World Journal of Hepatology

Online Submissions: http://www.wjgnet.com/1948-5182office
wjh@wjgnet.com
doi:10.4254/wjh.v3.i3.72

*World J Hepatol* 2011 March 27; 3(3): 72-78
ISSN 1948-5182 (online)


ORIGINAL ARTICLE

# Polymorphisms in programmed death-1 gene are not associated with chronic HBV infection in Chinese patients

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Supported by the Grants from the National Natural Science Foundation of China, No.30700698 and 30771907 and the Foundation of Pre-973 Program Projects, No. 2009CB526411
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Received: November 26, 2010 Revised: December 23, 2010
Accepted: December 30, 2010
Published online: March 27, 2011

## Abstract

**AIM:** To investigate the association between the programmed death-1(PD-1) polymorphisms and genetic susceptibility of chronic hepatitis B virus (HBV) infection in Chinese patients.

**METHODS:** Two single nucleotide polymorphisms (SNPs), PD-1.1 G > A and PD-1.2 G > A, were genotyped in 539 patients with chronic HBV infection and 353 other family members (HbsAg-) from 256 nuclear families using polymerase chain reactiorestriction fragment length polymorphisms assay. The associations between PD-1 polymorphisms and genetic susceptibility of chronic HBV infection were analyzed usng the family-based association analysis method.

**RESULTS:** No association or linkage was detected among 539 patients. Univariate (single-marker) family-based association tests demonstrated that PD-1 genotypes, alleles and transmitted haplotypes are not associated with chronic HBV infection (all with *P* value more than 0.05). Transmission/disequilibrium test and sibship disequilibrium test analysis showed no excess of the alleles from heterozygous parents to affected offspring (*P* = 0.688880, *P* = 1.000000 respectively).

**CONCLUSION:** The data demonstrated that PD-1.1 and PD-1.2 polymorphisms are not associated with chronic HBV infection in Chinese patients.



**Key words:** Programmed death-1; Hepatitis B virus; Single nucleotide polymorphism; Genetic association study; Family-based association test

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Lv F, Gao YF, Zhang ZH, Zhang TC, Pan FM, Cui MF, Xia SL, Li X, Yin HF. Polymorphisms in programmed death-1 gene are not associated with chronic HBV infection in Chinese patients. *World J Hepatol* 2011; 3(3): 72-78 Available from: URL: http://www.wjgnet.com/1948-5182/full/v3/i3/72.htm DOI: http://dx.doi.org/10.4254/wjh.v3.i3.72

## INTRODUCTION

Chronic hepatitis B virus (HBV) infection has been considered a multi-factorial and polygenic disorder with viral, environmental, genetic and immune components[1-3]. However, segregation analysis and twin studies strongly support the role of host genetic components in determining the chronicity of HBV infection[4-6]. Genetic association analyses have implicated that some human leukocyte antigen and non-human leukocyte antigen loci, including interferon-γ (IFN-γ)[7], tumor necrosis factor-α (TNF-α)[8], cytotoxic T lymphocyte antigen 4 (CTLA-4)[9], estrogen receptor-α (ESR1)[10], type 1 interferon receptor 1(IFNR-1)[11] and so on, are associated with chronic HBV infection or HBV clearance. Susceptibility to infectious diseases is considered to be determined at different functional levels such as cytokine production, antigen presentation and receptor recognition. Therefore, studies on the chronicity of HBV infection association with certain genes involved in the immune response may highlight the genetic background of HBV infection.

Programmed cell death 1 (PD-1) is an inhibitory immunoreceptor expressed on T-cells, B-cells and myeloid cells. It belongs to the immunoglobulin super family B7/CD28 and is mainly involved in T cells and B cells activation[12,13]. In patients with HBV infection, PD-1 is significantly up-regulated on the virus specific T cells and leads to inhibition of T cell receptor-mediated proliferation and cytokine production[14,15], thereby playing a crucial role in the down-regulation of immune responses. The human gene encoding PD-1, i.e. PDCD1, is located on chromosome 2q37.3[16]. Until now, more than 30 single nucleotide polymorphisms (SNPs) have been identified within PD-1 gene (found in the NCBI-Entrez SNP database). Many reports have highlighted that some regulatory polymorphisms in PD-1 gene might affect the expression and transcription of the gene[17,18] such as in the promoter or intron; they have been studied as a part of attempts to identify the pathogenesis of several immune relevant diseases including systemic lupus erythematosus (SLE)[17,19], rheumatoid arthritis (RA)[20] and type1 diabetes (T1D)[21]. Further work has shown that other SNPs in the PD-1 gene were studied in multiple sclerosis (MS)[22], ankylosing spondylitis (AS)[23], Graves' disease[24] and so on. However, no such studies have, until now, been performed within chronic HBV infection with a sufficiently large sample population. Hence, considering the important role of PD-1 in HBV-specific T cells response and the possible effects of expression or functional alteration of PD-1 due to gene polymorphisms on the immune response, our present study was firstly designed to investigate the association of PD-1 SNPs including PD-1.1 (position -531 in the promoter) and PD-1.2 (position 6438 in the intron 2) with genetic susceptibility of chronic HBV infection. Therefore, we performed a family-based association study in Chinese nuclear families to ascertain whether there is a susceptible gene locus for chronic HBV infection.

## MATERIALS AND METHODS

### Subjects

A family-based association study of chronic HBV infection was conducted in Anhui province, China between 2008 and 2009. All probands were diagnosed with chronic HBV infection and fulfilled the diagnostic criteria of the Proposal of Prevention and Treatment of Viral Hepatitis, Xi'an, 2000, issued by the Chinese Society of Infectious Diseases and Parasitology and the Chinese Society of Hepatology of the Chinese Medical Association[25]; they were positive for HBsAg and anti-HBc but negative for antibodies (Abs) to HCV, HDV, HIV-1 and -2, and excluded other symptoms of chronic liver damage. In a total of 892 subjects [539 patients (aged 36.5±14.6 years) and 353 other family members (aged 44.1±15.5 years)] from 256 qualified nuclear families [114 (44.53%) families had both parents, 94 (36.72%) families had one single parent and 48 (18.75%) families had no available parents] were finally recruited. The family criteria used for subject selection were as follows: (1) proband's parents, (2) at least two siblings, and (3) at least one parent or one additional sibling. All subjects gave their written informed consent and blood and this study obtained ethical committee approval from the Department of Infectious Diseases, Anhui Medical University. Peripheral whole blood samples were collected in 5-ml vacationer tubes containing EDTA and detected serum HBsAg by enzyme-linked immunosorbent assay (ELIAS) kits (Kehua Technologies Co., Ltd. Shanghai, China).

### Genomic DNA extraction

Genomic DNA was extracted from the peripheral blood leucocytes pellet using a DNA extraction kit (Yuan Pinghao Biotechnology Co., Ltd. Tianjin, China). The DNA samples were stored at -80℃ with a concentration of 100 ng/μL.

### Genotyping by polymerase chain reaction and restriction fragment length polymorphisms analysis

Two SNPs (PD-1.1 G > A and PD-1.2 G > A) were selected as targets and were genotyped using the TaqMan system (Applied Biosystems). The PD-1.1 G > A primers were forward, 5'-GATCTGGAACTGTGGCCATGGT-3' and reverse, 5'-CCCCCTCTGGGCTCAGGTT-3', and those for PD-1.2 G > A were 5'-CGGTCCTGGGGTGG GTGTCC-3' and 5'-GCTGGGGTGGGCTGTGGGCA -3' respectively. They were amplified in a 25 μL reaction mixture which consisted of 3 μL DNA (100 ng/μL), 1 μL deoxynucleoside triphosphates (dNTP, 10 mmol/μL), 2.5 μL 10 × buffer, 0.5 μL of Taq polymerase (5 U/μL, TaKaRa Biotechnology, Co., Ltd. Dalian, China), 1 μL each primer (10 μmol/μL, Invitrogen Biotechnology Co., Ltd. Shanghai, China) and $H_2O$ 16 μL. The amplification conditions for determining PD-1.1G > A polymorphisms were initial denaturation at 95℃ for 5 min and 30 cycles of denaturation at 95℃ for 15 s, annealing at 67℃ for 15 s, extension at 72℃ for 15 s and then a final extension phase at 72℃ for 10 min. The polymerase chain reaction

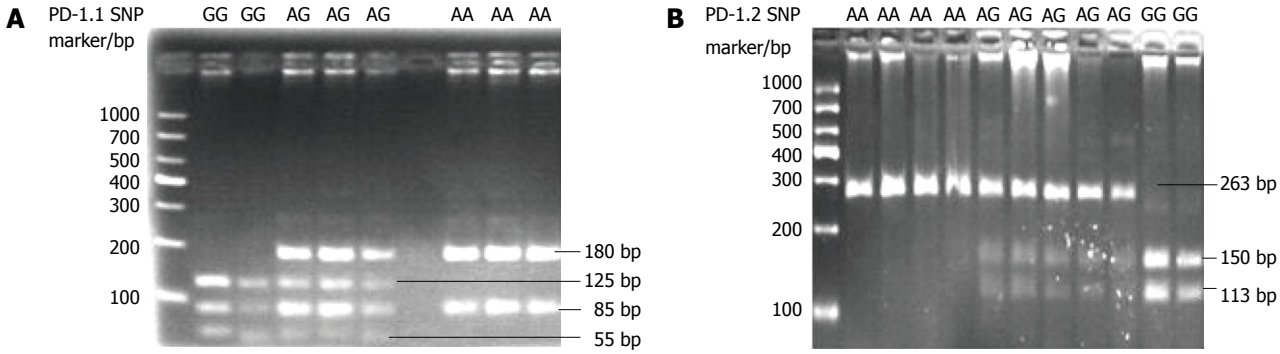


**Figure 1 Gel electrophoresis patterns of PD-1.1 and PD-1.2.** A: The amplified fragments of PD-1.1 were digested with MspI: the polymerase chain reaction (PCR) product size was 265 base pairs (bp) which was digested to 180 and 85 bp, the genotype was identified as AA; if not, it was digested to 180, 125, 85 and 55 bp and was identified as AG; and if only digested to 125, 85 and 55 bp, its genotype was identified as GG. B: The amplified fragments of PD-1.2 were also digested with MspI: the PCR product size was 263 bp which was digested to 150 and 113 bp; if the product was digested, the genotype was identified as GG; if not, it was identified as AA, the genotype AG appeared as 263, 150 and 113 bp.

(PCR) product 265 bp was digested with MspI (TaKaRa Biotechnology, Co., Ltd. Dalian, China) according to the manufacturer's instructions [total reaction volume 20 μL, including PCR product 10 μL, 10× T buffer 2 μL, 0.1% BSA 2 μL, restriction enzyme (10 U/μL) 1μL, $H_2O$ 5 μL] and separated by electrophoresis on 3% agarose gels. The AA genotype was cleaved and appeared as 180 and 85 bp fragments and GG genotype appeared as 125, 85 and 55 bp fragments, whereas the AG genotype appeared as 180, 125, 85 and 55 bp fragments (Figure 1). To determine the polymorphisms of PD-1.2G > A, PCR was carried out under the following conditions: initial denaturation at 94℃ for 5 min and 30 cycles of denaturation at 94℃ for 40 s, annealing at 70℃ for 15 s and extension at 72℃ for 15 s. A final extension phase was performed at 72℃ for 7 min. Then the PCR product was digested with MspI (TaKaRa Biotechnology Co., Ltd. Dalian, China) and separated by electrophoresis on 3% agarose gel. The AA genotype lacking the MspI site migrated as a 263 bp fragment whereas the GG genotype was cleaved and appeared as 150 and 113 bp. The AG genotype was digested into three fragments 263, 150 and 113 bp (Figure 1). To improve the genotyping quality and validate our results, a random selection of 1% of all samples were analyzed by direct sequencing (Invitrogen Biotechnology Co., Ltd, Shanghai, China) and the results found no discrepancies.

### *Statistical analysis*

In order to avoid the bias of population admixture arising from population-based association study, an extension of the transmission/ disequilibrium test and unified family-based association test (FBAT)[26] were performed under dominant, recessive and additive genetic models. The number of informative families was dependent on the genetic models. The statistical power of FBAT depended on the number of informative families. The single-marker FBAT analysis was used to estimate the single loci frequencies. Each test counted how often a specific locus was present in informative families with HBV infection. Positive *Z* statistic of single locus FBAT indicated a specific single locus was more frequently transmitted to patients with HBV infection in informative families than expected under the null hypothesis of no linkage and no association. To test transmission disequilibrium in families, we applied the conventional TDT statistic (disequilibrium of transmission of alleles from heterozygous parents to affected children) and the SDT (considering patient and other normal siblings). Statistic is calculated as $(b - c)^2/(b + c)$, where b and c are, respectively, the number of transmissions and non-transmissions of the alleles. Haplotype FBAT software[27] was also used to estimate haplotype frequencies. For families with ambiguous phase, expectation-maximization algorithm[28] was used to estimate genotype frequencies under Hardy-Weinberg equilibrium.

## RESULTS

A total of 539 patients with chronic HBV infection from 256 nuclear families that contained complete genotype were employed in the data analysis. At the same time, 353 family members of these patients were genotyped. Stratified by PD-1.1 G > A and PD-1.2 G > A genotypes among 539 patients, the frequencies of PD-1.1 G > A, AA, AG and GG genotypes were 16.6%, 63.7% and 19.7% respectively and the A and G allele frequencies were 48.4% and 51.6% respectively. PD-1.2 G > A, AA, AG and GG genotype frequencies were 22.8%, 59.8% and 17.4% respectively and the A and G allele frequencies were 52.7% and 47.3% respectively (Table 1). The genotype distributions of PD-1.1 and PD-1.2 polymorphisms were in Hardy-Weinberg equilibrium (PD-1.1, $\chi^2 = 3.032$, $P = 0.082$; PD-1.2, $\chi^2 = 0.25$, $P = 0.616$).

Univariate (single-marker) FBAT demonstrated that there were no associations between genotypes and chronic HBV infection ($P > 0.05$). In PD-1.1 G > A and PD-1.2 G > A, single loci analysis by FBAT showed that the alleles of A and G were also not associated with chronic HBV infection in the additive model [(PD-1.1, $Z = -0.265$, $P = 0.790993$; $Z = 0.265$, $P = 0.790993$ respectively); (PD-1.2, $Z = 0.640$, $Z = -0.640$, $P = 0.522127$ respectively)], the

**Table 1 The associations between PD-1 genotype and chronic HBV infection by family-based association test**

| Marker | Genotype | Frequency | Family[a] | S | E(S) | Var(S) | Z[b] | P |
|---|---|---|---|---|---|---|---|---|
| PD-1.1 | A/A | 0.166 | 46 | 30 | 29.311 | 12.718 | 0.193 | 0.847 |
| | A/G | 0.637 | 72 | 47 | 49.794 | 20.122 | -0.623 | 0.533 |
| | G/G | 0.197 | 42 | 30 | 27.895 | 11.630 | 0.617 | 0.537 |
| PD-1.2 | A/A | 0.228 | 53 | 38 | 34.228 | 14.206 | 1.001 | 0.317 |
| | A/G | 0.598 | 78 | 50 | 54.044 | 21.696 | -0.868 | 0.385 |
| | G/G | 0.174 | 41 | 27 | 26.728 | 11.593 | 0.080 | 0.936 |

[a]Number of nuclear families informative for that allele; S is the test statistic; E(S) and Var(S) are the expected value and variance of the test statistic, respectively; [b]The negative sign of Z statistic indicates that the frequency of transmitted genotype is negative association with susceptibility of chronic HBV infection. PD-1: programmed death-1; HBV: hepatitis B virus.

**Table 2 The associations between PD-1 gene polymorphism and chronic HBV infection by family-based association test (single-marker FBAT analysis) in additive or dominant or recessive model**

| Marker | Allele | Additive model | | | Dominant model | | | Recessive model | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Families[a] | Z[b] | P | Families[a] | Z[b] | P | Families[a] | Z[b] | P |
| PD1.1 | A | 72 | -0.265 | 0.791 | 42 | -0.617 | 0.537 | 46 | 0.193 | 0.847 |
| | G | 72 | 0.265 | 0.791 | 46 | -0.193 | 0.847 | 42 | 0.617 | 0.537 |
| PD1.2 | A | 79 | 0.640 | 0.522 | 41 | -0.080 | 0.936 | 53 | 1.001 | 0.317 |
| | G | 79 | -0.640 | 0.522 | 53 | -1.001 | 0.317 | 41 | 0.080 | 0.936 |

[a]Informative families: families may have two heterozygote parents or multiple offspring. When the number of informative families is less than 10, the test will not be computed; [b]The negative sign of Z statistic indicates that the frequency of transmitted allele is negative association with susceptibility of chronic HBV infection. PD-1: programmed death-1; HBV: hepatitis B virus.

**Table 3 TDT of association with HBV infection-affection status in 539 trios selected from the HBV infectious nuclear families**

| SNP | Frequency | Allele | Trio TDT (*n* probands with allele) | | |
|---|---|---|---|---|---|
| | | | Transmitted | Not transmitted | P |
| PD1.1 | 0.484 | A | 26 | 30 | 0.689 |
| | 0.516 | G | 30 | 26 | 0.689 |
| PD1.2 | 0.527 | A | 32 | 33 | 1.000 |
| | 0.473 | G | 33 | 32 | 1.000 |

The TDT analysis revealed no increased transmission for the major alleles to HBV infection offspring. TDT: transmission/disequilibrium test; HBV: hepatitis B virus.

dominant model [(PD-1.1, $Z$ = -0.617, $P$ = 0.537018; $Z$ = -0.193, $P$ = 0.846892 respectively); (PD-1.2, $Z$ = -0.080, $P$ = 0.936343; $Z$ = -1.001, $P$ = 0.316939 respectively)] and the recessive model [(PD-1.1, $Z$ = 0.193, $P$ = 0.846892; $Z$ = 0.617, $P$ = 0.537018 respectively); (PD-1.2, $Z$ = 1.001, $P$ = 0.316939; $Z$ = 0.080, $P$ = 0.936343 respectively)]. The results of single-marker FBAT analyses were summarized in Table 2. TDT and SDT analysis revealed no increased transmission for the major alleles from heterozygous parents to affected offspring ($P$ = 0.688880, $P$ = 1.000000 respectively) (Table 3).

In addition, haplotype analysis showed that four haplotypes, PD-1.1 A/PD-1.2 A (46.3%), PD-1.1 G/PD-1.2 G (45.4%), PD-1.1 G/PD-1.2 A (4.7%) and PD-1.1 A/PD-1.2 G (3.5%), were reconstructed. FBAT was utilized in analyzing the data from 256 nuclear families. As shown in Table 4, the transmitted haplotypes were not associated with genetic susceptibility of chronic HBV infection in each of additive, dominant and recessive models ($P > 0.05$).

## DISCUSSION

In humans, the molecular mechanisms of the association between HBV susceptibility and the PD-1 gene polymorphisms remain to be clarified. One possible mechanism is that selected PD-1 SNPs may be associated with an alteration in the level of expression of the PD-1 gene or possibly as a result of linkage disequilibrium with other PD-1 gene polymorphisms which differentially control PD-1 gene transcription. This possibility is supported by the identification of several SNPs in SLE, including in the promoter, introns, exon 5 and 3'-untranslated region of the PD-1 gene, and finding that the SNP PD-1.3 was involved in susceptibility to SLE in the European population while PD-1.1 and PD-1.2 was not[17]. However, the SNP PD-1.3 is not polymorphic in the Chinese population[20] and such discrepancies are worth further research. Another possible explanation is the connection between this SNP and the functional change in the PD-1 protein *via* linkage disequilibrium with other nucleotide polymorphisms that alter the sequence and structure of the PD-1 protein. This possibility may be tested by evaluating the ability of PD-1 protein to transduce inhibitory signals in response to ligand stimulation in individuals with different genotypes. However, it still cannot exclude the possibility that another

**Table 4 The associations between PD-1 haplotypes and chronic HBV infection by family-based association test**

| Haplotype | Additive model | | | Dominant model | | | Recessive model | | |
|---|---|---|---|---|---|---|---|---|---|
| PD-1.1/1.2 | Families[a] | $Z^b$ | *P* | Families[a] | $Z^b$ | *P* | Families[a] | $Z^b$ | *P* |
| A/A | 70.8 | 0.306 | 0.760 | 48.8 | -0.692 | 0.489 | 35 | 1.243 | 0.214 |
| G/G | 66.5 | 0.001 | 0.999 | 45.5 | -0.188 | 0.851 | 32 | 0.207 | 0.836 |
| G/A | 20.2 | 0.270 | 0.787 | 19.2 | 0.330 | 0.741 | - | - | - |
| A/G | 14.5 | -1.090 | 0.276 | 14.5 | -1.090 | 0.276 | - | - | - |

[a]The number of informative families. When the number of informative families is less than 10, the test will not be computed; [b]The negative sign of Z statistic indicates that the frequency of transmitted haplotype is negative association with susceptibility of chronic HBV infection. PD-1: programmed death-1; HBV: hepatitis B virus.

genuine HBV susceptibility gene is located adjacent to the PD-1 gene that affects the association between the PD-1 gene and chronic HBV susceptibility. For instance, CTLA-4 gene was associated with chronic HBV infection[9, 29] and the location of PD-1 is 2q37.3 in chromosome 2, almost nearer to CTLA-4 gene at 2q33. Therefore, based on the above considerations, our study employed a family-based association study in evaluating the genetic association of PD-1 to chronic HBV infection in Chinese patients; we suspected an immense role of PD-1 SNPs in chronic HBV infection.

However, in the present study, we have found no association of SNPs PD-1.1 A > G and PD-1.2 A > G in patients with chronic HBV infection. What is more, the major A or G allele from heterozygous parents did not transmit to affected offspring ($P > 0.05$). At the same time, we constructed four haplotypes comprising alleles of marker PD-1.1 G/A and PD-1.2 G/A and none of these haplotypes demonstrated an association ($P > 0.05$). In earlier studies, polymorphism of PD-1 SNPs (PD-1.1 and PD-1.2) in SLE, RA and T1D patients has been reported. Prokunina *et al*[17] reported PD-1.1 G > A and PD-1.2 G > A were not associated with SLE in European, Wang *et al*[19] validated their results in Taiwan Chinese. In addition, Asad *et al*[21] reported that PD-1.2 SNPs was not associated with T1D in Swedish. In these studies, no significant association was observed which is similar to our results. Conversely, Kong *et al*[20] reported that AA genotype of SNP PD-1.1 was associated with a decreased risk for developing RA in Hong Kong and that the PD-1.1 A allele can be considered a protective allele but the PD-1.1 G allele can not be called a risk allele by itself as it is a major allele in Caucasians (99%) and only a part of PD-1.1G-containing haplotypes are risk haplotypes. There may be several reasons for the differences between reported associations. For example, the underlying genetic factors involved in disease susceptibility might not be the same for chronic HBV infection as for RA or SLE[30-35]. The most likely reason for discrepancy between the other studies and ours is that our cohorts were larger than the Kong's RA cohort which only consisted of 180 patients with RA in a case-control association study[20]. In addition, Kong's study was based on case-control. Studies of case-control could lead to false-positive results because the control subjects were not ideally matched to the patients whereas the family-based study could avoid it by population stratification and reduced heterogeneity[36]. In some of the latest research, Zhang[37] and Zheng *et al*[38], investigated the association between chronic HBV infection and SNPs of the PD-1 gene and, although they used a large cohort of chronically HBV-infected patient samples and healthy controls, the results also indicated that neither PD-1.1 SNP nor P7209C > T, P8737A > G site in PD-1 gene was associated with the susceptibility of chronic HBV infection, validating our experimental results indirectly. Although Zhang *et al*[37] reported that PD-1.6 polymorphism may have a predisposing role in the disease progress, this needs further research. Therefore, the precision in estimating population frequencies among patients should be better in our investigation. Another reason for discrepancies is due to a large variation in the frequencies of PD-1 polymorphisms among different ethnic groups. For example, the PD-1.1 A allele is common in the Chinese population (49%) and in Mexican Indians (63%) while it is rare in Europeans (1%) and Africans (4%) (Prokunina-Olsson *et al*: unpublished observations); even James *et al*[34] reported PD-1.1 and PD-1.2 SNPs were not found in British Caucasians from southwest England. Moreover, it is known that complicated disorders like HBV depend on the interaction of multiple factors and no particular gene is uniquely responsible for the disease. Sometimes environmental factors may play a role in influencing susceptibility in different populations[39]. That could be an important reason why there are negative results in some research. For this reason, we cannot rule out the susceptibility of these genes even though the results are negative.

In conclusion, the present study has demonstrated that PD-1 gene polymorphism has no correlation with genetic susceptibility of chronic HBV infection in Chinese patients. Further genetic studies need to be replicated in populations of ancestries other than Chinese to estimate the polymorphisms and the information of the linkage disequilibrium (LD) between the other SNPs and the studied SNP.

## COMMENTS

### *Background*

Hepatitis B virus (HBV) infection remains a major public health problem. After infection, the diversity of the HBV disease spectrum and clinical course was attributed, to a very large extent, to the host immunological and genetic factors including single nucleotide polymorphisms (SNPs) of a variety of genes. Related gene polymorphism has important role in the understanding of the pathogenesis

of disease and formulation of the prevention and treatment.

### Research frontiers

In recent years, the association between the immune related gene polymorphism and HBV infection has been hot research, ranging from classic human leukocyte antigen (HLA), cytokines and some protein genes to a large study of genetic pathways and the whole genome scanning.

### Innovations and breakthroughs

Recent reports have highlighted the importance of the PD-1 pathway in T-cell response in different stages of chronic HBV infection. This study firstly investigated the association between the polymorphisms of PD-1 (PD-1.1 and PD-1.2) and genetic susceptibility of chronic HBV infection by family-based association analysis method. The family based research excluded the effect of environment which could not be avoided in case-control design.

### Applications

By discussing the association between the polymorphisms of PD-1 (PD-1.1 and PD-1.2) and genetic susceptibility of chronic HBV infection, this study may represent a future strategy for researching HBV infection by family-based association analysis method.

### Terminology

PD-1: A 55 kDa transmembrane protein containing an immunological receptor tyrosine-based inhibitory motif which was originally isolated from a T-cell line exhibiting a high sensitivity to apoptosis. As a negative co-stimulatory receptor, PD-1 interacts with its ligands, PD-L1 and PD-L2, to attenuate T-cell, B-cell and APC-cell responses and appears to be particularly important for regulating immune cells tolerance.

### Peer review

The manuscript described interesting data on the potential link between SNP in PD-1 and chronic HBV. As the data are contradictory to other reported findings, the authors are encouraged to analyze more carefully their experimental approach and interpret differences with others' data objectively.

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S- Editor Zhang HN L- Editor Roemmele A E- Editor Zhang L