mutated clones were verified by sequencing both strands of DNA within the regions of interest. To purify each plasmid, *E. coli* transformation was performed by Qiagen plasmid mini or midi kits (Qiagen) according to manufacturer's protocols.

5. Cell cultures and transfection into Huh7 cells

Huh7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA) at 37℃ in 5% CO_2. The cells were transiently transfected with plasmids pGEM4Z, HBx wild type, HBx5 mutant, HBx double mutant, or HBx triple mutant construct by Lipofectamine LTX and PLUS reagents (Invitrogen, Carlsbad, CA, USA). All experiments were performed in triplicate. The cell viability was observed daily during four consecutive days.

6. Detection of HBV replication by HBsAg and HBeAg expressions in culture supernatants

Total protein concentration in cell culture supernatants of each transfected Huh7 cells was determined by using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Then relative levels of HBsAg and HBeAg in culture supernatants were calculated according to the concentration of the proteins. The HBsAg and HBeAg expression levels were determined by Architect *i*2000

analyzer (Abbott Diagnostics, Abbott Park, IL, USA). All experiments were performed in triplicate and expressed as mean±SD.

7. Detection of HBV DNA replication by real-time PCR in cell extracts

Real-time PCR was used to determine HBV DNA replication activity in each transfected cell.

DNA was extracted from cells transfected with each construct, then purity and quantity of DNA were measured by Nanodrop (Thermo Fisher Scientific Inc., Wilmington, DE, USA).

Real-time PCR amplifications were carried out using ABI 7000 System (Applied Biosystems). The 100 ng/μl template was processed with following primers.

HBs-F: 5'-CCTCTTCATCCTGCTGCT-3',

HBs-R: 5'-AACTGAAAGCCAAACAGTG-3' with SYBR Green I with 2×SYBR Green mix (Applied Biosystems) to 20 μl total reaction volume. The thermal condition for SYBR real-time PCR was 95℃ for 2 min followed by 40 cycles of 95℃ for 15 sec and 60℃ for 1 min.

Data were analyzed according to the standard curve in order to relatively quantitate the cycle threshold (Ct) values of samples. The values were normalized to that of GAPDH expression in each sample.

8. NF-κB luciferase reporter gene assay

To determine NF-κB activity, cotransfection with NF-κB luciferase vector and HBx constructs were performed by Lipofectamine LTX and PLUS reagents (Invitrogen). Cells were harvested two days after transfection, then the luciferase activity was detected by the Dual luciferase reporter assay system (Promega, San Luis Obispo, CA, USA) on a VICTOR™ X4 Multilabel Plate Reader (Perkin-Elmer Life Sciences, Shelton, CT, USA). All the luciferase data were normalized with corresponding *Renilla* luciferase activity. Each experiment was performed in triplicate.

9. Statistical analysis

SPSS 12.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Differences between categorical variables were analyzed using the chi-square test or Fisher's exact test. Student's t-test was used for continuous variables when the data showed a normal distribution, and the Mann–Whitney *U* test was used when the data was not normally distributed. *P* values less than 0.05 were considered significant.

III. RESULTS

1. Characteristics of study subjects

Characteristics and status of HBx mutations of enrolled patients were summarized in Table 1. HCC patients' ages were higher than CHB patients (P < 0.001) but were not different from LC patients (P = 0.275). The levels of HBV DNA in HCC patients were lower than those of CHB patients (P = 0.002) but were not different from those of LC patients (P = 0.872). HBeAg status was different among three clinical phases of HBV infections (P = 0.002). Frequent HBx mutations in HCC patients were HBx131, HBx130, HBx5, and HBx94, in that order. All mutation profiles were summarized in Table 2. The incidence of HBx130+HBx131 double mutations and HBx5+HBx130+HBx131 triple mutations in HCC patients tended to increase with age (Table 3).

Table 1. Characteristics of enrolled 96 patients according to clinical diagnosis

	CHB			LC			HCC			*P* value
No. of patients	42			23			31			
Age (Mean±SD year)	43.6±10.7			51.7±7.8			55.6±7.8			0.007*
Male/Female	8/14			16/7			26/5			0.241†
HBV DNA (IU/ml)	$1.63\times10^{8}\pm2.23\times10^{8}$			$2.18\times10^{7}\pm7.72\times10^{7}$			$8.42\times10^{5}\pm1.56\times10^{6}$			<0.001*
HBeAg positive (%)	31(73.8%)			12(52.2%)			10(32.3%)			0.002†
Mean±SD count of HBx mutation	5.95±5.04			7.26±4.7			6±2.37			0.455*
Min-Max count of HBx mutation	1-25			3-27			1-11			
Frequent HBx mutations (≥4 times incidence)	Mutation site	Sum	%	Mutation site	Sum	%	Mutation site	Sum	%	
	HBx131	28	66.7	HBx131	23	100.0	HBx131	28	90.3	
	HBx130	27	64.3	HBx130	20	87.0	HBx130	27	87.1	
	HBx42	9	21.4	HBx94	13	56.5	HBx5	13	41.9	
	HBx36	8	19.0	HBx5	10	43.5	HBx94	12	38.7	
	HBx48	7	16.7	HBx79	7	30.4	HBx38	8	25.8	
	HBx79	7	16.7	HBx42	6	26.1	HBx36	6	19.4	
	HBx92	7	16.7	HBx36	6	26.1	HBx127	5	16.1	
	HBx94	7	16.7	HBx38	5	21.7	HBx101	4	12.9	
	HBx52	6	14.3	HBx127	4	17.4	HBx52	4	12.9	
	HBx106	6	14.3	HBx12	4	17.4				
	HBx127	6	14.3	HBx101	4	17.4				
	HBx5	5	11.9	HBx102	4	17.4				
	HBx38	5	11.9							
	HBx12	4	9.5							
	HBx80	4	9.5							
	HBx101	4	9.5							
	HBx116	4	9.5							

Abbreviations: CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma.
*One-way ANOVA analysis, †Chi-square test.

Table 2. HBx mutations according to clinical diagnosis

	Chronic hepatitis B (n=42)					Liver cirrhosis (n=23)					Hepatocellular carcinoma (n=31)			
	Mutation	No.	Sum	%		Mutation	No.	Sum	%		Mutation	No.	Sum	%
HBx1	M → X		1	2.4	HBx1	M →		0	0.0	HBx1	M → I		1	3.2
HBx2	A →		0	0.0	HBx2	A →		0	0.0	HBx2	A →		0	0.0
HBx3	A →		0	0.0	HBx3	A →		0	0.0	HBx3	A →		0	0.0
HBx4	R → X		2	4.8	HBx4	R →		0	0.0	HBx4	R → G		1	3.2
HBx5	V → M/X	3/2	5	11.9	HBx5	V → L/M/X	2/7/1	10	43.5	HBx5	V → M/X	8/5	13	41.9
HBx6	C →		0	0.0	HBx6	C →		0	0.0	HBx6	C → X/Y	1/1	2	6.5
HBx7	C → X	1	1	2.4	HBx7	C →		0	0.0	HBx7	C →		0	0.0
HBx8	Q → X	1	1	2.4	HBx8	Q →		0	0.0	HBx8	Q →		0	0.0
HBx9	L →		0	0.0	HBx9	L →		0	0.0	HBx9	L →		0	0.0
HBx10	D →		0	0.0	HBx10	D →		0	0.0	HBx10	D →		0	0.0
HBx11	P → X		3	7.1	HBx11	P →		0	0.0	HBx11	P →		0	0.0
HBx12	A → T/X	2/2	4	9.5	HBx12	A → T/X	3/1	4	17.4	HBx12	A →		0	0.0
HBx13	R →		0	0.0	HBx13	R →		0	0.0	HBx13	R →		0	0.0
HBx14	D → X	1	1	2.4	HBx14	D →		0	0.0	HBx14	D →		0	0.0
HBx15	V → I/X	1/1	2	4.8	HBx15	V →		0	0.0	HBx15	V → X	1	1	3.2
HBx16	L →		0	0.0	HBx16	L →		0	0.0	HBx16	L →		0	0.0
HBx17	C → R	1	1	2.4	HBx17	C →		0	0.0	HBx17	C → X	1	1	3.2
HBx18	L →		0	0.0	HBx18	L →		0	0.0	HBx18	L →		0	0.0
HBx19	R → X	2	2	4.8	HBx19	R →		0	0.0	HBx19	R →		0	0.0
HBx20	P → X	1	1	2.4	HBx20	P →		0	0.0	HBx20	P →		0	0.0
HBx21	V → X	1	1	2.4	HBx21	V →		0	0.0	HBx21	V →		0	0.0
HBx22	G → S/X	1/2	3	7.1	HBx22	G → S	2	2	8.7	HBx22	G →		0	0.0
HBx23	A →		0	0.0	HBx23	A →		0	0.0	HBx23	A → T	1	1	3.2
HBx24	E →		0	0.0	HBx24	E →		0	0.0	HBx24	E →		0	0.0
HBx25	S →		0	0.0	HBx25	S →		0	0.0	HBx25	S →		0	0.0
HBx26	R → P	1	1	2.4	HBx26	R →		0	0.0	HBx26	R →		0	0.0
HBx27	G →		0	0.0	HBx27	G →		0	0.0	HBx27	G →		0	0.0
HBx28	R → Q/X	1/1	2	4.8	HBx28	R →		0	0.0	HBx28	R →		0	0.0
HBx29	P →		0	0.0	HBx29	P →		0	0.0	HBx29	P →		0	0.0
HBx30	V → F/X	1/1	2	4.8	HBx30	V →		0	0.0	HBx30	V → I	1	1	3.2
HBx31	S → X	1	1	2.4	HBx31	S → A	1	1	4.3	HBx31	S → A	1	1	3.2
HBx32	G →		0	0.0	HBx32	G → X	1	1	4.3	HBx32	G →		0	0.0
HBx33	P →		0	0.0	HBx33	P →		0	0.0	HBx33	P →		0	0.0
HBx34	F → L	2	2	4.8	HBx34	F → L	1	1	4.3	HBx34	F → L/V	2/1	3	9.7
HBx35	G →		0	0.0	HBx35	G →		0	0.0	HBx35	G →		0	0.0
HBx36	P → A/S/T/X	1/2/4/1	8	19.0	HBx36	P → A/T	2/4	6	26.1	HBx36	P → G/T/X	1/3/2	6	19.4
HBx37	L →		0	0.0	HBx37	L → V	1	1	4.3	HBx37	L →		0	0.0
HBx38	P → S/X	3/2	5	11.9	HBx38	P → S/X	3/2	5	21.7	HBx38	P → S/X	7/1	8	25.8
HBx39	S →		0	0.0	HBx39	S →		0	0.0	HBx39	S →		0	0.0
HBx40	P →		0	0.0	HBx40	P →		0	0.0	HBx40	P → X	1	1	3.2
HBx41	S → A/C	1/1	2	4.8	HBx41	S → T	1	1	4.3	HBx41	S → T	1	1	3.2
HBx42	S → A/T/X	4//1/4	9	21.4	HBx42	S → A/X	4	6	26.1	HBx42	S → A/T	2/1	3	9.7
HBx43	S → A/X	1/1	2	4.8	HBx43	S → P	1	1	4.3	HBx43	S →		0	0.0
HBx44	A → V/X	1/1	2	4.8	HBx44	A →		0	0.0	HBx44	A →		0	0.0
HBx45	V →		0	0.0	HBx45	V →		0	0.0	HBx45	V → X	1	1	3.2
HBx46	P →		0	0.0	HBx46	P → X	2	2	8.7	HBx46	P →		0	0.0
HBx47	A → T	2	2	4.8	HBx47	A → X	1	1	4.3	HBx47	A → T	1	1	3.2
HBx48	D → N/V/X/Y	3/1/2/1	7	16.7	HBx48	D → N	2	2	8.7	HBx48	D → N/V	2/1	3	9.7
HBx49	H →		0	0.0	HBx49	H → D	1	1	4.3	HBx49	H → Y	1	1	3.2
HBx50	G → X	1	1	2.4	HBx50	G →		0	0.0	HBx50	G →		0	0.0
HBx51	A → T	1	1	2.4	HBx51	A →		0	0.0	HBx51	A → P	1	1	3.2
HBx52	H → X/Y	1/5	6	14.3	HBx52	H →		0	0.0	HBx52	H → Y	4	4	12.9
HBx53	L →		0	0.0	HBx53	L →		0	0.0	HBx53	L →		0	0.0
HBx54	S →		0	0.0	HBx54	S →		0	0.0	HBx54	S →		0	0.0
HBx55	L →		0	0.0	HBx55	L →		0	0.0	HBx55	L →		0	0.0
HBx56	R →		0	0.0	HBx56	R →		0	0.0	HBx56	R →		0	0.0
HBx57	G → X	1	1	2.4	HBx57	G → X	1	1	4.3	HBx57	G →		0	0.0
HBx58	L → X	2	2	4.8	HBx58	L →		0	0.0	HBx58	L →		0	0.0
HBx59	P →		0	0.0	HBx59	P →		0	0.0	HBx59	P →		0	0.0
HBx60	V →		0	0.0	HBx60	V →		0	0.0	HBx60	V →		0	0.0
HBx61	C → X	1	1	2.4	HBx61	C →		0	0.0	HBx61	C →		0	0.0
HBx62	A →		0	0.0	HBx62	A →		0	0.0	HBx62	A →		0	0.0
HBx63	F →		0	0.0	HBx63	F →		0	0.0	HBx63	F →		0	0.0
HBx64	S → X	1	1	2.4	HBx64	S →		0	0.0	HBx64	S →		0	0.0
HBx65	S → X	1	1	2.4	HBx65	S → P	1	1	4.3	HBx65	S → P	1	1	3.2
HBx66	A → X	1	1	2.4	HBx66	A →		0	0.0	HBx66	A →		0	0.0
HBx67	G → X	1	1	2.4	HBx67	G → X	1	1	4.3	HBx67	G →		0	0.0
HBx68	P →		0	0.0	HBx68	P →		0	0.0	HBx68	P → S	1	1	3.2
HBx69	C → X	2	2	4.8	HBx69	C →		0	0.0	HBx69	C →		0	0.0
HBx70	A →		0	0.0	HBx70	A →		0	0.0	HBx70	A →		0	0.0
HBx71	L → X	1	1	2.4	HBx71	L →		0	0.0	HBx71	L →		0	0.0
HBx72	R → X	1	1	2.4	HBx72	R → X	1	1	4.3	HBx72	R →		0	0.0
HBx73	F →		0	0.0	HBx73	F →		0	0.0	HBx73	F →		0	0.0
HBx74	T →		0	0.0	HBx74	T →		0	0.0	HBx74	T →		0	0.0
HBx75	S →		0	0.0	HBx75	S →		0	0.0	HBx75	S →		0	0.0
HBx76	A →		0	0.0	HBx76	A → X	1	1	4.3	HBx76	A →		0	0.0
HBx77	R →		0	0.0	HBx77	R →		0	0.0	HBx77	R →		0	0.0

		Chronic hepatitis B (n=42)						Liver cirrhosis (n=23)						Hepatocellular carcinoma (n=31)			
		Mutation	No.	Sum	%			Mutation	No.	Sum	%			Mutation	No.	Sum	%
HBx78	R	→		0	0.0	HBx78	R	→ H/X	1/1	2	8.7	HBx78	R	→		0	0.0
HBx79	M	→ X	7	7	16.7	HBx79	M	→ X	7	7	30.4	HBx79	M	→ X	3	3	9.7
HBx80	E	→ A/X	1/3	4	9.5	HBx80	E	→ A/X	1/1	2	8.7	HBx80	E	→ X	1	1	3.2
HBx81	T	→		0	0.0	HBx81	T	→		0	0.0	HBx81	T	→ X	1	1	3.2
HBx82	T	→		0	0.0	HBx82	T	→ S/X	1/1	2	8.7	HBx82	T	→ X	1	1	3.2
HBx83	V	→ M	2	2	4.8	HBx83	V	→ X	1	1	4.3	HBx83	V	→ M	1	1	3.2
HBx84	N	→		0	0.0	HBx84	N	→		0	0.0	HBx84	N	→		0	0.0
HBx85	A	→ T/V	1/1	2	4.8	HBx85	A	→		0	0.0	HBx85	A	→		0	0.0
HBx86	H	→ R/X	1/1	2	4.8	HBx86	H	→ R	3	3	13.0	HBx86	H	→ P/R/X	1/1/1	3	9.7
HBx87	Q	→		0	0.0	HBx87	Q	→		0	0.0	HBx87	Q	→		0	0.0
HBx88	V	→		0	0.0	HBx88	V	→ A/X	1/1	2	8.7	HBx88	V	→ X	2	2	6.5
HBx89	L	→		0	0.0	HBx89	L	→		0	0.0	HBx89	L	→		0	0.0
HBx90	P	→		0	0.0	HBx90	P	→		0	0.0	HBx90	P	→		0	0.0
HBx91	K	→		0	0.0	HBx91	K	→		0	0.0	HBx91	K	→		0	0.0
HBx92	V	→ L	7	7	16.7	HBx92	V	→		0	0.0	HBx92	V	→ L	2	2	6.5
HBx93	L	→		0	0.0	HBx93	L	→		0	0.0	HBx93	L	→		0	0.0
HBx94	H	→ X/Y	1/6	7	16.7	HBx94	H	→ N/X/Y	1/3/9	13	56.5	HBx94	H	→ X/Y	1/11	12	38.7
HBx95	K	→		0	0.0	HBx95	K	→		0	0.0	HBx95	K	→ X	1	1	3.2
HBx96	R	→		0	0.0	HBx96	R	→ X	2	2	8.7	HBx96	R	→ X	1	1	3.2
HBx97	T	→ X	1	1	2.4	HBx97	T	→		0	0.0	HBx97	T	→		0	0.0
HBx98	L	→		0	0.0	HBx98	L	→		0	0.0	HBx98	L	→		0	0.0
HBx99	G	→		0	0.0	HBx99	G	→ X	1	1	4.3	HBx99	G	→ X	1	1	3.2
HBx100	L	→ X	1	1	2.4	HBx100	L	→		0	0.0	HBx100	L	→		0	0.0
HBx101	S	→ P/X	2/2	4	9.5	HBx101	S	→ P/X	1/3	4	17.4	HBx101	S	→ P/X	2/2	4	12.9
HBx102	A	→ T	1	1	2.4	HBx102	A	→ S/T/V/X	1/1/1/1	4	17.4	HBx102	A	→ T	3	3	9.7
HBx103	M	→ L	2	2	4.8	HBx103	M	→		0	0.0	HBx103	M	→		0	0.0
HBx104	S	→		0	0.0	HBx104	S	→ X	1	1	4.3	HBx104	S	→		0	0.0
HBx105	T	→		0	0.0	HBx105	T	→ X	1	1	4.3	HBx105	T	→		0	0.0
HBx106	T	→ S	6	6	14.3	HBx106	T	→ X	1	1	4.3	HBx106	T	→		0	0.0
HBx107	D	→		0	0.0	HBx107	D	→		0	0.0	HBx107	D	→		0	0.0
HBx108	L	→		0	0.0	HBx108	L	→		0	0.0	HBx108	L	→		0	0.0
HBx109	E	→		0	0.0	HBx109	E	→		0	0.0	HBx109	E	→		0	0.0
HBx110	A	→		0	0.0	HBx110	A	→ X	1	1	4.3	HBx110	A	→ V	1	1	3.2
HBx111	Y	→		0	0.0	HBx111	Y	→ X	1	1	4.3	HBx111	Y	→		0	0.0
HBx112	F	→ X	1	1	2.4	HBx112	F	→ V/X	1/1	2	8.7	HBx112	F	→		0	0.0
HBx113	K	→ T	1	1	2.4	HBx113	K	→		0	0.0	HBx113	K	→		0	0.0
HBx114	D	→		0	0.0	HBx114	D	→		0	0.0	HBx114	D	→		0	0.0
HBx115	C	→ X	1	1	2.4	HBx115	C	→		0	0.0	HBx115	C	→ R	1	1	3.2
HBx116	L	→ V/X	1/3	4	9.5	HBx116	L	→ V	1	1	4.3	HBx116	L	→ V	1	1	3.2
HBx117	F	→ X	1	1	2.4	HBx117	F	→		0	0.0	HBx117	F	→ X	1	1	3.2
HBx118	K	→ X	1	1	2.4	HBx118	K	→ X	1	1	4.3	HBx118	K	→		0	0.0
HBx119	D	→		0	0.0	HBx119	D	→ E	2	2	8.7	HBx119	D	→		0	0.0
HBx120	W	→		0	0.0	HBx120	W	→		0	0.0	HBx120	W	→		0	0.0
HBx121	E	→		0	0.0	HBx121	E	→ X	1	1	4.3	HBx121	E	→		0	0.0
HBx122	E	→		0	0.0	HBx122	E	→		0	0.0	HBx122	E	→		0	0.0
HBx123	L	→ S	2	2	4.8	HBx123	L	→ X	1	1	4.3	HBx123	L	→ S	2	2	6.5
HBx124	G	→ X	1	1	2.4	HBx124	G	→		0	0.0	HBx124	G	→		0	0.0
HBx125	E	→ K/X	1/1	2	4.8	HBx125	E	→		0	0.0	HBx125	E	→ X	1	1	3.2
HBx126	E	→ K/X	1/1	2	4.8	HBx126	E	→		0	0.0	HBx126	E	→		0	0.0
HBx127	I	→ L/T/X	1/2/3	6	14.3	HBx127	I	→ S/T	3/1	4	17.4	HBx127	I	→ N/T/X/(-)	1/2/2/1	6	19.4
HBx128	R	→ S/X	1/1	2	4.8	HBx128	R	→		0	0.0	HBx128	R	→ (-)	2	2	6.5
HBx129	L	→ E/stop codon(TAA)/(-)	1/1/1	3	7.1	HBx129	L	→ X	1	1	4.3	HBx129	L	→ (-)	2	2	6.5
HBx130	K	→ M/X/(-)	25/2/1	28	66.7	HBx130	K	→ M/X/(-)	17/2/1	20	87.0	HBx130	K	→ M/(-)	27/2	29	93.5
HBx131	V	→ F/I/X(-)	1/26/1/1	29	69.0	HBx131	V	→ I/T	22/1	23	100.0	HBx131	V	→ I/(-)	28/2	30	96.8
HBx132	F	→ L/X/(-)	1/1/1	3	7.1	HBx132	F	→ X/Y	2/1	3	13.0	HBx132	F	→ X/Y/(-)	1/2/2	5	16.1
HBx133	V	→ (-)	1	1	2.4	HBx133	V	→		0	0.0	HBx133	V	→ X	2	2	6.5
HBx134	L	→ X/(-)	1/1	2	4.8	HBx134	L	→		0	0.0	HBx134	L	→ X	1	1	3.2
HBx135	G	→ X	1	1	2.4	HBx135	G	→ X	1	1	4.3	HBx135	G	→ X	1	1	3.2
HBx136	G	→		0	0.0	HBx136	G	→ X	1	1	4.3	HBx136	G	→		0	0.0
HBx137	C	→ X	2	2	4.8	HBx137	C	→		0	0.0	HBx137	C	→ X	1	1	3.2
HBx138	R	→ X	1	1	2.4	HBx138	R	→		0	0.0	HBx138	R	→ X	2	2	6.5
HBx139	H	→		0	0.0	HBx139	H	→		0	0.0	HBx139	H	→		0	0.0
HBx140	K	→		0	0.0	HBx140	K	→ X	1	1	4.3	HBx140	K	→		0	0.0
HBx141	L	→ K/X	1/2	3	7.1	HBx141	L	→		0	0.0	HBx141	L	→ V	1	1	3.2
HBx142	V	→ X	1	1	2.4	HBx142	V	→		0	0.0	HBx142	V	→		0	0.0
HBx143	C	→ X	1	1	2.4	HBx143	C	→ R	1	1	4.3	HBx143	C	→		0	0.0
HBx144	S	→ A	1	1	2.4	HBx144	S	→ X	1	1	4.3	HBx144	S	→ X	1	1	3.2
HBx145	P	→		0	0.0	HBx145	P	→		0	0.0	HBx145	P	→		0	0.0
HBx146	A	→ T/X	1/1	2	4.8	HBx146	A	→ X	1	1	4.3	HBx146	A	→		0	0.0
HBx147	P	→ X	3	3	7.1	HBx147	P	→ X	1	1	4.3	HBx147	P	→		0	0.0
HBx148	C	→ X	3	3	7.1	HBx148	C	→		0	0.0	HBx148	C	→ X	1	1	3.2
HBx149	N	→		0	0.0	HBx149	N	→ X	1	1	4.3	HBx149	N	→		0	0.0
HBx150	F	→		0	0.0	HBx150	F	→		0	0.0	HBx150	F	→		0	0.0
HBx151	F	→		0	0.0	HBx151	F	→		0	0.0	HBx151	F	→		0	0.0
HBx152	T	→ P/X	1/1	2	4.8	HBx152	T	→ H/X	1/1	2	8.7	HBx152	T	→ X	1	1	3.2
HBx153	S	→ X	1	1	2.4	HBx153	S	→		0	0.0	HBx153	S	→		0	0.0
HBx154	A	→		0	0.0	HBx154	A	→		0	0.0	HBx154	A	→ P	1	1	3.2

Abbreviations: A, alanine; R, arginine; N, asparagine; D, aspartic acid; C, cysteine; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; L, leucine; I, isoleucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine; X, any amino acid; HBx, Hepatitis B virus x protein.

Table 3. Incidence of double and triple HBx mutations by patients

	CHB (n=42)		LC (n=23)		HCC (n=31)	
Age range	No.	No.(%) of double and triple HBx mutations	No.	No.(%) of double and triple HBx mutations	No.	No.(%) of double and triple HBx mutations
20-30	5	Double=3(60%), Triple=0(0%)	0		0	
31-40	10	Double=4(40%), Triple=1(10%)	1	Double=0(0%), Triple=1(100%)	1	Double=1(100%), Triple=0(0%)
41-50	15	Double=5(33.3%), Triple=4(26.7%)	9	Double=5(55.6%), Triple=3(33.3%)	7	Double=3(42.9%), Triple=3(42.9%)
51-60	10	Double=8(80%), Triple=0(0%)	11	Double=4(36.4%), Triple=5(45.5%)	14	Double=6(42.9%), Triple=5(35.7%)
61-70	2	Double=2(100%), Triple=0(0%)	2	Double=1(50%), Triple=1(50%)	9	Double=4(44.4%), Triple=5(55.6%)

Abbreviations: CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; Double, HBx130+HBx131 double mutations; Triple, HBx5+ HBx130+HBx131 triple mutations.

2. HBx mutations according to the status of HBV infection

Enrolled 96 patients had total number of 603 mutations ranged from minimum 1 to maximum 27 amino acids mutations. The total frequency of HBx mutation in one patient was found in 6.28±4.27 (mean±SD). The incidence of each HBx mutation site regarding to clinical diagnosis of HBV infections was described in Figure 2. A total of 603 HBx mutations were noted: 250 in CHB (n = 42), 167 in LC (n = 23), and 186 in HCC (n = 31) patients. The number (mean±SD) of HBx mutations according to clinical diagnosis was 5.95±5.04 in CHB, 7.26±4.7 in LC, and 6±2.37 in HCC patients, respectively. Amino acid deletions were noted in one CHB patient (deletion from HBx129 to HBx134) and two HCC patients (deletion from HBx127 to HBx131; deletion from HBx128 to HBx132). Number of each HBx mutation according to the status of HBV infection was illustrated in Figure 1.

Figure 1. Incidence of HBx mutations according to clinical diagnosis of HBV infections. Abbreviations: CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

3. HBV genotype analysis

A neighbor joining method was used to analyze HBV genotype. All patients were determined as HBV genotype C2 which is known as the most frequent genotype in Korea. A circular type phylogenetic tree is illustrated in Figure 2.

Figure 2. Phylogenetic tree of patients based on HBV X gene sequences. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 4.0.2 (The Biodesign Institute, Tempe, AZ, USA). The genetic distances were estimated by Kimura's two-parameter method. The reliability of the phylogenetic tree analysis was assessed by the interior branch test with 1,000 replicates. Abbreviations: CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

4. Significant HBx mutations in HCC patients

The most frequent HBx mutation in HCC was HBx130+HBx131 double mutations which were detected in 71% HCC patients. In addition, HBx5+HBx130+HBx131 triple mutations were observed in 41.9% of HCC patients and HBx94+HBx130+HBx131 triple mutations in 35.5%. No patients had HBx130 or HBx131 or HBx5, or HBx94 single mutations. Quadruple HBx mutations of HBx5+HBx94+HBx130+HBx131 showed in 12.9% of HCC patients (Table 4). HBx mutations showed significant differences by clinical phase (Table 5). HCC patients had a more frequent incidence of HBx5+HBx130+HBx131 triple mutations than CHB patients.

Table 4. Single and compound mutation profiles of HBx based on HBx5, HBx94, HBx130, and HBx131 mutations

	CHB			LC			HCC		
No. of patients	42	Sum	%	23	Sum	%	31	Sum	%
Single mutation	HBx130 single	0	0.0	HBx130 single	0	0.0	HBx130 single	0	0.0
	HBx131 single	1	2.4	HBx131 single	3	13.0	HBx131 single	1	3.2
	HBx5 single	0	0.0	HBx5 single	0	0.0	HBx5 single	0	0.0
	HBx94 single	0	0.0	HBx94 single	0	0.0	HBx94 single	0	0.0
Double mutations	HBx94+HBx131	0	0.0	HBx94+HBx131	3	13.0	HBx94+HBx131	1	3.2
	HBx130+HBx131	27	64.3	HBx130+HBx131	20	87.0	HBx130+HBx131	27	87.1
Triple mutations	HBx5+HBx130+HBx131	5	11.9	HBx5+HBx130+HBx131	10	43.5	HBx5+HBx130+HBx131	13	41.9
	HBx94+HBx130+HBx131	7	16.7	HBx94+HBx130+HBx131	10	43.5	HBx94+HBx130+HBx131	11	35.5
Quadruple mutations	HBx5+HBx94+HBx130+HBx131	0	0.0	HBx5+HBx94+HBx130+HBx131	5	21.7	HBx5+HBx94+HBx130+HBx131	4	12.9

Abbreviations: CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma.

Table 5. Significant HBx mutations according to the status of chronic HBV infection

			Chronic hepatitis B (n=42)						Liver cirrhosis (n=23)						Hepatocellular carcinoma (n=31)			
			Mutation	No.	Sum				Mutation	No.	Sum				Mutation	No.	Sum	*P* value
HBx5	V	→	M/X	3/2	5	HBx5	V	→	L/M/X	2/7/1	10	HBx5	V	→	M/X	8/5	13	**0.005***
HBx12	A	→	T/X	2/2	4	HBx12	A	→	T/X	3/1	4	HBx12	A	→			0	0.055†
HBx36	P	→	A/S/T/X	1/2/4/1	8	HBx36	P	→	A/T	2/4	6	HBx36	P	→	G/T/X	1/3/2	6	0.808*
HBx38	P	→	S/X	3/2	5	HBx38	P	→	S/X	3/2	5	HBx38	P	→	S/X	7/1	8	0.335*
HBx42	S	→	A/T/X	4//1/4	9	HBx42	S	→	A/X	4	6	HBx42	S	→	A/T	2/1	3	0.39†
HBx48	D	→	N/V/X/Y	3/1/2/1	7	HBx48	D	→	N	2	2	HBx48	D	→	N/V	2/1	3	0.574†
HBx52	H	→	X/Y	1/5	6	HBx52	H	→			0	HBx52	H	→	Y	4	4	0.148†
HBx79	M	→	X	7	7	HBx79	M	→	X	7	7	HBx79	M	→	X	3	3	0.171†
HBx80	E	→	A/X	1/3	4	HBx80	E	→	A/X	1/1	2	HBx80	E	→	X	1	1	0.605†
HBx92	V	→	L	7	7	HBx92	V	→			0	HBx92	V	→	L	2	2	0.085†
HBx94	H	→	X/Y	1/6	7	HBx94	H	→	N/X/Y	1/3/9	13	HBx94	H	→	X/Y	1/11	12	**0.003***
HBx101	S	→	P/X	2/2	4	HBx101	S	→	P/X	1/3	4	HBx101	S	→	P/X	2/2	4	0.624†
HBx102	A	→	T	1	1	HBx102	A	→	S/T/V/X	1/1/1/1	4	HBx102	A	→	T	3	3	0.089†
HBx106	T	→	S	6	6	HBx106	T	→	X	1	1	HBx106	T	→			0	**0.045**†
HBx116	L	→	V/X	1/3	4	HBx116	L	→	V	1	1	HBx116	L	→	V	1	1	0.565†
HBx127	I	→	L/T/X	1/2/3	6	HBx127	I	→	S/T	3/1	4	HBx127	I	→	N/T/X/(-)	1/2/2/1	6	0.832†
HBx130	K	→	M/X/(-)	25/2/1	28	HBx130	K	→	M/X/(-)	17/2/1	20	HBx130	K	→	M/(-)	27/2	29	**0.014**†
HBx131	V	→	F/I/X(-)	1/26/1/1	29	HBx131	V	→	I/T	22/1	23	HBx131	V	→	I/(-)	28/2	30	**<0.001**†
HBx132	F	→	L/X/(-)	1/1/1	3	HBx132	F	→	X/Y	2/1	3	HBx132	F	→	X/Y/(-)	1/2/2	5	0.471†

Abbreviations: A, alanine; N, asparagine; D, aspartic acid; E, glutamic acid; G, glycine; H, histidine; L, leucine; I, isoleucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; Y, tyrosine; V, valine; X, any amino acid; HBx, Hepatitis B virus x protein. *P* values were calculated by using Pearson's chi-square test* or Fisher's exact test†.

5. Risk assessment of double mutations and triple mutations

Odds ratios for HCC risk were calculated based on the number of patients with double or triple mutations. The CHB patients who had HBx5+HBx130+HBx131 triple mutations showed significant risk for HCC development. Also CHB patients with HBx5+HBx130+HBx131 triple mutations showed a somewhat higher risk for HCC than those with HBx130+HBx131 double mutations. The odds ratio of HCC in patients with triple mutations was higher than that with double mutations. Double or triple mutations were significant risk factors for the HCC incidence when cirrhosis patients were ruled out. When LC is considered as a pre-HCC status, double or triple mutations were also significant for the HCC incidence (Table 6). There was no statistical significance when HCC patients were compared with all CHB patients regardless of cirrhosis status.

Table 6. Odds ratio for HCC risk according to double and triple mutations

HCC Vs. CHB			(HCC+LC) Vs. CHB			HCC Vs. (CHB+LC)		
	HCC	No HCC		HCC+LC	No (HCC+LC)		HCC	No HCC
Double mutations[*]	27	27	Double mutations[*]	47	22	Double mutations[*]	27	47
No double mutations	4	15	No double mutations	7	15	No double mutations	4	18
Odds ratio	3.75 (95% CI 1.101-12.768, ***P* = 0.033**)		Odds ratio	4.578 (95% CI 1.634-12.825, ***P* = 0.003**)		Odds ratio	2.585 (95% CI 0.793-8.432, *P* = 0.126)	
Triple mutations[*]	13	5	Triple mutations[*]	23	5	Triple mutations[*]	13	15
No triple mutations[†]	18	37	No triple mutations[†]	31	37	No triple mutations[†]	18	50
Odds ratio	5.344 (95% CI 1.65-17.309, ***P* = 0.005**)		Odds ratio	5.49 (95% CI 1.867-16.142, ***P* = 0.001**)		Odds ratio	2.407 (95% CI 0.962-6.026, *P* = 0.091)	

Abbreviations: CHB, chronic hepatitis B; LC, liver cirrhosis; HCC, hepatocellular carcinoma; CI, confidence interval.

[*]Double mutations: HBx130+HBx131 double mutations, [†]Triple mutations: HBx5+HBx130+HBx131 triple mutations.

6. Effect of HBx mutation on the HBV protein expression

To determine the effect of HBx mutation on the HBV protein expression, HBsAg and HBeAg assays were performed in triplicate. Negative control (Huh7 cells transfected with pGEM4Z) showed negative HBsAg and HBeAg results. Relative expression levels of HBsAg and HBeAg were calculated according to the concentration of total protein of HBx wild type. There was no significant difference from wild type at the protein level of each cell culture supernatants. The levels of HBsAg and HBeAg were shown in Figure 3. These results suggested that specific HBx mutations did not affect HBV protein expression.

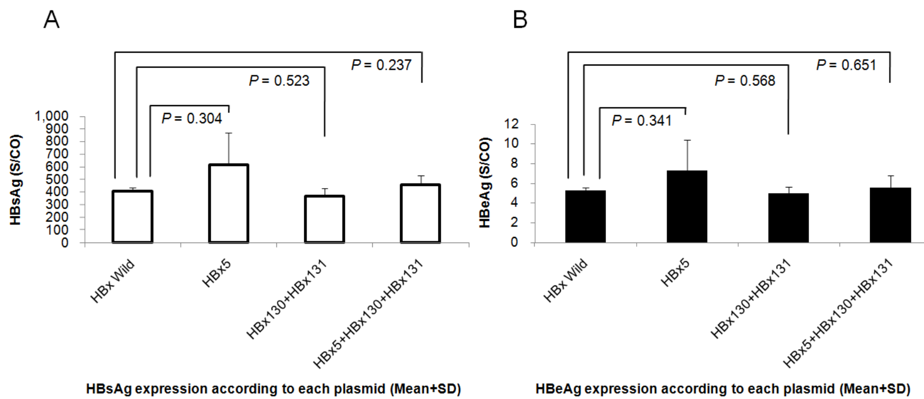

Figure 3. HBsAg (A) and HBeAg (B) expression in cell supernatants transfected with HBx mutant construct. Relative HBsAg and HBeAg of each cell culture supernatant were analyzed by Architect *i*2000 (Abbott Diagnostics, Abbott Park, IL, USA). Total protein concentration in cell culture supernatants of each transfected Huh7 cells was determined by using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). All experiments were performed in triplicate and expressed as mean±SD.

7. Effect of HBx mutation on the HBV DNA replication

To determine the HBx mutation on the HBV DNA replication, total HBV DNA levels of cell extract from transfectant with HBx wild type and three mutant types were analyzed (Fig. 4). There were no significant differences in HBV DNA levels between each HBx mutant and HBx wild type (P > 0.05). This result indicated that specific HBx mutations did not affect HBV DNA replication.

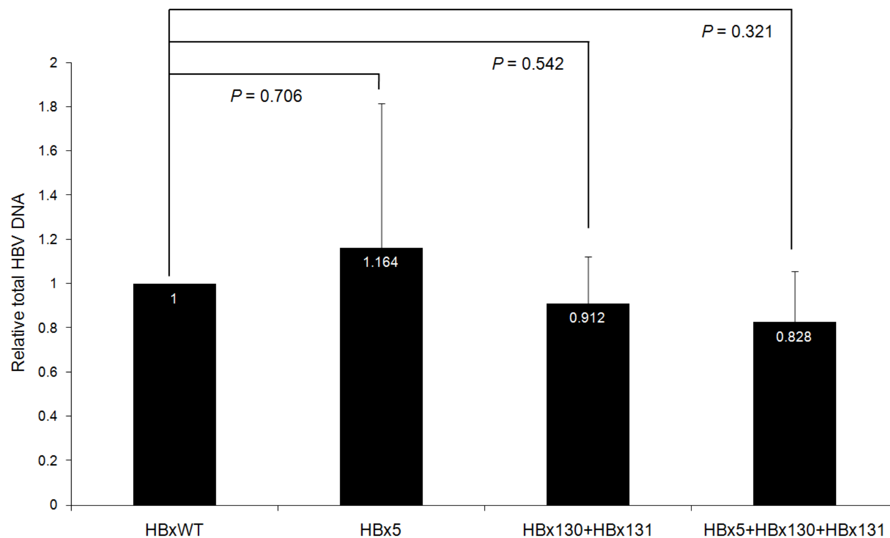

Figure 4. HBV DNA quantitation by real-time PCR in cell extracts of each HBx mutant. DNA was extracted from cells transfected with each construct, then purity and quantity of DNA were measured by Nanodrop (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Real-time PCR amplifications were carried out using ABI 7000 System (Applied Biosystems). Data were analyzed according to the standard curve in order to relatively quantitate the cycle threshold (Ct) values of samples. The values were normalized to that of GAPDH expression in each sample.

8. Effect of HBx mutation on NF-κB activity

NF-κB signaling pathway seems to have a central function in liver homeostasis, pathophysiology and regulation of the inflammation–fibrosis–cancer axis.[23] There were also studies on the role of NF-κB in the development

of HCC.[24] HBV and HBx have been known to activate NF-κB[25-26] but it is unclear whether specific HBx mutation can affect NF-κB activity. So, this study focused whether significant HBx mutation can affect NF-κB activity or not.

Huh7 cells were cotransfected with *Renilla* and each HBx mutant construct. Then, luciferase activity was analyzed. Luciferase activity was normalized by taking the activity of wild-type HBx-transfected cell lysates (relative luc). Results are expressed as the mean (bar)+SD (line) of at least three experiments (Fig. 5). There were significantly higher levels of NF-κB activity in cells with HBx5 mutation and HBx130+HBx131 double mutation than those of wild type and HBx5+ HBx130+HBx131 triple mutation. Cells with triple mutation did not show a significant difference in NF-κB activity comparing to that of wild type cells.

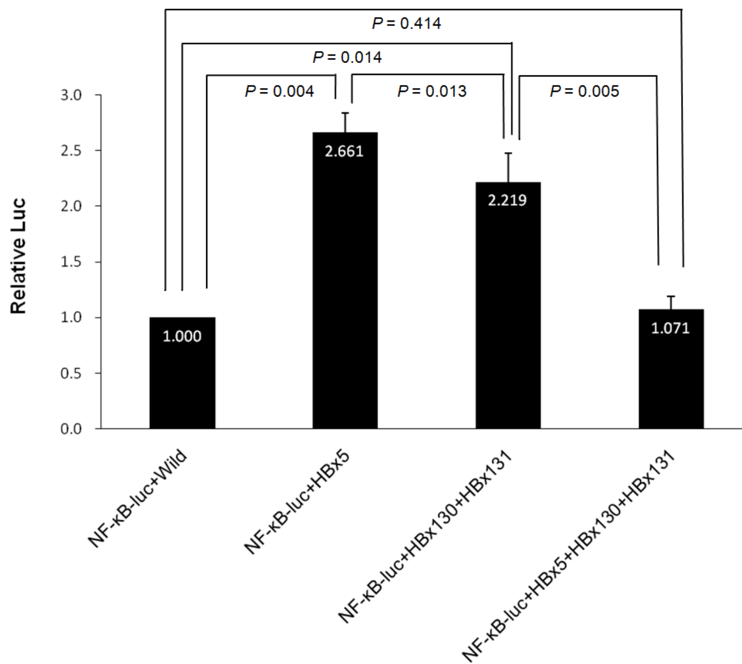

Figure 5. Relative luciferase assays showing the activity of NF-κB by HBx mutant types in Huh7 cells. Cotransfection with NF-κB luciferase vector and HBx constructs were performed by Lipofectamine LTX and PLUS reagents (Invitrogen). Cells were harvested two days after transfection, then the luciferase activity was detected by the Dual luciferase reporter assay system (Promega, San Luis Obispo, CA, USA) on a VICTOR™ X4 Multilabel Plate Reader (Perkin-Elmer Life Sciences, Shelton, CT, USA). All the luciferase data were normalized with corresponding *Renilla* luciferase activity. Each experiment was performed in triplicate.

IV. DISCUSSION

Although HBx cannot fully explain HCC pathogenesis, HBx has several clues associated with tumorigenesis. HBx is encoded by the smallest open reading frame of mammalian hepadnaviruses and translated from a small mRNA controlled by the HBx promoter.[27] HBx is also known to be a multifunctional protein that not only activates transcriptional transactivation but also mediates cell growth via regulating proliferation and apotosis.[9] Moreover, the HBx protein is potentially oncogenic via multistep carcinogenesis, modifies apoptosis, inhibits nucleotide excision, repair of damaged cellular DNA, and modulates transcriptional activation of cellular growth regulating genes.[16] Hepato-carcinogenesis has been partly explained by HBx participating various roles of such as suppressor p53[28], promoting cell cycle[29], promoting oncogene[30], interfering effect on DNA repair[21], Fas-related anti-apoptosis[31], activation of transcription factors such as NF-κB[32], activator protein 1 (AP-1)[33], AP-2[34], nuclear factor of activated T-cells (NF-AT)[35], and activating transcription factor/cAMP response element binding protein (ATF/CREB).[36] To date, clinical impact of HBx mutation has not been fully defined. Specific regions of HBx mutations or deletions in the sera[22,37] or liver tissues[38] in chronic HBV infected patients were reported to have an association with HBV pathogenesis. However, the exact impact of specific HBx mutation on chronicity or carcinogenesis remains unclear.

This study aimed to elucidate whether HBx mutation can affect development of HCC. Enrolled
96 patients showed 603 mutations; 250 mutations in CHB patients, 167 mutations in LC patients, and 186 mutations in HCC patients.

Interestingly, in this study it was found that the incidence of HBx5 mutation was significantly higher in HCC patients than CHB patients. All patients with HBx5 mutation also had HBx130+HBx131 double mutations. However, the frequency and function of HBx5 mutation have not been determined by the aspect of oncogenic potential. Further study of HBx5 mutation with or without HBx130+HBx131 double mutations will be needed to understand the role of HBx5 mutation.

Several reports dealt with the association between HBx mutation and specific diseases. Yuasa et al.[39] found that fulminant hepatitis patients were mostly infected by HBV genotype B and that fulminant genotype B strains differed from non-fulminant strains by a specific mutation in HBx41. Because HBV DNA sequences differ between HBV genotypes or subgenotypes, HBx5 mutation might be characteristically related with HBV genotype C2 in Korean HCC patients. HBV C2 is the most prevalent strain of Korean HBV infected patients.[40] Unfortunately, patients infected with genotype C2 HBV tends to rapidly progress to cirrhosis or HCC, and thus, it can commonly cause complications in younger patients.[41] Therefore, a well designed genotype specific mutation study is needed to identify the functional role of HBx.

There have been several studies on the clinical correlation between HBx mutation and HCC. Yeh et al.[42] reported that HBx codon-31 mutation was detected more frequently in patients with HCC. Muroyama et al.[43] reported that an HBx codon-38 change in genotype C is an independent risk factor for HCC development. Shinkai et al.[44] reported that regardless of HBeAg status, the T1653 mutation increases the risk of HCC in Japanese patients with HBV/C2. Zhu et al.[45] reported deletion at nt 382-400 of the X gene might play a role in carcinogenesis of HCC in southern China. Kim et al.[22] reported a novel mutation at codon 5 was found to be significantly associated with HCC in Korean patients (HBV C2) without functional study. Choi et al.[46] reported that only the B1499 (C or G or T1499) mutation was significantly associated with HCC.

Asim et al.[47] reported that TT1504, V1753, A1762T/G1764A mutations significantly increased the risk of HCC development. However, there has been no established consensus on or mechanism of which HBx mutation directly affects the pathogenesis of HCC. This might be attributed to differences in HBV genotype, level of HBV DNA, coinfection of other viruses, and effects of host factors.

To understand the role of HBx mutations, functional structure of HBx needs to be defined. However, no good structural studies of HBx based on X-ray crystallography or NMR have been performed.[48] Since HBx protein does not directly bind to DNA, it can affect transcription through association with

transcriptional transactivators.[49] Several groups reported the functional transactivating domain of HBx.[50-52] Murakami et al.[50] suggested that functional domain of HBx (HBx1-50) serve as a negative regulatory domain, and another (HBx51-154) as a transactivating domain. The HBx5 region resides near the N-terminal residues of HBx overlapping polymerase gene (765-766 codon translated from 2295-2297th polymerase gene). Murakami et al.[50] suggested that HBx52–148 domain is separated from the negative regulatory domain. Kumar et al.[51] also described that HBx58–140 is a transactivation domain, although there was some discrepancy on the N-terminal border. Runkel et al.[52] reported that two regions, one around HBx69, and another between HBx110 and HBx139 are critical for transactivation. The N-terminal of HBx has the ability to down regulate 'transactivation', and was defined as the negative regulatory domain.[50] Also, HBx1-50 was reported to be important for cell transformation.[12]

This current study showed that double mutations of HBV at nt 1762 (A→T) and 1764 (G→A) in the basal core promoter (BCP) region was the most frequent mutation type in HCC patients. Because double mutations of the BCP region also exist in the coding sequence of HBV X gene, it converts K to M at position 130 and V to I at 131 in the overlapping X-open reading frame (ORF) gene product. To date, these double mutations have been suggested to arrest the transcription of the precore RNA but not seriously affect the transcription of the pregenomic RNA.[53] In various cell transfection experiments, the T1762/A1764 double mutations have been known to produce conflicting results whether or not

increase viral replication.[54] However, BCP double mutations were suggested to be associated with the HCC development[55-56] and this finding has been supported by one prospective cohort study.[57]

The fifth amino acid of HBx is located in the negative regulatory region which is known as a dispensable region for transactivation. HBx5 transfectant showed no differences in HBV DNA replication activity, neither expression of HBsAg nor HBeAg whether they had double mutations or HBx5 mutation alone. These results showed that HBx mutation did not influence the viral replication or protein expression of HBV. However, relative transcriptional activity of NF-κB in HBx5 single mutation and HBx130+HBx131 double mutations was significantly higher than that of wild type and HBx5+HBx130+HBx131 triple mutations. This might suggest that NF-κB was not significantly activated by triple mutations of HCC patients.

NF-κB is the major player in virus-associated carcinogensis.[58] HBV and HBx have been known to activate NF-κB[25-26] but it is unclear that specific HBx mutation can affect NF-κB activity. This study showed that HBx5 and HBx130+HBx131 double mutations could activate the NF-κB signaling pathway. This phenomenon implies that HBx5 mutation does not directly influence HBV replication but might be associated with modulation of cellular signaling pathway. This study showed that NF-κB activity was characteristically affected by the existence of HBx5 mutation. In fact, HBx5 mutation of patients always coexisted with double mutations regardless of clinical phases of HBV

infection. This also implies that independent outbreak of HBx5 mutation may burden HBV life cycle and may not be affordable for viral immune escape from host defense system.

HBx was known to activate two transcription factors, NF-κB and NFAT, which are associated with the regulation of cytokine expression (IL-6, IL-8, and TNF-α).[59] Therefore, HBx5 mutation may enhance the oncogenic potential to progress from a chronic liver disease to HCC. Human NF-κB has been shown to be associated with tumorigenesis in mammalian cells through the regulation of a number of oncogenic genes.[60] Genes regulated by NF-κB activation include those encoding TNF-α, IL-6, E-selectin, ICAM-1, cyclin D1 and Bcl-XL.[61] HBV and HBx can activate NF-κB in liver cells.[62-63] In addition, Yu et al.[64] suggested that IL-6, IL-8R, and IL-2R were up-regulated by HBx expressed in the hepatoma cell line Huh7 microarray analysis. HBx may play a role in hepatic inflammation and disease by up-regulating IL-6, leading to cirrhosis and HCC.[65] Thus, this study suggests that a specific HBx mutation could modulate the NF-κB signaling pathway which is possibly associated with tumorigenesis.

However, NF-κB activity did not show difference between wild type HBx and HBx5+HBx130+HBx131 triple mutant type, there seems to be additional mechanisms to explain the development of HCC. Also older HCC patients tend to have more HBx130+HBx131 double mutations and HBx5+HBx130+HBx131 triple mutations than younger HCC patients. So, not only HBx mutations but also age factor may have some effect to develop HCC.

V. CONCLUSION

The most frequent HBx mutation was HBx130+HBx131 double mutations in HCC patients with chronic HBV infection and then followed by HBx5+HBx130+HBx131 triple mutations. HBx5 mutation was frequently observed in HCC patients than CHB patients. HBV construct containing wild type of HBx and 3 types of HBx mutation was transiently transfected and the effect of HBx mutation on the viral replication, HBV protein expression, and NF-κB activity was observed. The results showed that HBx5 single mutation, HBx130+HBx131 double mutations, and HBx5+HBx130+HBx131 triple mutations were neither directly associated with viral protein expression nor viral replication activity. However, HBx5 mutation and HBx130+HBx131 double mutations appear to modulate transcriptional transactivating function of the NF-κB signaling pathway.

This study showed that only HBx5 single mutation and HBx130+HBx131 double mutations increased NF-κB activity, but HBx5+HBx130+HBx131 triple mutations did not increase NF-κB activity, so there seems to be another pathway to regulate NF-κB activation by HBx mutation. Also older HCC patients tend to have more HBx130+HBx131 double mutations and HBx5+HBx130+HBx131 triple mutations than younger HCC patients. Therefore, age factor of patients should be considered to effect development of HCC. HBx5 mutation in genotype C2 may be a risk factor for HCC development and appears to serve as

a potential molecular marker for anticipating clinical outcomes of patients with chronic HBV infection.

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만성 B 형 간염 바이러스 감염에 의한 간세포암 발생에 미치는

B 형 간염 바이러스 X 단백질 돌연변이의 영향

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이종한

간세포암의 주 원인은 만성 B 형 간염 바이러스의 감염으로 알려져 있다. B 형 간염 바이러스의 X 단백질 (HBx)은 다양한 조절을 수행하는 단백질로 직접 또는 간접적으로 세포주기조절, 전사활성, 신호체계조절, 세포괴사, 혈관신생, DNA 복구 기전 등에 다양하게 관여 및 중개하는 것으로 알려져 있다. 본 연구는 간세포암의 발생에 관한 B 형 간염 바이러스 HBx 돌연변이의 영향을 규명하고자 하였다.

총 96 명의 만성 B 형 간염 바이러스 감염자들 즉, 42 명의 만성 B 형 간염 환자들, 23 명의 간경변 환자들, 31 명의 간암환자들을 대상으로 연구를 진행하였다. B 형 간염 바이러스 X 유전자의 염기순서분석과

그 결과 도출된 중요 돌연변이의 역할 규명을 위하여 체외 기능 연구를 수행하였다. 유전자형 분석 결과 모든 환자의 B 형 간염 바이러스는 유전자형 C2 이었다. 간세포암환자에서는 HBx131, HBx130, HBx5, HBx94, HBx38 단백질의 변이가 순서대로 비교적 높게 도출되었다. 다양한 변이 형태 중 HBx130+HBx131 중복변이와 HBx5+HBx130+HBx131 삼중변이가 간세포암에서 유의하게 높게 나타났다. 고연령층 간세포암 환자는 저연령층 간세포암 환자에 비하여 HBx130+HBx131 중복변이와 HBx5+HBx130+HBx131 삼중변이를 더 많이 가지고 있는 경향을 보여주었다. HBx130+HBx131 중복변이와 HBx5+HBx130+HBx131 삼중변이는 간세포암환자와 간경변 없는 단순 B 형 간염환자를 비교 시 각각 3.75 배, 5.34 배 간세포암의 위험도를 높이는 것으로 분석되었다.

기능적으로 야생형, HBx5 단일변이, HBx130+HBx131 중복변이, HBx5+HBx130+HBx131 삼중변이를 비교해 본 결과 감염 배양 상층액의 HBsAg, HBeAg 의 발현 정도는 모두 차이가 없었으며, 감염 세포 추출물의 B 형 간염 바이러스 DNA 수준 역시 전혀 차이가 없었다. 하지만, HBx5 단일변이, HBx130+HBx131 중복변이의 경우 야생형이나 HBx5+HBx130+HBx131 삼중변이보다 NF-κB 의 전사활성을 증가시킴을 확인하였다. HBx5 단일변이, HBx130+HBx131

중복변이의 경우 NF-κB 신호체계의 전사활성을 조절하는 것으로 생각된다. 그러나 HBx5+HBx130+HBx131 삼중변이는 NF-κB 활성도를 증가시키지 않았기에 NF-κB 활성을 조절하는 다른 종류의 신호전달체계가 작용하리라고 생각된다.

결론적으로, B 형 간염의 특정 HBx 돌연변이가 NF-κB 전사활성 과정을 통하여 간세포암의 발생을 촉진하는 것으로 보인다. B 형 간염 바이러스 유전자형 C2 형에서 HBx5 변이는 간세포암 발생의 위험 인자로서의 가능성이 있으며, 만성 B 형 간염 환자의 임상 결과를 예측하는 표지자로서의 기여가 가능할 수도 있을 것으로 판단된다.

핵심되는 말 : B 형 간염 바이러스, 간세포암, X 단백질, 돌연변이