

RESEARCH ARTICLE

T118N Substitution of Hepatitis B X Protein Reduces Colony Formation of HepG2 Cells

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Abstract

BACKGROUND: The acute Hepatitis B virus (HBV) infection usually ceases before six months, but chronic infection that lasts for more than six months might develop into liver cirrhosis and hepatocellular carcinoma (HCC). Viral particle load, HBV genotypes and association to the HBV x (HBx) gene mutations are the probable factors related to HCC occurrence. The mutation which leads to HBx T118N was found as the second most common HBx mutation in Indonesia, as compared to the known cancer-related HBx K130M/V131I mutant. However, the effect of T118N mutation and its combination with K130M/V131I on human hepatoma cells has not been elucidated well. Hence, this study was conducted to dissect the role of HBx T118N and its mutant combination in colony formation, as compared to the wild type HBx and cancer-related HBx K130M/V131I.

METHODS: In this study, the genes encoding wild type HBx, HBx T118N, and HBx K130M/V131I mutations were obtained as synthetic gene. Meanwhile, the gene encoding

HBx T118N/K130M/V131I mutations was successfully generated using site-directed mutagenesis. The optimum condition for colony formation assays was determined through Zeocin sensitivity test of HepG2 cells.

RESULTS: Selection of HepG2 cells using Zeocin was determined at 200 µg/mL. Colony formation assays performed upon expression of HBx T118N and HBx T118N/K130M/V131I mutant proteins showed reduced colony numbers as compared to the expression of wild type HBx, similar to the effect from HBx K130M/V131I mutant expression.

CONCLUSION: The HBx T118N and HBx T118N/K130M/V131I mutation caused less colony formation of HepG2 cells, similar to the K130M/V131I mutation. This indicates a possible role of the T118N mutation in liver cancer development.

KEYWORDS: colony formation assay, hepatitis B virus, HBx, T118N, K130M/V131I

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Introduction

Liver cancer is known to have the 2nd highest high mortality rate after lung cancer (1), and occurs mainly as hepatocellular carcinoma (HCC). Chronic infection of hepatitis B virus poses higher risk of HCC development, with or without incidence of liver cirrhosis. Hepatitis B virus (HBV) belongs to the Hepadnaviridae family and

classified into genotype A – H. Incidence of liver cirrhosis and HCC are also correlated with the HBV genotype. The HBV genotype C is known to cause severe liver disease, cirrhosis and HCC as compared to the HBV genotype B. However, HBV genotype B correlates with HCC incidence without cirrhosis.(2,3)

HCC development in the case of chronic HBV infection involves a complex mechanism. It might involve viral DNA integration, truncated form of HBsAg, inability

of host immune response to clear the infection, or even the role of HBV x protein (HBx).(4,5) The HBx may cause epigenetic change, act as a transcriptional activator of many cellular genes, activate certain cell signalling pathways, interact with host protein.(4,6,7) Modulation of signalling transduction pathways through Ras, Raf, mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF- κ B) and janus kinase/signal transducers and activators of transcription (JAK-STAT) by HBx has been shown to stimulate hepatocyte proliferation.(6) Presence of HBx mutants have been found to correlate with HCC incidence, i.e., V5M/L, P38S, H94Y, I127T/N and K130M/V131I. (8-10) The K130M/V131I HBx mutant protein has been shown to differentially influence cell proliferation.(11,12) Moreover, the C-terminally truncated HBx has been found in 30-40% tumor samples from HCC patients.(13-15)

The presence of HBx T118N mutation in HBV infected patients in Indonesia has been observed as the second most common mutant after the HBx K130M/V131I.(16,17) The HBx T118N mutation occurs only in the HBV genotype B. However, to date, there are no reports about the effect of T118N mutation and its combination with K130M/V131I on human hepatoma cells at the molecular level albeit its presence in samples from chronic infection of hepatitis B and HCC patients has been reported.(16) Hence, in this study, the role of HBx T118N and HBx T118N/K130M/V131I mutations on HepG2 cells colony formation was investigated, in comparison to the wild type HBx and HBx K130M/V131I mutation.

Methods

Cells and Plasmids

Human hepatoma cells, HepG2, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 4mM L-Glutamine and 1 mM sodium pyruvate. The wild type HBx gene (NCBI accession number: KX429626.1) was synthesized by DNA2.0 in pD609 plasmid. Meanwhile, the HBx T118N and HBx K130M/V131I mutations were synthesized by Genescript in pcDNA3.1/Zeo(+), further named pcDNA-HBx_T118N and pcDNA-HBx_K130M/V131I, respectively.

Construction of pcDNA-wtHBx

The wild type HBx gene was first subcloned from pD609_HBx-wt into pcDNA3.1/Zeo(+). The HBx-wt gene was amplified by polymerase chain reaction

(PCR), using primers that contain extensions with *Eco*R1 restriction sites: 0.2 μ M primer cl_HBx_For (5'-AGTGAT[GAATTC]ATGGCTGCTAGGCTGTG-3') and 0.2 μ M primer cl_HBx_Rev (5'-GCGATT[GAATTC]TTAGGCAGAGGTGAAAAAGTTG-3'), 100 ng pD609_wtHBx, 12,5 μ L DreamTaq Green PCR Master Mix 2x (Thermo Scientific, Massachusetts, USA). The PCR reaction was done at 95°C for 5 min for initial denaturation, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR product was characterized using 1% agarose gel electrophoresis and then cut with *Eco*RI.

The pcDNA3.1/Zeo(+) plasmid was prepared by cutting the pcDNA-HBx_K130M/V131I using *Eco*RI and extracting it after agarose electrophoresis from the gel using Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan). Ligation of pcDNA3.1/Zeo(+) was performed using T4 DNA ligase (Thermo Scientific, Massachusetts, USA) at 4°C for 18 h. The ligation result was transformed into *Escherichia coli* TOP10 and characterized by PCR and HBx gene sequencing.

Site-directed Mutagenesis

Construction of the HBx T118N/K130M/V131I mutant gene was performed on pHBx_K130M/V131I using single-primer site-directed mutagenesis.(18) Mutagenic primers were designed for HBx T118N (C1726A) (Table 1). Briefly, each PCR reaction was performed in two different tubes with forward primer in the first tube and reverse primer in the second tube, including addition of 500 ng of pcDNA-K130M/V131I, dNTP, buffer and DNA polymerase. Optimization of PCR was performed by varying the primer concentration and using *Pwo* and KAPA HiFi DNA Polymerase. After 30 cycles, the PCR products from two tubes were combined and annealed by incubation at 95°C for 5 min, 90°C for 1 min, 80°C for 1 min, 70°C for 30 sec, 60°C for 30 sec, 50°C for 30 sec, 40°C for 30 sec and 37°C for 10 min. The product was then treated with *Dpn*I enzyme, to digest the DNA template, and incubated at 37°C for 2 days and transformed into *E. coli* TOP10. Plasmids from transformed colonies were characterized and confirmed by nucleotide sequencing.

Determination of HepG2 Cells Sensitivity

HepG2 cells were seeded at 1000 cells per well in 6-well plates for 24 h. Next day, the growth media was replaced with growth media containing Zeocin (Thermo Scientific, Waltham, MA, USA) at various concentrations, ranging

Table 1. Sequences of the primers used for site-directed mutagenesis.

Primers	Sequences (5'→3') ¹	Nucleotide mutation ²	Amino acid substitution ³
T118N_Forward	GACTGTGTGTTTAA <u>T</u> GTAGTGGGAGGAGTT		
T118N_Reverse	TCCCACTCA <u>T</u> TAAACACACAGTCTTTGAAGTAT	1726C→A	T118N

Mutated nucleotides are shown as underlined; 1726 indicates the position of mutated nucleotides in the HBV genome; 118 is position of substituted amino acids in HBx.

from 100 µg/mL up to 500 µg/mL, and incubated for 14 days at 37°C, 5% CO₂. Selection media replacement was done every 2-3 days. Prior to observation, cells were washed once with DPBS and fixed with 3.7% formaldehyde. The cell colonies were then stained using 0.1% crystal violet. Determination of the colony number was performed using ImageJ program (National Institute of Health, Bethesda, MD, USA).

Colony Formation Assay

The colony formation assays were performed using optimum Zeocin concentration for selection of transfected HepG2 cells. HepG2 cells were seeded at 100,000 cells per well in 12-well plate for 24 h. Next day, 1 µg plasmid was diluted in 100 µL OptiMEM (Thermo Scientific) and mixed with 3 µL XtremeGene HP DNA Transfection Reagent (Roche, Basel, Switzerland). After 30 min incubation at room temperature, transfection mixture was added to HepG2 cells. After 24 h of transfection, cells were trypsinized and seeded at 1000 cells per well in 6-well plates. The growth media was then replaced with growth media containing 100 µg/mL Zeocin on the next day. The cells were incubated at 37°C, 5% CO₂ for 14 days and the growth media containing Zeocin was replaced every 2-3 days. After 14 days of incubation, cells were washed once with Dulbecco's phosphate-buffered saline (DPBS) and fixed with 3.7% formaldehyde for

30 min at room temperature. The cell colonies were then stained using 0.1% crystal violet. Determination of the colony number was performed using ImageJ program.

Results

Characterization and Generation of HBx Mutations

The HBx T118N and K130M/V131I mutations were obtained as synthetic genes in mammalian expression vector pcDNA3.1/Zeo(+), while the wild type HBx gene was subcloned into pcDNA3.1/Zeo(+) plasmid. The preparation of linear pcDNA3.1/Zeo(+) was obtained by cutting pcDNA-HBx_K130M/V131I with *EcoRI*. The result showed two bands with the size of 5000 bp and 500 bp, which was the plasmid backbone and the HBx gene, respectively (Figure 1A). The wildtype HBx gene was amplified by PCR and the resulting PCR product showed the size of HBx gene, *i.e.*, 465 bp (Figure 1B). After ligation and transformation, the resulting transformants were screened using PCR, and the positive clone was obtained from colony 2 (Figure 1C). The plasmid was then isolated and confirmed by sequencing.

To confirm the plasmids carrying genes encoding wild type HBx, HBx T118N and K130M/V131I mutations, restriction analyses were performed using *EcoRI* restriction enzyme. The results showed two bands with size

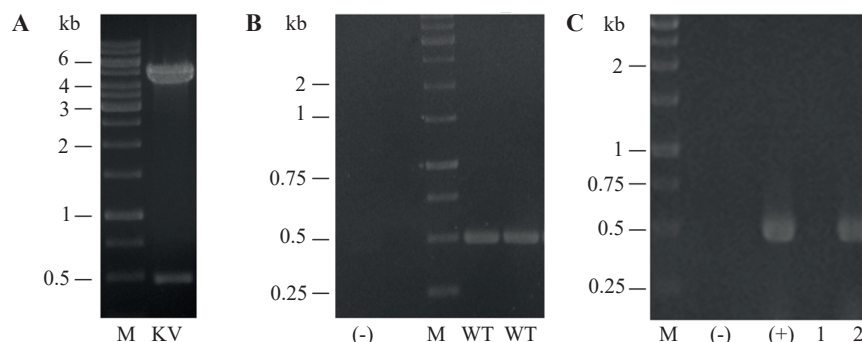


Figure 1. Subcloning of wildtype HBx gene into pcDNA3.1/Zeo(+). A: pcDNA3.1/Zeo(+) backbone was obtained by cutting pcDNA_{3.1}-HBx K130M/V131I with *EcoRI*, followed by gel extraction; B: wildtype HBx gene was amplified using PCR and then purified; C: screening of transformants using PCR from colony 1 and 2. M: DNA 1 kb ladder; (-): PCR negative control; (+) PCR positive control; KV: pcDNA-HBxKV + *EcoRI*; WT: wild type HBx; 1: colony 1; 2: colony 2.

of 5000 bp and 500 bp, which correspond to the size of pcDNA3.1/Zeo(+) and HBx gene, respectively (Figure 2A). DNA sequencing was also performed using T7-promoter primer and the result confirmed the sequences of the wild type and mutant HBx genes (Figure 2B).

To generate the HBx T118N/K130M/V131I mutant gene, single-primer site-directed mutagenesis approach was performed using T118N mutagenic primer pair and pcDNA-HBx_K130M/V131I as template. The site-directed mutagenesis was performed using KAPA HiFi DNA Polymerase with addition of ethylene glycol. The plasmids obtained from transformed cells were then isolated and sequenced using T7-promoter primer. The sequencing result of HBx wildtype showed the same sequence as in the genbank database (NCBI Acc. No. KX429626.1). The sequencing result of the HBx T118N constructs showed the ACT→AAT substitution at codon 118, with no substitution at codons 130 and 131. The sequencing result of the HBx K130M/V131I construct showed AAG→ATG substitution at codon 130 and GTC→ATC at codon 131. Meanwhile, the HBx mutant combination harbored the three substitutions that encode HBx T118N/K130M/V131I (Figure 2B).

Determination of HepG2 Cell Sensitivity and Colony Formation Assay

In the colony formation assay, the transfected HepG2 cells were selected using Zeocin. Therefore, the sensitivity of HepG2 cells was determined by propagation of cells in media with Zeocin at various concentrations, 0-500 µg/mL. The cells were grown for 14 days and the number of

the colonies was counted. The results showed that 200 µg/mL Zeocin was the minimum concentration that resulted in complete growth inhibition of HepG2 cells (Figure 3A). This concentration was used in the colony formation assay. Determination of cell survival upon HBx wild type and mutant proteins expression was carried out using colony formation assay or clonogenic assay. The transfected HepG2 cells were reseeded at density of 1000 cells/well and then grown for 14 days in media containing 200 µg/mL Zeocin. The results showed that there was increase of cell colonies in the HBx wild type protein expression as compared to the vector control (Figure 3B). However, there was reduction of cell colonies upon expression of HBx K130M/V131I in comparison to the HBx wild type protein expression (Figure 3B). The HBx T118N and HBx T118N/K130M/V131I expression also showed a lower number of cell colonies as compared to the wildtype HBx expression, similar to HBx K130M/V131I (Figure 3B).

Discussion

The role of HBx protein has been intensively studied in relation to development of HCC. HBx is known for its role in transcriptional activation, modulation of cell cycle regulation and apoptosis, as well as its ability to interact with cellular proteins.(4) Many studies have performed diverse investigation of HBx activity in various hallmarks of liver cancer. HBx activity in sustaining proliferative signaling was shown through the effect on c-myc expression, upregulation

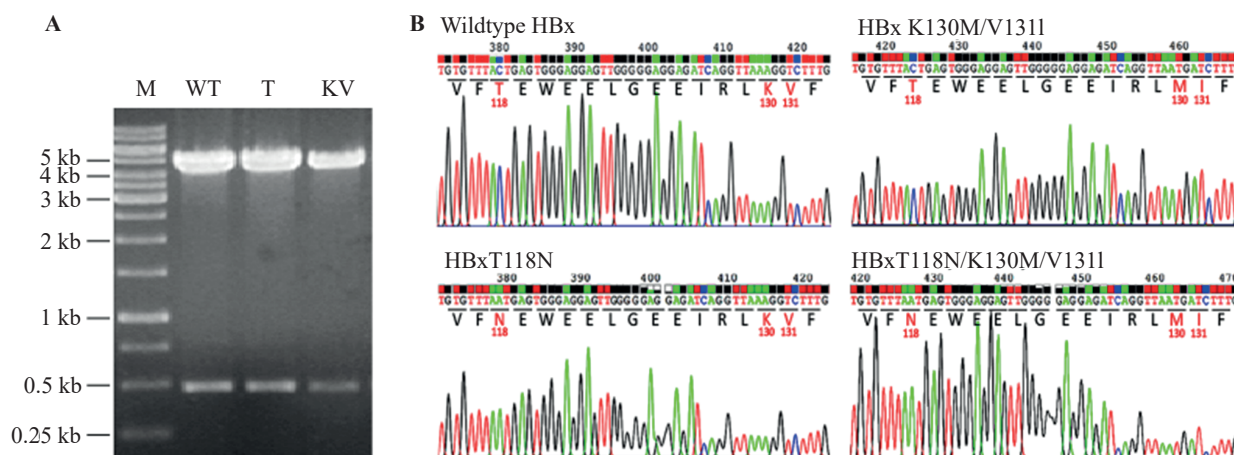


Figure 2. Plasmid confirmation by restriction analysis and DNA sequencing. A: Restriction analysis of pcDNA-HBx_{wt}, pcDNA-HBx_{T118N} and pcDNA-HBx_{K130M/V131I} using *Eco*RI. Digested plasmid was analysed using 1% agarose gel electrophoresis; B: The HBx gene sequences obtained as synthetic genes and site-directed mutagenesis were determined using DNA sequencing analysis. The sequencing was performed using T7 promoter primer at Macrogen, Seoul, Korea. M: DNA 1 kb ladder; WT: pcDNA-HBx_{wt}; T: pcDNA-HBx_{T118N}; KV: pcDNA-HBx_{K130M/V131I}.

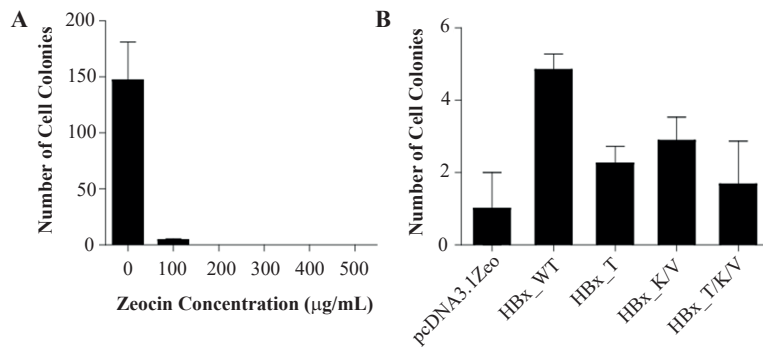


Figure 3. Determination of HepG2 cells sensitivity and colony formation assay. A: HepG2 cells were grown in 6-well plate at 1000 cells/well in media containing 0-500 µg/ml Zeocin for 14 days. B: HepG2 cells were transfected with pcDNA3.1/Zeo(+), plasmids carrying gene encoding for HBx wildtype, T118N, K130M, K130M/V131I, T118N/K130M/V131I or left untransfected.

of p21 and p27, activation of Ras and Src kinase, interaction with SMYD3, and many more. In terms of evading growth suppressors, HBx was known to inhibit p53 response element, inactivation of Rb gene promoter, suppression of E-cadherin. Meanwhile, HBx also can induce angiogenesis, through overexpression of MMPs and stimulation of Ang-2 isoform, and is known to trigger invasion and metastasis through various possible mechanisms.(19)

Several HBx mutations have been found to be associated with HCC incidence, such as V5M/L, P38S, H94Y, I127T/N and K130M/V131I.(8-10) Detection of HBx gene mutants in Indonesia revealed the presence of the T118N mutation in HBV genotype B. The prevalence of this mutant was also quite high in patients having chronic hepatitis and HCC.(16) Therefore, further study on the clinical significance of HBx T118N is of importance. In this study, we first generated the combination of HBx T118N with K130M/V131I mutations using site-directed mutagenesis. Study on cell survival was then performed upon expression of all the HBx mutants. Our result revealed that the HBx wildtype caused an increase of cell colonies. This result was in accordance with previous reports, showing that in HepG2 and in Chang cells, HBx protein increased the formation of colonies as compared to the vector control or mock transfected cells.(11,12,20)

The HBx K130M/V131I mutation showed reduction of colony formation as compared to the HBx wildtype. Additionally, the effects of HBx T118N and T118N/K130M/V131I mutations were similar to the effect of HBx K130M/V131I in colony formation assays. The HCC-related HBx K130M/V131I has been reported to cause less colony formation as compared to the HBx wildtype in HepG2 cells.(11) This slow induction of growth by HBx K130M/V131I was hypothesized to be correlated with presence of p53. In the cells lacking p53 expression, Hep3B cells, the effect of HBx K130M/V131I on colony formation was the same as the HBx wildtype. The HBx K130M/V131I also better stabilizes p53 than HBx wildtype based on the increase of Phospho-Ser15.(11) One study in Indonesia also revealed that the mutation in codon 118 of HBx occurred

in 7% of chronic liver infected patients.(21) As the HBx T118N and HBx T118N/K130M/V131I mutants showed similar effects on colony formation as the HBx K130M/V131I, the putative roles of HBx T118N and HBx T118N/K130M/V131I mutants in HCC development need to be further elucidated. It is unclear whether the amino acid 118 is important for interaction of HBx with p53, however, it is part of the p53 binding domain which is located at 101-154.(22) The mode of interaction between HBx T118N and HBx T118N/K130M/V131I mutants with p53 and its effect on apoptosis and cell cycle regulation will also be investigated further. The amino acid 118 is also part of the transactivation domain of HBx (23), and studying the transactivation ability of HBx T118N and T118N/K130M/V131I is also of importance for understanding the role of these mutants in HCC development.

Development of HCC due to HBV infection involves complex mechanisms. The effect of HBx activity and its mutations was only one factor that contributed to the HCC formation. One of the known predictors for HCC in liver cirrhosis patient was the expression level of vascular endothelial growth factor (VEGF) (24), which was known to contribute to angiogenesis in cancer development. Modulation of players in apoptosis induction, such as Caspase-3, could also contribute to the development of liver cirrhosis and toxicity.(25) Another contributing factor is diet. It has been shown that choline-deficient and high-fat diets contributed to steatohepatitis.(26)

Conclusion

Our findings showed that T118N substitution in HBx protein, either as single substitution or in combination with the K130M/V131I substitutions, reduced colony formation in HepG2 cells, similar to the effect of the cancer-related K130M/V131I mutation. This finding serves as initial indication that the T118N mutation poses a risk for HCC development.

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Authors Contribution

AA, CR, RRT, DSR were involved in conceptualizing and planning the research; DRN and SCP performed the data acquisition/collection and data analysis; AA, DRN, SCP and DSR performed the result interpretation; AA and CR drafted the manuscript and designed the figures; AA, CR and DSR acquired the funding. All authors took parts in giving critical revision of the manuscript.

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