Impact of Nucleotide Mutations at the HNF3- and HNF4-Binding Sites in Enhancer 1 on Viral Replication in Patients with Chronic Hepatitis B Virus Infection

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Background/Aims: The hepatitis B virus (HBV) genome contains binding sites for hepatocyte nuclear factors (HNF) 3 and 4 in the core domain of enhancer 1 (Enh1), and mutations in this domain have a strong impact on virus replication. We aimed to identify frequent base-mutation sites in the core domain of Enh1 and to examine the impact of these mutations on viral replication. **Methods:** We studied virological characteristics and genetic sequences in 387 patients with chronic hepatitis B. We evaluated functional differences associated with specific mutations within the core domain of Enh1. **Results:** Mutations in the core domain were found with significant frequency in C1126 (122/387 [31.5%], the binding site for HNF3) and in C1134 (106/387 [27.4%], the binding site for HNF4). A single mutation at nt 1126 (C1126) was identified in 17/123 (13.8%), and 105/123 (85.4%) had double mutations (C1126/1134). The level of HBV DNA ($\log_{10}$ copies/mL) was lower in single mutants (C1126, 5.81±1.25) than in wild (6.80±1.65) and double mutants (C1126/1134, 6.81±1.54). Similarly, the relative luciferase activity of C1126 and C1126/C1134 was 0.18 and 1.12 times that of the wild-type virus, respectively. **Conclusions:** Mutations in the HNF3 binding site inhibit viral replication, whereas mutations at the HNF4 binding site restore viral replication. **(Gut Liver 2013;7:569-575)**

Key Words: Hepatocyte nuclear factors; Hepatitis B virus; Virus replication

INTRODUCTION

Hepatitis B virus (HBV) infection is the most common cause of cirrhosis of the liver and hepatocellular carcinoma (HCC), and its prevalence in Korea is rather high, that is, 3.2%.[1] After infection, HBV follows either of two pathways: 1) the virus will be eliminated by T-cell and B-cell responses specific to HBV proteins, and as a result, the host will acquire permanent immunity; 2) the virus will not be eliminated because of problems with the immune system, and as a result, HBV will remain in the host, who becomes a chronic carrier.[2] Due to the lack of the proofreading function of HBV polymerase, mutant viruses will frequently arise.

The HBV genome is a 3.2-kb DNA molecule with a partial double-stranded helix structure containing four open reading frames (X, precore/core, pre-S/S gene, and Pol), four promoters (pre-S1, pre-S2, core, and X), and two enhancers (enhancer 1 [Enh1] and enhancer 2). Enh1, which is located just upstream of the X promoter, has three domains (a modulator element, a core domain, and the 3' end, which overlaps with the X promoter), along with eight known functional sites.[3-5] Among these functional sites are the binding sequences for the liver-enriched transcription factors hepatocyte nuclear factor 3 (HNF3) and hepatocyte nuclear factor 4 (HNF4), which are located in the enhancer core domain and play important roles in regulating HBV replication,[5,6] as evidenced by experiments in transgenic HBV mouse models and cell culture systems in which HNF3 was found to inhibit nuclear hormone receptor-dependent viral replication and HNF4 was shown to stimulate viral replication.[6-9] Further, it has been reported that base substitutions (i.e., point mutations and deletions) in these sites alter the replication activity of HBV.[5] Here, we have examined the nucleotide substitutions that occur in the HNF3 and HNF4 binding sites of Enh1 and have analyzed their significance by identifying the mutations that correlated with HBV DNA levels.

MATERIALS AND METHODS

1. Patients and clinical samples

The 387 patients in this study were selected from among patients who had chronic hepatitis B, had visited Wonkwang University Hospital from January 2004 to September 2007, and who met the criteria for inclusion, which were as follows: 1) no infection with other viruses; 2) no exposure to drugs and no significant history of drinking (>20 g/day); 3) no use of antiviral agents; and 4) data available for the HBV DNA sequence and consent obtained for use of the blood sample. Cirrhosis of the liver was diagnosed by histology or imaging studies (computed tomography [CT] or ultrasonography); biochemical and endoscopic findings (e.g., esophageal varices, congestive gastropathy); and clinical features of portal hypertension, such as thrombocytopenia, high-albumin gradient ascites, and hepatic encephalopathy. HCC was diagnosed by histology or a combination of ultrasonography, CT, or magnetic resonance imaging and/or hepatic angiography as well as α-fetoprotein levels >200 ng/mL, in accordance with the European Association of the Study of the Liver guidelines for the diagnosis of HCC. Serum samples from each patient were maintained at -20°C until use. The study protocol was approved by the ethics committee of our institution.

2. Laboratory assays

The chemical laboratory tests for transaminases were performed according to the protocol recommended by the International Federation of Clinical Chemistry. Serological testing for HBV (hepatitis B surface antigen, antihepatitis B core antigen [anti-HBc], anti-HBc immunoglobulin M, hepatitis B e antigen [HBeAg], and anti-HBe) was performed with commercial kits of electrochemiluminescence assay (Abbott, North Chicago, IL, USA).

The HBV viral load was assayed using the RealArt-PCR kit (RealArt HBV LC PCR Reagents; Artus GmbH, Hamburg, Germany). However, before February 2003, the HBV viral load was measured with a hybridization assay (Digene, Gaithersburg, MD, USA), and the frozen sera from these patients, which had been stored at -20°C, were reevaluated for the present study using the RealArt-PCR kit.

3. Method for direct sequencing and definition of significant nucleotide mutations

For sequencing, HBV DNA was extracted from serum using a commercial kit (QIAmp DNA Blood Mini Kit; Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instructions. The sequences of the first PCR primer set were S_1 (953→968): 5'-AAC TKC CTG TAA AYC AG-3' and AS_1 (1433→1416): 5'-GGG ACG TAA RAC AAA GGA C-3'. The sequences of the nested PCR primers were S_2 (970→988): 5'-CCT ATT GAT TGG AAA GTW TG-3' and AS_2 (1430→1413): 5'-ACG TAR ACA

AAG GAC GTC-3'. Agarose gel electrophoresis was performed to sequence the PCR products, and the band corresponding to the expected size was excised from the gel. The products were purified using a commercial kit (QIAquick®; Qiagen) to remove the residual primers and deoxyribonucleotide triphosphates. The purified PCR products were directly sequenced by the dideoxy method by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit V2 and analyzed on an ABI PRISM® 377 analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences of the Enh1/X promoter (Enh1/X promoter) region were aligned using the SeqMan software (DNASTAR, Madison, WI, USA). The sequences of the 387 isolates from our study subjects were compared to those of genotype C HBV and nongenotype C HBV isolates retrieved from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/Genbank). The HBV genomic sequences were aligned using the ClustalW software (Dublin, Ireland). Genetic distances were estimated by Kimura's 2-parameter method, and phylogenetic trees were constructed by the neighbor-joining method.[10,11] Nucleotide variation was defined according to differences from the consensus sequences within the same subgenotype. Moreover, we defined a hotspot as a site with nucleotide variability 5-fold greater than the average.

4. Constructs and site-directed mutagenesis

We selected a sequence that corresponded to the consensus (wild-type) sequence and then inserted the Enh1/X promoter region (nt 950-1373 of genotype C2) from this sequence into the multicloning site of the pGL3 basic vector (Promega, Madison, WI, USA). The NheI and HindIII sites in the multicloning site were selected for cloning. For cleavage and ligation, the following primers were used: sense, 5'-CTA GCT AGC GGA AAC TGC CTG TAA AT-3' (starting at nt 950 and including the NheI site); antisense, 5'-GGT GCA AGC TTG GGA AGG AGG TGT ATT TCC G-3' (terminating at nt 1373 and including the HindIII site). Using the wild-type construct, three mutant constructs were generated. The first carried a point mutation at nt 1126 (A→C; C1126A), the second carried a mutation at nt 1134 (T→C; C1124T), and the third carried mutations at both sites (nt 1126 and nt 1134; C1126A/C1134T). The three mutant constructs were confirmed by direct sequencing.

5. Cells, transfection, and the luciferase assay

HepG2 cells (American Type Culture Collection, Manassas, VA, USA) were chosen for use in functional assays. Cells were cultured in DMEM (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum. Approximately 2×10^5 cells were plated in 6-well plates 24 hours before transfection. The cells were transfected with 2 μg of the pGL3-HBV constructs and 0.5 μg of pcDNA1-galactosidase (Stratagene, La Jolla, CA, USA) by using the FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. After 48 hours in culture, total cell extract was obtained

by lysing the cells. Luciferase activity was measured with a luciferase assay system (Promega) according to the manufacturer's instructions. Luciferase activity was measured in triplicate, averaged, and then normalized to β-galactosidase activity using the galactosidase assay system (Galacto-Light; Tropix, Bedford, MA, USA) according to the manufacturer's instructions.

6. Statistical analysis

The data were analyzed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). To compare continuous data, statistical analyses were performed with the Kruskal-Wallis test. To compare the categorical data, statistical analyses were performed with the chi-squired test, and Fisher exact test was used when a cell value was <5 in 2-by-2 tables. All p-values were 2-sided, and a p<0.05 was considered statistically significant.

RESULTS

1. Patient characteristics

A total of 387 patients were recruited during the study period; 163 patients had chronic hepatitis B, 69 had cirrhosis of the liver, and 155 had HCC. The mean±standard deviation value for the age of the patients was 45.15±16.71 years. Of the 387 patients, 267 (69%) were men and 120 (31%) were women. There were 191 (49.4%) who were HBeAg/anti-HBe +/-; 136 (35.1%) were HBeAg/anti-HBe -/+; 5 (1.3%) were HBeAg/anti-HBe -/-; and 55 (14.2%) were HBeAg/anti-HBe +/+. The mean serum HBV DNA level was 6.76±1.62 $\log_{10}$ copies/mL (Table 1).

2. Nucleotide mutations in Enh1/X promoter and relationships among multiple mutation sites

The nucleotide divergence of the Enh1/X promoter sequences from the 387 HBV patients sampled in this study was 1.69%. Based on this, we defined a hot spot as a site with a mutation rate greater than 8.45%, and we identified 19 sites that met this definition: T/A1050, A1053, G1078, T1102, C1126, C1134, C/T1167, C/T1206, A1229, C1272, G/T1284, G1287, G1314, G/T1317, C1320, C/G1323, C/G/A1341, C/A1356, and T/A1368. The site with the highest rate of mutation in the HNF3 binding

Table 1. Baseline Characteristics of Study Patients

Characteristic	Value (n=387)
CH:LC:HCC	163 (42.1):69 (17.8):155 (40.1)
Sex, male:female	267 (69):120 (31)
Age, yr	45.15±16.71
HBeAg, +:-	246 (63.6):141 (36.4)
Anti-HBe, +:-	191 (49.4):196 (50.6)
AST, U/L	106.71±181.81
Platelet, /mm³	160.76±83.65
HBV DNA, $\log_{10}$ copies/mL	6.76±1.62

Data are presented as number (%) or mean±SD.
CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; anti-HBe, hepatitis B e antibody; AST, aspartate aminotransferase; HBV, hepatitis B virus.

Table 2. Patient Characteristics according to the Nucleotide Mutations in the Hepatocyte Nuclear Factor Binding Sites

Characteristic	Wild-type (n=265)	C1126 (n=17)	C1126/C1134 (n=105)	p-value
CH:LC:HCC	109 (41.1):44 (16.9):112 (42.2)	6 (35.3):5 (29.4):6 (35.3)	48 (45.7):20 (19.0):37 (35.3)	0.395
Sex, male:female	179 (67.5):86 (32.5)	12 (70.6):5 (29.4)	76 (72.4):29 (27.6)	0.656
Age, yr				
<20	26	0	14	
20–29	32	1	16	
30–39	30	1	12	0.293
40–49	57	6	19	
50–59	62	4	21	
≥60	58	5	23	
HBeAg, +:-	168 (63.4):97 (36.6)	10 (58.8):7 (41.2)	68 (64.8):37 (35.2)	0.890
Anti-HBe, +:-	131 (49.4):134 (50.6)	11 (64.7):6 (35.3)	49 (46.6):56 (53.4)	0.385
HBV DNA, $\log_{10}$ copies/mL	6.80±1.65	5.81±1.25	6.81±1.54	0.049*
AST, U/L	119.94±212.96	75.06±58.36	78.45±74.97	0.108
Platelet, /mm³	156.06±79.84	145.38±71.99	174.93±93.14	0.111

Data are presented as number (%) or mean±SD. The significance of the differences (p-value) in the indicated group is shown. The p-values <0.05 are shown in italic type.
CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; anti-HBe, hepatitis B e antibody; HBV, hepatitis B virus; AST, aspartate aminotransferase.
*Wild-type vs C1126, p=0.040; Wild-type vs C1126/C1134, p=0.999; C1126 vs C1126/C1134, p=0.050.

region was C1126 (122/387, 31.5%), and that in the HNF4 binding region was C1134 (106/387, 27.4%). Interestingly, concomitant mutation of C1126 and C1134 was found at a significantly high frequency, occurring in 85.4% (105/123) of the samples evaluated, while the single mutation in C1134 occurred in 0.8% of the samples (1/123) tested, and the single mutation in C1126 was present in 13.8% of the samples tested (17/123) (Table 2). The structure of Enh1/X promoter in the hepatitis B virus genome was demonstrated in Fig. 1.

3. Relationship between concomitant C1126/C1134 mutation and clinical profiles of patients

In order to determine the factors that contributed to the significantly high frequency of the concomitant C1126 and C1134 mutation, we analyzed the differences in clinical characteristics of patients according to the pattern of the C1126 and/or C1134 mutations. No difference was observed in the distribution of diseases, age, serum aspartate aminotransferase levels, serum platelet levels, the HBeAg positive rate, or the anti-HBe positive rate (Table 2). However, serum HBV DNA levels differed according to these nucleotide mutations. The C1126 single mutation was associated with the lowest serum HBV DNA levels, at 5.81 ± 1.25 $\log_{10}$ copies/mL, while the wild-type virus, C1134 single mutation, and C1126/C1134 concomitant mutation were all associated with similar serum HBV DNA levels of 6.80 ± 1.65, 8.58, and 6.81 ± 1.54 $\log_{10}$ copies/mL, respectively (Table 2).

Because the C1126 single mutation group showed higher positive ratios in anti-HBe, although not statistically significant, we evaluated the differences in HBV DNA, AST, and platelet levels were according to mutation patterns, classifying affected patients into HBeAg-positive and anti-HBe-positive groups. Be-cause the HBeAg/anti-HBe -/- and HBeAg/anti-HBe +/+ types involved too few patients, we compared the clinical profiles of patients according to mutation patterns in HBeAg/anti-HBe +/- group and HBeAg/anti-HBe -/+ groups only. While patients in the HBeAg + group showed different HBV DNA values according to the mutation pattern, those in the anti-HBe + group did not show differences in the HBV DNA value (p=0.012 vs 0.862) (Tables 3 and 4).

4. Difference in luciferase activity according to C1126 and C1134 mutations

Since we found a significant difference in serum HBV DNA levels according to the C1126 and C1134 mutation pattern, we performed a functional study based on this finding. First, we generated C1126, C1134, and C1126/C1134 vector constructs through site-directed mutagenesis of the wild-type vector, and then we performed luciferase analysis using these vectors. The activity of each mutant vector was determined and compared to that of the wild-type vector. In HepG2 cells, the activity of the C1126 vector was 0.18-fold that of wild-type, the activity of the C1134 vector was 1.16-fold that of wild-type, and the C1126/C1134 vector was 1.12-fold that of wild-type (Fig. 2). The activity decreased with the C1126 mutation, while activity increased with the C1134 mutation and with the C1126/C1134 concomitant mutation.

DISCUSSION

The progression of chronic HBV infection is determined by the interaction of viral factors with host factors.[12-14] The viral factors include HBV DNA levels,[15-17] the presence of HBeAg,

Table 3. Laboratory Findings according to Hepatitis B e Antigen Positive Group

	Wild-type (n=168)	C1126 (n=10)	C1126/C1134 (n=68)	p-value
HBV DNA, $\log_{10}$ copies/mL	7.39 ± 1.40	6.04 ± 0.99	7.31 ± 1.35	0.012*
AST, U/L	130.71 ± 242.23	73.50 ± 57.74	74.85 ± 66.07	0.137
Platelet, /mm^3	163.75 ± 76.21	144.90 ± 65.92	194.91 ± 94.95	0.018†

Data are presented as mean±SD. The significance of the differences (p-value) among the groups is shown. A p<0.05 is indicated in italic type.
HBV, hepatitis B virus; AST, aspartate aminotransferase.
*Wild-type vs C1126, p=0.008; Wild-type vs C1126/1134, p=0.914; C1126 vs C1126/1134, p=0.019; †Wild-type vs C1126, p=0.523; Wild-type vs C1126/1134, p=0.023; C1126 vs C1126/1134, p=0.078.

Table 4. Laboratory Findings according to Anti-hepatitis B e Positive Group

	Wild-type (n=94)	C1126 (n=7)	C1126/C1134 (n=35)	p-value
HBV DNA, $\log_{10}$ copies/mL	5.73 ± 1.55	5.50 ± 1.58	5.83 ± 1.49	0.862
AST, U/L	101.56 ± 150.71	77.29 ± 63.81	76.15 ± 80.57	0.595
Platelet, /mm^3	139.10 ± 83.12	152.83 ± 84.51	141.49 ± 79.31	0.749

Data are presented as mean±SD.
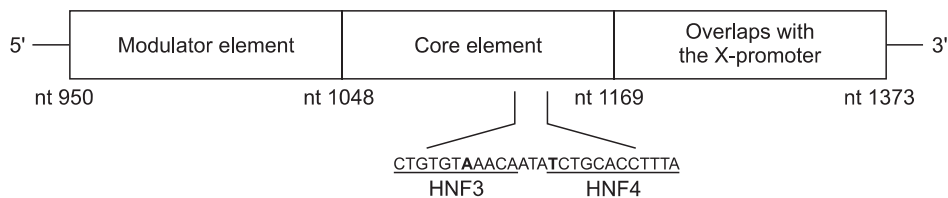HBV, hepatitis B virus; AST, aspartate aminotransferase.

Fig. 1. The structure of enhancer 1 and the X-promoter in the hepatitis B virus genome. The sequences of binding sites for hepatocyte nuclear factor (HNF) 3 and HNF 4 are shown. The mutation sites of nt 1126 and nt 1134 are highlighted (bold font).
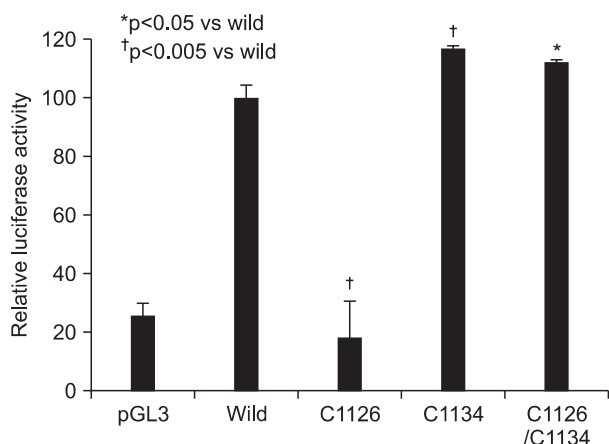

Fig. 2. Luciferase activity of wild-type and mutant vectors in HepG2 cells. The activity of each mutant vector was determined and compared to that of the wild-type vector in HepG2 cells. The activity of the C1126 vector was 0.18-fold that of the wild-type vector; the activity of the C1134 vector was 1.16-fold that of the wild-type vector and the activity of the C1126/C1134 vector was 1.12-fold that of the wild-type vector.

viral mutation,[18-24] and the HBV genotype.[25,26] The host factors include the host's immune state (the function of cytotoxic T-cells),[13,14] the duration of hepatitis B infection, age, and gender (male).[27,28] Among these factors, the HBV DNA level is important not only as a factor influencing disease progression but also as an indicator of treatment response to antiviral agents. A high HBV DNA level is associated with increased risk of liver cirrhosis and HCC[25,27,29-31] and increased liver failure-related mortality.[32]

Nucleotide mutations that occur during the natural course of HBV infection affect HBV DNA levels, the presence of HBeAg, and liver disease activity. The nucleotide mutations that affect these functions, such as precore mutations and core promoter mutations, have been studied most frequently, while the results from studies regarding the effects of T1762/A1764 mutations on viral replication have been inconsistent. One study reported that the T1762/A1764 mutations occurred frequently in patients with viral loads rising to over 10^6 copies/mL regardless of the presence of HBeAg,[33] while another study reported that the effect of T1762/A1764 mutation on viral replication differed according to the presence of HBeAg. That is, T1762/A1764 mutations were associated with reduced HBV DNA levels in HBeAg-positive pa-

tients but not in HBeAg-negative patients.[34] Another significant naturally double mutation is A1896/A1899. Some studies have reported that this mutation induces seroconversion of HBeAg and is present in inactive carriers,[35] that it is associated with severe hepatitis (fulminant hepatitis), or that it is related with disease progression.[36,37] Both these naturally occurring double mutations are strongly related to the replication capacity of HBV and the status of HBeAg/anti-HBe, although the findings have been somewhat inconsistent. In our study, the natural double mutation C1126/C1134 was identified. Interestingly, the single mutation of C1126 resulted in remarkably lower replication activities than other mutations, and this difference was more conspicuous in the HBeAg-positive group. There is little possibility of sequential change from a C1126 single mutation to a C1126/C1134 double mutation as the distribution of disease groups and the platelet values did not differ much between the two groups. Rather, single mutations and double mutations seem to occur independently. In the anti-HBe-positive group in the present study, the HBV DNA values had not changed according to the mutation pattern, probably because the replication status had already changed because of the precore or core promoter mutation. Therefore, the C1126 and/or C1134 mutations, which were identified in this paper for the first time, did not seem to have as strong an influence on the replication activity of virus as precore mutations or core promoter mutations and seemed to have only a minor influence on the replication activity of HBV, independent of the stronger mutations. As with our results, previous studies reported that HBV DNA levels in the liver tissue of HBV-infected patients were high if HNF4 was strongly expressed and low if HNF3 was strongly expressed, suggesting that HNF3 and HNF4 are related to viral replication.[6] Another previous study reported that both HNF3 and HNF4 mutants strongly suppressed HBV replication activity.

To the best of our knowledge, this study is the first specific report focusing on a point mutation of a nucleotide in Enh1 that affects HBV DNA levels. In our study, nucleotide mutations in the HNF3 and HNF4 binding regions were found to affect HBV DNA levels. A mutation HNF3 was associated with low levels of HBV DNA, while a mutation in the HNF4 region was associated with high HBV DNA levels. Interestingly, in all but one of the cases, the nucleotide mutation in the HNF4 site was concomitant with the nucleotide mutation in the HNF3 region, and the

HBV DNA level, which decreased with a mutation in the HNF3 region, increased when this mutation and the mutation in the HNF4 region occurred together. A similar result was obtained in the functional study, in which vectors containing the mutation in the HNF3 site showed decreased luciferase activity while vectors with the mutation in the HNF3 site concomitant with the mutation in the HNF4 site had increased luciferase activity similar to that of the wild-type vector. Accordingly, the C1134 mutation in the HNF4 site is believed to compensate for the decreased viral replication ability that results from the C1126 mutation in the HNF3 site.

This study has some limitations. It included only a small number of patients. In addition, it is a cross sectional study and as such, we did not obtain serial samples from the patients and could not check for serial mutational changes. Therefore, this study was limited in its ability to explain the significance of these mutations as they relate to disease progression; therefore, an additional longitudinal study may be necessary.

In conclusion, nucleotide mutations in the binding sequence for HNF3 may inhibit viral replication, but this activity may be restored by a nucleotide mutation in the binding sequence for HNF 4 in human hepatocytes *in vitro* and *in vivo*.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

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