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Lack of Association of BIRC5 Polymorphisms with Clearance of HBV Infection and HCC Occurrence in a Korean Population

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Abstract

BIRC5 (Survivin) belongs to the inhibitor of apoptosis gene family. The BIRC5 protein inhibits caspases and consequently blocks apoptosis. Thus, BIRC5 contributes to the progression of cancer allowing for continued cell proliferation and survival. In this study, we identified eight sequence variants of *BIRC5* through direct DNA sequencing. Among the eight single nucleotide polymorphisms (SNPs), six common variants with frequencies higher than 0.05 were selected for larger-scale genotyping (n=1,066). Results of the study did not show any association between the promoter region polymorphisms and the clearance of hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC) occurrence. This is in line with a previous study in which polymorphisms in the promoter region does not influence the function of BIRC5. Initially, we were able to detect a signal with the *+9194A>G*, which disappeared after multiple corrections but led to a change in amino acid. Similarly, we were also able to detect an association signal between two haplotypes (*haplotype-2* and *haplotype-5*) on the onset age of HCC and/or HCC occurrence, but the signals also disappeared after multiple corrections. As a result, we concluded that there was no association between *BIRC5* polymorphisms and the clearance HBV infection and/or HCC occurrence. However, our results might useful to future studies.

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Introduction

Hepatitis B virus (HBV) infection, one of the most common virus infections among humans, is the major cause of acute and chronic liver diseases (Lin and Kao, 2008). It affects approximately 350 million people every year, especially those in Asia, Africa, Southern Europe, and Latin America (Lok and McMahon, 2007).

The clinical courses of HBV infection are diverse, ranging from spontaneous recovery after hepatitis to a chronic infection. The risk of developing liver cirrhosis (LC) or hepatocellular carcinoma (HCC) is higher for those who happen to be HBV chronic carriers as compared to the uninfected ones (Merican, Guan et al. 2000). Several previous studies on HBV infection have reported that polymorphisms were associated with the risk of HCC and/or clearance of HBV (Kida *et al*., 2007; Kim *et al*., 2006; Park *et al*., 2006; Shin *et al*., 2003).

BIRC5 (baculoviral inhibitor of apoptosis repeat-contaning 5), also known as survivin, is a protein encoded by the *BIRC5* (Altieri 1994a; Altieri 1994b). It belongs to the inhibitor of apoptosis (IAP) gene family. The BIRC5 protein functions to inhibit caspase activation, thus leading to a decrease in apoptosis or programmed cell death. This has been shown by increase in apoptosis and decrease in tumor growth by disruption of BIRC5 induction pathways. In addition, the BIRC5 protein is highly expressed in most tumor cells (Sah *et al*., 2006). Because of this, *BIRC5* is considered as one of the potent target for cancer therapy (Altieri, 2003).

A previous study demonstrates that BIRC5 is associated with microtubules of the mitotic spindle at the start of mitosis through the disruption of microtubule formation after BIRC5 protein in cancer cells are knocked out. This appearance leads to polyploidy as well as massive apoptosis (Castedo *et al*., 2004). Another study involves how the BIRC5 depleted cells exit mitosis without achieving proper chromosome alignment and then reforms single tetraploid nuclei. It suggests that BIRC5 protein is needed for sustaining mitotic arrest upon encounter with mitosis problems. The previous studies im-

plicate that BIRC5 protein plays an important regulatory role both in the progression of mitosis and sustaining mitotic arrest (Castedo *et al*., 2004).

Based on those observations in cancer development, we hypothesized that *BIRC5* polymorphisms may affect the function of BIRC5 protein and influence the clearance of HBV and HCC progression among HBV-infected patients thus, conducting a case-control study on the *BIRC5* gene.

Methods

Study population and outcomes

A total of 1,066 Korean subjects having either present or past evidences of HBV infection were enrolled from the outpatient clinic of the liver unit or from the Center for Health Promotion of Seoul National University Hospital. Subjects were divided into two different groups according to serologic markers: the chronic carrier (CC) group, and the spontaneous recovery (SR) group. The CC and SR cohorts consisted of 632 and 434 subjects, respectively (Table 1). The diagnoses of the CC and SR subjects were established by repeated seropositivity for the hepatitis B surface antigen (HBsAg) (Enzygnost® HBsAg 5.0; Dade Behring, Marburg, Germany) over a 6-month period, and for both anti-HBs (Enzygnost® Anti-HBs II; Dade Behring, Marburg, Germany) and anti-HBc (AB-Corek; DiaSorin s.r.l., Saluggia, Italy) of the IgG type without HBsAg, respectively. Asymptomatic HBV carriers, which usually involve patients with inactive liver disease on liver biopsy, were also included the in CC group. However, it has been known that, HBV continues to replicate, albeit at very low levels, in some patients that have residual liver disease, which in most cases leads to the development of HCC. We excluded subjects who were positive for anti-HBs but 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). Patients who had any other types of liver diseases such as autoimmune hepatitis, toxic hepatitis, primary biliary cirrhosis, and Budd-Chiari syndrome were also excluded from the study. None of the patients had any previous history of immunosuppression or anti-viral treatment. Informed consent was obtained from each patient, and the Institutional Review Board of Human Research at Seoul National University Hospital approved the study protocol. The clinical parameters are summarized in Table 1.

Sequence analysis of the human BIRC5

Using the ABI PRISM 3700 DNA analyzer (Applied Biosystems, Foster City, CA), we sequenced exons, introns, and the promoter region (~1.5) to discover variants from 24 unrelated individual' s DNA samples. Primer sets for the amplification and sequencing analysis of *BIRC5* were designed based on GenBank sequences. Sequence analysis was carried out using SeqMan® software.

Genotyping with fluorescence polarization detection

Amplifying primers and probes were designed for TaqMan® (An *et al*., 2002) which was used for genotyping of the six polymorphic sites. Primer Express (Applied Biosystems) was used to design both the PCR primers and the MGB TaqMan probes. One allelic probe was labeled with FAM dye and the other with fluorescent VIC dye. Typically, PCR was run in the TaqMan Universal Master mix without UNG (Applied Biosystems) at primer concentration of 900 nM and TaqMan MGB-probe con-

Table 1. Clinical profile of the study subjects

	SR	CC	
		CH or LC	HCC
No. of subjects	434	325	307
Age (mean (range))	55.1 (22~79)	49.4 (22~81)	59.2 (24~79)
Sex (male/female)	243/191	265/60	261/46
HBeAg (positive rate, %)	0	34.2	20.5
HBeAb (positive rate, %)	0	30.8	45.9
HBsAg (positive rate, %)	0	100	100
HBsAb (positive rate, %)	100	0	0
U albumin (positive rate, %)	0	5.8	13.0
U blood (positive rate, %)	28.1	11.7	21.5

SR: spontaneous recovery, CC: chronic carrier, CH: chronic hepatitis, LC: liver cirrhosis, HCC: hepatocellular carcinoma.

centration of 200 nM. The reaction was performed in a 384-well format with a total reaction volume of 5 ul using 20 ng of genomic DNA. The plate was then placed in a thermal cycler (PE 9,700, Applied Biosystems) and was heated for 2 min at 50°C, then for 10 min at 95°C, followed by 40 cycles of 95°C for 15 sec, and finally 60°C for 1 min. The TaqMan assay plate was then transferred to a Prism 7900HT instrument (Applied Biosystems) where the fluorescence intensity of each well was read. Fluorescence data files from each plate were analyzed by automated software (SDS 2.1). Detailed information concerning the primers can be obtained at the website mentioned above.

Statistics

We examined Lewontin's D' (|D'|) and LD coefficient r^2 between all pairs of biallelic loci (Hedrick, 1987). Haplotypes of each individual were determined using the algorithm developed by Stephens *et al.*(Stephens *et al.*, 2001), which (PHASE) uses a Bayesian approach incorporating priori expectations of haplotypic structure from population genetic and coalescent theory. Genetic effects of inferred haplotypes were analyzed in the same way as SNPs. Logistic regression models were used for calculating odds ratios (95% confidential interval) and corresponding P-values controlling for age (continuous value) and sex (male=0, female=1) as covariates. In our analysis on HCC occurrence, LC (LC=1, no LC=0) and HBeAg (negative=0, blank=1, positive=2) were also used as covariates. Statistical powers were also calculated using PGA matlab application (Menashe, Rosenberg et al. 2008). PGA is designed to calculate statistical power and other values of case-control genetic association studies. In this study, a co-dominant (1df) model, relative risk 1.3, disease prevalence value 7.1% (Lee, Kim

A. Map of *BIRC5* (baculoviral IAP repeat-containing 5 (survivin)) on 17q25 (12.5 kb)

B. Haplotypes in *BIRC5*

Hap.	-1547T>C	-644T>C	-625G>C	+9194A>G	+9386T>C	+9809T>C	Freq.
ht1	T	C	C	A	T	C	0.252
ht2	T	T	G	G	T	T	0.229
ht3	C	T	G	A	C	T	0.215
ht4	T	T	G	A	T	T	0.157
ht5	T	T	G	A	T	C	0.117
Others	.	.	.	.	.	.	0.031

C. LDs among *BIRC5* polymorphisms

		D'					
		-1547T>C	-644T>C	-625G>C	+9194A>G	+9386T>C	+9809T>C
r^2	-1547T>C	-	1	0.984	1	0.991	0.983
	-644T>C	0.108	-	0.993	1	0.73	0.987
	-625G>C	0.101	0.969	-	1	0.71	0.99
	+9194A>G	0.084	0.114	0.113	-	1	0.992
	+9386T>C	0.885	0.063	0.058	0.093	-	0.819
	+9809T>C	0.176	0.576	0.574	0.193	0.135	-

Fig. 1. Gene maps and haplotypes of the *BIRC5*. (A) Polymorphisms identified in *BIRC5*. Coding exons are marked by shaded blocks and 5' and 3'UTR by white blocks. Asterisks (*) indicate SNPs that were genotyped in the larger population. The frequencies of SNPs without larger-scale genotyping were based on sequencing data. The first base of the translational start site is denoted as nucleotide +1. (B) Haplotypes of *BIRC5* in the Korean population. Only those with frequencies ≥0.05 are shown. Others (1) contain rare haplotypes: TCACCC, TCATGC, TTATGC, TCGCGT, TCATGT, CTATGT, CTACCC, CTATGC, TCATCT, TTACGT and TTGTGC. C. LD coefficients (|D'| and r^2) among SNPs in *BIRC5*.

et al. 1998), EDF (Effective degree of freedom) 2, and alpha error level 5% were used to calculate the statistical power.

Results

We identified eight genetic variants in *BIRC5* using direct sequencing from 24 unrelated individuals. The range of direct sequencing included the promoter region, as well as the introns and exons of BIRC5. Among the eight genetic variants, four SNPs were located in the promoter region (*−1547T>C, −644T>C, −625G>C, −241C>T*), one in exon 4 (*+9194A>G*) and three in 3' UTR (*+9386T>C, +9625G>A, +9809T>C*), respectively. The frequencies of each SNPs were 0.216 (*−1547T>C*), 0.277 (*−644T>C*), 0.272 (*−625G>C*), 0.021 (*−241C>T*), 0.227 (*+9194A>G*), 0.236 (*+9386T>C*), 0.022 (*+9625G>A*), and 0.395 (*+9809T>C*) (Fig. 1A, Table 2).

The logistic analysis for clearance of HBV infection, HCC occurrence, and onset age of HCC occurrence, as well as the p-values of each polymorphisms, haplotypes, and statistical powers are displayed in Table 3. In the case of SNPs, among the eight variants detected by direct sequencing, six were selected for the association analysis of the clearance of HBV infection and HCC occurrence. Two SNPs (*−241C>T* and *+9625G>A*) were excluded from the analysis due to low frequencies, 0.021 and 0.022 respectively. Among the six polymorphisms, *−644T>C* in the promoter region showed an association signal with HCC occurrence (p=0.04) and onset age of HCC (p=0.05) before correction was conducted, whereas the other SNPs in the promoter regions showed no association signals in all the analysis. An association with the clearance of HBV infection before correction was also detected in *+9194A>G* (p=0.04), which led to a change in amino acid lysine to glutamate.

Another association analysis was conducted for haplotypes. Five haplotypes with frequencies greater than 5% were selected for analysis. The selected haplotypes were able to explain more than 96% of the distribution (Fig 1B). Linkage disequilibrium coefficients (|D'|) between all SNP pairs were also shown (Fig. 1C). Among the haplotypes that we selected for analysis, *haplotype-5 [T-T-G-A-T-C]* showed an association with the clearance of HBV infection (p=0.01) and *haplotype-2 [T-T-G-G-T-T]* showed an association in onset age of HCC analysis (p=0.03) before correction Table 3.

Discussion

BIRC5 serves as a major factor in cancer development by contributing to the resistance of cancer cells to apoptosis (Hedrick, 1987). BIRC5 has been known to be involved in cell-cycle progression by bypassing cell-cycle checkpoints leading to continued proliferation (Ambrosini *et al.*, 1998). This can be explained by the fact that tumor cells can divide without breaking and eventually survive. In addition, a previous study has reported that the expression level of BIRC5 in hepatocellular carcinoma cells is higher than in other cancer-causing cells (Montorsi *et al.*, 2007). With the role played by BIRC5 in cancer development, we hypothesized that *BIRC5* polymorphisms were associated with the clearance of HBV infection and/or HCC occurrence.

Previous studies have reported three polymorphisms (*−644T>C, −625G>C, −31C>G*) involved in the study of lung cancer in a Korean population (n=582) (Jang *et al.*, 2008) and seven polymorphisms (*−267G>*

Table 2. Frequency of BIRC5 gene polymorphisms in this study (n=1,066)

Loci	Position	rs#	Amino acid change	Genotype				Frequency	Heterozygosity	HWE
−1547T>C	Promoter	*rs3764383*	.	T	CT	C	N	0.216	0.339	0.107
				677	351	60	1,088			
−644T>C	Promoter	*rs8073903*	.	T	CT	C	N	0.277	0.400	0.651
				556	434	79	1,069			
−625G>C	Promoter	*rs8073069*	.	G	CG	C	N	0.272	0.396	0.789
				561	415	80	1,056			
+9194A>G	Exon4	*rs17886532*	K129E	A	AG	G	N	0.227	0.351	0.195
				654	364	63	1,081			
+9386T>C	Exon4	*rs2239680*	.	T	CT	C	N	0.236	0.360	0.135
				640	372	69	1,081			
+9809T>C	Exon4	*rs1042489*	.	T	CT	C	N	0.395	0.478	0.253
				401	494	176	1,071			

p-values of deviation from Hardy-Weinberg Equilibrium (HWE).

Table 3. Association analysis of *BIRC5* SNPs and haplotypes with clearance of HBV infection, HCC occurrence and the onset age of HCC

Loci	rs#	Amino acid change	Clearance of HBV infection						HCC occurrence						Onset age of HCC				
			MAF		OR (95%CI)	p	P^{corr}	Statistical power (%)	MAF		OR (95%CI)	p	P^{corr}	Statistical power (%)	N/Event	χ^2	p	P^{corr}	RH
			CC (n=632)	SR (n=434)					HCC (n=307)	CH/LC (n=325)									
−1547T>C	*rs3764383*	.	0.204	0.237	0.84 (0.68~1.04)	0.1	0.47	91.84	0.206	0.205	0.98 (0.72~1.34)	0.92	−	78.74	649/308	1.6	0.21	0.96	1.14
−644T>C	*rs8073903*	.	0.28	0.273	1.02 (0.83~1.26)	0.82	−	93.96	0.298	0.263	1.38 (1.02~1.88)	**0.04**	0.18	85.15	630/298	6.04	**0.01**	0.06	1.24
−625G>C	*rs8073069*	.	0.272	0.273	0.97 (0.79~1.19)	0.79	−	93.96	0.284	0.26	1.26 (0.92~1.72)	0.14	0.66	84.9	616/292	3.22	0.07	0.32	1.17
+9194A>G	*rs17886532*	K129E	0.21	0.25	0.80 (0.65~0.99)	**0.04**	0.18	92.72	0.187	0.233	0.74 (0.53~1.02)	0.07	0.31	82.3	641/301	6.25	**0.01**	0.06	0.78
+9386T>C	*rs2239680*	.	0.223	0.256	0.86 (0.70~1.06)	0.15	0.69	93.08	0.231	0.22	1.05 (0.77~1.41)	0.78	−	80.77	640/296	0.97	0.33	−	1.1
+9809T>C	*rs1042489*	.	0.411	0.372	1.19 (0.99~1.43)	0.06	0.28	96.6	0.444	0.378	1.29 (0.98~1.70)	0.06	0.3	90.55	631/296	0.94	0.33	−	1.08
ht1	.	.	0.243	0.249	0.94 (0.77~1.16)	0.59	−	92.66	0.248	0.239	1.15 (0.84~1.58)	0.39	1.79	82.94	627/296	2.92	0.09	0.41	1.17
ht2	.	.	0.219	0.251	0.84 (0.68~1.04)	0.11	0.49	92.78	0.2	0.238	0.78 (0.56~1.07)	0.12	0.57	82.84	627/296	4.67	**0.03**	0.14	0.81
ht3	.	.	0.201	0.234	0.84 (0.68~1.04)	0.11	0.49	91.62	0.201	0.205	0.96 (0.70~1.31)	0.79	−	78.74	627/296	0.88	0.35	−	1.1
ht4	.	.	0.159	0.141	1.11 (0.86~1.43)	0.41	−	77.94	0.145	0.17	0.89 (0.63~1.27)	0.52	−	72.63	627/296	0.14	0.71	−	0.96
ht5	.	.	0.13	0.098	1.46 (1.08~1.96)	**0.01**	0.06	65.52	0.145	0.111	1.14 (0.75~1.72)	0.55	−	56.3	627/296	0.59	0.44	−	0.92

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. Age (continuous value) and sex (male=0, female=1) were adjusted by including logistic analysis as covariables. All patients included in this study were HBsAg-positive (chronic hepatitis). Cox models were used for calculating onset age of HCC and P-values for SNPs and haplotypes, controlling age, sex, adjusted age (age<40, adjage=0; 40<=age<60, adjage=1; age>60, adjage=2), LC (LC=0, no LC=1), and HBeAg (negative=0, positive=1) by SAS. All patients also included in this Table were HBsAg-positive (chronic HBV).

*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 (4.58) in BIRC5 was calculated using the software SNPSpD (http://genepi.qimr.edu.au/general/daleN/SNPSpD/) on the basis of spectral decomposition (SpD) of matrices of pair-wise LD between the SNPs. Statistical power of single associations was calculated with alpha error level of 5%, disease prevalence of 7.1%, given minor allele frequencies and sample sizes, and assuming a relative risk of 1.3, using PGA (Power for Genetic Association Analyses) software (http://dceg.cancer.gov/bb/tools/pga).

Bold values indicate the case of $p<0.05$

A, −241C>T, −235G>A, −198, −191, −141 and *−31C>G*) studied for breast carcinoma in a French population (n=191) (Boidot *et al*., 2008). Among the promoter region polymorphisms that were studied in both Korean and French populations, *−31C>G* has been considered to play an important role in cancer development by affecting the expression level of mRNA of BIRC5 (Yang *et al*., 2009). Furthermore, two studies concerning cancer in Taiwan and China populations also examined the *−31C>G* polymorphism (Wang *et al*., 2009; Yang *et al*., 2009). In contrast, one study reported that the polymorphisms in the promoter region, including *−31C<G* does not have any influence on the BIRC5 activity (Boidot *et al*., 2008). Although *−31C>G* was not identified in our analysis, findings of this study on the promoter region polymorphisms (*−1547T>C, −644T>C, −625G>C*) showed no association signals with the clearance of HBV infection and/or HCC occurrence and is thus, consistent with one of the previous studies.

Among the six SNPs that were analyzed for large-scale genotyping, *+9194A>G* initially showed an association signal with the clearance of HBV infection before multiple testing corrections. The polymorphism also demonstrated a protective effect on the clearance of HBV infection and led to the amino acid modification from lysine to glutamate. Marusawa *et al*. has demonstrated that BIRC5 forms complexes with a cellular protein called hepatitis B X-interacting protein (HBXIP). This protein's pro-caspase-9 is activated by Apaf1, a cytoplasmic protein recognized for associating the X protein of hepatitis B virus (HBX) and BIRC5. Complexes of BIRC5-HBXIP prevents the recruitment of components that can initiate apoptosis (Marusawa *et al*., 2003). Moreover, in another SNP study, Jang *et al*. reported that *+9194A>G* might be related with cancer (Jang *et al*., 2008). According to the study if alteration of amino acid by *+9194A>G* in exon4 affects protein formation, then it will also affect the interaction of HBXIP and BIRC5. In this study, *+9194A>G* initially showed an association signal with clearance of HBV infection. Although the signal disappeared after multiple testing corrections, it might provide valuable meaning to studies on the function of BIRC5.

In summary, we have identified eight polymorphisms in the human *BIRC5* gene that were used to locate six common polymorphic sites selected for large-scale genotyping. After conducting a test on the functions of the promoter region polymorphisms, statistical analysis displayed no association among polymorphisms in the promoter region with the clearance of HBV infection and/or HCC occurrence. In addition, *+9194A>G* initially showed an association signal with the clearance of HBV infection and led to transformation of amino acid. We also found that haplotype-2 and haplotype-5 initially showed association signals with HBV infection and the onset age of HCC analysis, respectively. However, all association signals disappeared after multiple testing corrections, which led us to conclude that there was no association between BIRC5 polymorphisms and the clearance of HBV infection and/or HCC occurrence. Although our p-values did not undergo multiple testing corrections, results from this study might be useful for future researches which should include additional investigation on the function and/or expression of the polymorphisms of *BIRC5*.

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Table S1. Sequences of amplifying, Taqman probe, and extension primer for BIRC5 SNP genotyping

Loci	rs#		Probe sequence
−1547T>C	*rs3764383*	Forward	GAGGAAGAAGCAGAGAGTGAATGTT
		Reverse	ACTCATATACCCTTCACCCAGATTTTCT
		VIC	TGTTTTGCCTATTTCCTT
		FAM	TTTTGCCTGTTTCCTT
−644T>C	*rs8073903*	Forward	CGATGTCTGCTGCACTCCAT
		Reverse	CACCTCTGCAAAAAGCATCTACTC
		VIC	ATGAAGGACAAATGAACAG
		FAM	ATGAAGGACAAATGGACAG
−625G>C	*rs8073069*	Forward	AAAGACAGTGGAGGCACCAG
		Reverse	GCATGCCTGTAATCCCAACT
		Extension	ATGATTCCCCTGTTCATTTGTCCTTCATGCCC
+9194A>G	*rs17886532*	Forward	CAGGCAAAGGAAACCAACAATAAGA
		Reverse	GCAGCCAGCTGCTCGAT
		VIC	AACTGCGAAGAAAGT
		FAM	CTGCGGAGAAAGT
+9386T>C	*rs2239680*	Forward	GCAATGTCTTAGGAAAGGAGATCAACAT
		Reverse	CGCTGCACAGGCAGAAG
		VIC	AAGACAAAACAAGAGCACA
		FAM	AAGACAAAACAGGAGCACA
+9809T>C	*rs1042489*	Forward	GTTTTGATTCCCGGGCTTAC
		Reverse	AGCATCGAGCCAAGTCATTT
		Extension	TTGACTTGTGTGTGATGAGAGAATGGAGACAGAGTCCC