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Association of HLA-DQB1 gene polymorphisms with outcome of HBV infection in a Chinese Han population

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Summary

Background: Host genetic factors and environmental factors including hepatitis B virus (HBV) genotype are widely studied for the different outcomes of HBV infection. Human leukocyte antigen (HLA) plays an important role in the immunological reaction to HBV infection.

Aims: To explore whether the HLA-DQB1 allele polymorphisms are associated with the outcome of HBV infection in a Chinese Han population.

Patients: One hundred and thirty three HBV subjects with spontaneous recovery and 151 chronic hepatitis B patients were recruited into this case-control study in the Beijing area of China.

Methods: Sequence specific primer-polymerase chain reaction (SSP-PCR) was used to detect 13 alleles of HLA-DQB1 gene and 13 alleles of HLA-DRB1 gene. Multivariate logistic regression model was performed to detect the association of candidate factors with outcome of HBV infection by SAS 9.1.2 software package.

Results: The frequency of HLA-DQB1*0502 allele in the chronic hepatitis B group was significantly higher than that in the group with spontaneous recovery independent of HLA-DRB1 (odds ratio 95%CI 1.8–190). In this study there was no evidence to indicate that cigarette smoking or alcohol consumption was associated with the outcome of HBV infection.

Conclusion: HLA-DQB1*0502 is independently associated with the outcome of HBV infection and is one host genetic factor affecting HBV infection outcome. At the same time, we can not rule out the possibility that excluded genes and alleles may also affect outcome.

Keywords: Hepatitis B; HLA-DQB1 gene; polymorphisms

Introduction

Chronic hepatitis B virus (HBV) infection is a serious public health problem affecting 350 million people worldwide [1]. A complex combination of environmental, pathogen and host genetic factors plays a role in determining both susceptibility to HBV and the course of the infection [2]. Highly polymorphic human leukocyte antigen (HLA) gene, a cluster of closely linked genes including class I, class II, and class III , is located on the short arm of chromosome 6, and is postulated to have arisen as a host strategy to counter antigenic diversity in infectious organisms [3, 4]. Moreover, HLA gene is located alongside a remarkably large number of other genes that are either known or predicted to have immunological functions. These include genes for tumour necrosis factor-alpha, a key mediator of inflammatory response to infection and for various complement and heat shock proteins [5].

HLA class II molecules present antigens to $CD4^+$ T lymphocytes, which modulate the $CD8^+$ CTL response and are crucial to the production of neutralizing antibodies. Since HBV clearance may be mediated by both eliminating infected cells through CTL and preventing infection of additional cells through antibody, it is plausible that

Abbreviations of amino acid residuals: A, Ala; Y, Tyr; R, Arg; S, Ser; G, Gly; T, Thr; H, His; V, Val; M, Met; I, Ile.

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differences in the efficiency of the class II interaction with HBV antigens might be important [6]. Vigorous HLA class II restricted $CD4^+$ T cell responses towards the hepatitis B core antigen (HBcAg) result in acute HBV infections and in chronic hepatitis patients weak or no responses are observed.

Previous studies have highlighted that HLA-DQB1 polymorphism influence individual immune response and thus affect the outcome of diseases [7, 8]. Many diseases, especially autoimmune disorders are related to HLA class II gene polymorphisms [9]. The relationship between the HLA gene polymorphisms and diseases shows racial, ethnic and geographic differences [10].

In spite of the pivotal role that the polymorphic HLA antigens play in immunosurveillance and the immune response and the multitude of studies performed, conclusive evidence of association with outcomes of HBV infection has remained elusive. In this study, a case-control design was used to explore whether HLA-DQB1 gene polymorphism is associated with the outcome of HBV infection by comparing the frequency distribution differences of 13 HLA-DQB1 alleles between a group with spontaneous recovery and a chronic hepatitis B group. Environmental risk factors for chronic hepatitis B were investigated simultaneously. Hence, it is expected that the evidence accrued from this study will be useful in identifying susceptible genes for chronic hepatitis B and predicting the outcome of HBV infection.

Patients and methods

Patients

A total of 180 patients diagnosed with chronic hepatitis B and 155 hepatitis B subjects with spontaneous recovery from Beijing Ditan Hospital and Beijing Shunyi District Hospital were enrolled in this study between November 2001 and August 2002. 170 chronic hepatitis B patients and 149 hepatitis B subjects with spontaneous recovery completed case report forms and signed an informed consent form according to the standards of the ethics committee. During the analysis 7 subjects from the chronic hepatitis B group and 11 subjects from the spontaneously recovered HBV group were excluded as they were not Han race. 8 chronic hepatitis B patients and 9 spontaneously recovered hepatitis B subjects failed to be

Table 1
Outline of HLA-DQB1 allele genotyping.

DQB1 alleles	Specific primers		PCR product (bp)
DQB1*0501	5’	5’CGGAGCGCGTGCGGGG3’	128
	3’	5’GCTGTTCCAGTACTCGGCAA3’	
DQB1*0502	5’	5’ TGCGGGGTGTGACCAGAC3’	117
	3’	5’ TGTTCCAGTACTCGGCGCT3’	
DQB1*0503	5’	5’TGCGGGGTGTGACCAGAC 3’	87
	3’	5’GCGGCGTCACCGCCCGA 3’	
DQB1*0601	5’	5’ GCCATGTGCTACTTCACCAAT3’	198
	3’	5’ CACCGTGTCCAACTCCGCT3’	
DQB1*0602	5’	5’ CGTGCGTCTTGTGACCAGAT3’	121
	3’	5’GCTGTTCCAGTACTCGGCAT 3’	
DQB1*0603/8	5’	5’GGAGCGCGTGCGTCTTGTA 3’	127
	3’	5’ GCTGTTCCAGTACTCGGCAT 3’	
DQB1*0604	5’	5’ CGTGTACCAGTTTAAGGGCA3’	254
	3’	5’GCAGGATCCCGCGGTACC 3’	
DQB1*0201	5’	5’GTGCGTATTGTGAGCAGAAG 3’	205
	3’	5’GCAAGGTCGTGCGGAGCT 3’	
DQB1*0201/0302	5’	5’GACGGAGCGCGTGCGTCT 3’	129
	3’	5’ CTGTTCCAGTACTCGGCGG3’	
DQB1*0301/4	5’	5’ GACGGAGCGCGTGCGTTA3’	122
	3’	5’AGTACTCGGCGTCAGGCG 3’	
DQB1*0302/3	5’	5’ GACGGAGCGCGTGCGTCT 3’	122
	3’	5’ AGTACTCGGCGTCAGGCG 3’	
DQB1*0303	5’	5’ GACGGAGCGCGTGCGTCT 3’	129
	3’	5’CTGTTCCAGTACTCGGCGT 3’	
DQB1*0401	5’	5’ CACCAACGGGACCGAGCT3’	200
	3’	5’ GGTAGTTGTGTCTGCATACG3’	
DQB1*0402	5’	5’ CACCAACGGGACCGAGCG 3’	200
	3’	5’ GGTAGTTGTGTCTGCATACG 3’	

genotyped. Thus a total of 155 chronic hepatitis B patients and 133 spontaneously recovered hepatitis B subjects were included in the analysis. The criteria for spontaneous recovery from infection were as follows: positive for both anti-HBs and anti-HBc antibodies, negative for hepatitis B surface antigen (HBsAg), normal liver function tests and no medical history of dominant hepatitis B and HBV vaccination. Chronic hepatitis B was diagnosed if the serum levels of ALT and AST were continuously abnormal, HBsAg and/or HBeAg seropositive, anti-HBs seronegative sixth months after acute infection.

As a further study objective was to analyze the association of behavioural risk factors (smoking and drinking) with progression of disease, these factors were included in the CRF, in which smoking is defined as "at least one cigarette was consumed per day, over a period of at least 6 months", and drinking was "an alcoholic beverage was consumed at least once per month for 6 months or more".

Sample size

When planning this case control study, we calculated the necessary sample size using EpiInfo (version 5). We set to 95% the probability that if the two samples differ this reflects a true difference in the two populations (i.e. 1-α), and to 80% the probability that if the two populations differ, the two samples will show a significant difference (i.e. 1-β). We assumed that the frequency of associated allele (DQB1*0301) in the control population would be about 20% [4] and we wanted to detect an odds ratio of 2.0 or greater. These requirements gave a sample size for each group of 186.

Table 2

Outline of HLA-DRB1 allele genotyping.

DRB1 alleles	Specific primers		PCR product (bp)
DRB1*01	5'	5' TTG TGG CAG CTT AAG TTT GAAT 3'	168
	3'	5' GCT GTTCCAGTA CTC GGC AT 3'	
DRB1*15/16	5'	5' TCC TGT GGC AGC CTA AGA G 3'	259
	3'	5' CGC TGC ACT GTG AAG CTC TC 3'	
DRB1*03	5'	5' GTTTCTTGGAGTACTCTAGGTC 3'	222
	3'	5' TGCAGTAGTTGTCCACCCG 3'	
DRB1*04	5'	5' GTTTCTTGGAGCAGGTTA AAC A 3'	262
	3'	5' CGC TGC ACT GTG AAG CTC TC 3'	
DRB1*11	5'	5' CAC GTT TCT TGG AGT ACT CTA C 3'	179
	3'	5' CTG GCT GTT CCA GTA CTC CT 3'	
DRB1*12	5'	5' AGT ACT CTA CGG GTG AGTGTT 3'	163
	3'	5' CTG TTC CAG GAC TCG GCG A 3'	
DRB1*13/14/11	5'	5' GTTTCTTGG AGT ACT CTA CGTC 3'	234
	3'	5' CGTAGTTGTGTCTGCA(GA)TAGG 3'	
DRB1*07	5'	5' CCTGTGGCAGGG AAGTATA 3'	232
	3'	5' CCC GTAGTTGTG TCT GCA CAC 3'	
DRB1*08	5'	5' AGT ACT CTA CGG GTG AGT GTT 3'	227
	3'	5' CCC GTA TTG TGT CTG CAG 3'	
DRB1*09	5'	5' GTTTCTTGAAGCAGGATA AGTTT 3'	236
	3'	5' CCC GTA GTT GTG TCT GCA CAC 3'	
DRB1*10	5'	5' CGG TTG CTG GAA AGA CGCG 3'	204
	3'	5' CTG CAC TGT GAA GCT CTC AC 3'	
DRB1-Exon2	5'	5' TTCGTGTCCCCACAGCACGTTTC	295
	3'	3' 5' GCC GCT GCA CTG TGA AGC TCTC 3'	

Table 3

Outline of HLA-DRB1*13/14 and *15/16 allele genotyping.

DRB1 alleles	Specific primers		PCR product (bp)	Annealing temperature
13.1	5'	5' TACTTCCATAACCAGGAGGAGA 3'	130	64 °C
	3'	5' CCCGCTCGTCTTCCAGGAT3'		
13.2	5'	5' GTTTCTTGGAGTACTCTACGTC 3'	171	64 °C
	3'	5' TGTTCCAGTACTCGGCGCT		
14.1	5'-1	5' GTTTCTTGGAGTACTCTACGTC 3'	224	64 °C
	5'-2	5' AGTACTCTACGGGTGAGTGTT 3'	215	62 °C
	3'	5' TCTGCAATAGGTGTCCACCT 3'		
14.2	5'	5' TACTTCCATAACCAGGAGGAGA 3'	140	64 °C
	3'	5' TCCACCGCGGCCCGCC 3'		
15	5'	5' TCCTGTGGCAGCCTAAGAG 3'	197	60 °C
	3'	5' CCGCGCCTGCTCCAGGAT 3'		
16	5'	5' TCCTGTGGCAGCCTAAGAG 3'	213	60 °C
	3'	5' AGGTGTCCACCGCGGCG 3'		

Serological testing

Enzyme-linked immunoadsorbent assay (ELISA) was used for detection of serum HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc (IMX; Abbott Diagnostics, North Chicago, IL).

HLA-DQB1 and HLA-DRB1 polymorphisms genotyping

Genomic DNA was extracted from 5 mL of peripheral blood by standard phenol-chloroform method, dissolved in 300 µL of Tris-HCl buffer (10 mmol /L, pH 8.0) containing 1 mmol/L EDTA, and stored at –70 °C until use. Genotypes were detected by sequence specific primer-polymerase chain reaction (SSP-PCR) [11, 12]. Primers sequences and PCR product size are shown in table 1 and table 2.

Positive internal control primers with concentration as one fifth of specific primers concentration were pooled into a reaction system in order to eradicate pseudonegative results. The internal control was a fragment of human growth hormone gene1 (chr 17) with 439 bp. The sequences of control primers C5 and C3 were 5' CAGTGCCTTCCCAACCATTCCCTTA 3' and 5' ATCCACTCACGGATTTCTGTTGTGTTTC 3' respectively. PCR was performed in 25 µL reaction mixture containing 1 U DNA Taq polymerase (Shanghai Biocolor, China), 50 ng genomic DNA, 1× buffer and 1.5 mmol/L of $MgCl_2$, 0.2 µmol/L primers, 0.04 mmol/L internal control primers C5 and C3, and 0.08 mmol/L dNTPs. The following cycling conditions were employed: 94 °C for 2 min, 30 cycles of 94 °C for 30 sec, annealing temperature for 30 sec (66 °C for DQB1 genotyping and 64.5 °C for DRB1), 72 °C for 30 sec, and a single final extension at 72 °C for 10 min in Perkin Elmer thermocycler (2700, applied biosystems, Foster City, CA). After amplification, products were identified by ultraviolet light after electrophoresis in 2% agarose gel stained by ethidium bromide (0.5 µg/mL). The allelic type was determined according to the presence or absence of the desired length PCR products.

In HLA-DRB1, another 7 reactions were needed to distinguish DRB1*13 from *14 and *15 from *16. The reaction pools were the same as above. The primers' sequences, PCR products sizes, and annealing temperature are listed in table 3.

In order to ensure the stability and sensitivity of genotype detection, annealing temperature and primers concentrations were modified during the determination of PCR conditions. During genotyping we maintained stable reaction conditions and 50 percent of samples were repeated.

Statistical analysis of data

All statistical analyses for the association of candidate factors with the outcome of HBV infection were performed using an unconditional logistic regression model in SAS 9.1.2 software package. All studied exposure factors (containing all DQB1 and DRB1 alleles, age, gender, smoking and drinking) were included in one regression model. Alleles with a frequency less than 3% were excluded and the remaining alleles of HLA-DQB1 and DRB1 were switched to dummy variables, which were used to find the contribution of alleles to outcome of disease. The alleles with fairly low frequencies and balanced distribution between two groups (DQB1*0401 and DRB1*03, respectively) were selected as the blank control alleles. The results were described by OR and 95% CI. We defined $\alpha = 0.05$ (two-side) as statistical significance level.

Results

Analysis of subject demographics

As shown in table 4. The percentage of men in the chronic hepatitis group was clearly higher than that in the group with spontaneous recovery. The mean ages of the chronic hepatitis group and spontaneously recovered group were not significantly different. The frequency of exposure to cigarette smoking or alcohol consumption in patients with chronic hepatitis B was higher than that in spontaneously recovered subjects, without statistical significance.

Unconditional logistic regression analysis

As shown in table 5. After adjustment of all confounding factors by unconditional logistic regression, it was shown that male sex (OR 7.3, 95%CI 4.5–12), increasing age (OR 1.3 per 10 years, 95%CI 1.1–1.5), and the HLA-DQB1*0502 allele (OR 18, 95%CI 1.8–190) were associated with a greater susceptibility to chronic hepatitis B independent of other studied exposure factors.

Discussion

Among the 13 HLA-DQB1 alleles detected in the present study, the alleles *0303 and *0301/*0304 were detected most frequently, with a frequency of 23% and 21%, respectively. It was shown from association analysis that HLA-DQB1*0502 allele was significantly related to persistence of hepatitis B independent of other confounding factors including gender, age, smok-

Table 4
Patients' demographics.

Variable	Spontaneously recovered group (n = 133)	Chronic hepatitis B group (n = 151)
Age: mean (sd)	37 (13)	40 (15)
Males: n (%)	63 (47)	120 (79)
Smokers: n (%)	38 (29)	55 (36)
Drinkers: n (%)	38 (29)	48 (32)

Table 5

Logistic regression model for chronic hepatitis B given patient demographics and alleles of HLA-DQB1 and HLA-DRB1.

Variable	Spontaneously recovered group n = 133 (%)	Chronic hepatitis B group n = 151 (%)	OR	95%CI
Age per 10 years			1.3	1.1–1.5
Male	63 (47)	120 (79)	7.3	4.5–12
Smokers	38 (29)	55 (36)	0.68	0.40–1.1
Drinkers	38 (29)	48 (32)	0.62	0.37–1.1
DQB1*0501	18 (6.8)	15 (5.0)	1.2	0.25–5.4
DQB1*0502	3 (1.1)	18 (6.0)	18	1.8–190
DQB1*0601	15 (5.6)	26 (8.6)	2.2	0.60–8.0
DQB1*0602	25 (9.4)	22 (7.3)	1.4	0.33–5.8
DQB1*0201	38 (14)	51 (17)	1.9	0.56–6.4
DQB1*0302	20 (7.5)	12 (4.0)	0.93	0.24–3.6
DQB1*0301/4	56 (21)	79 (26)	1.8	0.57–5.8
DQB1*0303	61 (23)	61 (20)	1.4	0.42–4.9
DQB1*0401	10 (3.8)	8 (2.7)	1	
Other DQB1	20 (7.5)	10 (3.3)	1.1	0.25–4.3
DRB1*01	13 (4.9)	7 (2.3)	0.40	0.07–2.4
DRB1*03	8 (3.0)	11 (3.6)	1	
DRB1*04	30 (11)	27 (8.9)	0.66	0.18–2.3
DRB1*07	32 (12)	43 (14)	0.89	0.28–2.9
DRB1*08	22 (8.3)	29 (9.6)	1.0	0.29–3.5
DRB1*09	45 (17)	43 (14)	0.79	0.21–2.9
DRB1*10	2 (0.75)	1 (0.33)	0.14	0.010–2.0
DRB1*11	17 (6.4)	22 (7.3)	1.2	0.32–4.2
DRB1*12	37 (14)	52 (17)	0.92	0.28–3.0
DRB1*13	7 (2.6)	1 (0.33)	0.11	0.0080–1.5
DRB1*14	16 (6.0)	29 (9.6)	1.0	0.28–3.8
DRB1*15	32 (12)	36 (12)	0.76	0.20–2.9
DRB1*16	5 (1.9)	1 (0.33)	0.045	0.0020–1.1

ing, drinking and DRB1 alleles. In this study, we found male sex and increased age increased the risk of chronic hepatitis B, while an association of cigarette smoking and alcohol consumption with outcome of HBV infection could not be shown.

The association of male sex with susceptibility to chronic hepatitis B cannot be conclusively determined in such a case control study because this difference may result from a mismatch during subject recruiting. However, to a certain extent this result can provide complementary evidence that that male individual are more susceptible to chronic hepatitis B, as reported in the literature [13].

There have been several studies of the association between HLA-DQB1 allele and viral hepatitis B. In 1999 Thio CL demonstrated [4] that in African Americans DQB1*0301 was significantly associated with persistence of HBV. In 2003 Thio CL found that DQB1*0201 was clearly associated with HBV persistence in Caucasian individuals [14] and in the same year a study in a Chinese Han population by Jiang YG indicated that individuals carrying DQB1*0301 were susceptible to chronic hepatitis B [15]. In 2002 Lindermann M. [16] reported that the mean antibody titres after HBV vaccination in individuals in Germany carrying HLA-DQB1*0301 were clearly higher than those in other people. Thus it can be seen that the results from different studies are distinct. This may be due to differences in race, inclusion criteria of subjects, sample size, genotyping method, statistical analysis strategy, and may even be caused by chance. It is noticeable that in this study the subjects, who recovered spontaneously, were selected as the control group, which ensured a similar exposure to HBV between case and control group.

Since the strength of the immune response plays an important role in the outcome of HBV infection, association of HLA class II genetic polymorphisms with the outcome of HBV infection might result from the variance in allelic specific HLA antigen peptide binding pocket structures and promoter regions affecting HLA expression level. HLA genetic polymorphisms cause differences in HLA molecular structure, which focuses on antigen peptide binding pockets and determines the selectivity of individual HLA molecule binding to antigen peptides [17]. α chain and β chain encoded by HLA-DQA1 and DQB1 genes constitute antigen peptide binding pocket on cellular surface, which consists of 9 peptide binding motifs, P1~P9. Sequences of amino acid residuals of P1, P2, P3, P4, P5, P6, P7, and P9 are encoded

Table 6
Variability of the X box in DQB1.

X box motifs	Sequence (5'-3')	DQB1 variants included
X-A	AAAAAAAA//TCTGCCCAGAGACAGATGAGGTCCA	*0301
X-B	————————-TG————-T————————-T————	*0302,*0303,*0401, *0402
X-C	————————-TG————————————————————	All others

Note: The bases underlined construct palindromes

by DQB1*0502 are AY, R, S, GGTAS, GAS, YHG, HYVGA, and YHYVS, respectively, most of which are hydrophilic amino acids. P8 is invariant across all known DQB1 sequences. P9 amino acid residuals encoded by DQB1*0502 are unique. Amino acids of corresponding α chain are IM*, which is encoded by DQA1*01. This unique P9 amino acid sequence may be related to the ability to present antigen, and influence the outcomes of HBV infection by changing the HLA class II restricted immune response. Moreover, the expression level of HLA class II molecule depends on the allelic specific promoter sequence. HLA class II gene expression requires coordinate binding of transcription factors to the W-X-Y box region of the proximal promoter [18]. Within this region allelic differences exist for DQB1, as shown in table 6. Different types of X box consist of different palindrome sequences, which affect the expression at transcriptional level by distinct binding to the transcription factors. DQB1*0502 allele constructs an X-C box [7]. The allele-specific differences in the rate of expression of individual DQB1 alleles may affect the synthetic rate of the α-β heterodimers and variances in the composition of the active heterodimers mediated by the differential expression of individual α and β chains may give rise to differences in the ability of binding and presenting antigen peptides. In summary, HLA-DQB1 genetic polymorphisms affect the immune response to HBV by influencing the structure and quantity of HLA class II molecules.

However, we know there are several limitations in the present study. Firstly we used an unconditional logistic regression model simulating multiple variables based on maximum likelihood estimation to analyze relative parameters. Such estimates are known to be inefficient in the case of large numbers of parameters with sparse data, and complications arise when the candidate gene is highly polymorphic, such as in the HLA region. For example, the largest observed relative risks are often those based on the smallest number of expected events and are quite unstable. The research performed by Thomas DC in 1992 indicated that multivariate maximum likelihood estimation is superior to the univariate estimation, but somewhat more unstable, whereas in most instances the empirical Bayes estimates were closer to the simulated values [22].

Secondly, Wald intervals are adopted as 95% confidence intervals in this model, by taking the point estimate ±1.96 estimated standard errors. Wald method was only used for a large sample approximation when dealing with non-linear models and has the less accurate coverage among approximate intervals. In multilevel modelling it tends to suffer additional inaccuracy by falling more frequently to one side than the other of the true parameter [23]. From the results in the literature [4, 15] described above, it can be found that DQB1*0301 is a hot-spot allele that is associated with chronic hepatitis B in several populations including Chinese. We could not confirm this result, however, 95%CI of exposure to DQB1*0301 was too wide, 0.59–6.1. Therefore we can not rule out the possibility that this allele has an appreciable effect in our studied population. In this point our results did not conflict with other studies. Due to the small sample size, the rare positive allele DQB1*0502 and the wide 95% confidence intervals in this present study, there was an instability when using maximum likelihood to estimate the contribution of a highly polymorphic gene to the clinical outcome. Therefore, our results do not rule out the possibility of excluded genes and alleles also affecting outcome.

Thirdly, this present study did not show that cigarette smoking and alcohol consumption were associated with chronic hepatitis B, as indicated in previous epidemiological research [19, 20]. However, we can not rule out that it was a shortcoming of our study that category variables were used to define cigarette smoking and alcohol consumption that may disguise the association of these factors with outcomes of HBV infection.

It was suggested in this present study that the HLA-DQB1*0502 allele may be an independent susceptible factor for chronic hepatitis B and this may be mediated by regulation of the structure and expression level by specific sequence. Moreover, it is not definite that HLA class II allele polymorphisms determine the susceptibility to disease, and it is possible that HLA class II gene was linked closely with the real susceptible genes, such as TNF-α [21].

Our findings implicate variation in the genes governing the immune response of the host as one factor that may explain the variable outcome of hepatitis B infection and offer an approach to elucidating the molecular mechanisms of viral clearance. Further analysis of this association may help identify strategies for the prevention and management of chronic HBV infection.

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