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Core promoter mutation of nucleotides A1762T and G1764A of hepatitis B virus increases core promoter transactivation by hepatocyte nuclear factor 1

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Hepatitis B virus (HBV) infection highly increases the risk for liver cirrhosis and hepatocellular carcinoma (HCC). The clinical manifestation of HBV infection is determined by the mutual interplay of the viral genotype, host genetic factors, mode of transmission, adaptive mutations, and environmental factors. Core promoter activation plays a critical role in the pre-genomic RNA transcription of HBV for HBV replication. The mutations of core promoter have been implicated in HCC development. We had obtained HBV genes from Myanmar HBV infectants and identified gene variations at the core promoter region. For measuring the relative transactivation activity on core promoter, we prepared the core-promoter reporter construct. Both of A1762T and G1764A mutation were consistently found in the HBV genes with hepatocellular carcinoma. The A1762T/G1764A mutation was corresponding to K130M/V131I of HBx protein. We prepared the core promoter-luciferase reporter construct containing the double A1762T/G1764A mutation and the K130M/V131I HBx protein expression construct. The A1762T/G1764A mutation highly was responsive to core promoter transactivation by HBx, regardless of HBx mutation. The A1762T/G1764A mutation newly created hepatocyte nuclear factor 1 (HNF1) responsive element. Ectopic expression of HNF1 largely increased the HBV core promoter containing A1762T/G1764A mutation. In addition, hepatic rich fatty acid, palmitic acid and oleic acid, increased K130M/V131I HBx level by core promoter activation. These results provide biological properties and clinical significance of specific HBV core promoter mutants related with HCC development.

Keywords: HBV, HBV core promoter, HBx, virus mutation, HNF1

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Introduction

Chronic infection of hepatitis B virus (HBV) causes liver diseases including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Kao and Chen, 2002). About 350 million people was infected with HBV chronically worldwide (Schweitzer *et el.*, 2015). HBV infection have shown distinct differential geographical appearance. In the Europe and United States, HBV prevalence is low at 0.1–0.2% whereas, in the Mediterranean coast, Japan, and Korea HBV prevalence is about 2.0–7.0%. HBV prevalence is high and ranges from 8.0% to 20.0% in China, Southeast Asia, and Sub-Saharan Africa (MacLachlan and Cowie, 2015). Hepatitis B is one of the most common and serious disease in Myanmar. HBV can be classified into ten genotypes, A through J, based on a sequence divergence of > 8%. Within the viral genotypes are sub genotypes that differ above 4%. The mutation rate of HBV is higher than that of other DNA viruses owing to the reverse transcription in its life cycle. Myanmar has a moderate to high endemicity of hepatitis B infection. According to the nationwide seroprevalence survey in 2015, 6.5% of general population was infected with viral hepatitis B. The prevalence was found to be varied with geographic variation with highest prevalence in Yangon Region (10%) and lowest in Kayah State (4.2%) and differential genotype variation with various mutations.

After chronic HBV infection, covalent closed circular DNA (cccDNA) of HBV is used as a template for the transcription of mRNA (Tang *et al.*, 2001). The transactivation of HBV core promoter is essential for HBV replication (Karayiannis, 2017), and is found at the start site of the genomic RNA (Quarleri, 2014). HBV transcribes a 3.5 kb pre-Core (pre-C) mRNA, a 3.5 kb pre-genomic (pg) RNA, a 2.4 kb large (L), a 2.1 kb middle (M), small (S) mRNA, and a 0.7 kb X mRNA. Pre-C mRNA is a template for pre-C protein translation, producing serological hepatitis B e antigen (HBeAg). L, M, and S mRNA are translated to produce envelope proteins and HBx mRNA is translated to HBx protein. Pre-genomic RNA is little shorter than pre-core mRNA. The two AUG start codons for the transcription of pre-genomic RNA and pre-core mRNA are present in same reading frame (Quarleri, 2014; Lamontagne *et al.*, 2016).

Although HBeAg is not a necessary component for HBV virion formation, the level of HBeAg expression is measured as HBV replication efficiency. Low levels of HBeAg and high levels of Anti-HBe are associated with low levels of HBV replication (Hadziyannis and Laras, 2018). A high mutation rate during HBV replication results in resistance to antiviral

therapies (Mirandola *et al.*, 2012). Chronic HBV showing HBeAg-negative was seen in 24% of patients when HBeAg seroconversion was observed for 8.5 years. Moreover, HBeAg-negative chronic HBV reappeared in the period of HBV retention. Precore mutation or core promoter mutation was detected in most HBeAg-negative chronic hepatitis B patients (Choi *et al.*, 2009). Pre-core and core promoter mutations resulted in the inhibition of HBeAg synthesis. The report suggested that these mutations are associated with clinical reactivation; however, precore mutations are also detected during the non-proliferative period. This study indicates that the sporadic core promoter mutations of HBV in Myanmar HCC patients can increase HBV gene expression, resulting from new creation of liver-specific transcription factor binding sites and increased HBx transactivation.

Materials and Methods

Serum samples

This study used serum samples of 96 HBsAg positive patients that were received for routine HBV DNA detection from Department Medical Research (DMR) in Yangon, Myanmar. The serum samples were stored at -80°C. This study was approved by Ethics Review Committee of Department of Medical Research. The approval number is 22/ Ethics 2015, dated 25.3.2015. Informed consent was obtained from the study participants who were at or more than 16 years and from the parents/guardians of the study participants who were under 16 years.

Amplification of viral DNA by the PCR

HBV DNA was extracted from 96 serum of HBV HBsAg positive patients. PBS the quadruple of serum mixed with 40 µl of HBsAg positive serum. The mixed samples were incubated for 3 min at 95°C and 5 min at 4°C. After centrifugation is used at 12,000 rpm for 20 min, the supernatants 100 µl transfer to 1.5 ml Microcentrifuge tubes. Collected supernatant is used as DNA template for PCR. HBV core promoter and preS region was amplified using specific gene primers. PCR of HBV core promoter region was performed using nTaq DNA polymerase (Enzynomics) and primary and nested primer. Amplified PCR products of HBV DNA were examined by electrophoresis on a 1% agarose gel at 150 V for 25 min and stained with ethidium bromide.

Sequencing and HBV mutation analysis

The purified PCR products of HBV DNA were sent to a DNA sequencing company (COSMO GENETECH) for sequencing. HBV core promoter mutation sites were analyzed using the BioEdit and multiple sequence alignment service on the CLUSTALW home page (http://www.genome.jp/tools/clustalw/) in the HBV nucleotide sequences.

Plasmid constructs and reagents

To construct HBV core promoter A1762T and G1764A mutations, the promoter fragments from the serum of HBV infected patients were amplified by PCR using cloning primers contacting restriction enzyme site, *Kpn*I and *Hin*dIII: forward, 5′-GCC GGT ACC ACG CCC ACC AAA TAT-3′ and reverse, 5′-CGC AAG CTT TAC AAG AGA TGA TTA-3′. The PCR products were digested with *Kpn*I and *Hin*dIII and were cloned into pGL3B vector. The sequences were confirmed by COSMO GENETECH. pcDNA3-HA-HBx was constructed by inserting the PCR fragments of HBx ORF into *Eco*RI and *Xho*I cloning sites of pcDNA3-HA. 1.3x Cp-luciferase HBV was generously provided by Y. Shaul (Weizmann Institute of Science). The 1.2 mer HBV (HBx+) replicon and HBV 3xflag (1.2 mer HBV constructs including N-terminal 3xflagged HBx) were kindly provided by W.S. Ryu. The transfection reagent jetPEI was purchased from Polyplus Transfection.

Cell culture

Hep3B cells (all obtained from the American Type Culture Collection) were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL) and 1% (v/v) penicillin-streptomycin (Gibco BRL) at 37°C in a humid atmosphere containing 5% CO_2.

Treatment of fatty acids

Each fatty acid was dissolved in ethanol and diluted in Dulbecco's modified Eagle's medium containing 0.1% (w/v) fatty-acid-free BSA. Fatty acid–BSA complexes were added to culture plates at the indicated specific concentrations of fatty acids. Controls were incubated with equal concentrations of fatty acid-free BSA containing ethanol.

Luciferase assay

Hep3B cells were seeded in a 24-well culture plate and transfected with reporter vector and β-galactosidase expression plasmid, along with each indicated expression plasmids using jetPEI (PolyPlus Transfection SA). The pcDNA3 plasmid DNA was added to accomplish the same amount of plasmid DNA transfection. After 24 h of transfection, the cells were washed with ice-cold PBS and lysed with the cell culture lysis buffer (Promega). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were conducted at least in triplicate.

SDS-PAGE and western blotting

The cells were prepared by washing with cold PBS before lysing with lysis buffer (150 mM NaCl, 1% Nonidet P-40 [NP-40], 1 mM EDTA, 50 mM Tris; pH 7.5, 10% glycerol, 20 mM NaF and 5 mM Na_3VO_3) containing protease inhibitor and 1 mM PMSF (Phenylmethylsulfonyl fluoride). The protein concentration was determined using the Bradford reagent (Bio-Rad) and BSA (Bovine serum albumin) was used as a standard. An equal amount of proteins was loaded and separated by SDS-PAGE (15%), and the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). For Western blotting, the membranes were incubated with anti-β-actin (A2066, Sigma) and anti-HA (1 867 423, Roche)

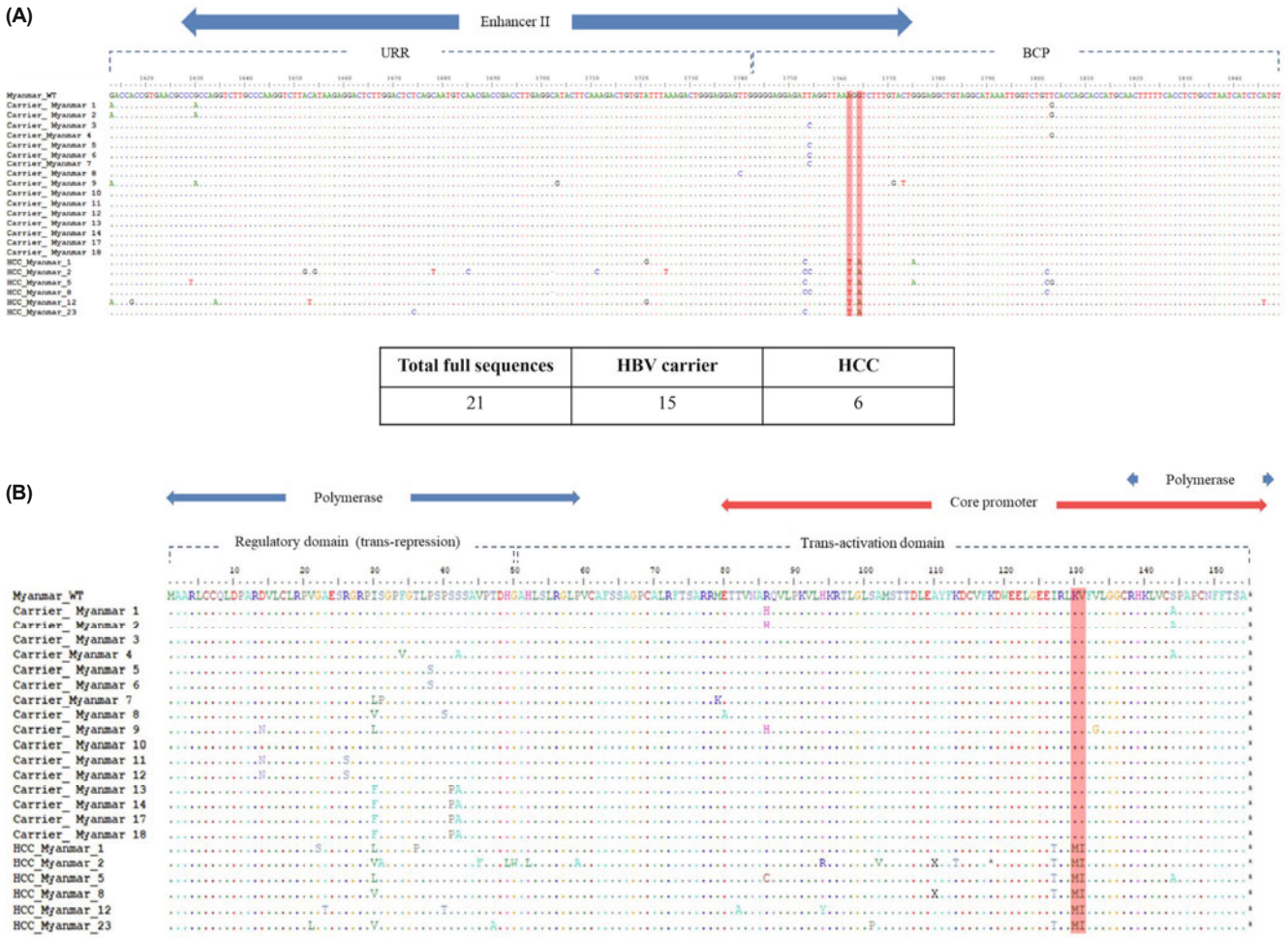
antibodies, in TBST (Tris-buffered saline containing 1% Tween 20) supplemented with 3% non-fat dried skim milk overnight at 4°C. Next, after washing three times with TBST, the blotted membranes were incubated with peroxidase-conjugated secondary antibodies (Enzo) for 40 min at room temperature. Following this, the proteins were visualized by the ECL (Enhanced chemiluminescence system) development reagent (GE Healthcare).

ChIP (chromatin immunoprecipitation) assays

Cells were seeded and transfected with the indicated plasmid using jetPEI. After 36 h of transfection, cells were washed twice with PBS and cross-linked with 1% (w/v) formaldehyde for 10 min at 37°C. Glycine (125 mM) was added for 5 min at room temperature to stop the reaction. Chromatin solution was sonicated 5 times at 10 sec intervals with a power output setting of 10 and incubated with the anti-HA (Roche) or control IgG, overnight at 4°C with rotation. The immune complexes were collected with Protein G–Sepharose slurry (Invitrogen) and salmon-sperm DNA for 4 h with rotational washing and then incubated overnight at 65°C to reverse the cross-linking. Purified DNA was subjected to PCR using primers flanking the HNF1α-binding motif in the HBV core promoter. The following PCR primers were used: 5′- CAC CAG GTC TTG CCC AAG GTC -3′ (forward) and 5′- GCA TGG TGC TGG TGA ACA GAC C -3′ (reverse). The PCR products were 189 bp in length.

EMSA

His-tagged HNF1 protein (50 ng) was used in each binding reaction mixture contained 2 μg of poly(dA-dT)· poly(dA-dT) in 15 μl of 1 mM DTT–20% glycerol–1 mM EDTA–10 mM Tris-HCl (pH 7.5)–15 mM NaCl. The reaction mixtures were incubated at room temperature for 20 min. Competition assays were done with unlabeled DNA at 40-fold molar excess over labeled probe DNA. After addition of ^{32}P-labeled probe (2×10^6 cpm), the reaction mixture was incubated for an additional 20 min at room temperature. Samples were subjected



Total full sequences	HBV carrier	HCC
21	15	6

Fig. 1. Comparison of gene variation of core promoter and HBx protein from Myanmar HBV-infected patients. (A) Nucleotide variation of HBV core promoter in HCC-related HBV genes. After obtaining HBV gene from Myanmar HBV-infected patients, the core promoter region (nt 1,595–nt 1,880) was sequenced. The conserved core promoter sequence of Myanmar HBV was used as a wild type and control for comparing with other the mutated core promoter sequences. HCC_Myanmar indicates HBV DNA sequence obtained from HBV-positive patients with hepatocellular carcinoma. (B) Amino acid variation of HBx protein in HCC-related HBV genes. Carrier designates a non-clinical sign with HBV infection and HCC indicates a patient having hepatocellular carcinoma in the presence of HBV infection.

to electrophoresis in a 5% polyacrylamide gel (36:1 acrylamide/bisacrylamide) containing 45 mM Tris-HCl (pH 8.3), 45 mM boric acid, and 1 mM EDTA.

Transcription factor search

Blast searches of GenBank were performed using the BLAST service at the National Center for Biotechnology Information (NCBI) home page (http://www.ncbi.nlm.nih.gov/). For analyzing transcription factor binding sites in the HBV core promoter region, the PROMO3 service was used.

Statistical analysis

Statistical software GraphPad Prism 5.03 (GraphPad Software, Inc.) was used for analysis. All experiments are presented as mean ± standard deviation (SD) and analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. p-value of < 0.05 was considered statistically significant.

Results

HBV genes obtained from Myanmar hepatocellular carcinoma patients provide HCC-related core promoter mutations

HBV core promoter activation for synthesis of pre-genomic RNA is important to regulate efficient HBV replication (Karayiannis, 2017). Mutations in the core promoter result in dysregulated HBV replication. An increased viral load by core promoter mutation in the HBV-infected liver cells triggers liver pathogenesis. We have collaborated Myanmar researchers, who are members of Department of Medical Research of Myanmar, for analyzing HBV gene variation from HBV-infected patients in Myanmar. Myanmar has high frequency of liver diseases, such as hepatitis, cirrhosis, and hepatocellular carcinoma (HCC), resulting from HBV infection.

After obtaining HBV genes of 22 Myanmar patients, including 16 no-clinical signs and 6 HCC, we analyzed gene sequences of core promoter. To identify mutations in the core promoter, genes including the core promoter site were amplified using PCR. Sequencing and alignment reactions were conducted on the PCR products. Among a variety of core promoter mutations, A1762T and G1764A double mutations were found in all core promoter DNA obtained from six Myanmar HCC with HBV infection (Fig. 1A). The A1762T/G1764A core promoter mutation leads to two overlapped HBx amino acid changes, K130M and V131I (Fig. 1B). The exact mechanism underlying the role of this mutation in hepatocarcinogenesis is still unknown. The mutation can cause a substantial decrease in HBeAg expression and enhancement of viral genome replication, which contribute to the liver disease progression via increased inflammation and viral invasion.

A1762T and G1764A double mutations increase the transactivation of HBV core promoter and HBx-mediated activation

Figure 2A shows the overlapping mutations of core promoter nucleotides and HBx amino acids from Myanmar HBV-positive patients with HCC. Among the mutations, we evaluated the reciprocal enhancement effect of the A1762T/G1764A mutation and two overlapped HBx amino acid changes, K130M and V131I, on the core promoter transactivation. In order to analyzing the core promoter activity quantitatively, we constructed two luciferase reporter systems containing HBV core promoter wild type and mutation (Fig. 2B). In addition, two HBx protein expression constructs were also prepared for examining the HBx-mediated core promoter transactivation. We transfected with HBV core promoter con-

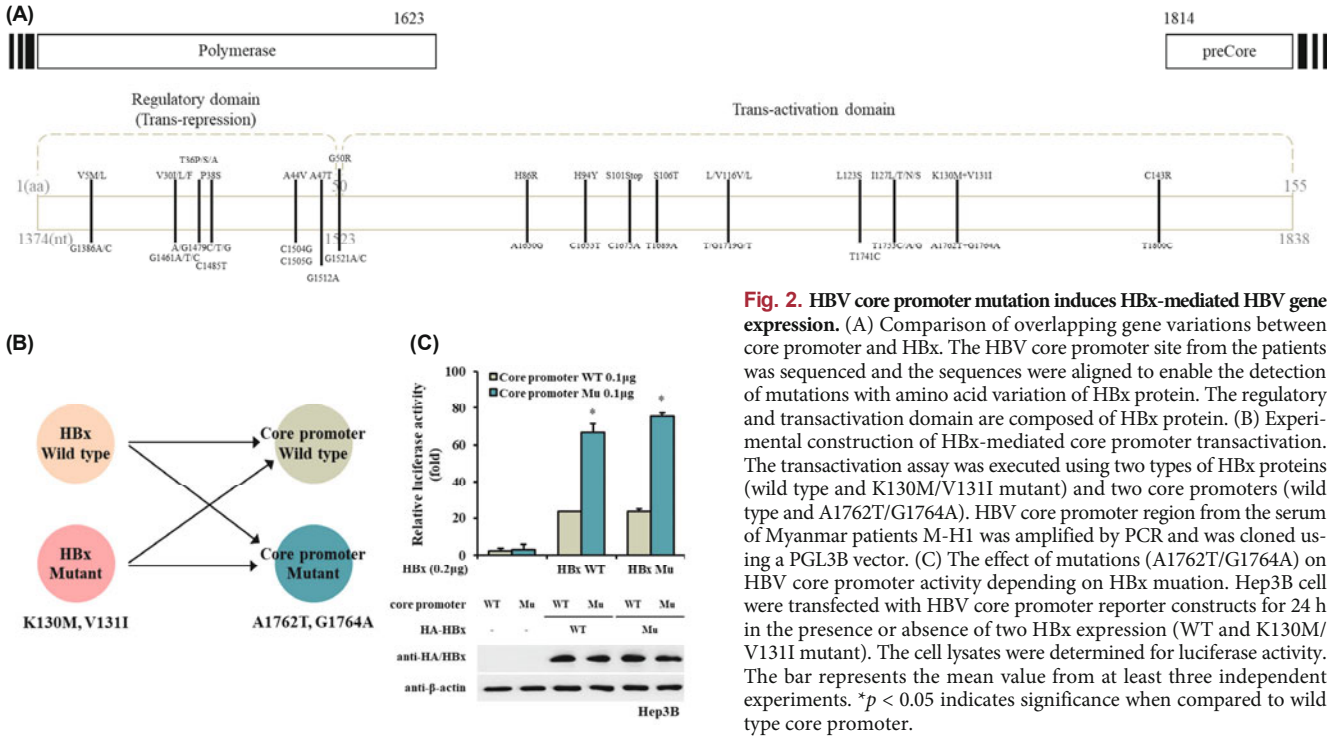


Fig. 2. HBV core promoter mutation induces HBx-mediated HBV gene expression. (A) Comparison of overlapping gene variations between core promoter and HBx. The HBV core promoter site from the patients was sequenced and the sequences were aligned to enable the detection of mutations with amino acid variation of HBx protein. The regulatory and transactivation domain are composed of HBx protein. (B) Experimental construction of HBx-mediated core promoter transactivation. The transactivation assay was executed using two types of HBx proteins (wild type and K130M/V131I mutant) and two core promoters (wild type and A1762T/G1764A). HBV core promoter region from the serum of Myanmar patients M-H1 was amplified by PCR and was cloned using a PGL3B vector. (C) The effect of mutations (A1762T/G1764A) on HBV core promoter activity depending on HBx muation. Hep3B cell were transfected with HBV core promoter reporter constructs for 24 h in the presence or absence of two HBx expression (WT and K130M/V131I mutant). The cell lysates were determined for luciferase activity. The bar represents the mean value from at least three independent experiments. *$p < 0.05$ indicates significance when compared to wild type core promoter.

structs of wild type and double mutant into Hep3B cells and measured the luciferase activity for evaluating the relative promoter activity of core promoter variation. The transactivation activity of core promoter containing A1762T/G1764A mutation (M-H1) showed much stronger than wild type core promoter (M-WT) activation in Hep3B cells (Fig. 2B). This result indicated that HCC-related gene variation in the core promoter increases the transactivation of HBV core promoter.

Since the core promoter region overlaps with HBx gene, which protein is critical to induce hepatocarcinogenesis, we also prepared the HBx mutants on K130M and V131I. In order to examine the effect of this double amino acid variation on the HBx-mediated transactivation, we transfected HBx wild type and mutant genes into Hep3B cells together with the HBV core promoter and measured differential HBx transactivation through the luciferase reporter expression. As shown in Fig. 2B, the double K130M/V131I HBx mutant did not increase the HBV core promoter transactivation comparted to the wild type HBx. These results indicate that the HBV core promoter activation is highly dependent on the gene variation of core promoter at A1762T/G1764A by increasing responsiveness of the hepatic specific transcription factor.

Hepatic rich transcription factor HNF1 increases the A1762T/G1764A mutated core promoter transactivation

For the pre-genomic transcription of HBV, liver cell-specific transcription factors should bind to the core promoter and induce the core promoter activation. We searched the tentative binding sites of specific transcription factors in the control and the mutated promoters using PROMO program. The results show that HNF4 bind to the HBV core promoter control at region covering from nt 1,762 to 1,771 (Fig. 3A). Even HNF4 is known to bind to the HBV core promoter and regulates HBV gene expression, there is no report for HNF1 to regulate HBV core promoter yet. However, the transcription factor HNF1 was reported to respond to the A1762T/G1764A

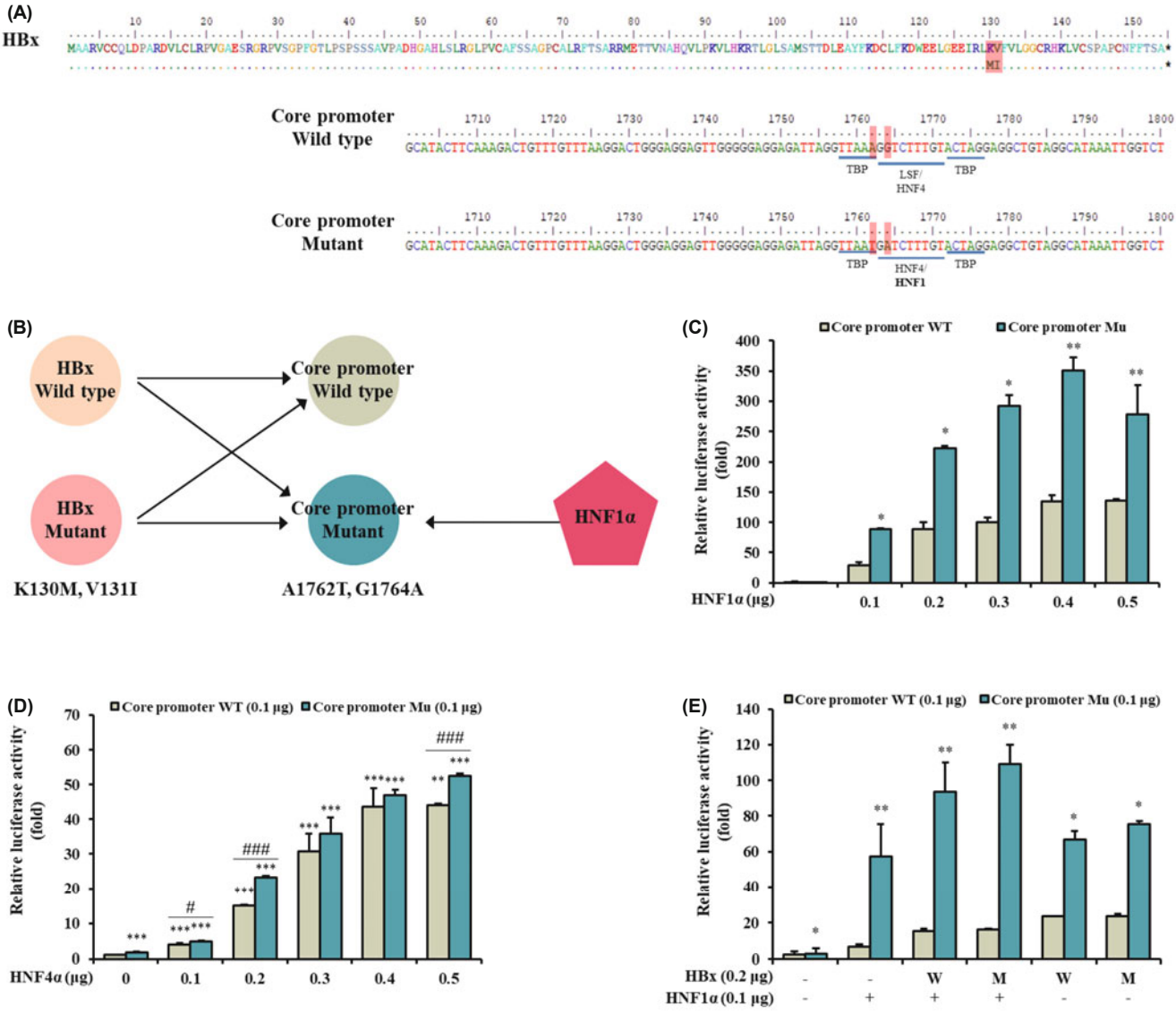


Fig. 3. Continued

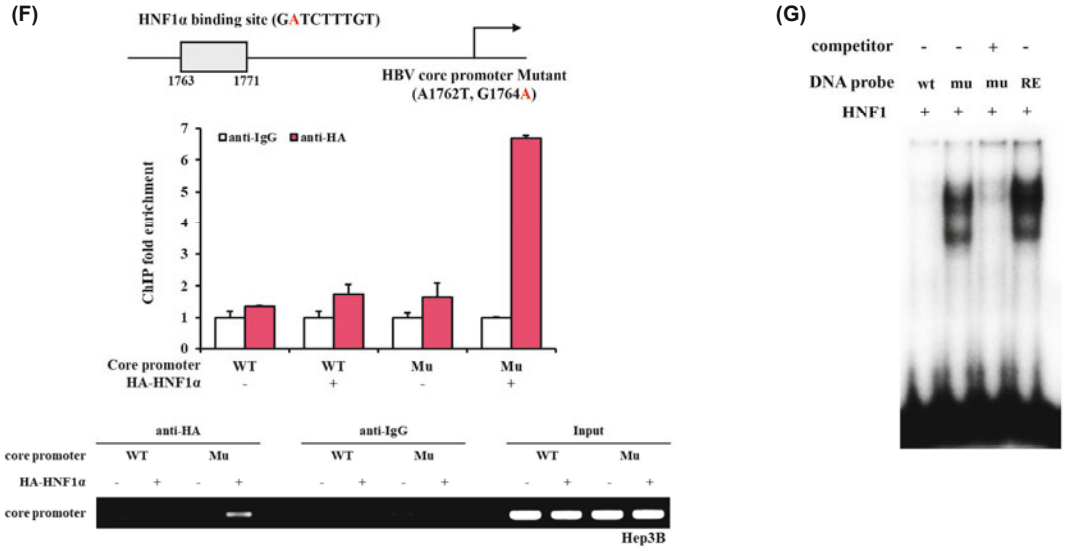


Fig. 3. HNF1 is responsible to increase HBV core promoter activity of A1762T/G1764A mutation. (A) Creation of HNF1 binding site on the A1762T/G1764A core promoter of M-H1. The transcription factor binding sites to the HBV core promoter mutation sites at A1762T/G1764A was searched through PROMO (http://alggenlsi.upc.es/cgibin/promo_V3/promo/Promoinit.cgi?-dirDB=TF_8.3). (B) Experimental construction of HNF1-mediated core promoter transactivation. The transactivation assay was executed using two types of HBx proteins (wild type and K130M/V131I mutant) and two core promoters (wild type and A1762T/G1764A). (C) The effect of HNF1 binding on HBV core promoter activity. Hep3B cells were transfected with HBV core promoter wild type and M-H1 construct for 24 h in the presence or absence of HNF1 transfection. The cell lysates were applied for luciferase activity. The bar represents the mean value from at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significance when compared to wild type core promoter. (D) The effect of HNF4α binding on HBV core promoter activity. Hep3B cells were transfected with HBV core promoter wild type and M-H1 construct for 24 h in the presence or absence of HNF4α transfection. The cell lysates were applied for luciferase activity. The bar represents the mean value from at least three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ indicate significance when compared to control. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared between indicated groups. (E) The effect of HNF1 and HBx on HBV core promoter activity. Hep3B cells were transfected with HBV core promoter wild type and M-H1 construct for 24 h with HNF1 expression plasmid in the presence or absence of HBx transfection. The cell lysates were applied for luciferase activity. The bar represents the mean value from at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significance when compared to wild type core promoter. (F) ChIP analysis on the double A1762T/G1764A mutant core promoter. Hep3B cells were transfected with mammalian expression vectors for 36 h as indicated, then harvested for ChIP analysis with anti-HA or control IgG. The precipitated genomic fragments were amplified using primers flanking the mutant site on the core promoters. (G) EMSA to confirm that the HBV core promoter putative HNF1 binding region is binding to HNF1. Isolated His-HNF1 protein was applied to labeled core promoter DNA (Lanes: 1, wild type; 2, mutant) probe. The concentration of the unlabeled competitors was in 40x molar excess relative to the labeled probe (Lane 3). HNF1-responsive element sequence DNA probe was applied to HNF1 binding as a positive control.

mutation site of HBV core promoter (Li *et al.*, 2002). The A1762T/G1764A double mutation sites might contribute to higher transactivation of HBV core promoter via HNF1α and the mutant HBx containing K130M and V131I amino acid variation (Fig. 3B).

To examine whether HNF1 is responsible to increase the core promoter activation of M-H1, we tried to transfect each reporter plasmids (M-WT and M-H1) into Hep3B cells in the presence or absence of HNF1 co-transfection and measured the luciferase activity for evaluating relative transactivation of core promoter variation. As shown in Fig. 3C, HNF1 largely increased the core promoter activity of M-H1 compared to M-WT promoter activity. HNF4α has been known as a master regulator for core promoter activity. We determined the effect of HNF4α on A1762G/G1764A mutation to show that A1762G/G1764A mutation is solely dependent on HNF1. Ectopic HNF4α expression increased both the wild type core promoter and the mutated core promoter similarly (Fig. 3D). In the result of Fig. 2, HBx expression increased the transactivation of the mutated core promoter compared to wild type. In order to examine whether HNF1α affects the HBx-mediated core promoter activation, we applied co-transfection of HBx and HNF1α genes with either wild type promoter or mutated promoter. As shown in Fig. 3E, HNF1α synergistically increased the mutated core promoter, but not the wild type. These results indicated that the enriched liver transcription factor HNF1 plays a critical role to regulate the core promoter activity including A1762T/G1764A gene variation and possibly to increase production of pre-genomic RNA for upregulation of HBV replication.

To confirm a transcriptional protein complex of HNF1 on the core promoters, we conducted chromatin immunoprecipitation (ChIP) assays. As shown in Fig. 3F, HNF1 was detected in a transcriptional complex to the double A1762T/G1764A mutant core promoter, but not on the wild type core promoter. No PCR products were detected when control IgG was utilized in immunoprecipitation. These results showed that HNF1 performs an important function in the formation of an active transcriptional complex of the double A1762T/G1764A mutant core promoter in the occurrence of HBV infection. In order to further examine the direct interaction of HNF1 protein and the double mutant core promoter, we examined EMSA using His-tagged HNF1 protein and core promoter DNA probe (1,745–1,780) with either the wild type or double mutant sequences. While HNF1 protein did not bind to the wild type core promoter, it specifically bound to the double mutant DNA probe (Fig. 3G, Lanes 1 and 2). Addition of competitor DNA in the binding mixture of

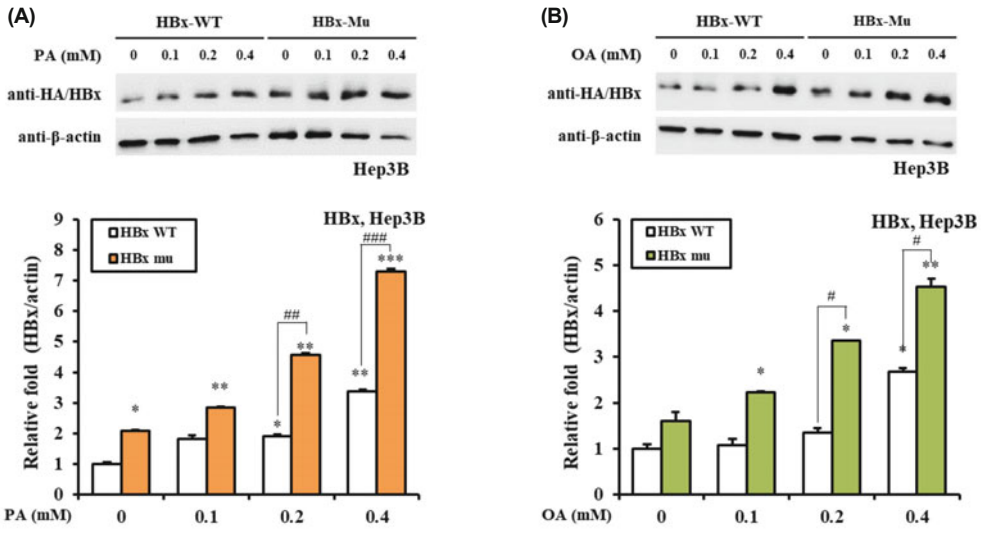


Fig. 4. Hepatic fatty acid increases HBx protein level. (A and B) Hep3B cells were plated and treated with mock, palmitic acid (A) or oleic acid (B) for 24 h in the co-transfection of two HBx expression (WT and K130M/V131I mutant). Western blotting was performed on the cell extracts using anti-HA antibody. The equivalence of protein loading in the lanes was verified using anti-actin serum. Relative band intensity was shown to be normalized to actin. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significance when compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with wild type HBx transfectant.

HNF1 protein and core promoter DNA probe significantly inhibited the molecular interaction of HNF1 protein and the mutant DNA probe (Fig. 3G, Lane 3). In Lane 4, HNF1-responsive element DNA (TGTGGTTAATGATTAACCG TT) probe was used to EMSA for positive control of HNF1-DNA binding.

Fatty acid increases the transactivation of core promoter M-H1 and the protein expression of HBx

In previous study, we reported that fatty acid, such as palmitic acid and oleic acid, increase HBV core promoter activation and stabilization of HBx protein (Cho *et al.*, 2014). To examine whether the double mutation in the core promoter and HBx affects core promoter activity and HBx protein stabilization, we treated palmitic acid and oleic acid to Hep3B cells with HBx transfection. The protein stabilization of HBx (M-H1) was increased more in the palmitic acid treatment than the wild type HBx protein (Fig. 4A). The mutated HBx protein was stabilized by oleic acid more than the wild type HBx (Fig. 4B). The band intensities of HA-HBx of Fig. 4A and B were added at the bottom of the blotting membranes. These results indicate that the double mutation of HBx might increase HBx protein stabilization to hepatic fatty acid composition.

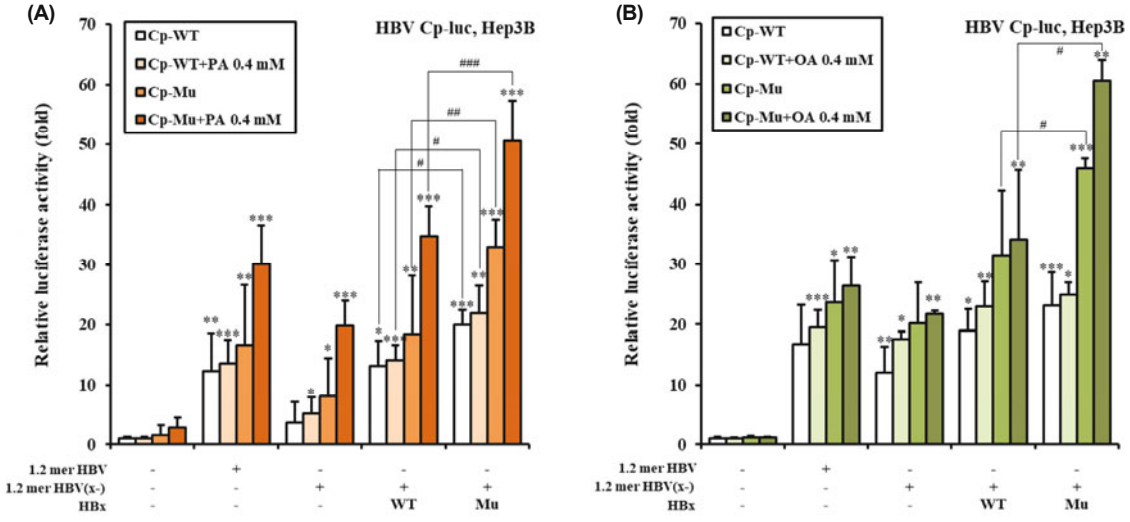


Fig. 5. Hepatic fatty acid increases the the A1762T/G1764A core promoter activation by HBx. (A and B) Hep3B cells were plated and treated with mock, palmitic acid (A) or oleic acid (B) in the co-transfection of HBV full-length replicon or HBx-lacking HBV replicon and two core promoter constructs (wild type and A1762T/G1764A mutant) in the presence or absence of two HBx constructs (wild type and K130M/V131I mutant). The cell lysates were applied for luciferase activity. The bar represents the mean value from at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ indicate significance when compared to control. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared between indicated groups.

To further confirm whether fatty acid-mediated HBx induction is consistent with transactivation of core promoter, we treated palmitic acid or oleic acid to Hep3B cells after co-transfection of core promoter constructs with 1.2mer HBV full replicon expressing HBx or nullifying HBx expression. In correlated with the results of Fig. 4, both fatty acids strongly increased the M-H1 core promoter than the M-WT promoter dependent on HBx expression and the mutated HBx protein increased the mutated core promoter stronger than the wild type HBx and wild type core promoter (Fig. 5). These results indicate that hepatic fatty acids increase the promoter responsive activation on the A1762T/G1764A mutation of core promoter, resulting in protein induction of HBV proteins including HBx.

Discussion

In this study, the effect of mutations in the core promoter and HBx on HBV DNA gene expression was investigated. The development of HCC is caused by various factors such as route of viral transmission, the immune response of the host and the interaction of numerous transcription factors. Previous studies showed that HBV transcription was regulated by transcription factors such as C/EBP, FOXA, HNF1, HNF4, and AP-1 (Reese *et al*., 2011; Kim *et al*., 2016). Hepatic HNF1 induction is known to regulate inflammation in the liver cell though production of various cytokines including IL-1β, IL-6, IL-8, IL-12, and TNFα (Zhou *et al*., 2016) Therefore, HNF1 binding to mutation sites in the HBV core promoter results in the induction of the inflammatory response in the liver, leading to development of chronic hepatitis.

Two previous references presented the HNF1 binding to the double A1762T and G1764A mutant core promoter. One publication showed that HBx protein can stimulate the different DNA binding activity of HNF-1 dependent on HBx mutation (Li *et al*., 2002). The other reference indicated that the functional differences of double mutation core promoter are related to the differential activities of HNF1, HNF4, and HBx on the core promote variation (Zheng *et al*., 2004). The following points are novel findings in this study compared to the previous references. First, we found that HBV variants with the double A1762T and G1764A mutation of HBV core promoter was exclusively detected in hepatocellular carcinoma patients of Myanmar, not asymptomatic HBV infected people. Second, in this study, we had prepared both core promoter mutations and HBx mutants containing the double mutation. The double mutant HBx showed stronger transactivation of the double mutation core promoter than wild type HBx. Third, hepatic fatty acids, palmitic acid and oleic acid, increased the protein stability of HBx mutant higher than the wild type HBx protein. Fourth, hepatic fatty acids increased strongly the double mutation core promoter activation compared to the wild type core promoter.

Chronic hepatitis B therapies lead to reduce liver damage and HCC production by reducing viral copy number and inhibiting HBV DNA replication (Alonso *et al.*, 2017). A variety of nucleotide analogues inhibits reverse transcription by HBV DNA polymerase and plays a critical role in the inhibition of viral replication and the normalization of ALT levels (Ohsaki *et al.*, 2021). Long-term maintenance therapy of nucleotide analogues is essential for continued antiviral effects against HBV, however nucleotide analogues treatment often induce drug-resistance mutation of HBV gene.

Both A1762T and G1764A mutations are frequently found in the core promoter, associating with HCC development. Clinical examinations have indicated that HBV core promoter mutations generally accumulate in a stepwise fashion before the development of HCC, often early stage with the TA double mutation. The double mutation expression reduced precore RNA transcription and HBeAg expression (Jiang *et al*., 2016; Lau *et al.*, 2020). In addition, it moderately increased genome replication through up regulation of pgRNA levels. However, there is no clear molecular mechanism how the A1762T and G1764A mutations induce precore RNA transcription.

In addition to A1762T/G1764A, mutations can be detected at nearby positions such as 1,753, 1,757, 1,766, and 1,768 (Tong *et al.*, 2013). Site-directed mutagenesis experiments have suggested that the additional mutations at 1,753, 1,766, and 1,788 further reduce HBeAg expression and enhance genome replication, with the A1762T/G1764A/C1766T triple mutant having greater than 10 folds higher replication capacity than the wild-type virus. In this study, we newly reported that two core promoter mutation, C1713T and G1806A, were closely related to upregulate the core promoter activity by producing novel promoter-specific transcription factor, C/EBPβ and XBP1, respectively.

Hypoxia is regularly attributed to various tumours linked by disease progression along with treatment resistance (Marignol *et al*., 2008). Hypoxia-inducible factor 1 (HIF-1) complex regulates many oxygen-responsive genes. HIF-1α overexpression, evident with accumulated immunostaining, has been described in various human cancers during their metastases (Zhong *et al*., 1999). Current evidence proposes that HIF-1 is engaged with notch-responsive promoters in hypoxic conditions to initiate transcriptional targets (Gustafsson *et al*., 2005). Hypoxia induces initial up-regulation of *HNF1B* from 1 to 24 h *in vitro*, independent of the HIF-1α expression (Faguer *et al.*, 2013). The hypoxia-driven HNF1 might increase stronger the double A1762T/G1764A mutation core promoter activation than the wild type core promoter. This indicates that HNF-1-mediated transactivation of the double A1762T/G1764A mutation core promoter provides higher HCC incidence in the presence of hypoxic condition.

HBx is known to influence apoptotic and cell cycle regulatory pathways, but the results of these activities are likely affected by the systems in which the studies have been conducted (Bouchard and Schneider, 2004). Several results reported that HBx can induce apoptosis, sensitize cells to proapoptotic stimuli, prevent apoptosis, or promote cell proliferation (Madden *et al.*, 2001; Marusawa *et al.*, 2003). But the underlying mechanism upon hepatocarcinogenesis and core promoter mutation is not cleared yet. Since mutations in the core promoter region in patients with HCC can alter the coding sequence of the overlapping HBx gene, and HBx protein has been previously incriminated in hepatocarcinogenesis, we suggest the concomitant core promoter and HBX mutations on the biologic function of HBx protein might endow entire progression of hepatocarcinogenesis.

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Conflict of Interest

The authors declare no conflicts of interest.

Ethics Statement

This study was approved by Ethics Review Committee of Department of Medical Research. The approval number is 22/Ethics 2015, dated 25.3.2015. Informed consent was obtained from the study participants who were at or more than 16 years and from the parents/guardians of the study participants who were under 16 years.

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