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Original Article

Killer Ig-like receptor (KIR) genetic polymorphism in chronic hepatitis B susceptibility in a Chinese population

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Abstract: Natural killer and $CD8^+$ T cells are identified as cleaners to be involved in the immune protection against virus, bacteria and tumor cells. Their function may be regulated by a cohort of receptors such as killer immunogloblin-like receptors (KIRS) and their counterpart HLA class I ligands. In this study, we analyzed the influence of *KIR* genes and *KIR/HLA-Cw* combinations on chronia hepatitis B susceptibility in a Han Chinese population. For the purpose, KIR and HLA-C genotyping by PCR-SSP were performed in 175 patients with chronic hepatitis B and 125 patients who cleared HBV spontaneously. The results showed a significantly lower frequency of KIR2DL2 and KIR2DS2 in the case group which named patients with chronic hepatitis B, in who was found a higher frequency of KIR2DL3. However, KIR2DL2, -2DL3 and -2DS2 in the presence of their ligands HLA-C1 were failed to reach significance. In conclusion, the study of KIR genes and HLA ligands may contribute to the genetic susceptibility of chronic hepatitis B, and help in assessing chronic hepatitis B risk and diognosis.

Keywords: Chronic hepatitis B, genetic polymorphism, killer immunogloblin-like receptors, HLA

Introduction

Chronic hepatitis B is a serious public health problem worldwide which were caused by hepatitis B virus (HBV) infection which may be either cleared spontaneously or become chronic hepatitis B [1-3]. Chronic hepatitis B is liver chronic inflammation resulting cirrhosis after several years and increasing the incident of hepatocellular carcinoma (HCC) [4, 5]. Different from other infection, the host immune response to HBV infection lead to not only viral response but also hepatocellular damage. The outcome of HBV infection was affected by many factors, such as the type of virus, the host immune response and the environment. There were robust evidences showed that the genetic variation in human population contributes to progression to chronic hepatitis B proved by genetic epidemiological. Many studies have sought a possible association between genetic polymorphisms in immune response related genes and the risk of chronic HBV infection [6, 7]. Genetic polymorphisms in the human leukocyte antigens (HLA), interleukin (IL)-10, tumor necrosis factor-alpha (TNF-α), natural killer (NK) cell receptor NKG2D and IFN-γ receptor 1 (IFNGR1) genes are correlate with susceptibility to chronic hepatitis B [8-17].

The effector function of NK and $CD8^+$ T cells after infection may be regulated by a balance between inhibitory and activating signals mediated by a group of receptors after the interaction with their cognate ligands expressed on target cells [18-20]. The killer immunoglobulin-like receptor (KIR) is a class of NK receptors whose genes are also highly polymorphic, so as to their protein products display variegated expression on NK cells and some T-cell subsets. Their gene family is located within the IBD6 linkage region at chromosome 19q13.4. The family comprises 14 genes and two pseudogenes, although the number of loci varies on different KIR haplotypes. Only four loci are common to all KIR haplotypes (the so-called 'framework' genes KIR2DL4, -3DL1, -3DL3, and the pseudogene -3DP1), whereas the presence of

Table 1. Phenotype frequencies of KIR2DL2, -2DL3, -2DS2 and their ligand HLA-C1 in case group and control group

	KIR2DL2	KIR2DL2 with HLA-C1	KIR2DL3	KIR2DL3 with HLA-C1	KIR2DS2	KIR2DS2 with HLA-C1	HLA-C1
Case	0.23±0.15	0.21±0.06	0.90±0.04	0.91±0.09	0.22±0.10	0.23±0.09	0.71±0.06
Control	0.19±0.11	0.20±0.07	0.99±0.07	0.92±0.07	0.19±0.11	0.21±0.11	0.70±0.04
t value	2.53	1.33	14.09	1.03	2.46	1.73	1.62
P value	0.01	0.19	< 0.001	0.30	0.01	0.08	0.11

Table 2. Characteristics of the patients

	Case	Control	*P* value
Number	175	125	-
Age (years)	37.14±8.53	36.79±9.72	0.7357
Gender (male/female)	86/89	53/72	0.2482
Body weight (kg)	151.04±9.26	149.68±8.97	0.1638
Height (cm)	171.28±10.14	169.63±11.37	0.1903

Values are mean ± SD. *P* value were calculated by the *t* test or χ^2 test. Case: patients with chronic hepatitis B. Control: patients who cleared HBV spontaneously.

remaining 'non-framework' loci can vary between haplotypes [21, 22]. KIR molecules interact with specific epitopes of human leucocyte antigen (HLA)-C and -B alleles, providing information on the HLA class I surface expression of target cells. Engagement of KIR with the appropriate HLA ligand induces either inhibitory or activating signaling depending on the presence of intracellular immunoregulatory tyrosine-based inhibitory motifs or activation motifs [23, 24]. Analysis of KIR/HLA ligand combination is increasingly of interest in the genetic study of infectious, antoimmune, tumoral diseases and even allogeneic hematopoietic stem cell transplantation.

Based on the previous research, this present study evaluated the association between the KIR and their HLA ligands genetic polymorphisms and susceptibility to chronic hepatitis B by carrying out a genetic study in a Han Chinese population.

Methods

Patients and controls

A total of 300 patients infected with HBV were enrolled in the Qingdao Sixth People's Hospital between December 2013 and October 2015. All the subjects were randomly divided into two groups: 175 patients with chronic hepatitis B in case group and 125 patients who cleared HBV spontaneously in control group. Patients were diagnosed with chronic hepatitis B if they were hepatitis B surface antigen, (HBsAg)-positive for more than 6 months with elevated alanine amino transferase (ALT) and aspartate aminotransferase (AST) [2 times the upper limit of normal], and/or had persistence of HBsAg for more than 6 months with liver biopsies showing signs of chronic hepatitis B, confirmed by a pathologist. The patients who cleared HBV spontaneously were HBsAg-negative, hepatitis B e antigen (HBeAg)-negative, hepatitis B surface antibody (anti-HBs)-positive, hepatitis B core antibody (antiH-Bc)-positive, and had recovered from HBV infection [16]. No difference between the two groups of patients with basic data comparison (**Table 2**). The patients who were infected with other hepatitis viruses or hepatitis were not caused by HBV and were not Han ethnicity but were excluded.

The protocol of the study was approved by the local Ethics Committee.

DNA extraction and genotyping

Genomic DNA was extracted from blood by using a salting-out protocal and stored at -20°C.

KIR typing of genomic DNA was performed by a PCR-SSP (polymerase chain reaction-sequence specific primer) method as described by DC Jones et al [25]. Primers were designed using sequence alignments comprising KIR2DL1-5 and KIR2DS1-5 allelic variants present in the immuno-polymorphism database (IPD) KIR sequence database (http://www.ebi.ac.uk/ipd/kir/) [25, 26].

Reactions were also designed for the detection of the HLA-C class I ligands of KIR. epitopes C1

Table 3. Primer mixes

KIR alleles	Sense primers (sequence 5'-3')	Concentration (μM)	Antesense primers (sequence 5'-3')	Concentration (μM)	Amplicon (bp)
2DL1	TgTTggTCAgATgTCATgTTTgAA	2.4	TCCCTgCCAggTCTTgCg	2.4	143
2DL2 (except 2DL2*004)	ggTCgCCTggTgAAATCAgA	2.4	gACCgATggAgAAgTTggCT	2.4	160
2DL2*004	CAggAAACAgAACAgCgAATAT	1.2	TTTggATCTggACTCAgCAC	1.2	284
2DL3 (except 2DL3*003)	gACCCTCAggAggTgACATAT	1.8	gCAggAgACAACTTTggATCA	1.8	156
2DL3*003	CCCTCAggAggTgACATACA	1.8			155
2DL4	gTCTATATgAgAAACCTTCgCTTA	3.0	TCTCCgTgggTggCAggA	3.0	210
2DL5	CTgCAgCTCCAggAgCTCA	1.8	ACTCATAgggTgAgTCATggAg	1.8	182
22DS1 (except 2DS1*001)	TgTTggTCAgATgTCATgTTTgAA	2.4	TCCCTgCCAggTCTTgCT	2.4	144
2DS1*001		2.4	TCCCTgCCAggTCTTgCC	2.4	144
2DS2	gCCgggCCCCACggTTT	1.8	CACTCgAgTTTgACCACTCA	1.8	243
2DS3	TTCTgCACAgAgAggggAC	1.8	AgggTCACTgggAgCTgAA	1.8	176
2DS4	CCTggCCCTCCCAggTCA	1.2	gACAggCATCATgggACCA	1.2	187
2DS5	CAgAgAggggACgTTTAACC	1.2	gTgATCACgATgTCCAgAggg	1.2	186
3DL1	CCTggTgAAATCAggAgAgAg	1.8	TAggTCCCTgCAAgggCAA	1.8	182
3DL2	TCCTggCCCACCCAggg	1.8	ggAgTgAggAACAgAACCATAA	1.8	230
3DL3	CTgCACAgAgAggggATCA	1.2	gACAACTCATAgggTAAgTgAgTg	1.2	158
3DS1	CCTggTgAAATCAggAgAgAg	2.4	CAAgggCACgCATCATggA	2.4	173
HLA class I epitopea/alleles					
HLA-Cw C1	CCgCgAgTCCgAgAggg	1.2	gCgCAggTTCCgCAggC	1.2	128
	gCCgCgAgTCCAAgAgg)	1.2			129
HLA-Cw C2	CCgCgAgTCCgAgAggg	1.2	CgCgCAgTTTCCgCAggT	1.2	129
	gCCgCgAgTCCAAgAgg	1.2			130

Abbreviations: HLA, human leucocyte antigen; KIR, killer immunoglobulin-like receptor.

(defined by the presence of Asn80 in the a1 domain of the HLA-C molecule, recognized by KIR2DL2, -2DL3 and -2DS247) and C2 (Lys80, recognized by KIR2DL1 and -2DS147). The reaction to detect HLA-Cw C1 epitope will also coamplify the rare HLA-Cw alleles *0114, *0119, *0329, *0749 and *1221. All primers were synthesized by Sigma Genosys (Haverhill, UK); PCR-SSP primer sequences are listed in **Table 3**.

The PCR reaction was composed of 10 μL Biomix (containing Biotaq DNA polymerase) (Westchester, PA, USA). Each batch of PCR expansion took Genovision KIR PCR/SSP classification identification for the masculine and feminine DNA samples for the quality control.

Statistical analysis

A comparison of the phenotype distribution of KIR genes and HLA-Cw epitopes polymorphism between the two cohorts was performed using chi-square and Fisher's exact test as appropriate. The influence of KIR genes both in combination with, and independent from their HLA-C ligands, was assessed jointly using stepwise binary logistical regression analysis. All of the data were calculated using SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

Results

To assess the genetic contribution of the NK receptor gene KIR in chronic hepatitis B, the frequency of the KIR genotypes of case and control group were shown in **Table 4**. We also made genotypes of all 14 KIR and their HLA-Cw ligand epitopes. The phenotype frequencies of non-framework KIR loci and HLA-Cw epitopes are displayed in **Table 5**. The frame work genes KIR2DL4, -3DL2 and -3DL3 were absent in all individuals, and probably on both haplotypes in each individual. The genotype frequencies of HLA-Cw epitopes fell within Hardy-Weinberg distribution in both groups (Supplementary Table 1). Fisher's exact test were adopted to analyse the data which showed that phenotype frequencies of KIR2DL2, -2DL3 and -2DS2 were significantly changed in the chronic hepatitis B patients (**Tables 1** and **5**). Otherwise, the differences of frequencies of KIR2DL2, -2DL3 and -2DS2 between case and control group were related to Chronic Hepatitis B through univariate and multivariate regression (**Tables 6** and **7**).

Table 4. The frequency of KIR genotypes of case and control group

Geno type	Haplotype combination	Case group (n=175)		Control group (n=125)		*P* value
		Number	Frequency	Number	Frequency	
C	3.5 or 1.14	3	0.017	2	0.016	0.939
E	1.3	5	0.028	3	0.024	0.808
F	2.3 or 1.4	3	0.017	2	0.016	0.939
G	4.5	3	0.017	3	0.024	0.676
P	2.17	2	0.011	2	0.016	0.734
AE	1.6	3	0.017	1	0.008	0.496
AF	1.2	44	0.251	19	0.152	0.037
AG	1.1	5	0.028	4	0.032	0.864
AH	2.5	13	0.074	25	0.200	0.703
AI	1.5	4	0.023	2	0.016	0.224
AJ	2.2	54	0.308	31	0.248	0.001
NN1	1.18	2	0.011	2	0.016	0.734
NN2	2.6	7	0.040	3	0.024	0.447
NN3	2.15 or 1.16	2	0.011	1	0.008	0.769
NN4	2.18	2	0.011	2	0.016	0.734
NN5	4.14	2	0.011	4	0.032	0.224
NN6	5.5	2	0.011	1	0.008	0.769
NN7	5.6	2	0.011	2	0.016	0.734
NN8	5.14	2	0.011	3	0.024	0.402
NN9	5.19	2	0.011	1	0.008	0.769
NN10	5.22	2	0.011	3	0.024	0.402
NN11	6.6	3	0.017	3	0.024	0.676
NN12	?	2	0.011	1	0.008	0.769
NN13	?	3	0.017	2	0.016	0.939
NN14	?	2	0.011	2	0.016	0.734
NN15	?	2	0.011	1	0.008	0.769

#, ? mean this result had not obtained accurate conclusion.

We next performed logistic regression analysis of KIR phenotype (both in combination with, and independent of, their corresponding HLA-Cw ligands) to assess the effect of KIR gene and HLA-Cw ligand interaction on chronic hepatitis B susceptibility, as known risk factor in several inflammatory or autoimmunity diseases [25, 27-30]. Analysis revealed KIR2DL3 in the presence of HLA-C1 as the dominant association, a little reduced in chronic hepatitis B patients which was failed to reach significance (OR=0.633, 95% CI=0.250-1.601, **Table 1**). KIR2DL2 and -2DS2 in the presence of their shared ligand HLA-C1 also were not significant (-2DL2/HLA-C1: OR=1.056, 95% CI=-0.602-1.853; -2DS2/HLA-C1: OR=1.240, 95% CI=0.714-2.155). Analysis of KIR2DL2, -2DL3 and -2DS2 ligand interactions stratified by HLA-C1 or -C2 homozygosity did not cause significant results (data not shown).

Discussion

In the present work, we investigate the influence of KIR polymorphism and KIR-HLA class I ligand combinations on chronic hepatitis B susceptibility and prognosis in a population of 175 patients with chronic hepatitis B and 125 patients who cleared HBV spontaneously from Chinese Han population. Our statistical analysis indicated a possible involvement of the activating KIR2DS2 and the inhibitory KIR2DL2 and -2DL3 in the evolution of chronic hepatitis B. All the genes showed a high level of linkage disequilibrium with each other, occurring independently in only three of the samples genotyped in this study. However, logistic regression analysis didn't find any correction of chronic hepatitis B with KIR2DS2, -2DL2 or -2DL3 in combination with their appropriate ligand HLA-C.

KIR2DL3 is present on the 'A' haplotypes, whereas KIR2DL2 and -2DS2 are located on the opposing 'B' haplotypes. In principle, increase in KIR2DL2 and -2DS2 will cause a decrease in the frequency of KIR2DL3. But the decrease fails to reach significance. Moreover, logistical regression revealed no association with KIR2DS2, -2DL2 and -2DL3 when in combination with HLA-Cw1 [31]. The result suggests that the interaction between KIR2DL2, -2DL3 and -2DS2 exerts protective effect in infection with HBV.

KIRs are predominantly expressed on NK cells, main innate immune cells in liver which have been found to produce both protective and detrimental effects in the native immunity [28]. The activation of NK cells occurs in parallel with flares of liver inflammation and enrichment of activated NK cells in the liver infected with HBV [32-34]. In human populations, there is a variable balance between 'A' and 'B' KIR haplotypes, which maintain the balance of inhibitory and activating functions. But the interaction of

Table 5. Phenotype frequencies of individual KIR genes and HLA-Cw ligands

	KIR											HLA	
	2DL1	2DL2	2DL3	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DS1	HLA-C1	HLA-C2
Case	0.9948	0.2642	0.9031	0.4693	0.3724	0.2581	0.2792	0.9521	0.2937	0.9438	0.4035	0.7136	0.3026
Control	0.9987	0.1633	0.9998	0.4528	0.3673	0.1635	0.2594	0.9483	0.2862	0.9583	0.4018	0.7089	0.2909
χ^2 value	0.0583	4.606	12.77	0.0436	0.0037	4.054	0.2131	0.0302	0.0004	0.1207	0.0100	0.0019	0.0771
P value	0.8092	0.0319	< 0.001	0.8296	0.9516	0.0441	0.6444	0.8621	0.9486	0.7283	0.9208	0.9656	0.7812

Abbreviations: HLA, human leucocyte antigen; KIR, killer immunoglobulin-like receptor.

Table 6. Univariate regression of individual KIR genes frequencies as risk factors of CHB

KIR	*P* value	χ^2 value	OR	95% CI
2DL1	0.8092	0.0583	0.331	0.013-8.198
2DL2	0.0319*	0.4606	1.872	1.043-3.360
2DL3	< 0.001***	12.77	0.075	0.010-0.571
2DL5	0.8296	0.0436	1.071	0.703-1.632
2DS1	0.9516	0.0037	1.025	0.663-1.582
2DS2	0.0441*	4.054	1.817	1.011-3.266
2DS3	0.6444	0.2131	1.123	0.699-1.803
2DS4	0.8621	0.0302	1.132	0.426-3.005
2DS5	0.9486	0.0004	1.028	0.647-1.633
3DL1	0.7283	0.1207	1.132	0.426-3.005
3DS1	0.9208	0.0100	1.024	0.668-1.570

*P<0.05, **P<0.01, ***P<0.001.

Table 7. Multivariate regression of individual KIR genes frequencies as risk factors of CHB

KIR	*P* value	χ^2 value	OR	95% CI
2DL2	0.0362	4.390	1.730	1.036-2.890
2DL3	< 0.001	14.17	2.289	1.487-3.524
2DS2	0.0374	4.351	1.483	1.024-2.147

inhibitory KIR with their HLA class I ligands look more predominate mostly. Therefore, also the affinity of KIR2DL3 to its ligand is the weakest known of all inhibitory KIR [35], KIR2DL3-positive NK cells may theoretically have a greater potential for HBV clearance and protection for liver.

In summary, the present study confirmed influence of KIRs and their HLA-C ligands to the development of chronic hepatitis B and HBV clearance. KIR2DL3 provided a strongest protective effect, so as to a possible influence of KIR2DL2/-2DS2 in chronic hepatitis B susceptibility cannot be ruled out. All the present results and conclusion derived from the relatively small sample size and the moderate levels of significance obtained, so further studies are needed to make sure of these conclusions.

Disclosure of conflict of interest

None.

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Supplementary Table 1. *P* value for HWE for both groups

	2DL1	2DL2	2DL3	2DL5	2DS1	2DS2	2DS3	2DS4	2DS5	3DL1	3DS1
Case	0.924	0.579	0.759	0.427	0.148	0.512	0.184	0.169	0.347	0.848	0.273
Control	0.793	0.420	0.668	0.391	0.195	0.497	0.199	0.153	0.294	0.795	0.305