Article

Association of KIR Genotypes and Haplotypes with Susceptibility to Chronic Hepatitis B Virus Infection in Chinese Han Population

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Killer immunoglobulin-like receptor (KIR) genes can regulate the activation of NK and T cells upon interaction with HLA class I molecules. Hepatitis B virus (HBV) infection has been regarded as a multi-factorial disorder disease. Previous studies revealed that KIRs were involved in HCV and HIV infection or clearance. The aim of this study was to explore the possibility of the inheritance of KIR genotypes and haplotypes as a candidate for susceptibility to persistent HBV infection or HBV clearance. The sequence specific primer polymerase chain reaction (SSP-PCR) was employed to identify the KIR genes and pseudogenes in 150 chronic hepatitis B (CHB) patients, 251 spontaneously recovered (SR) controls, and 412 healthy controls. The frequencies of genotype G, M, FZ1 increased in CHB patients compared with healthy control subjects. The frequency of genotype AH was higher in SR controls than that in both CHB patients and healthy controls. The carriage frequencies of genotype G and AH were higher; while, the frequencies of AF and AJ were lower in SR controls than those in healthy control subjects. The frequency of A haplotype was lower, whereas, the frequency of B haplotype was higher in CHB patients and SR controls than those in healthy controls. In healthy controls, haplotype 4 was found lower compared with that in CHB patients and SR controls and the frequency of haplotype 5 was higher in SR controls than that in other two groups. Based on these findings, it seems that the genotypes M and FZ1 are HBV susceptive genotypes; AH, on the other hand, may be protective genotypes that facilitate the clearance of HBV. It appears that the haplotype 4 is HBV susceptive haplotype, whereas, haplotype 5 may be the protective haplotype that facilitates the clearance of HBV. *Cellular & Molecular Immunology*. 2008;5(6):457-463.

Key Words: HBV, KIR, KIR genotype, KIR haplotype

Introduction

The immune system, especially autoantibodies with agonist-like activity, KIR molecules are encoded by the KIR gene family that clusters within the leukocyte receptor complex on chromosome 19q13.4 (1, 2). KIR genes exhibit allelic, haplotypic, and gene content variability in both the number and type of genes presented on a haplotype (3-6). The haplotypes have a framework of three conserved blocks containing KIR3DL3, KIR2DL4 and KIR3DL2 and differ in the number and type of KIR genes. In general, most KIR haplotypes belong to one of two broad groups, termed A and B. The latest haplotype definition (14th International HLA and Immunogenetics Workshop, 2005) has identified that haplotype A is composed of KIR3DL3, KIR2DL3, KIR2DP1, KIR2DL1, KIR3DP1, KIR2DL4, KIR3DL1, KIR2DS4 and KIR3DL2 genes, while, all other haplotypes are described as haplotype B. Both groups of genotypes have been found in all populations analyzed so far, but, their distribution varies considerably among ethnic groups (3-5).

Group A haplotypes have a fixed content of seven KIR genes and two pseudogenes, and are diversified through allelic polymorphism. The genes include those specifying inhibitory receptors for each of the four KIR ligands

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(HLA-A, -B, -C, and -G), KIR2DL4 and KIR3DL3, as well as KIR2DS4. The group B haplotypes are diversified by both gene content and allelic polymorphism. Although all genes of the group A haplotype are represented in the group B haplotypes, what distinguishes group B haplotypes is presence of a variable number of KIR genes that are not components of the group A haplotypes, i.e., KIR2DS1, 2DS2, 2DS3, 2DS5, 3DS1, and 2DL5. Most of the activating KIRs are associated only with the group B haplotypes, whereas, both group A and group B haplotypes have comparable complements of inhibitory receptors. Thus, the group A and B KIR haplotypes provide distinct and complementary functions in the biology of NK cells and T cells. This means the group A and B KIR heliotypes may play different roles in hepatitis B virus persistence and clearance.

Hepatitis B virus (HBV) infection still remains a major health issue worldwide despite the availability of an effective vaccine. It has been documented that approximately 350 million people are chronically infected with the virus worldwide, with more than 200 million in China. Thus, HBV infection, as a leading contagious disease, constitutes a threat to our county. Clinically, HBV infection can persist in the host for the whole life, often leading to severe consequences such as liver failure, fibrosis, cirrhosis, and hepatocellular carcinoma. However, the molecular mechanism for the elimination of and host susceptibility to HBV remains largely unsolved because numerous factors are responsible for the chronicity of HBV infection.

It has been reported that the polymorphisms of certain genes, such as CXCL10, intercellular adhesion molecule-1 (ICAM-1), TNF-α, etc., are associated with susceptibility to chronic hepatitis B virus infection (7-9). Of note, polymorphisms of KIR gene have generated considerable interest in disease association studies in recent years (10). Chen et al. found that the expression of HBV in human hepatoplastoma cell line significantly down-regulated the expressions of MHC class I molecules and it was further observed that in murine chronic HBsAg carriers the expression of classical MHC-I molecules on hepatocytes was down-regulated (11). Yang et al. revealed both anti-HBc IgG1 and IgG3 were significantly higher in chronic carriers than those in recovered individuals (12). Recently, we have demonstrated that KIR2DS2 and KIR2DS3 may act as HBV susceptive genes of chronic hepatitis B; whereas, KIR2DS1, KIR3DS1, and KIR2DL5 may be the protective genes that facilitate the clearance of HBV (13). This promotes us to further analyze the differences in frequency of genotypes and halotypes between patient groups and control group so as to investigate whether KIR genotypes and haplotypes participate in the HBV infection or HBV clearance.

Materials and Methods

Study subjects

Eight hundred and thirteen subjects, comprising 150 patients with chronic hepatitis B (CHB), 251 spontaneously recovered (SR) controls, and 412 healthy Chinese adults from Shandong Provincial Hospital and Jinan Infectious Disease Hospital between October 2004 and August 2006, were recruited in this study. Only the CHB patients, who had a history of HBV infection for more than one year and the elevated levels of alanine aminotransferase/aspartate aminotransferase or total bilirubin, could meet our inclusion criteria. Those who were negative for hepatitis B surface antigen (HBsAg) and positive for both hepatitis B surface antibody (HBsAb) and hepatitis B core antibody (HBcAb) were defined as SR controls. All the recruited subjects had excluded from the other disorders, such as infection of hepatitis C virus, hepatitis D virus and HIV, as well as diabetes, malignant tumor, or any autoimmune diseases (14). Prior to the study, informed consent was obtained from each individual.

Genome DNA extraction

Genomic DNA sample was extracted from 5 ml EDTA anticoagulated peripheral blood with a standard salting-out procedure (15) and stored at -20°C before use.

KIR genotyping

KIR genotyping was performed by SSP-PCR in all the recruited subjects. In the present study, KIR locus typing was performed to detect the presence or absence of all of 18 known KIR genes, such as 2DL1-5, 2DS1-5, 3DL1-3, 3DS1, KIR1D and the pseudogenes X, Xv and Z (KIR2DP1). Among them, 8 KIR genes (2DLI, 2DL2, 2DL3, 2DL4, 2DL5, 3DL1, 3DL2, and 3DL3) were responsible for inhibitory functions and 6 KIR genes (2DS1, 2DS2, 2DS3, 2DS4, 2DS5, and 3DS1) for conveying activating signals. The SSP-PCR primers used for the detection of KIR loci were based on primer sites that had been previously described (16). Among the 29 formatted couple primers (Shanghai Boya Biotechnology Co. Ltd, China), 2DS5 gene used one couple primer and each of the 14 surplus genes used two couple primers, so as to ensure a detectable rate of positive gene (KIR genes primer in Table 1). The framework genes (2DL4, 3DL2, and 3DL3) served as a positive marker of PCR. PCR was conducted on the Gene Amp PCR system 9700-R (Applied Biosystems, Foster City, CA, USA). Briefly, 1 μl of genomic DNA was amplified in a volume of approximately 20 μl system including 6 μl primers, 2 μl 10 × PCR buffer, 1.6 μl $MgCl_2$ (25 mM), 0.4 μl dNTP (10 mM), 0.125 μl Taq polymerase (5 U/μl), and 8.875 μl dH_2O. After the initial denaturation for 1 min at 96°C, the samples were amplified in the following way: 5 cycles of 25 sec at 96°C, 45 sec at 65°C, and 30 sec at 72°C; 21 cycles of 25 sec at 96°C, 45 sec at 60°C, and 30 sec at 72°C; 5 cycles of 25 seconds at 96°C, 1 min at 55°C, 2 min at 72°C; and a prolongation of 10 min at 72°C. The PCR products, together with approximately 3 μl 100 base pairs (bp) DNA ladder as molecular weight marker (MBI, San Francisco, CA, USA), were electrophoresed on 1.5% agarose gels with bromophenol blue, keeping voltage at 160 V for 30 min. After electrophoresis, the agarose gel was scanned and imaged by Alphaimager TM 2200 instrument (Alpha Innotech

Table 1. Sequence specific PCR primers of KIR genes

KIR gene	Forward (5' – 3')	Reverse (5' – 3')	Length (bp)
2DL1	GTT GGT CAG ATG TCA TGT TTG AA	GGT CCC TGC CAG GTC TTG CG	127
	TGG ACC AAG AGT CTG CAG GA	TGT TGT CTC CCT AGA AGA CG	330
2DL2	CTG GCC CAC CCA GGT CG	GGA CCG ATG GAG AAG TTG GCT	173
	GAG GGG GAG GCC CAT GAA T	TCG AGT TTG ACC ACT CGT AT	150
2DL3	CTT CAT CGC TGG TGC TG	AGG CTC TTG GTC CAT TAC AA	550
	TCC TTC ATC GCT GGT GCT G	GGC AGG AGA CAA CTT TGG ATC A	800
2DL4	CAG GAC AAG CCC TTC TGC	CTG GGT GCC GAC CAC T	254
	ACC TTC GCT TAC AGC CCG	GGG TTT CCT GTG ACA GAA ACA G	288
2DL5	GCG CTG TGG TGC CTC G	GAC CAC TCA ATG GGG GAG C	214
	TGC AGC TCC AGG AGC TCA	GGG TCT GAC CAC TCA TAG GGT	194
3DL1	CGC TGT GGT GCC TCG A	GGT GTG AAC CCC GAC ATG	197
	CCC TGG TGA AAT CAG GAG AGA G	TGT AGG TCC CTG CAA GGG CAA	181
3DL2	CAA ACC CTT CCT GTC TGC CC	GTG CCG ACC ACC CAG TGA	245
	CCC ATG AAC GTA GGC TCC G	CAC ACG CAG GGC AGG G	130
3DL3	GTC AGA TGT CAG GTT TGA GCG	CAT GGA ATA GTT GAC CTG GGA AC	112
	GCA GCT CCC GGA GCT TG	GGG TCT GAC CAC GCG TG	190
2DS1	CTT CTC CAT CAG TCG CAT GAA	CTT CTC CAT CAG TCG CAT GAG	102
	CTT CTC CAT CAG TCG CAT GAA	AGA GGG TCA CTG GGA GCT GAC	102
2DS2	TTC TGC ACA GAG AGG GGA AGT A	AGG TCA CTG GGA GCT GAC AA	173
	CGG GCC CCA CGG TTT	GGT CAC TCG AGT TTG ACC ACT CA	240
2DS3	TGG CCC ACC CAG GTC G	TGA AAA CTG ATA GGG GGA GTG AGG	242
	CTA TGA CAT GTA CCA TCT ATC CAC	AAG CAG TGG GTC ACT TGA C	190
2DS4	CTG GCC CTC CCA GGT CA	TCT GTA GGT TCC TGC AAG GAC AG	204
	CTG GCC CTC CCA GGT CA	GGA ATG TTC CGT TGA TGC	2000
2DS5	TGA TGG GGT CTC CAA GGG	TCC AGA GGG TCA CTG GGC	125
3DS1	AGC CTG CAG GGA ACA GAA G	GCC TGA CTG TGG TGC TCG	300
	CCT GGT GAA ATC AGG AGA GAG	GTC CCT GCA AGG GCA C	177
2DP1	GTC TGC CTG GCC CAG CT	GTG TGA ACC CCG ACA TCT GTA C	205
	CCA TCG GTC CCA TGA TGG	CAC TGG GAG CTG ACA ACT GAT G	90
3DP1(X)	ATC CTG TGC GCT GCT GAG CTG AG	GCC TAT GAA AAC GGT GTT TCG GAA TAC	344
3DP1v(Xv)	ATC CTG TGC GCT GCT GAG CTG AG	GCC TAT GAA AAC GGT GTT TCG GAA TAC	1817
KIR1D	ATC CTG CAA TGT TGG TCG	CTG GAT GAG TGG AGC TGC AG	1885

Corporation, San Leandro, CA, USA) and each sample was genotyped. All primers were validated to be gene-specific by PCR product sequencing.

Haplotype analysis

Each genotype was given the putative haplotype combination according to the model of Hsu et al. (6). In assigning genes to specific haplotypes, the following assumptions were made: 1) all haplotypes contained KIR3DL3, 2DL4, and 3DL2; 2) haplotypes contained either 2DL2 or 2DL3, but not both; 3) haplotypes contained either 3DP1 or 3DP1 variant (3DP1v), but not both (6).

In the assessment of the KIR haplotypes, group B haplotypes were defined by the presence of one or more of the following genes: KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, and KIR3DS1 (17). Conversely, group A haplotypes were defined by the absence of all these genes.

Statistical analysis

Genotype frequencies were determined by direct counting of the individual positive for some particular KIR phenotype specificity. Each genotype was given the putative haplotype combination according to the model of Hsu et al. (6). Chi Square was used to test for statistical significance of the genotypes or haplotypes between each two groups observed. Analysis was performed by the SPSS13.0 statistical package.

Table 2. The frequency of KIR genotypes in patients with CHB, SR controls and healthy controls

Genotype	Haplotype	Chronic hepatitis B (n = 150)			Spontaneously recovered controls (n = 251)			Healthy controls (n = 412)		
		+	pf (%)	Pch	+	pf (%)	Pcs	+	pf (%)	Psh
C	3, 5	1	0.67	0.577	2	0.79	0.884	5	1.21	0.611
D	3, 12	3	2	0.091	1	0.39	0.118	2	0.49	0.871
E	1, 3	1	0.67	0.455	3	1.19	0.606	6	1.46	0.778
F	1, 4	1	0.67	0.287	1	0.39	0.712	8	1.94	0.096
G	4, 5	24	16	0.014	40	15.94	0.987	36	8.74	0.005
H	2, 4	0	0	0.137	1	0.39	0.439	6	1.46	0.196
I	5, 8	1	0.67	0.934	2	0.79	0.884	3	0.73	0.921
M	2, 8	5	3.3	0.048	2	0.79	0.061	4	0.97	0.818
N	1, 8	2	1.33	0.499	2	0.79	0.601	3	0.73	0.921
O	5, 15	1	0.67	0.794	2	0.79	0.884	2	0.49	0.616
P	2, 17	2	1.33	0.290	4	1.59	0.835	2	0.49	0.144
AE	1, 6	8	5.33	0.052	9	3.59	0.401	9	2.18	0.282
AF	1, 2	24	16	0.090	35	13.94	0.574	93	22.57	0.006
AG	1, 1	7	4.67	0.309	14	5.58	0.692	12	2.91	0.080
AH	2, 5	15	10	0.159	53	21.12	0.004	60	14.56	0.030
AI	1, 5	12	8	0.774	22	8.76	0.79	30	7.28	0.491
AJ	2, 2	29	19.33	0.161	38	15.14	0.276	103	25	0.003
FZ1	2, 9	5	3.3	0.048	2	0.79	0.061	4	0.97	0.818
FZ2	1, 16	0	0	0.393	3	1.19	0.179	2	0.49	0.306
FZ3	6, 17	1	0.67	0.794	1	0.39	0.712	2	0.49	0.871
FZ4	4, 13	0	0	0.226	3	1.19	0.179	4	0.97	0.784
FZ5	2, 6	1	0.67	0.455	3	1.19	0.606	6	1.46	0.778
FZ6	5, 6	1	0.67	0.794	1	0.39	0.712	2	0.49	0.871
FZ7	2, 23	1	0.67	0.455	0	0	0.195	1	0.24	0.435
FZ8	3, 4	0	0	0.000	1	0.39	0.439	0	0	0.200
FZ9	4, 8	2	1.33	0.290	0	0	0.067	2	0.49	0.269
FZ10	1, 4	1	0.67	0.097	1	0.39	0.712	0	0	0.200
FZ11	4, 9	0	0	0.393	1	0.39	0.439	2	0.49	0.871
FZ12	5, 22	0	0	0.393	1	0.39	0.439	2	0.49	0.871
FZ13	6, 6	1	0.67	0.097	1	0.39	0.712	0	0	0.200
FZ14	5, 19	0	0	0.546	1	0.39	0.439	1	0.24	0.723
FZ15	5, 14	1	0.67	0.097	1	0.39	0.712	0	0	0.200

Abbreviations: Pch, *p* value for the comparison between chronic hepatitis patients and healthy control; Psh, *p* value for the comparison between spontaneously recovered controls and healthy controls; Pcs, *p* value for the comparison between spontaneously recovered controls and chronic hepatitis patients.

The value of $p < 0.05$ was considered to be statistically significant.

Results

KIR genotype frequency in patients and control subjects

In this study, all the tested KIR genes were present in patient groups and control groups in different frequencies. Framework genes KIR2DL4, KIR3DL2, KIR3DL3, and KIRZ were present in all individuals. All KIR genotypes and putative haplotypes were determined in this study according to the model of Hsu et al. (6).

As reported previously, we found 32 genotypes, including 15 new genotypes of FZ1~FZ15, which had not been observed in Caucasians so far (9). Among these genotypes, 28 were determined in healthy controls, 25 in chronic hepatitis B, and 30 in SR controls (Table 2). In health controls, the three genotypes with higher frequency in rank order were AJ (25.00%), AF (22.57%) and AH (14.56%). In CHB patients, genotypes AJ (19.33%), G (16.00%) and AF (16.00%) were the three higher genotypes. In SR patients, genotypes AH (21.12%), G (15.94%) and AJ (15.14%) held the three leading places.

Table 3. The frequency of haplotypes in patients with CHB, SR controls and healthy controls

Haplotype	Chronic hepatitis B (n = 150)			Spontaneously recovered controls (n = 251)			Healthy controls (n = 412)		
	+	pf (%)	Pch	+	pf (%)	Pcs	+	pf (%)	Psh
1	63	21	0.112	104	20.72	0.924	211	25.61	0.042
2	111	37	0.000	176	35.06	0.579	382	46.36	0.000
3	5	1.67	0.916	7	1.39	0.759	13	1.58	0.791
4	28	9.33	0.000	48	9.56	0.915	22	3.4	0.000
5	56	18.67	0.544	125	24.9	0.041	141	17.11	0.001
6	13	4.33	0.071	16	3.19	0.400	19	2.31	0.331
8	10	3.33	0.044	6	1.2	0.036	12	1.46	0.690
9	5	1.67	0.157	3	0.6	0.140	6	0.728	0.779
12	3	1	0.091	1	0.2	0.119	2	0.24	0.871
13	0	0	0.227	3	0.6	0.180	4	0.48	0.785
14	1	0.33	0.097	1	0.2	0.712	0	0	0.200
15	1	0.33	0.795	2	0.4	0.884	2	0.24	0.616
16	0	0	0.393	3	0.6	0.180	2	0.24	0.306
17	3	1	0.332	5	1	0.996	4	0.48	0.272
19	0	0	0.546	1	0.2	0.439	1	0.12	0.723
22	0	0	0.393	1	0.2	0.439	2	0.24	0.871
23	1	0.33	0.456	1	0.2	0.196	1	0.12	0.435

Abbreviations: Pch, *p* value for the comparison between chronic hepatitis patients and healthy control; Psh, *p* value for the comparison between spontaneously recovered controls and healthy controls; Pcs, *p* value for the comparison between spontaneously recovered controls and chronic hepatitis patients.

In CHB patients, the total carriage frequency of genotype G was higher than that in healthy control subjects ($p = 0.014$), while the frequency of genotype AH was lower than that in SR controls ($p = 0.004$). In SR patients, the total carriage frequency of genotype G and AH was higher ($p = 0.005$, $p = 0.030$), while the frequency of AF and AJ was lower ($p = 0.006$, $p = 0.003$) than those in healthy control. In this study, other genotypes including 15 new identified genotypes had low frequencies with no values in statistics, except FZ1, which increased in chronic hepatitis B patients compared with that in healthy control.

KIR haplotype frequency in patients and control subjects

In the current study, the KIR genotypes were analyzed for deducing the KIR hapotypes based on the hapotypes characterized by other researchers (6, 18). All the 32 genotypes could be resolved into corresponding pairs of hapotypes as shown in Table 3. There were 17 different haplotypes obtained from SR controls, 13 haplotypes in CHB patients, and 16 in healthy controls. As shown in Table 3, haplotype 2 was the most frequent, followed by haplotype 1, 5 and 4 in both CHB patients and healthy controls; while in SR controls, haplotype 2 still remained the most frequent one, followed by haplotype 5, 1 and 4 in rank order. However, haplotype 2 and 1 were found higher in healthy controls than those in CHB patients and in SR controls; while in health controls, haplotype 4 was found lower compared with those in CHB patients and SR controls. The frequency of haplotype 5 was higher and that of haplotype 8 was lower in SR controls than that in other two groups.

The frequencies of A and B haplotypes were illustrated in Table 4. The frequency of A haplotype was lower in CHB patients and SR controls than that in healthy controls, while the frequency of B haplotype was higher. There were no

Table 4. The haplotype A and B observed in patients with CHB, SR controls and healthy controls

Haplotype	Chronic hepatitis B (n = 150)			Spontaneously recovered controls (N = 251)			Healthy controls (N = 412)		
	+	pf (%)	Pch	+	pf (%)	Pcs	+	pf (%)	Psh
A	174	58	0.000	280	55.78	0.539	593	71.97	0.000
B	126	42	0.000	222	44.22	0.539	231	28.03	0.000

Abbreviations: Pch, *p* value for the comparison between chronic hepatitis patients and healthy control; Psh, *p* value for the comparison between spontaneously recovered controls and healthy controls; Pcs, *p* value for the comparison between spontaneously recovered controls and chronic hepatitis patients.

significant differences with haplotypes A and B between CHB patients and SR controls.

Discussion

Previously, we explored the KIR gene polymorphisms in this cohort of normal controls and chronic hepatitis B patients by means of PCR-SSP, with special attention given to association of the frequencies of KIR gene polymorphisms with susceptibility to persistent HBV infection or HBV clearance. In order to further investigate the roles of KIR gene underlying HBV action with a different point of view, in the present study, more subtypes of KIR gene were examined, such as KIR3DP1(X), KIR3DP1v (Xv), KIR1D by the same approaches. Meanwhile, the relationship between the KIR genotypes and haplotypes with susceptibility to chronic hepatitis B virus infection was further investigated. In this work, we found that genotypes G, M and FZ1 were significantly different in the CHB patients in comparison to healthy controls and genotype AH was significantly different as compared with SR controls. And also, the considerable differences between the SR controls and healthy controls were found in relation to genotypes G, AF, AH and AJ. In the current study 15 new genotypes FZ1~FZ15 which had not been observed in Caucasians so far were identified. This suggests that there are distinctive frequencies of KIR genotypes in Chinese Han population in Shandong area. In addition, we found that the frequency of haplotype B was higher in patients with CHB and SR controls comparison with healthy controls. The combinations of human leukocyte antigen (HLA) and KIR genes have been associated with autoimmunity, viral infections, reproductive failure and cancer (19-24). It is highly relevant that polymorphism studies precede a detailed knowledge of HLA-KIR combination, KIR haplotype, genes and allele distribution in populations. In human populations, there is a variable balance between group A and group B KIR haplotypes, which appears maintained by balancing selection for inhibitory and activating functions (3, 25). This selection is mediated, in part, by the interaction of inhibitory KIR with their HLA class I ligands. Because of the biologic significance of the A/B haplotype difference, it is conceivably inferred whether combinations of A and B haplotypes can influence the HBV infection or HBV clearance. In deed, the present study has clearly confirmed that such a situation occurred in this series of patients.

In conclusion, based on the findings of this study, we propose that genotypes and haplotypes containing more activating genes may play an important role in the infection or clearance of HBV. Further research is required to uncover the molecular mechanisms by which they participate in the infection and clearance of HBV.

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