Putative association of DNA methyltransferase 1 (DNMT1) polymorphisms with clearance of HBV infection

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DNA methyltransferase (DNMT) 1 is the key enzyme responsible for DNA methylation, which often occurs in CpG islands located near the regulatory regions of genes and affects transcription of specific genes. In this study, we examined the possible association of *DNMT1* polymorphisms with HBV clearance and the risk of hepatocellular carcinoma (HCC). Seven common polymorphic sites were selected by considering their allele frequencies, haplotype-tagging status and LDs for genotyping in larger-scale subjects (n = 1,100). Statistical analysis demonstrated that two intron polymorphisms of *DNMT1*, +34542G > C and +38565G > T, showed significant association with HBV clearance in a co-dominant model (OR = 1.30, P^{corr} = 0.03) and co-dominant/recessive model (OR = 1.34-1.74, P^{corr} = 0.01-0.03), respectively. These results suggest that two intron polymorphisms of *DNMT1*, +34542G > C and +38565G > T, might affect HBV clearance. [BMB reports 2009; 42(12): 834-839]

INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most contagious diseases with more than 350 million chronic carriers worldwide (1). HBV is known to increase the risk of chronic hepatitis (CH), liver cirrhosis (LC) and the development of hepatocellular carcinoma (HCC) (1, 2). Chronicity of HBV infection among a group of patients presumed infected at the same age did not appear to be the same outcomes of the infection. The various outcomes were not determined by viral strain types, but instead were likely altered after infection by allelic differences in the human genome (3). Thus, it is imaginable that genetic differences play another role.
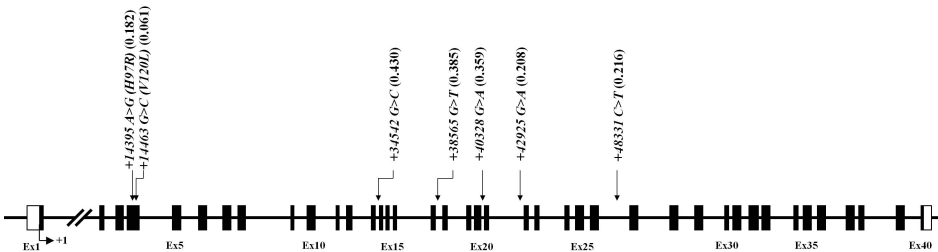
Generally, the level of DNA methylation in various cancers is lower than in normal tissues (4, 5). However, some loci do exhibit increased DNA methylation in certain types of cancer (6-8). DNA methylation may play a role in carcinogenesis through the following three mechanisms: (i) DNA cytosine methylation promotes gene mutation as 5-methylcytosine is deaminated to thymine (9); (ii) aberrant DNA methylation may be related with allelic loss (5, 10, 11), and (iii) DNA methylation often occurs in CpG islands located near the regulatory regions of genes and affects transcription of specific genes (12-15). Among them, gene silencing brought on by aberrant DNA methylation of promoter CpG islands is one of the most consistent epigenetic changes observed in human cancers, including HCC (12-15). In normal cells, the methylation of CpG islands by DNMT is related to the stringent maintenance of DNA conditions after DNA replication and mitosis. Hypermethylation, however, of specific CpG island sequences has been frequently observed in malignancies (13, 16, 17). The hypermethylation of promoter CpG islands has been implicated in hepatocarcinogenesis, and the list of tumor suppressor genes affected by such epigenetic silencing in hepatocellular carcinomas is expanding (18-23). Moreover, the number of methylating events in HCCs is higher than in cirrhotic livers, providing evidence that stepwise methylation may be a feature of human hepatocarcinogenesis (18, 19, 23).

DNMT is organized by 3 enzymes, DNMT1 (24), DNMT3a and DNMT3b (25), of which DNMT1 is the most well known and the major one. We have examined the hypothesis that polymorphisms within the *DNMT1* can affect the progression of HCC among HBV-infected patients. Thus, we performed screening of *DNMT1* in order to examine its genetic association with HBV clearance and HCC progression.

RESULTS

In this study, we examined the association of *DNMT1* polymorphisms with persistent HBV infection and HCC occurrence. We analyzed and selected seven polymorphisms (+14395A > G, +14463G > C, +34542G > C, +38565G > T, +40328G > A, +42925G > A and +48331C > T) in *DNMT1* for larg-

A. Map of DNA methyltransferase 1 (*DNMT1*) on chromosome 19p13.3-p13.2

B. Haplotypes of *DNMT1* gene

Hap.	+14395A>G	+14463G>C	+34542G>C	+38565G>T	+40328G>A	+42925G>A	+48331C>T	Freq.
ht1	A	G	G	G	G	G	T	0.215
ht2	A	G	G	G	A	A	C	0.201
ht3	G	G	C	T	G	G	C	0.180
ht4	A	G	G	G	A	G	C	0.148
ht5	A	G	C	T	G	G	C	0.140
ht6	A	C	C	T	G	G	C	0.063
Others.	.	.	.	.	.	.	.	0.052

C. LDs among *DNMT1* polymorphisms

		+14395A>G	+14463G>C	+34542G>C	+38565G>T	+40328G>A	+42925G>A	+48331C>T
					|D'|			
	+14395A>G	-	1	1	0.974	1	0.936	1
	+14463G>C	0.015	-	1	1	1	1	0.992
r^2	+34542G>C	0.086	0.086	-	0.996	0.958	0.890	0.992
	+38565G>T	0.344	0.104	0.822	-	0.991	1	1
	+40328G>A	0.126	0.037	0.389	0.344	-	0.976	0.980
	+42925G>A	0.053	0.018	0.159	0.166	0.455	-	0.979
	+48331C>T	0.062	0.018	0.207	0.174	0.149	0.071	-

Fig. 1. Gene maps and haplotypes of the DNMT1 gene. (A) Polymorphisms of *DNMT1*. Coding exons are marked by shaded blocks and the 5′ and 3′ UTRs by white blocks. The first base of the translational start site is denoted as nucleotide +1. Seven polymorphisms were genotyped in a larger population (n = 1,093). (B) Haplotypes of *DNMT1*. Only those with frequencies over 0.05 are shown. Others (1) contain the rare haplotypes: AGCCGAAC, GGCCGGAC, AGCCGAGT, AGCCGGGT, AGTGGGGT, and AGCGGGGC. (C) Linkage disequilibrium coefficients (|D'| and r^2) among DNMT1 polymorphisms.

Table 1. Allele frequencies of DNMT1 polymorphisms among various ethnic groups

Loci	Position	Amino acid change	rs#	HWE[a]	Heterozygosity[b]	Minor allele frequency				
						Korean	Caucasian[c]	Japanese[c]	Chinese[c]	African[c]
+14395A > G	Exon4	His97Arg	rs16999593	0.587	0.298	0.182	0	0.194	0.175	0.000
+14463G > C	Exon4	Val120Leu	novel	0.120	0.115	0.061	NA	NA	NA	NA
+34542G > C	Intron13	.	rs2241531	0.222	0.490	0.430	0.05	0.398	0.478	0.258
+38565G > T	Intron17	.	rs4804490	0.497	0.474	0.385	0.058	0.329	0.446	0.181
+40328G > A	Intron20	.	rs2114724	0.011	0.460	0.359	0.492	0.384	0.267	0.467
+42925G > A	Intron21	.	rs1863771	0.175	0.329	0.208	NA	NA	NA	NA
+48331C > T	Intron26	.	rs2288349	0.170	0.339	0.216	0.412	0.194	0.244	0.179

NA: not available. [a]P values of deviation from Hardy-Weinberg equilibrium in the Korean population, [b]Heterozygosity calculated in Korean population, [c]Data from international HapMap (http://www.hapmap.org/index.html.en/).

er-scale genotyping (n = 1,093) by considering their allele frequencies, haplotype-tagging status and LDs from the previous study. The minor allele frequencies of these seven polymorphisms in the Korean population were 0.182 (+14395A > G), 0.061 (+14463G > C), 0.430 (+34542G > C), 0.385 (+38565G > T), 0.359 (+40328G > A), 0.208 (+42925G > A) and 0.216 (+48331C > T) (Fig. 1A). Genotype distributions of all loci were in Hardy-Weinberg equilibrium, except for +40328G > A (P > 0.05, Table 1). The allele frequencies of the *DNMT1* polymorphisms were compared among different ethnic groups (Table 1) and were found to have distinct differences. Seven SNPs showed tight LDs (Fig. 1C), and six major haplotypes accounted for over 94.8% of the distribution (Fig. 1B). *DNMT1* -ht1, -ht2, -ht3, and -ht6 were nearly equivalent with +48331C

> T, +42925G > A, +14395A > G and +14463G > C, respectively. Therefore only *DNMT1* -ht4 and -ht5 were used for haplotype association analysis (Fig. 1B).

The genotype frequencies for each *DNMT1* polymorphism and haplotype among 'chronic carrier' (CC) and 'spontaneously recovered' (SR) groups were analyzed through logistic regression models (Table 2) by controlling age and sex as co-variates (gender and age were significantly associated with HBV clearance; P < 0.05, data not shown) in our Korean HBV study (667 CC and 433 SR individuals).

In the analysis, the frequency of individuals bearing the *DNMT1* +34542G > C in the CC group was higher than in the SR group (OR = 1.30, P = 0.005, P^corr = 0.03 in co-dominant model; Table 2), suggesting that *DNMT1* +34542G > C

Table 2. Association analysis of DNMT1 polymorphisms with HBV clearance

Locus	Position	Amino acid change	CC (n = 667)	SR (n = 433)	Co-dominant			Dominant			Recessive		
					OR (95%CI)	P	P^{corr}*	OR (95%CI)	P	P^{corr}*	OR (95%CI)	P	P^{corr}*
+14395A > G	Exon4	His97Arg	0.188	0.174	1.07 (0.85-1.36)	0.56	1	1.09 (0.83-1.43)	0.56	1	1.09 (0.52-2.30)	0.82	1
+14463G > C	Exon4	Val120Leu	0.065	0.054	1.37 (0.93-2.00)	0.11	0.77	1.39 (0.92-2.09)	0.12	0.81	1.76 (0.31-9.83)	0.52	1
+34542G > T	Intron13	.	**0.449**	**0.400**	**1.30 (1.08-1.55)**	**0.005**	**0.03**	**1.33 (1.02-1.74)**	**0.04**	0.27	**1.56 (1.12-2.18)**	**0.009**	0.06
+38565G > T	Intron17	.	**0.409**	**0.348**	**1.34 (1.12-1.62)**	**0.002**	**0.01**	**1.36 (1.05-1.76)**	**0.02**	0.15	**1.74 (1.20-2.53)**	**0.004**	**0.03**
+40328G > A	Intron20	.	0.349	0.375	0.86 (0.72-1.03)	0.11	0.77	**0.77 (0.60-1.00)**	**0.05**	0.36	0.92 (0.64-1.32)	0.65	1
+42925G > A	Intron21	.	0.202	0.218	0.90 (0.72-1.12)	0.34	1	0.87 (0.66-1.13)	0.28	1	0.94 (0.53-1.69)	0.85	1
+48331C > T	Intron26	.	0.206	0.230	0.84 (0.68-1.05)	0.12	0.83	0.77 (0.60-1.01)	0.06	0.40	0.99 (0.56-1.75)	0.98	1
ht1	.	.	0.205	0.229	0.85 (0.69-1.05)	0.13	0.93	0.79 (0.61-1.03)	0.08	0.57	0.94 (0.54-1.64)	0.82	1
ht2	.	.	0.197	0.209	0.92 (0.74-1.16)	0.49	1	0.92 (0.71-1.20)	0.53	1	0.86 (0.46-1.61)	0.64	1
ht3	.	.	0.187	0.171	1.09 (0.86-1.39)	0.46	1	1.11 (0.84-1.45)	0.47	1	1.15 (0.53-2.48)	0.73	1
ht4	.	.	0.144	0.156	0.85 (0.66-1.09)	0.20	1	0.79 (0.60-1.05)	0.11	0.77	1.29 (0.50-3.29)	0.60	1
ht5	.	.	**0.154**	**0.120**	**1.38 (1.06-1.80)**	**0.02**	0.11	**1.46 (1.09-1.97)**	**0.01**	0.09	1.38 (0.58-3.30)	0.47	1
ht6	.	.	0.067	0.055	1.38 (0.94-2.01)	0.10	0.68	1.40 (0.93-2.10)	0.10	0.73	1.79 (0.32-9.98)	0.51	1

Logistic regression models were used for calculating odds ratios (95% confidential interval) and corresponding P-values for each SNP site and haplotype controlling age and sex as covariates using SAS. P values of co-dominant, dominant and recessive models are also given. Age (continuous value) and sex (male = 0, female = 1) were adjusted as co-variables in the logistic analysis. All patients included in study were HBsAg-positive (chronic hepatitis). CC: chronic carrier group, SR: spontaneous recovered group, P^{corr}: corrected P value. *To achieve the optimal correction for multiple testing of single-nucleotide polymorphisms (SNPs) in linkage disequilibrium (LD) with each other, the effective number of independent marker loci (5.82) in DNMT1 were calculated using the SNPSpD software (http://genepi.qimr.edu.au/general/daleN/SNPSpD/) on the basis of the spectral decomposition (SpD) of matrices of pair-wise LD between SNPs (44).

shows significant association with HBV clearance. A similar association was observed for *DNMT1 +38565G > T* (OR = 1.34-1.74, P = 0.002-0.004, P^{corr} = 0.01-0.03 in co-dominant model; Table 2). A marginal association was identified between *DNMT1 +40328G > A* and HBV clearance (OR = 0.77, P = 0.05 in dominant model); however, the significance diminished after multiple test correction (P^{corr} = 0.36). No other loci showed significant association with HBV clearance. Upon further analysis, one common haplotype, *DNMT1-ht5* (A-G-C-T-G-G-C), was found to be significantly associated with HBV clearance in co-dominant and dominant models (OR = 0.55-0.58, P = 0.009-0.01 and OR = 1.31-1.65, P = 0.001-0.007, respectively; Table 2), but lost significance after multiple test correction. No DNMT1 polymorphisms showed association with HCC occurrence among HBV patients.

DISCUSSION

Several recent studies have indicated that HBV X protein (HBx)

induces the expression of DNMT1 by stimulating its transcription through the E2F1 pathway (26). Moreover, it was observed that DNMT1 can be upregulated as an innate immune response to the CpG islands of HBV DNA during infection (27). Moreover, DNMT1 catalyzes the methylation of the 50-CpG di-nucleotide of the cytosine ring in mammalian cells (28). Hepatocarcinogenesis involves the increased expression of DNMT1, DNMT3a and DNMT3b mRNA accompanied by a progressive increase in the number of methylated genes from normal liver, chronic hepatitis/cirrhosis to HCC (29). In addition, the genome-wide hypomethylation commonly observed in HCC is a continuing process that persists throughout the lifetime of the tumor cells, rather than a historical event occurring at pre-cancer stages (30). These facts suggest that DMNT1 is related to the progression of HBV-positive HCC.

DNMT1 is known to play another major role in promoting a hemi-methylated state as opposed to an un-methylated state (31). It does this by targeting replication foci though binding to proliferating cell nuclear antigen (PCNA) (32). Therefore,

DNMT1 copies methylation patterns, such as "maintenance" processes, after DNA replication has been completed (33). For these reasons, DNMT1 has become a focus in the targeting of genes for cancer therapy.

We have demonstrated that the absence of the *DNMT1 +34542G > C* and *DNMT1 +38565G > T* polymorphisms was significantly associated with resolution of HBV infection in the co-dominant and co-dominant/recessive models, respectively. *DNMT1 +34542G > C* and *DNMT1 +38565G > T* are located in introns of the transcript variant. Polymorphisms located in introns may impact gene function by affecting the splice donor-acceptor site, regulatory motifs or these nearby regions. This could be the basis for explaining the association of *DNMT1* polymorphisms with HCC, although it still remains unclear. Moreover, these two polymorphisms showed different allele frequencies among the other populations (Table 1). Additional studies are needed to clarify the association of *DNMT1* with HBV clearance in other ethnic populations (Table 1), including the Caucasian population.

In summary, we have shown that the polymorphisms in the *DNMT1* are associated with HBV clearance in a large-scale study on Korean HBV (n = 1,100). These results may provide an approach for elucidating the molecular mechanisms of HBV clearance.

MATERIALS AND METHODS

Subjects
A total number of 1,100 Korean subjects with either present or past evidence of HBV infection were enrolled from the out-patient clinic of the liver unit or Center for Health Promotion of Seoul National University Hospital from January 2001 to August 2003. All study subjects were of Korean ethnicity. Subjects were classified into two different groups: CC (chronic carrier) and SR (spontaneously recovered), according to serological markers. The CC and SR cohorts consisted of 667 and 433 subjects, respectively, and the CC cohort was composed of 325 CH/LC and 342 HCC patients (Table 3). The diagnoses of the CC and SR subjects were established by repeated sero-positivity for hepatitis B surface antigen (HBsAg) (Enzygnost$^®$ HBsAg 5.0; Dade Behring, Marburg, Germany) over a six-month period using anti-HBs (Enzygnost$^®$ Anti-HBs II; Dade Behring, Marburg, Germany) and anti-HBc (AB-Corek; Dia-Sorin s.r.l., Saluggia, Italy) of the IgG type antibodies without HBsAg, respectively. We excluded subjects that were positive only for anti-HBs and not for anti-HBc, as well as those positive for anti-HCV or anti-HIV (GENEDIA$^®$; Greencross Life Science Corp., Yongin-shi, Korea, HCV$^®$3.2; Dong-A Pharmaceutical Co., Seoul, Korea). Subjects whose average alcohol consumption assessed by interview was >510 g/day or average cigarette smoking was >1 pack/day were excluded. Patients who had any other type of liver disease such as auto-immune hepatitis, toxic hepatitis, biliary cirrhosis and Budd-Chiari syndrome were also excluded. No patients in our study

Table 3. Clinical profiles of study subjects

Description	Spontaneously recovered (SR)	Chronic carrier (CC)	
		Chronic hepatitis (CH) or liver cirrhosis (LC)	Hepatocellular carcinoma (HCC)
No. of subjects	433	342	325
Age (mean, range)	54.95 (28-79)	49.78 (22-82)	58.43 (25-79)
Sex (male/female)	243/190	277/65	278/47
HBeAg (positive rate, %)	0	33.33	19.69
HBeAb (positive rate, %)	0	47.25	64.25
HBsAg (positive rate, %)	38.13	100	100
HBsAb (positive rate, %)	100	0	0
Urine albumin (positive rate, %)	0	8.42	14.47
Urine blood (positive rate, %)	28.54	14.49	24.75

had a previous history of immunosuppression or anti-viral treatment.

Informed consent was obtained from each patient and the study protocol was approved by the Institutional Review Board of Human Research at Seoul National University Hospital. All patients in the CC group were on regular medical follow-up and were evaluated with serum alpha-fetoprotein level assessment, abdominal ultrasonography and/or 2-phase spiral liver CT scan more than twice a year to detect early stages of HCC. We also performed abdominal MRI, bone scan, chest CT, brain MRI, brain CT, hepatic angiography or PET scan in some patients based on clinical examination. Liver cirrhosis was diagnosed pathologically or of the clinical presence of portal hypertension such as visible collateral vessels on the abdominal wall, esophageal varices on esophagogastroscopy, palpable splenomegaly and cirrhotic liver or ascites found on sonograph. HCC was diagnosed as previously described (the age of onset was determined by the date of the diagnosis.) (34).

Genotyping by fluorescence polarization
For genotyping of polymorphic sites, amplifying primers and probes were designed for TaqMan assay (35). One allelic probe was labeled with FAM dye and the other with fluorescent VIC dye. Information regarding the primers is attached in Supplementary Table 1. Primer Express (Applied Biosystems, Foster City, CA) was used to design the MGB TaqMan probes. PCRs were run in a TaqMan Universal Master Mix without UNG (Applied Biosystems), with PCR primer concentrations of 900 nM and TaqMan MGB-probe concentrations of 200 nM. Reactions were performed in 384-well format in a total reaction volume of 5 ml using 20 ng of genomic DNA. The plates were then placed in a thermal cycler (PE 9700, Applied Biosystems) and heated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1

min and a final soak at 25°C. The TaqMan assay plates were transferred to a Prism 7,900 HT instrument (Applied Biosystems) where the fluorescence intensity was read in each well of the plate. Fluorescence data files from each plate were analyzed by automated allele-calling software (SDS 2.1).

Statistics

We searched for a spine of strong |D′| and LD coefficient r^2 between all pairs of biallelic loci (36). Linkage disequilibrium (LD) was inferred using the algorithm developed by the Broad Institute (using the program Haploview) (37). Haplotypes of each individual were inferred using the algorithm (PHASE, version 2.0) developed by Stephens et al. (38). Subjects harboring missing genotypes were omitted in the analysis of individual single-nucleotide polymorphisms (SNPs) and haplotypes. The genotyping success rate was >99%, making it unlikely that bias was introduced by omitting a small number of individuals. Allele frequencies of the *DNMT1* polymorphisms were taken from the HapMap database (http://www.hapmap.org/ index.html.en/) and used for the comparison among ethnic groups. For the evaluation of viral clearance, logistic regression models were used for calculating odds ratios (95% confidential interval) and for corresponding P values controlling for age (continuous value) and sex (male = 0, female = 1) as covariates. HBV genotypes, HBV DNA and Alanine transaminase (ALT) levels are regarded as important factors influencing HBV clearance and the development of HCC. However, HBV genotype C predominates among CCs of the virus in Korea (39-43), and the levels of HBV DNA and ALT were found to fluctuate during the follow-up for the majority of our HBV cohort. Therefore, logistic models for HBV clearance were adjusted only for age and sex.

The effective number of independent marker loci in *DNMT1* was calculated to correct for multiple testing using the SNPSpD software (http://genepi.qimr.edu.au/general/daleN/SNPSpD/), which is based on the spectral decomposition (SpD) of matrices of pairwise LD between SNPs (44). The resulting number of independent marker loci was applied to correct for multiple testing.

Acknowledgements

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