# Polymorphism of IFNL3 rs12979860 associated immunity establishment in primary HBV infection: a multicenter study

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**Abstract**

The polymorphisms of IFNL3 associated with the outcomes of primary HBV infection remains controversial, the susceptibility has not been investigated across cohorts. So two cohorts, including 3874 participants, were enrolled to detect the association of IFNL3 genetic variants (rs12979860, rs12980275, and rs809917) with outcomes of primary HBV infection between groups of chronic HBV infection (CHB), Natural clearance (NC), Health control (HC), and a false-positive report probability (FPRP) test was applied to assess positive results, and function prediction of significant polymorphism was evaluated. Polymorphism of rs12979860 demonstrated a correlation to the outcomes of primary infection. In comparison between NC and CHB, the rs12979860-C significantly decreased the risk of CHB as compared to T allele in Hubei, Hainan and combined two cohorts, and in HC vs. NC comparison, the allele of rs12979860-C also refers immunity establishment against HBV in comparison to T allele in Hubei, Hainan, and combined two cohorts. In comparison between NC and CHB, the rs12979860-TT genotypes significantly increased the risk of CHB in Hubei, Hainan and combined two cohort. After adjusting the influence of age and sex, the results remain significant. FPRP test confirms the significance of rs12979860, moreover, rs12979860-C/T alteration brings the minimum free energy change in the centroid secondary structure. The polymorphism of rs12979860 in IFNL3 is associated with primary outcomes of HBV infection, and rs12979860-CC is associated with immunity establishment in primary HBV infection, serving as potential predictors against virus infection.

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**Abstract:** The polymorphisms of IFNL3 associated with the outcomes of primary HBV infection remains controversial, the susceptibility has not been investigated across cohorts. So two cohorts, including 3874 participants, were enrolled to detect the association of IFNL3 genetic variants (rs12979860, rs12980275, and rs809917) with outcomes of primary HBV infection between groups of chronic HBV infection (CHB), Natural clearance (NC), Health control (HC), and a false-positive report probability (FPRP) test was applied to assess positive results, and function prediction of significant polymorphism was evaluated. Polymorphism of rs12979860 demonstrated a correlation to the outcomes of primary infection. In comparison between NC and CHB, the rs12979860-C significantly decreased the risk of CHB as compared to T allele in Hubei, Hainan and combined two cohorts, and in HC vs. NC comparison, the allele of rs12979860-C also refers immunity establishment against HBV in comparison to T allele in Hubei, Hainan, and combined two cohorts. In comparison between NC and CHB, the rs12979860-TT genotypes significantly increased the risk of CHB in Hubei, Hainan and combined two cohort. After adjusting the influence of age and sex, the results remain significant. FPRP test confirms the significance of rs12979860, moreover, rs12979860-C/T alteration brings the minimum free energy change in the centroid secondary structure. The polymorphism of rs12979860 in IFNL3 is associated with primary outcomes of HBV infection, and rs12979860-CC is associated with immunity establishment in primary HBV infection, serving as potential predictors against virus infection.

**Keywords:** HBV, Natural clearance, IFNL3, Polymorphism, chronic HBV infection

## 1. Introduction

Primary hepatitis B virus infection could lead to three outcomes: chronic HBV infection (CHB), natural clearance (NC) and health control (health control is defined as no response to HBV). Compared to the favorable outcomes of HC and NC, CHB leads to liver disease globally and increased risks of developing cirrhosis, hepatocellular carcinoma (HCC) and liver failure, either of which can lead to liv-er-related death, thus imposing a burden on the health system globally [1], so CHB is the initial stage for progression among liver-related disease and death. Chronicity of hepatitis B virus infection has been considered a multifactorial and polygenic event that is associated with age, sex and genetic factors. However, accumulated association studies support the influence of host genetic factors in determining the chronicity of HBV infection [2,3]. The IFNL3 genetic variants have been previously characterized with HCV clearance [3] and the response during pegylated interferon therapy against HBV [4-6]. The role of polymorphisms has been frequently investigated in a wide spectrum of adverse outcomes of CHB progression [7-9], but the susceptibility of IFNL3 genetic variants to the outcomes of primary HBV infection remains controversial.

To uncover the susceptibility of genetic variants of IFNL3 to outcomes of primary HBV infection, samples from cohorts were enrolled to detect the association of genetic variants IFNL3 (rs12979860, rs12980275 and rs809917) with three outcomes of primary HBV infection.

## 2. Materials and Methods

### 2.1 Subjects

All unrelated Han Chinese subjects were recruited in Hubei province at the central region of China [7-9] and Hainan province at the southern region of China. Two cohorts of patients included 2164 subjects from four tertiary hospitals in Hubei cohort and 1710 subjects from three tertiary hospitals in Hainan cohort.

Participants enrolled according to the uniform predesigned requirements in these hospitals, the natural history and diagnosis were confirmed in detail by reviewing medical records and querying medical history. To keep the homogeneity, the chronic HBV infection (CHB) includes positive for serum HBsAg, or plus antibody to hepatitis B core antigen(anti-HBc) for more than 6 months, three times elevation of serum ALT level [$>$ 40U/L,1$\times$ upper limit of normal (ULN]). The patients had no serologic evidence for co-infection with hepatitis C virus (HCV), hepatitis E virus (HEV), hepatitis D virus (HDV), cytomegalovirus (CMV), HIV, autoimmune disease and medications associated with liver injuries. For the subjects of healthy individuals and natural clearances were negative for any of the following criteria (1) clear history of no HBV vaccination, (2) HCV and HIV infection, (3) history of other chronic liver diseases and malignant carcinoma, (4) immunosuppressants and systemic corticosteroids medications, and clinical criteria for HBV natural clearances(NC) were only positive for antibody to hepatitis B surface antigen (anti-HBs) or anti-HBs and anti-HBc (Hepatitis B core antigen) without hepatitis B virus vaccination. Health control (no response to HBV) was negative for all HBV serum makers without hepatitis B virus vaccination.

The study was approved by the Ethics Committee of all the recruitment hospitals and performed according to the Helsinki Declaration II. Written informed consent was obtained from all patients.

### 2.2 Genotyping

Genomic DNA was isolated from whole blood collected in EDTA tubes using a TIANamp Blood DNA Kit (Tiangen, China). The yield and purity of DNA were qualified by ultraviolet light spectroscopy and divided into three aliquots at -70°C for DNA analysis.

The allelic discrimination was carried out using ABI 9700. The rs12979860 (C$>$T) differentiated by predesigned commercial genotyping assay C_7820464_10. The rs12980275 (A$>$G) differentiated by predesigned commercial genotyping assay C__31438519_10, and rs809917(G$>$T)discriminated by predesigned commercial genotyping assay C_3084990_10. The conditions for Taqman genotyping were as follows: 2 times Taqman genotyping master mix (Applied Biosystems, ABI) 2.5 μL; 20 times SNP genotyping assay mix (ABI) 0.25 μL; template DNA 2.25 μL. The PCR reaction conditions were 95 ° C for 10 min, denaturation at 95 ° C for 15 s, and 60 ° C for 90 s. A total of 40 cycles were completed. After completion, the fluorescent signal was read and genotyping was completed.

### 2.3 Statistical analysis

Statistical analysis was performed using STATA 10.0SE. The characters between groups were compared by $\chi 2$ test and Wilcoxon rank sum test. The Hardy–Weinberg equilibrium (HWE) test was performed using the likelihood ratio $\chi 2$ test or Fisher exact test, when is necessary. The Chi-square analysis was used to examine the differences in allelic (major allele "M" vs. minor allele "m") and geno-typic frequencies, the Cochran-Armitage test was used for genotypic trend analysis (MM vs. Mm vs. mm), and genetic models fit the genetic susceptibility, dominant genetic model (MM vs. Mm+mm) and recessive model (MM +Mm vs. mm), and adjustments were performed regarding age and sex in logistic regression. The difference was considered as statistically significant when $P < 0.05$.

The false-positive report probability (FPRR) was calculated as described previously. Only the significant result with an FPRP value less than 0.2 was considered a noteworthy finding. The threshold was set at 0.2 and assigned a prior probability of 0.1 to detect an odds ra-tio (OR) of 0.67/1.5 (protective/risk effects) for an association. We also chose $P<0.05$ as a standard of statistical significance.

### 2.4 Genotypic Function Exploration by Web-Based Bioinformatics

We used RNAfold (http://rna.tbi.univie.ac.at//cgi-bin /RNAWebSuite/RNAfold.cgi) to predict effect of the significant SNPs on second RNA structure. RNAfold is a classic database to predict RNAs structure and

energy change in RNA formation according to RNA se-quence. Free energy represents the energy required to change the secondary structure from the current RNA structure.

## 3. Results

### 3.1 Subjects characteristics

The 3874 participants included 2164 subjects in the Hubei and 1710 subjects in the Hainan cohorts, the male significantly inclined to chronic HBV infection (60.48% in Hubei and 67.94 in Hainan) in compared with NC and HC in the two cohorts, while there was no statistical difference in age between the three groups of chronic HBV infection (CHB), no response to HBV infection (HC), natural clearance (NC).

### 3.2 Hardy–Weinberg equilibrium analysis

Rs12979860 (98.9%), rs12980275(98.8%) and rs809917 (99.6%) were successful genotyping rates in the Hubei cohort and rs12979860 (99.4%), rs12980275(99.3%) and rs809917 (98.7%) were successful genotyping rates in the Hainan cohort. The Hardy-Weinberg equilibrium test showed no significant difference among rs12979860, rs12980275 and rs809917 in the HC in Hubei and Hainan, indicating that control samples were from the same population (Table 1).

### 3.3 Association of IFNL3 genetic variants with NC, HC and CHB in Hubei cohort

In the Hubei cohort, the median age of 726 NC, 770 HC and 668 CHB is 48, 47, and 47, respectively. The male is more common in the group of CHB than HC($P$ <0.001) and NC($P$ <0.001), and age demonstrated no association with HC and NC in comparison between CHB. The C allele in rs12979860 (ref allele is C) is the favorable allele for the NC in compared with CHB (OR=0.58, 95%CI [0.42-0.78],$P$ =0.0002), and a significant difference in C allele frequency also existed between NC vs. HC (0.64, 95%CI [0.48-0.85],$P$ =0.0017). The homozygous CC genotype was a strong associated with immunity establishment in primary HBV infection as shown Table.2. We compared three genetic models between three groups of NC, CHB and HC. the significant difference was found in$P_{\text{additive}}$=0.0002 and$P_{\text{recessive}}$=0.0001 in NC vs. CHB; $P_{\text{additive}}$=0.0016 and $P_{\text{recessive}}$=0.001 in NC vs. HC. In contrast, no association of rs12980275 and rs80991 was found between NC, HC and CHB comparison in both genotypes and alleles.

### 3.4 Association of IFNL3 genetic variants with NC, HC and CHB in Hainan cohort

In the Hainan cohort, the median age of 528 NC, 530 HC and 652 CHB is 55, 55, and 41, respectively. As similar to Hubei cohort, the male is more predominant in the group of CHB, and the female is strongly inclined to HC($P$ <0.001) and NC($P$ <0.001) as compared to CHB (Table 3).

We compared the frequency of both allele and genotype between NC vs. CHB, HC vs. CHB, and NC vs. HC. There was a significantly higher proportion of rs12979860 C in NC. The C allele frequency was significantly higher in subjects with NC (93.7%) than patients with CHB (90.15%) and subjects with HC (91.14%). In genotypic analysis, the homozygous rs12979860-CC was strongly associated with NC in compared to CHB. Three genetic models used to detect association among three NC, CHB and HC groups. The significant difference was found in $P_{\text{additive}}$ =0.0017 in NC vs. CHB; $P_{\text{additive}}$ =0.0315 in NC vs. HC, $P_{\text{recessive}}$ =0.0007 in NC vs. CHB; $P_{\text{recessive}}$=0.034 in NC vs. HC. While genetic variants of rs809917 and rs12980275 were not associated with NC, CHB and HC in both genotypes and alleles comparison.

3.5 In combined data analysis of rs12979860

We combined all samples of Hubei and Hainan for further analysis, the male is predominant in chronic HBV infection between CHB vs. HC (OR=1.88 CI 95% [1.60-2.21], $P$ <0.001) and CHB vs. NC (OR=1.96 CI 95% [1.67-2.30], $P$ <0.001), No age distribution significance found between group comparisons of NC vs. CHB, NC vs. HC, and HC vs. CHB, as shown

In the allelic comparison between CHB vs. NC, the C allele of rs12979860 decreased the risk of CHB in compared to T allele (OR= 0.60, 95% CI [0.48-0.74], $P$ <0.001), and the C allele of rs12979860 also protective effect in comparison to T allele in NC vs. HC. In the genotypic analysis, the rs12979860-TT genotypes

demonstrated an increased risk of CHB in compared to NC (OR=1.75, CI 95% [1.42-2.17], $P$ <0.0001), and rs12979860-TT genotypes also showed the increased risk of failure to clear the virus in additive model and recessive model, the rs12979860-CC illustrated the favor effect in NC (OR=0.63 CI 95% [0.49-0.79], $P$ <0.001) in comparing to HC.

After adjusting influence of age and sex, the rs12979860-TT genotype showed chronicity predisposition after primary HBV infection (OR=1.66 CI 95% [1.34-2.06], $P$ <0.0001) in NC vs. CHB, (OR=1.49 CI 95% [1.21-1.85], $P$ <0.0001) in NC vs. HC.

3.6 Association of rs12979860 with primary outcome Stratified by Gender and Age

Stratification by age, the genotype of rs12979860-CC in age more than 40 shown significance in predisposing to develop immunity in Hubei, Hainan and combined cohort. Stratification by sex, the male with rs12979860-CC demonstrated substantially protective effect in Hubei, Hainan and combined cohort, irrespectively of sex, rs12979860-CC still shown the predisposition of development HBV immunity against HBV, as illustrated in Table 5.

### 3.7 False Positive Report Probability (FPRP) Analysis

The FPRP values for significant findings at different prior probability levels are il-lustrated in Table 6, as defined standard that assigned a prior probability of 0.1 to detect an odds ratio (OR) of 0.67/1.5 (protective/risk effects), the evident association for rs12979860 remained noteworthy in allelic comparison and genotypic comparison of an additive model and recessive model in comparison between NC vs. CHB and NC vs. HC as demonstrated in Table 6.

### 3.8 Web-based Functional analysis

In RNAfold analysis, the centroid secondary and minimum free energy (minimum free energy, MFE) structure of rs12979860 demonstrated in Figure 1. The genotype of rs12979860 changes centroid secondary in minimum free energy from -27.80 kcal/mol (rs12979860-T) to -46.07 kcal/mol (rs12979860-C), indicating rs12979860-C may increase the structural mRNA stability [Figure 1].

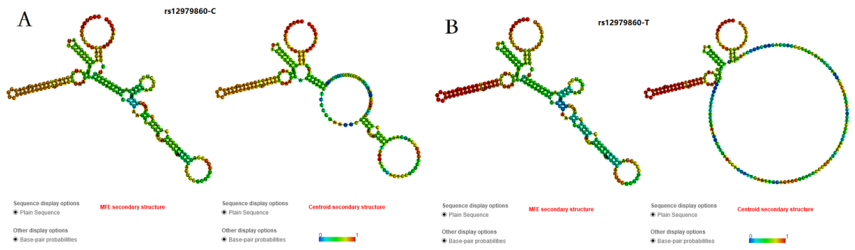


Figure 1. The RNAfold algorithm predicts the genotypic impact of rs12979860 on centroid secondary and minimum free energy. (A) MFE and Centroid secondary structure of rs12979860-C. (B) MFE and Centroid secondary structure of rs12979860-T

4. Discussion

The innate immune system response is the primary action in combating virus invasion [10-11]. Genetic variants of the IFNL3 gene locus are associated with virus infection outcomes by modulating almost all phases of the innate immune response to viral infection [12-13]. In the present study, we investigated the association of IFNL3 genetic variants (rs12979860, rs12980275 and rs809917) with heterogeneous outcomes of primary HBV infection in a large sample of participants from two cohorts within well-defined criteria in the Chinese Han population. Our results confirm that rs12979860-CC is particularly prone to a favorable outcome in primary infection in comparison with 12979860-TT and rs12979860-TC, suggesting that rs12979860 is involved in

autoimmunity in primary HBV clearance. rs12979860-CC is predisposed to immunity establishment; hence, the rs12979860 polymorphism might predict outcomes of primary HBV infection.

The polymorphism of IFNL3 (IFN-λ and IL28B) affects the immune process against viral infections. IFNL3 triggers a cascade through the JAK-STAT pathway that upregulates IFN-stimulated genes (ISGs). The genotypes of rs12979860-CC in the IFN-λ region are associated with an increase in IFNL3 expression compared to rs12979860-TT+CT in HCV and HBV infection [14-15]. The individuals from spontaneous HBV recovery and healthy controls bear a higher serum level of IFNL3 than those of chronic HBV infection [15], suggesting that the genotypes of rs12979860 are involved in the outcomes of primary infection by increasing the peripheral level of IFNL3. Rs12979860-CC might have higher IFNL3 expression, inflammation, clearance of HCV, and combination therapy against HCV [16]. Intricately elevated IFNL3 expression is associated with fibrosis in chronic hepatitis C [17]. IFNL3 expression levels induce higher baseline ISG expression, which may better inhibit the initial replication of a primary virus infection; however, a consistent immune response leads to disease progression in chronic infection, indicating that genotypic predisposition of a rapid and strong immune response in primary infection enables spontaneous clearance of the virus, while in chronic infection, the immune response mediates hepatic inflammatory injury. The function of IFNLs is macrophage and dendritic cell polarization, which prime helper T-cell activation and proliferation. SNPs in IFNLs might functionally affect IFNL signaling and thereby modulate the Th1/Th2 balance during infection, which may influence the subsequent priming of cytotoxic T cells versus antibody-secreting B cells [18]. The implications for the breadth and durability of the host response to HBV contributed to liver injuries, and the lasting immune response eliminated infected hepatic cells, causing deconstruction of the hepatic lobule.

The rs12979860-TT genotype may contribute to the chronicity and progression of HCV infection more than other genotypes (TC/CC) [19]. Patients with the rs12979860-TT genotype presumably have decreased intrahepatic expression of interferon-stimulated genes (ISGs), so they may fail to clear HCV infection and experience chronic disease [20,21]. Previous studies have shown that rs12979860-TT is frequently associated with disease progression for a broad range of viral infections [22], including an increased risk for developing hepatocellular carcinoma in hepatitis HCV patients [23]. The T allele of rs12979860 in the IFNL3 gene can reduce the expression of IFNL3-γ in liver and peripheral blood mononuclear cells [22]. Progression is consistent during immune-mediated injury and is influenced by the extent of intrahepatic inflammation induced by these ISGs. Additionally, the T allele of rs12979860 was associated with the presence of HCC among HBV patients in the China Han population [23], so combined with our findings, the T allele of rs12979860 is a risk factor for chronic infection and progression in HBV patients.

SNP rs12979860 comprises a CpG dinucleotide on the immune transcription factor site, which is necessary for methylation. Methylated DNA likely corresponds to reduced expression of IL28B and may lead to downregulation of IFN-stimulated gene (ISG). ISG levels correlate with the response to IFN, as has been confirmed in several studies [14, 15, 24]. Therefore, in the homozygous rs12979860CC variant, ISG expression may explain the increased IFN responsiveness. In contrast, ISG expression in carriers of the T allele corresponds with low responsiveness. Rs12979860 T carriers have also been shown to be associated with treatment unresponsiveness with increased CXCL10[16]. The rs12979860 genotype is associated with hepatic inflammation and fibrosis by modulating the immune response, irrespective of disease etiology [17]. The evidence above may partially explain the profound association of the rs12979860 genotype with virus clearance.

The rs12979860 maps onto human chromosome 19 and is in high linkage disequilibrium among IFNL4 variants (rs4803217, s368234815, rs117648444, and rs12979860) and the LD of these SNPs (rs4803217 $R^2$=0.93; rs368234815 $R^2$=0.96). The functional significance of those variants presents identical results to those for rs12979860, extending the implication as genetic markers to predict the function of this region. IFNL4 rs368234815 variants confer off-treatment HBsAg seroclearance in IFN-treated HBeAg-negative chronic hepatitis B patients [25]. The findings of interest were that age greater than 40 years and male sex, progressive factors in chronic HBV infection, are also protective factors in primary infection, suggesting that males above the age of 40 have immune capacity that assists with natural clearance in primary HBV infection. Consistent with previous findings, adults and males are predisposed to resolve spontaneously, while males

at earlier-onset puberty with primary HBV infection are shown to be associated with chronicity, immune tolerance, and greater HBV-DNA load [26].

To further confirm this association, FPRP analysis was used to evaluate the association between the genetic variant and the disease. The statistical power of the test was demonstrated across cohorts, and functional exploration proved the genotypic difference in energy required to change the centroid secondary structure.

The controversial associations might be inherited by the heterogeneity of studies in terms of sample size and inclusion criteria. The bias might come from the study design that determines the significance of the IFNL3 polymorphism on outcomes of primary HBV infection. A small sample size leads to results that are more likely to be false-positives because of the low sensitivity. Although the meta-analysis failed to confirm the association of the IFNL3 polymorphism [27], the results of the meta-analysis are dependent on the quality of each included study. Therefore, the large sample size, well-defined population and multiple centers are likely to provide a precise susceptible estimation.

The limitations in our study should be mentioned. First, although we included samples from two independent locations, the sample size in this study is comparatively median due to the low frequency of rs12979860-TT. Second, the age enrolled in our study was primarily above 40 due to predesigned standards to guarantee that aged individuals without HBV vaccination have a high chance of natural HBV exposure in HBsAg-positive epidemic places of Hubei and Hainan. Third, the genotypic function of rs12979860 was only investigated in web-based analysis. Therefore, it is important to further elucidate the mechanism by which the gene variants regulate the expression of IFN in primary HBV infection.

Our study highlighted that the rs12979860 polymorphism is associated with susceptibility to HBV clearance in primary infection. Future studies in multiple centers and functional analysis from a profound perspective are warranted to validate and extend clinical implications to promote the host immune response to virus clearance in unvaccinated individuals, even in emerging pandemics such as the COVID-19 pandemic and its variant [28].

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the

Declaration of Helsinki on human subjects, and approved by the Ethics Committee of Hainan general hospital.

**Data Availability Statement:** The data presented in this study are available on request from the corresponding author, the person privacy of subjects will be reserved.

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