Association between *KIR* Genes and Efficacy of Treatment of HBeAg-Positive Chronic Hepatitis B Patients with Entecavir

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ABSTRACT

Background: Entecavir (ETV) is commonly used to treat chronic hepatitis B (CHB) in China. However, certain percentages of e-Antigen (HBeAg) positive CHB patients do not respond to ETV therapy. **Objective:** To investigate whether the killer immunoglobulin-like receptor (*KIR*) genes were associated with seroconversion in HBeAg positive CHB responder patients treated with ETV. **Methods:** Polymerase chain reaction with sequence-specific primers (PCR-SSP) method was performed to genotype *KIR* genes in 200 healthy controls and 198 HBeAg-positive CHB patients which 59 were defined as the complete response group (CRG) to the treatment with ETV and 139 were defined as null or partial response group (NPRG). **Results:** The frequencies of *KIR2DS2* and *KIR2DS3* were significantly higher (P=0.030, OR=1.57，95%CI=2.36-1.05 and P=0.018, OR=1.773，95%CI=2.77-1.13, respectively), while, the frequencies of *KIR2DL3, KIR2DS1* and *KIR3DS1* were significantly lower (P=0.038, OR=0.525, 95%CI=0.96-0.29,and P=0.031, OR=0.640, 95%CI =0.95-0.43, and P=0.035, OR=0.641, 95%CI =0.96-0.43, respectively) in HBeAg-positive CHB patients than those in healthy controls. The frequency of *KIR2DS3* gene was significantly higher in NPRG than that in CRG (P=0.018, OR=0.402, 95%CI=0.83-0.20). The frequencies of *KIR2DL3* and *KIR3DS1* genes were significantly higher in CRG than those in NPRG (P=0.019, OR=3.625, 95%CI=10.83-1.21 and P=0.041, OR=1.949, 95%CI=3.65-1.04, respectively). **Conclusion:** Patients with *KIR2DS3* might have negative responses to anti-HBV therapy with ETV and patients with *KIR2DL3* and *KIR3DS1* might have advantage in the therapy with ETV.

Zhuang YL, et al. Iran J Immunol. 2018; 15(2):112-121.

Keywords: Entecavir, HBeAg-Positive CHB Patients, *KIR*Genes

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the global health concerns affecting about 350 million people (more than 200 million in China) around the world(1). Clinically, HBV infection involves severe consequences such as liver functional failure, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) since it can survive in the host for the whole life (2). Entecavir (ETV) is the drug of first choice recommendation in chronic hepatitis B (CHB) therapy because of its higher antiviral characteristics and higher genetic barriers than other antiviral agents (3,4). In this regard, the antiviral therapy could ameliorate the progression of liver injury, cirrhosis, and HCC (5-7). The amounts of HBV DNA were less than 300 copies/mL in 80% of HBeAg-positive patients after ETV therapy at 0.5 mg daily through 96 weeks (8). However, there are still some portions of HBeAg-positive CHB patients that do not respond to the ETV therapy, making the molecular mechanism remain largely unknown.

Natural killer (NK) cells play key roles in the control of viral hepatitis and the pathogenesis of liver injury and inflammation (9). The activations of NK cells are of paramount importance in liver inflammation during chronic HBV infection in both HBV transgenic mice and HBV-infected patients (10-12). NK cell activities are regulated by several kinds of regulatory receptors such as the killer immunoglobulin-like receptor (KIR) family (13). *KIR* gene family makes clusters on human chromosome 19q13.4, which contains 7 inhibitory *KIR* genes (*KIR3DL1-3*, *KIR2DL1-3*, and *KIR2DL5*), 6 activating *KIR* genes (*KIR3DS1* and *KIR2DS1-5*), and 1 (*KIR2DL4*) with both inhibitory and activating characteristics and 2 pseudogenes (*KIR2DP1* and *KIR3DP1*). KIR regulates the activations of NK cells and certain T cells by providing activating or inhibitory signals and possesses the potential for anti-virus applications (14). *KIR* gene polymorphisms are associated with susceptibility to or protection from infectious diseases such as *Treponema pallidum* (15), HIV(16),hepatitis C virus (HCV) (17), HBV (18),Ebola virus (19),and *Mycobacterium tuberculosis*(20). However, whether *KIR* genes are associated with seroconversion and HBV DNA suppression in HBeAg-positive CHB patients treated with ETV remains unclear.

In this study, we investigated the association of *KIR* genes with seroconversion in HBeAg-positive CHB patients with ETV therapy. For this purpose, we analyzed the frequency of *KIR* genes in 198 ETV-treated HBeAg-positive CHB patients and 200 healthy blood donors in Chinese Han population through polymerase chain reaction with sequence-specific primers (PCR-SSP) method.

MATERIALS AND METHODS

Patients and Controls. Between October 2010 and August 2015 (retrospective type study), a total of 198 consecutive ETV-treated HBeAg-positive CHB patients including 106 men and 92 women with mean age of 40.4±12.6 years were recruited and treated with ETV for 48 weeks. The mean HBV DNA copies were (7.22±1.20) log10 copies/mL in the HBsAg-positive CHB patients. Patients were excluded if they had previous antiviral therapy for HBV, HCV, hepatitis D virus, HIV infections, and liver cirrhosis or HCC.A 48-weeks ETV therapy (0.5 mg daily) was defined as the primary efficacy end point with the changes in seroconversion and HBV DNA suppression.

After the therapy, patients with negative HBeAg and HBV DNA copies less than 300 were defined as complete response group (CRG), those with positive HBeAg and HBV DNA copies more than 300 were defined as null response group (NRG), those with positive HBeAg and HBV DNA copies less than 300 were defined as partial response group (PRG), and the last two situations were together called null or partial response group (NPRG). Meanwhile, 200 regular blood donors were recruited as healthy controls (no HIV, HCV,HBV, syphilis infections, and normal alanine aminotransferase (ALT) level) including 102 males and 98 females with mean age of 43.5±6.1 years. The patients and healthy blood donors consent to participate in the study. We had the ethical statement (NO.2010017) approved by the Ethics Committee of the Blood Center of Shandong Province.

Laboratory Methods.HBV DNA copies in blood serum were examined by real-time PCR method with a linear dynamic detection range from 75 copies/mL to 5×10^9 copies/mL (Abbott Laboratories, Chicago, IL, USA). The characteristics of ALT HBeAg, HBsAb, HBeAg, HBeAb, and HBcAb in patients' serum were measured by enzyme immunoassays method (lower limit of detection (LLOD):0.05 IU/mL, Abbott Laboratories, Chicago, IL, USA).

***KIR* Gene Distributions.** The 16 *KIR* genes were examined by PCR-SSP method (15) in recruited subjects.When the DNA from frozen peripheral blood mononuclear cells was extracted (EZ Bead System-32 DNA workstation, Texas BioGene Inc., USA), the PCR-SSP typing of *KIR* genes were done as soon as possible to avoid false-negative results due to the longer KIR-specific amplicons. The human growth hormone gene (15) was used as positive control while water was as a negative control. All primers for the PCR-SSP were bought from BOYA. Bio Co., Ltd., Shanghai. The 10 µl volume PCR system contained 20-50 ng DNA ,0.2 mM dNTP, 0.5U *Taq* DNA polymerase (Promega), 0.4 µM primers (except for *KIR2DS1*, 0.8 µM), and 1X PCR buffer. The conditions of PCR amplification in a 9700 thermal cycler (PerkinElmer, Waltham, MA, USA) were as follows: initial denaturing at 94 □ for 4 min, followed by 30 cycles of 94□ for 30sec, 65□ for 30sec, 72□ for 90sec, plus a final extension at 72℃ for 10 min. The annealing temperatures of *KIR2DS2*, *KIR2DS3,*and *KIR2DS5* were 63□ while *KIR2DS4* was 61□. About 1-2% fluorescence-dyed agarose gels were used to analyze the PCR products (Gene Genius Bio Imaging System, Syngene Ltd., UK).

Statistical Analysis. *KIR* gene frequencies were analyzed according to the description given in (15). The direct counting method was used for the analysis of phenotypic frequencies (PF). The significance difference analysis of *KIR* genes' frequencies was performed by Yates' correction analysis ($P \leq 0.05$).The odds ratio (OR) and relative risk (RR) were analyzed using the SPSS13.0 software package.

RESULTS

Patients' Characteristics.

All the 198 HBeAg-positive CHB patients showed different outcomes after 48 weeks ETV therapy. Of this population, 29.8% of the patients were classified as CRG while others were classified as NPRG including NRG (10.1%) and PRG (60.1%), Table 1. The patients with reduced HBV DNA (<300 copies/mL) were 89.9%, including CRG and PRG. The ALT levels showed significant differences between CRG and NRG (P=0.00016).

Table1. The characteristics of HBeAg-positive CHB patients after 48-weeks-treatment with ETV.

	n (%)	ALT (U/L)	HBV DNA (copies/mL)	HBsAg	HbsAb	HBeAg	HBeAb	HBcAb
CRG	59(29.80)	30.21±7.25	＜300	+	-	-	+	+
NRG	20(10.10)	132.67±10.58	＞300	+	-	+	-	+
PRG	119(60.10)	48.29±9.17	＜300	+	-	+	-	+

CHB: Chronic Hepatitis B; ETV: Entecavir; n: numbers of patients; CRG: Complete Response Group Treated with ETV; NRG: null response group treated with ETV, PRG: partial response group treated with ETV; ALT: alanine aminotransferas;+ : positive; -: negative.

KIR Gene Frequencies in Healthy Controls and HBeAg-Positive CHB Patients.

A total of 16 *KIR* genes were detected in healthy controls and HBeAg-positive CHB patients(Table 2) while *KIR2DL4*, *KIR3DL2*, *KIR3DL3,*and *KIR3DP1* were found in all subjects as the framework genes. The inhibitory *KIR* genes presented commonly higher frequencies than activating types in the two groups except for *KIR2DL2* and *KIR2DL5*. The frequencies of activating *KIR2DS2* and *KIR2DS3* increased significantly in HBeAg-positive CHB patients than those in controls (P=0.030, OR=1.577, and 95%CI=2.36-1.05; P=0.018, OR=1.773, and 95%CI=2.77-1.13, respectively) while the frequencies of *KIR2DL3*, *KIR2DS1,*and*KIR3DS1* were significantly decreased in CHB patients (P=0.038, OR=0.525, and 95%CI=0.96-0.29;P=0.031, OR=0.640, and 95%CI=0.95-0.43; and P=0.035, OR=0.641, 95%CI =0.96-0.43, respectively). There was no statistically significant difference in distributions of other *KIR* genes in the healthy controls and HBeAg-positive CHB patients.

KIR Gene Frequencies in CRG and NPRG HBeAg-Positive CHB Patients with ETV Therapy.

After treatment with ETV for 48 weeks, 59 of 198 HBeAg-positive CHB patients were CRG responders and 139 were NPRG responders. The distributions of *KIR* genes between the CRG and NPRG patients are shown in Table 3.The results show a significant increase in the frequency of *KIR2DS3* significantly (P=0.018, OR=0.402, and 95%CI=0.83-0.20) and a significant decrease in the frequencies of *KIR2DL3* and *KIR3DS1* (P=0.019, OR=3.625, and 95%CI=10.83-1.21; P=0.041, OR=1.949, and 95%CI=3.65-1.04, respectively) in NPRG patients compared with CRG patients. There were no significantly different distributions of other *KIR* genes in the two groups.

Table 2. The frequencies of *KIR* genotypes in healthy controls and HBe Ag-positive CHB patients.

KIR genes	Healthy controls (n=200)		CHB patients (n=198)		*P*-value	OR	95%CI
	+	gf(%)	+	gf(%)			
2DL1	195	97.50	188	94.95	0.187	0.482	1.44-0.16
2DL2	53	26.50	46	23.23	0.456	0.839	1.32-0.53
2DL3	181	90.50	165	83.33	0.038*	0.525	0.96-0.29
2DL4	200	100.00	198	100.00	-	-	-
2DL5	64	32.00	76	38.38	0.185	1.324	2.00-0.88
3DL1	177	88.50	172	86.87	0.615	0.860	1.56-0.47
3DL2	200	100.00	198	100.00	-	-	-
3DL3	200	100.00	198	100.00	-	-	-
2DS1	106	53.00	83	41.92	0.031*	0.640	0.95-0.43
2DS2	71	35.50	92	46.46	0.030 *	1.577	2.36-1.05
2DS3	44	22.00	66	33.33	0.018*	1.773	2.77-1.13
2DS4	154	77.00	165	83.33	0.122	1.494	2.46-0.91
2DS5	63	31.50	56	28.28	0.476	0.858	1.32-0.56
3DS1	91	45.50	69	34.85	0.035*	0.641	0.96-0.43
2DP1	193	96.50	185	93.43	0.156	0.516	1.32-0.20
3DP1	200	100.00	198	100.00	-	-	-

+: numbers of each genotype; gf: genotype frequencies; *: indicates statistical significance (P < 0.05).

Table 3. The distributions of the frequencies of *KIR* genes in CRG and NPRG patients.

KIR genes	CRG (n=59)		NPRG (n=139)		*P*-value	OR	95%CI
	+	gf(%)	+	gf(%)			
2DL1	57	96.61	131	94.24	0.492	1.740	8.45-0.36
2DL2	17	28.81	29	20.86	0.231	1.535	3.08-0.77
2DL3	55	93.22	110	79.14	0.019*	3.625	10.83-1.21
2DL4	59	100.00	139	100.00	-	-	-
2DL5	27	45.76	49	35.25	0.169	1.550	2.88-0.83
3DL1	49	83.05	123	88.49	0.308	0.637	1.50-0.27
3DL2	59	100.00	139	100.00	-	-	-
3DL3	59	100.00	139	100.00	-	-	-
2DS1	25	42.37	58	41.73	0.939	1.027	1.90-0.55
2DS2	26	44.07	66	47.48	0.667	0.871	1.61-0.47
2DS3	12	20.34	52	38.85	0.018*	0.402	0.83-0.20
2DS4	46	77.97	119	85.61	0.192	0.595	1.29-0.27
2DS5	16	27.12	40	28.78	0.820	0.921	1.82-0.47
3DS1	27	45.76	42	30.22	0.041*	1.949	3.65-1.04
2DP1	53	89.83	132	94.96	0.189	0.468	1.46-0.15
3DP1	59	100.00	139	100.00	-	-	-

CRG: complete response group treated with ETV; NPRG:null or partial response group treated with ETV; +: numbers of each genotype; gf: genotype frequencies; *: indicates statistical significance (*P*< 0.05).

DISCUSSION

Through anti-HBV treatment, the progression of chronic liver diseases such as cirrhosis and HCC was ameliorated and HBV DNA replication was suppressed; otherwise,their HBV DNA levels increased gradually (21,22).Therefore, the reduction levels of HBV DNA copies are defined as a critically clinical therapeutic aim for CHB patients. Currently, ETV is one of the most common anti-HBV drugs used in China. During the therapy of HBV infection in the CHB patients, HBeAg seroconversion serves as an important landmark in the control of HBV infectious progression. Earlier HBeAg seroconversion with low HBV DNA copies usually confers a favorable clinical outcome. NK cells possess a critical role in anti-virus immunity. In this regard, ETV promotes NK cells functions (23,24). The numbers of NK cells increased in CHB patients treated with ETV(23). ETV therapy for HBeAg-positive CHB patients declined the number of HBV DNA and ALT levels became normal; besides, it led to the recovery of NK cell-mediated immunity (24).*KIRs* regulate the activations of NK cells(14). Therefore, the HBeAg-positive CHB patients undergoing 48 weeks ETV therapy were chosen to determine whether the suppression of HBV DNA replication and HBeAg seroconversion was associated with *KIR* genes and ETV treatment. To our knowledge, there has been no report on the effects of *KIR* genes on efficacies of HBeAg-positive CHB patients with ETV therapy.

ETV was effective to reduce HBV DNA (<300 copies/mL) in 89.9% and decrease HBeAg seroconversion in 29.8% of the HBeAg-positive CHB patients in this study (Table 1); in line with the results of previous reports (22,25). The amount of HBV DNA is one of the crucial components for evaluating the efficacies of anti-HBV treatment in the CHB patients. The efficacies of ETV therapy might be due to its potent functions in suppression of HBV replication. Further, these results indicate that there were still some patients with poor responses to ETV treatment in the early stage.

As the immune system plays important roles in the amelioration of HBV-infection, it is a promising area to investigate predictive markers of response to ETV therapy. The activities of NK cells and certain T cells has been shown to be important functions in reducing amounts of HBV and in controlling the development processes of liver injury(9,26-28).The *KIR* genes can regulate the activations of NK cells. Moreover, certain T cells responded to many kinds of microbial pathogens (14), suggesting that *KIR* gene diversities may affect susceptibilities to or protection from variable infections. The previous discoveries indicated that *KIR* genes were associated with infectious diseases caused by *Treponema pallidum* (15), HIV (16),HCV (17), HBV (18), Ebola virus(19), *Mycobacterium tuberculosis*(20), malaria(29),and *Mycobacterium leprae*(30)infection, indicating that different *KIR* genes might be of importance in the susceptibility or clearance to different infections.

The results of the present study showed that the *KIR2DS2* and *KIR2DS 3* were associated with susceptibility of, whereas the *KIR2DS1*,*KIR3DS1,*and *KIR2DL3* might be protective against HBV infection, suggesting that different *KIR* genes might show the variable immune responses to HBV infection. These results were partly different from a previous report(18), which showed *KIR2DL5* was protective, but not *KIR2DL3*.We considered that the main possible reasons were the different patient groups, which one was CHB patient group (18) and the other was HBeAg-positive CHB patient group in our study. It is of note that the HBeAg-positive CHB patients with *KIR* genes showed significantly different responses to ETV therapy. The patients with

KIR3DS1 and *KIR2DL3* were CRG that facilitated the seroconversion and reduction of the amount of HBV, whereas the patients with the *KIR2DS3* were NPRG. This study was the first to research *KIR* genes associated with efficacies of HBeAg-positive CHB patients with ETV therapy. These findings suggested that different *KIR* genes might use combinations of synergistic receptors to activate NK cells for benefit of HBV clearance. A recent study showed that KIRs expressed on NK cell surface play an important role in regulating immune responses through the transducing or activating inhibitory signals(31). Previous investigations have shown that the frequency of *KIR2DS3* was significantly increased in syphilis (15), hepatitis C (17), CHB (18), and hemorrhagic fever (19) patients compared to those in the respective controls. It was suggested that KIR2DS3 might involve in the physiopathological process by excessively destroying host's cells due to its favoring role in the host's NK cells activation, supporting a result that activating KIR has an association with disease susceptibility to infections (19). Interestingly, the variations of *KIR2DS3* and *IL28B* were strongly related with clinical responses to both pegylated-IFN and ribavirin treatments in an HIV-1/HCV co-infected patient. This result demonstrates that testing for host certain genetic genes would be useful for precision medicine in infected patients. Besides, it provides further evidence that the innate immune system is of importance in protection from HIV-1/HCV infections(32).The *KIR3DS1* with its *HLA-B* ligand gene was related with a slower development process for AIDS in HIV-infected individuals, suggesting that KIR3DS1 with and its ligand were involved protective responses of NK cells after HIV-1 infection (16). Moreover,*KIR3DS1* homozygotes showed the significantly slower progression of HIV infection than *KIR3DL1/S1* heterozygotes (33).HIV infection promotes the activations of NK cell receptors and its ligands, which in turn stimulates NK cells activities such as secreting chemokines to protect from HIV(34,35).The frequency of *KIR2DL3* was lower in CHB than in subjects with resolved HBV infection, which indicates a protective role of *KIR2DL3*(36).This result is in line with our findings.The mechanism responsible for the protective role of *KIR2DL3* is unclear, as this gene codes for an inhibitory receptor.Lisovsky *et al.* showed that KIR2DL3 $^{+}$ NK cells were mediators of HIV-specific responses (37).The interaction of the KIR2DL3-HLA-C1 was associated with resolved infection in HCV (38), *Treponema pallidum*(15), and HBV(39), suggesting a possible generalizability of the protective role of KIR2DL3 to different infections. KIR2DL3 has a weaker affinity to HLA-C1 than KIR2DL2. Therefore, it was suggested that this weaker interaction between the inhibitory receptor and its ligand (KIR2DL3-HLA-C1) would have protective properties because of the more facile overriding through activating signals (38).Those studies suggested that virus infection was associated with the host's *KIR* gene distributions and that NK cells might play a critical role in protection from or susceptible to the infectious diseases. The biological functions of *KIR* gene distributions in HBeAg-positive CHB pathogenesis, however, are still unclear and need further research.

In conclusion, the certain *KIR* genes might play a critical role in the sustain infection or cure of CHB patients undergoing ETV therapy. Our findings might be useful for predicting the precision medicine in HBeAg-positive CHB patients with ETV therapy. Future studies on the mechanism underlying these genetic associations might provide insights for new therapeutic strategies in HBV infected patients.

ACKNOWLEDGEMENTS

This work was supported by Shandong Provincial Natural Science Foundation (ZR201702210457, ZR2015HM027), Shandong Province Young and Middle-Aged Scientists Research Awards Fund (BS2011SW044), Medicine and Health Science and Technology Development Planning Projects of Shandong Province (2013WS0170), and Yantai science and Technology Development Projects (2013WS259).

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