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Genetic polymorphisms in the Wnt/β-catenin pathway genes as predictors of tumor development and survival in patients with hepatitis B virus-associated hepatocellular carcinoma

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ABSTRACT

Objectives: Wnt/β-catenin signaling has a pivotal role in the pathogenesis of hepatocellular carcinoma (HCC). The present study aimed to determine whether genetic variation in the Wnt/β-catenin signaling pathway is associated with the development and/or progression of HCC and the survival of patients with hepatitis B virus (HBV)-associated HCC.

Design and methods: We assessed seven single nucleotide polymorphisms (SNPs) of the *AXIN1*, *AXIN2*, *CTNNB1*, and *WNT2* genes in 245 patients with HBV-associated HCC and 483 chronic HBV carriers without HCC. We analyzed the association of each SNP with HCC development or progression and overall survival.

Results: The *CTNNB1* rs3864004 A allele was associated with a decreased risk of HCC development ($P = 0.049$). Haplotype analysis revealed a significantly higher frequency of *CTNNB1* G-A/G-A haplotype at rs3864004 and rs4135385 positions in patients with HCC than in chronic HBV carriers without HCC ($P = 0.042$). The *AXIN1* rs1805105 T > C SNP was associated with small tumor size and early tumor stage and the *WNT2* rs39315 G allele was associated with advanced tumor stage in HCC. In Kaplan–Meier analysis, carriers of the *AXIN1* rs214252 C allele showed longer survival than those with the TT genotype ($P = 0.020$). In multivariate Cox regression analysis, absence of *CTNNB1* haplotype A-A at rs3864004 and rs4135385 positions and advanced tumor stage were independent poor predictors of patient survival in patients with HCC.

Conclusion: These findings suggest that the genetic polymorphisms in *CTNNB1* gene might affect tumor development and survival in patients with HBV-associated HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1]. Hepatitis B virus (HBV) infection, hepatitis C virus (HCV) infection, excessive alcohol intake, and nonalcoholic fatty liver disease are major risk factors of HCC [2].

HCC develops at a rate of 2% to 3% annually in at-risk populations [2]. Risk factors for HCC development are cirrhosis [3–5], old age, male gender [3,6], and high inflammatory activity within the liver [7]. In addition to known risk factors, the identification of host genetic factors that define populations at an increased risk of HCC development would be extremely useful for personalized surveillance and management of patients with chronic liver disease. Single nucleotide polymorphisms (SNPs) have been extensively studied in order to identify host genetic factors affecting HCC pathogenesis. Several studies have demonstrated the association between SNPs of genes and HCC occurrence [8,9]. However, the results of these studies are not reproducible and the causative SNPs for the development of HCC are not yet identified.

Dysregulation of the Wnt/β-catenin signaling pathway has a critical role in the pathogenesis of HCC [10]. β-catenin protein, encoded by the *CTNNB1* gene, is a key component of Wnt signaling pathway [11]. Deregulation of Wnt signaling may lead to cancer development [11]. A considerable proportion of HCC cells shows nuclear accumulation of β-catenin, a hallmark of Wnt signaling pathway activation [12–14]. Recently, integrative analysis of somatic mutations and copy number aberrations showed that Wnt/β-catenin signaling might contribute to HCC tumorigenesis by both oxidative stress metabolism and Ras/mitogen-activated protein kinase pathways [15].

Abbreviations: AFP, alpha-fetoprotein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LD, linkage disequilibrium; SNP, single nucleotide polymorphism.

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The Wnt/β-catenin pathway contributes to the aggressive behavior of HCC by increasing cell proliferation and promoting cell migration and invasion. Previous investigations have suggested that the Wnt/β-catenin pathway is also involved in the self-renewal and expansion of liver cancer stem cells, which induce a more aggressive tumor phenotype [16,17].

In the clinical setting, prediction of prognosis relies exclusively on clinicopathologic data, and the use of molecular markers has not been yet integrated in the management of HCC patients. Even though the Wnt/β-catenin signaling pathway has been considered as an important component in HCC pathogenesis, only few studies have investigated the association between germline genetic variants in the Wnt/β-catenin pathway genes and the development or prognosis of HCC.

We hypothesized that germline variants in Wnt/β-catenin pathway genes may alter gene function and/or influence the activity of genes, thereby causing different outcomes in patients with HCC. In the present study, we evaluated the association between genetic variations in Wnt/β-catenin signaling genes and HCC development and the relationship between these SNPs and survival in patients with HCC caused by HBV infection.

2. Materials and methods

2.1. Study subjects

A total of 728 patients (483 chronic HBV carriers without HCC and 245 chronic HBV carriers with HCC), who had visited the outpatient clinics of the gastroenterology department of Ajou University Hospital (Suwon, South Korea) between June 2000 and February 2006, were enrolled in this study. The chronic HBV carriers were diagnosed based on positive results for HBsAg and HBV DNA for more than 6 months and the presence of elevated serum alanine aminotransferase levels at least once during the follow-up period. The diagnosis of HCC was confirmed if they had a tumor with a maximum diameter greater than 2 cm, a serum alpha-fetoprotein (AFP) level of greater than 200 ng/ml, and typical features of HCC observed on dynamic imaging. Nodules of 1–2 cm were investigated with two of the following dynamic imaging techniques: computed tomography, angiography, and contrast-enhanced magnetic resonance imaging. If the findings were not characteristic or if the vascular profile was not typical, a biopsy was performed [18]. All the enrolled patients were Korean.

Informed consent was obtained from all participants, and the study protocol was approved by the Institutional Review Board of Human Research of Ajou University Hospital (GN3-08-030).

2.2. DNA preparation

The blood samples were stored at −80 °C until analyses. The genomic DNA was purified using G-DEXTM blood genomic DNA purification kits (Intron Biotechnology Inc., Seongnam, South Korea) and the genomic DNA was quantified using the Picogreen double stranded DNA quantification reagent according to the standard protocol (Molecular Probes, Eugene, OR, USA). A Picogreen working solution was prepared by making 1:200 dilutions in TE buffer (pH 8.0). The λ DNA standard and samples were mixed with the Picogreen working solution followed by incubation for 5 min at room temperature. The plates were read using a Victor™ 3 multi-label counter (excitation 480 nm and emission 520 nm; PerkinElmer Inc., Waltham, MA, USA). A standard DNA concentration curve was calculated with the known concentrations of λ DNA.

Table 2
Baseline characteristics of the study subjects.

Parameters	Chronic HBV carriers with HCC (n = 245)	Chronic HBV carriers without HCC (n = 483)	*P* value
Age (mean ± SD, years)	53.8 ± 10.3	41.1 ± 10.3	<0.001
Male gender	186 (75.9%)	359 (74.3%)	0.640
Alpha fetoprotein			
≤ 400 ng/ml	157 (64.1%)		
> 400 ng/ml	88 (35.9%)		
Tumor size			
≤ 5 cm	136 (55.5%)		
> 5 cm	109 (44.5%)		
Major vessel invasion	78 (31.8%)		
Tumor stage (modified UICC)			
I	28 (11.4%)		
II	85 (34.7%)		
III	88 (35.9%)		
IV	44 (18.0%)		

HBV, hepatitis B virus; HCC, hepatocellular carcinoma; SD, standard deviation; and UICC, Union for International Cancer Control.

2.3. Genotyping

The validated SNPs in the *AXIN1*, *CTNNB1* and *WNT2* genes were searched from a public SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). We finally selected seven SNPs with literature review among SNPs with minor allele frequency ≥ 5% and $r^2 \geq 0.8$. Therefore, we analyzed SNPs at seven polymorphic sites: the *AXIN1* gene at positions rs387467 (C to A substitution), rs214252 (T to C substitution), and rs1805105 (T to C substitution); the *AXIN2* gene at position rs2240308 (C to T substitution); the *CTNNB1* gene at positions rs3864004 (G to A substitution) and rs4135385 (G to A substitution); and the *WNT2* gene at position rs39315 (A to G substitution) (Table 1). Genotyping was performed using the Golden gate genotyping assay kit according to the standard protocol (Illumina Inc., San Diego, CA, USA), as previously described [19]. Signal intensities were extracted and analyzed using Illumina's BEADSTUDIO software Version 3.0.22.

2.4. Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (SPSS, Chicago, IL, USA). Maintenance of the Hardy–Weinberg equilibrium for each SNP was tested to check for deviations in genotype frequencies using χ^2 tests. Genetic models that were used in association testing were the co-dominant, dominant, and recessive models.

We analyzed the association between genotypes and HCC development using a multiple logistic regression model, controlling for covariates such as age and gender. For this analysis, chronic HBV carriers without HCC were regarded as the control group, and their analysis

Table 1
SNP information of genes analyzed in this study.

Gene		Reference sequence	HWE	Major	Minor	Location	Position	Amino acid change
AXIN1	IVS10 + 25C/A	rs387467	0.907	C	A	16p13.3	Downstream	–
AXIN1	A609A	rs214252	0.792	T	C	16p13.3	Downstream	–
AXIN1	D254D	rs1805105	0.704	T	C	16p13.3	Coding exon	D/D
AXIN2	P50S	rs2240308	0.985	C	T	17q24.1	Coding exon	P/?
CTNNB1	−25,384 A/G	rs3864004	0.208	G	A	3p22.1	Promoter	–
CTNNB1	IVS13–66 A/G	rs4135385	0.884	G	A	3p22.1	Intron	–
WNT2	−520 A/G	rs39315	0.793	A	G	7q31.2	Promoter	–

SNP, single nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium.