J Clin Immunol (2010) 30:855–860
DOI 10.1007/s10875-010-9450-1

Association Between Hepatitis B Viral Burden in Chronic Infection and a Functional Single Nucleotide Polymorphism of the *PDCD1* Gene

Linghua Zheng · Dongling Li · Fusheng Wang · Hao Wu · Xiaozhu Li · Junliang Fu · Xinyue Chen · Lizhong Wang · Yang Liu · Shengdian Wang

Received: 20 May 2010 / Accepted: 26 July 2010 / Published online: 11 August 2010

Abstract
Background PD-1, encoded by *PDCD1*, is highly expressed on virus-specific T cells and plays critical roles in modulating anti-virus immune responses in chronic viral infection. It is unknown, however, whether polymorphisms of the *PDCD1* are associated with viral clearance during chronic viral infections.
Methodology and principal findings Here, we used the polymerase chain reaction-restriction fragment length polymorphism method to genotype two single nucleotide polymorphisms (SNPs) of *PDCD1* in 502 patients with chronic hepatitis B virus (HBV) infection and 359 healthy controls to determine the association between *PDCD1* genotypes and serum viral load as well as the risk of chronic infection. Our results showed that although neither the P7209$^{C/T}$ SNP site nor the P8737$^{A/G}$ site was associated with the risk of chronic HBV infection, the *P7209*^{T} allele in intron 4 is significantly associated with lower viral burden in the blood. Using a luciferase reporter assay, we demonstrated that the *P7209*^{T} allele creates a negative *cis*-element for gene transcription.
Conclusions and significance Our data provide the first evidence that *PDCD1* polymorphisms is a genetic factor in pathogenesis of chronic viral infection and reveal the functional significance of the *P7209* SNP of the *PDCD1*.

Keywords PD-1 · single-nucleotide polymorphism · hepatitis B virus · viral load

Electronic supplementary material The online version of this article (doi:10.1007/s10875-010-9450-1) contains supplementary material, which is available to authorized users.

L. Zheng · D. Li · F. Wang · X. Li · Y. Liu · S. Wang (✉)
Key Laboratory of Infection and Immunity,
Institute of Biophysics,
Beijing 100101, China
e-mail: sdwang@moon.ibp.ac.cn

L. Zheng
Graduate school, Chinese Academy of Sciences,
Beijing 100101, China

F. Wang · J. Fu
Research Center for Biological Therapy, Beijing Institute of Infectious Diseases,
Beijing 302 Hospital,
Beijing 100039, China

H. Wu · X. Chen
Departments of Infectious Diseases, Beijing You-An Hospital,
Capital Medical University,
Beijing 100054, China

L. Wang · Y. Liu (✉)
Division of Immunotherapy, University of Michigan,
Ann Arbor, MI 48109, USA
e-mail: yangl@umich.edu

Introduction

Chronic hepatitis B virus (HBV) infection is characterized by decades of high level viral replication and a leading cause of liver cirrhosis and hepatocellular carcinoma. HBV specific CD8^{+} T cell responses, which is critical for viral control, is markedly defective in chronic infection [1, 2]. PD-1, an inhibitory co-signaling receptor [3, 4], is expressed on exhausted virus-specific T cells at high levels and impair the virus-specific T cell response in chronic viral infections including HBV [5–7]. Blockade of B7-H1/PD-1 pathway restored the function of exhausted virus-specific T cells [5, 7].

The outcome of an HBV infection varies according to the vigor of the immune response [2]. Polymorphisms of many immune-related genes, such as HLA [8, 9], IFNγ [10], CTLA-4 [11], and CD24 [12] are associated with the risk or progression of chronic HBV infection. Although single nucleotide polymorphisms (SNPs) of *PDCD1* have been implicated in the pathogenesis of several inflammatory autoimmune disorders, such as systemic lupus erythematosus (SLE) [13, 14], rheumatoid arthritis [15–17], multiple sclerosis [18], and diabetes mellitus [19, 20], an effect of PDCD1 polymorphisms on chronic viral infection diseases has not been investigated.

A region of PDCD1 intron 4 was described as an enhancer-like structure containing binding sites for several transcription factors [13]. P7146$^{G/A}$ (PD-1.3) SNP in this region affected binding of the runt-related transcription factor 1 (RUNX1, also called AML1) and associated with development of SLE [13]. However, this nucleotide is not polymorphic among Chinese population [14, 16, 21, 22] P7209$^{C/T}$ SNP located in this region was also shown to be associated with SLE [14], although it is unclear whether this SNP can alter gene expression. Another SNP P8737$^{A/G}$ site is in 3′-UTR, which may affect mRNA stability. Using a large cohort of chronically HBV-infected patient samples and healthy controls, we investigated the effect of the SNP P7209$^{C/T}$ and P8737$^{A/G}$ in *PDCD1* on viral clearance as well as risk of chronic infection. Our data revealed a significant impact of a P7209$^{C/T}$ SNP on the blood HBV burden in chronic HBV infection. Interestingly, the P7209^{T} allele generated a new negative *cis*-element in gene transcription.

Results

The Genotypes of P7209$^{C/T}$ and P8737$^{A/G}$ Site in PDCD1 and the Susceptibility of Chronic HBV Infection

We found nine SNP sites in the genomic sequence of human PD-1 with the accession number AF363458 by searching the NCBI Genbank. Among these SNPs, we focused on the intronic region containing the enhancer-like structure because of its association with autoimmune diseases. Although P7146$^{G/A}$ (PD-1.3) and P7209$^{C/T}$ SNP in this region were shown to be associated with SLE [13, 14], the genotype distribution of P7146$^{G/A}$ (PD-1.3) is not polymorphic among Chinese population [14, 16, 21, 22]. In addition, another SNP P8737$^{A/G}$ site in 3′-UTR, which may affect mRNA stability, was also tested.

We used a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) method to genotype these two polymorphic sites in DNA samples from 502 patients with chronic HBV infection and 359 healthy controls. The P8737 genotyping was unsuccessful in 26 samples from the chronic HBV patients and 33 samples of the controls. The validity of the PCR-RFLP analysis was confirmed by direct sequencing of several PCR samples with each genotype.

We first analyzed the distribution of the P7209$^{C/T}$ and P8737$^{A/G}$ genotypes in these samples. The distribution of genotype frequencies of each SNP in the patients or healthy controls were in accordance with the Hardy–Weinberg equilibrium (Table S2). We next compared the distributions of the genotypes between normal controls and chronic HBV patients by the chi-square test. As shown in Table S2, the distribution of P7209$^{C/T}$ and P8737$^{A/G}$ genotypes among the chronic HBV patients did not significantly differed from that of the healthy controls. These results indicated that neither P7209$^{C/T}$ nor P8737$^{A/G}$ site was associated with the susceptibility of chronic HBV infection.

P7209$^{C/T}$ Polymorphism Associates with the Serum Viral Load of Chronic HBV Infection

We next analyzed association of P7209$^{C/T}$ or P8737$^{A/G}$ site with the serum viral burden of chronic HBV infection. Among the patient cohort that we studied, 274 patients have the record of the blood viral titer before therapy. These samples were used to study the correlation between the *PDCD1* genotypes and viral load. As shown in Fig. 2a, patients with the P7209$^{C/T}$ and P7209$^{T/T}$ genotypes showed significantly lower serum viral burden than patients with the P7209$^{C/C}$ genotype (P= 0.005). Allele analysis also showed that serum viral burden of patients with P7209 ^{T} allele was significantly lower than that of patients with P7209 ^{C} allele (P=0.009, Fig. 2b). However, there was no significant difference between different genotypes or alleles at the P8737$^{A/G}$ site (Fig. 2c, d).

P7209^{T} Allele Functions as a Negative *Cis*-element for Transcription

Since the PDCD1 intron 4 consisted of multiple *cis*-elements that may affect PD-1 expression [13], we tested the possibility that the P7209$^{C/T}$ alleles affected the function of the *cis*-elements. We cloned a 238-bp *PDCD1* genomic DNA including P7209 site with both P7209$^{T/T}$ and P7209$^{C/C}$ genotypes (Fig. 1) to measure enhancer (or repressor) activities using luciferase reporter assay. As shown in Fig. 3, no enhancer activity was found for either allele. Interestingly, the P7209^{T} allele contained a negative *cis*-element for transcription, as revealed by the reduction of the luciferase reporter activity. In contrast, no inhibition by the same fragment from the P7209^{C} allele was detected. Thus, P7209^{T} allele is expected to be expressed at a lower level than the P7209^{C} allele. As such, this allele may reduce serum viral burden of chronic HBV infection by reducing PD-1 expression to maintain a vigorous virus-specific T cell

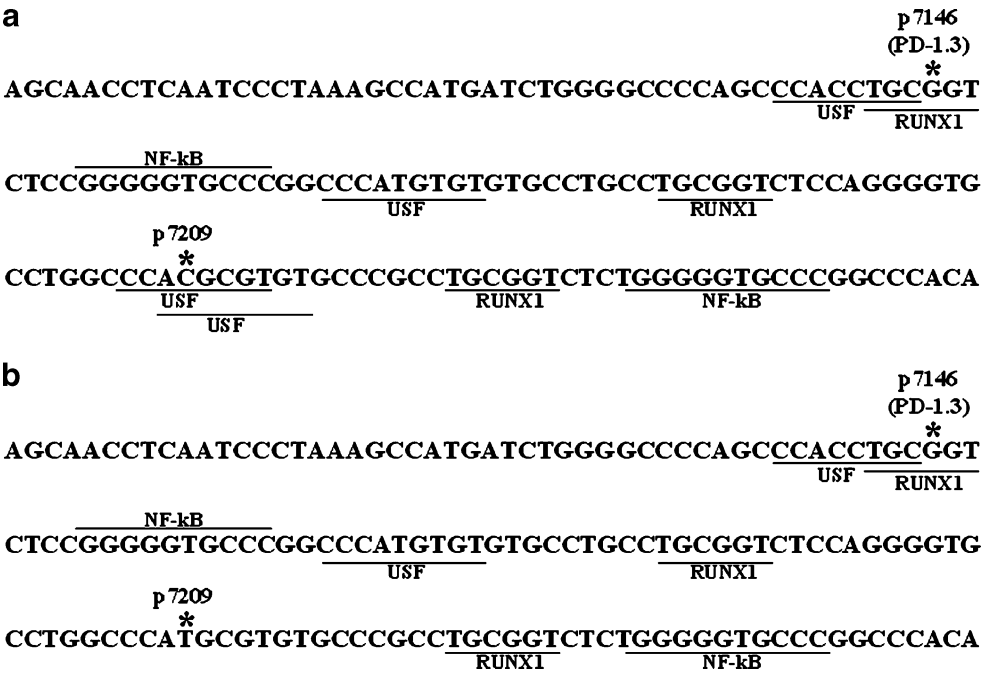

Fig. 1 Structure of the predicted binding sites in the cloned DNA fragment in the intron 4 of *PDCD1*. Predicted binding sites for the transcription factors RUNX1, NFκB and USF in the DNA fragment (7,098~7,250) are shown. P7209 is located in intronic enhancer region of PDCD1 and near the binding sites of transcription factors RUNX1 and NFκB. $P7209^{C}$ allele that contains two USF binding sites (**a**), while $P7209^{T}$ allele disrupts the USF binding sites (**b**). TFSEARCH database (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html) was used to predict binding sites of human transcription factors with the threshold 80.0

response. Since the $CD8^{+}$ T cells capable of recognizing HBV epitopes are barely detectable in most patients with high-level HBV replication [23, 24], it is not possible to compare the PD-1 levels on these cells from patients with different PDCD1 alleles.

Discussion

Patients with chronic hepatitis B always exhibit HBV specific T cell exhaustion and are unable to mount vigorous specific T cell responses [2]. Thus, human genetic variations that affect virus-specific T cell exhaustion may affect the risk or viral load of chronic HBV infection. In this study, we showed that $P7209^{T}$ allele in the PDCD1 intron 4 functions as a negative *cis*-element, which may reduce the expression of PD-1 to maintain vigorous virus-specific T cell responses, leading to reduction of serum viral burden of chronic HBV infection.

The $P7146^{G/A}$ (PD-1.3) SNP have been identified as a susceptibility factor for SLE and the region in PDCD1 intron 4 containing the $P7146^{G/A}$ was proposed to affect enhancer activity based on interaction with known transcription factors [13]. However, luciferase reporter assay showed that two alleles of $P7146^{G/A}$ have similar effect on reporter gene transcription [25]. The same study suggested that the nucleotides flanking the binding sequence negatively affect the RUNX1 binding to PDCD1 [25]. Here, we have shown that $P7209^{T}$ SNP in the putative enhancer-like region created a negative *cis*-element in the *PDCD1* gene and that this SNP is significantly associated with a lower serum viral burden of chronic HBV infection.

P7209 site has also been reported to be polymorphic in Korean and Polish populations and associate with two autoimmune diseases, Kawasaki disease and SLE [26, 27]. $P7209^{T}$ allele frequency was significantly higher in patient group in those studies. Our functional assay of $P7209^{T}$ allele may provide an explanation for their association finding. $P7209^{T}$ allele may act as a negative *cis*-element to repress PD-1 gene expression and lead to stronger self antigen-specific T cell response, thus conferring higher risk for autoimmune diseases.

Previous studies have implied a major role for $P7146^{A}$ in the risk of autoimmune diseases [13]. Since the function of P7209 was not evaluated in the early study, the possibility that the reported function of $P7146^{A}$ is due to linkage to $P7209^{T}$ has not been ruled out. In contrast, since P7146 is invariable in Chinese population, our data cannot be explained by linkage to P7146. In order to understand the functional difference, we compared the two alleles using the TFsearch software (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). Interestingly, a change of C to T at 7209 disrupts two binding site for upstream stimulating factor (USF), which are known to be involved in both stimulating and inhibiting gene transcription [28] (Fig. 1). Further studies are needed to determine whether disruption of binding to USF explains the negative *cis*-element activity.

In summary, our data have shown that $P7209^{T}$ allele in human PD-1 gene functioned as a negative *cis*-element for gene transcription and was associated with lower serum viral load of chronic HBV infection. The genetic impact may be achieved through regulating PD-1 expression, leading to stronger virus-specific T cell response. To our knowledge,

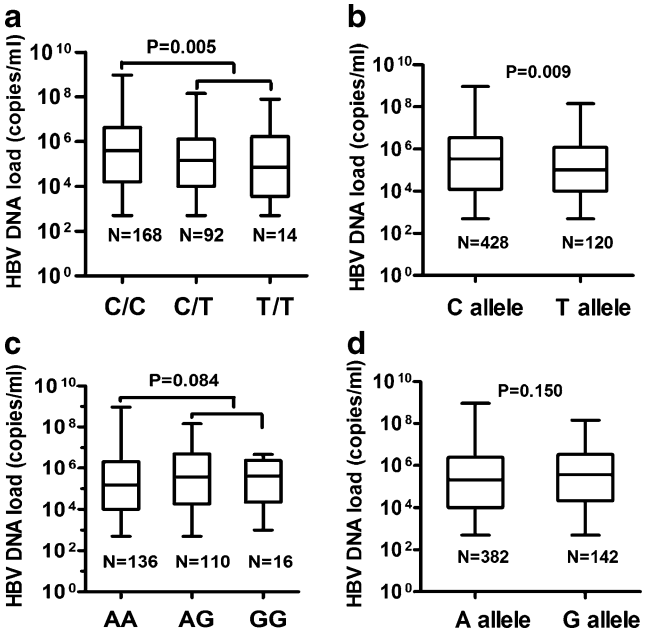

Fig. 2 Association analysis of PD-1 SNP genotypes with viral load in serum of chronic hepatitis B patients. **a** Patients with the $P7209^{T/T}$(median, 7.04×10^4; min~max, 500~7.71×10^7)+$P7209^{C/T}$ (median, 1.43×10^5; min~max, 500~1.42×10^8) genotypes were associated with lower viral load compared with those with the $P7209^{C/C}$ genotype (median, 3.915×10^5; min~max, 500~9.41×10^8); **b** patients with the $P7209^{T}$ allele (median, 1.0345×10^5; min~max, 500~1.42×10^8) were also associated with lower viral load compared with those with the $P7209^{C}$ allele (median, 3.42×10^5; min~max, 500~9.41×10^8); **c**–**d** no significant difference was found in viral load among patients with different $P8737^{A/G}$ genotypes($P8737^{A/A}$: median, 1.55×10^5, min~max, 500~9.41×10^8; $P8737^{A/G}$: median, 3.745×10^5, min~max, 500~1.45×10^8; $P8737^{G/G}$: median, 4.295×10^5, min~max, 1,000~4.65×10^6) or alleles ($P8737^{A}$ allele: median, 2.16×10^5, min~max, 500~9.41×10^8; $P8737^{G}$ allele: median, 3.745×10^5, min~max, 500~1.45×10^8). The copy numbers of serum HBV-DNA in each subgroup were presented as a logarithmic distribution. In **a** and **c**, HBV copies for each subgroup were $\log_{10}$ transformed and the distribution was changed to be a normal distribution ($p>0.05$ in One-Sample Kolmogorov–Smirnov test). *P* value was calculated by independent sample *t* test to compare the $\log_{10}$ (serum HBV load) value between the compared two groups. In **b** and **d**, after $\log_{10}$ transformation, **c** allele group and **a** allele group still did not show normal distribution ($P=0.029$ and $P=0.049$ in One-Sample Kolmogorov–Smirnov test). Thus, Mann–Whitney *U* test was used to compare the $\log_{10}$ (serum HBV load) value between the compared two groups and the corresponding *P* value was indicated

this is the only *PDCD1* SNP that generates a negative *cis*-element. It would be of interest to test the involvement of this SNP in other chronic viral infection and autoimmune diseases.

Materials and Methods

Human Subjects

Five hundred two patients with chronic HBV infection (389 males and 113 females), seropositive for HBsAg for at least six months, were recruited from the inpatients (336) and outpatients (166) of the Beijing 302 Hospital and You An Hospital. The clinical diagnosis and characteristics of the 502 patients was shown in Table S1. The patients with HIV and other types of chronic liver disease such as HCV, chronic hepatitis E, alcoholic liver disease, and steatohepatitis were excluded from the present study. Three hundred fifty-nine healthy individuals (216 males and 143 females) were enrolled as healthy controls (HBsAg negative, anti-HBc negative, and anti-HBe negative). All the study subjects were unrelated Chinese.

PCR Amplification and PCR-RFLP Analysis

Genomic DNA extraction and PCR-RFLP were performed as described [12]. For the P7209 site, the forward primer was CCC AAG TGT GTT TCT CTG and the reverse primer was GCA TTC TTG CAG ATT TAG. A PCR product of 499 bp with $P7209^{C}$ allele yielded a BstUI site that was absent in the $P7209^{T}$ allele. For the P8737 site, the forward primer was GAA GTT TCA GGG AAG GTC and the reverse primer was GGA TGT GAG GAG TGG ATA. A PCR product of 482 bp with $P8737^{A}$ allele yielded an NdeI site that was absent in the $P8737^{G}$ allele.

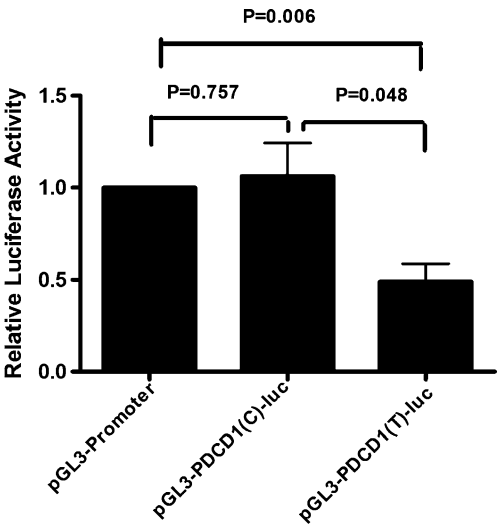

Fig. 3 Luciferase assay for the transcriptional activity of PD-1 genome fragments carrying $P7209^{C}$ and $P7209^{T}$ alleles. Two hundred ninety-three T cells were cotransfected in 24-well plates using phosphate calcium with renilla luciferase expressing vector pRL-CMV (internal control) and pGL-3 promoter vector (negative control), pGL-3-PDCD1(C)-luc, or pGL-3-PDCD1(T)-luc. After 24 h, the cells were lysed for measuring firefly and renilla luciferase activity in triplicate using dual-luciferase assays (Promega). Firefly luciferase activity in each group was normalized by renilla lucifrase activity, and presented relatively to that of the pGL-3 control group. Experiments were performed three times, and mean±SD of three experiments was shown. Independent sample *t* test was used to calculate the *P* value using SPSS

To confirm the PCR-RFLP results, 29 samples, including various genotypes of both sites, were randomly selected to be amplified by PCR and directly sequenced in ABI PRISM 310 Genetic Analyzer with the corresponding primers.

Luciferase Reporter Assay

Two hundred and thirty-eight bp bands (7,098–7,335) containing the $P7209^{C/T}$ site were amplified by PCR from DNA samples with $P7209^{C/C}$ genotype and $P7209^{T/T}$ genotype, respectively, and cloned into the pGL-3- promoter vector (Promega, USA) to generate the pGL-3-PDCD1(C)-luc and pGL-3-PDCD1(T)-luc vectors. The constructs were sequenced to confirm that there existed only one base C→T difference. 293T cells were seeded in 24-well plate, and after overnight growth, when the cell density reached around 80%, they were cotransfected with firefly luciferase expressing vector and renilla luciferase expressing vector (pRL-CMV vector, Promega). Luciferase activity was measured using dual-luciferase assays (Promega).

Statistical Analysis

Chi-square tests were performed to analyze case-control data with $P<0.05$ considered significant. P values were calculated in a multivariate regression model adjusted by gender as a potential confounding factor. Hardy–Weinberg equilibrium was tested using a chi-square test with one degree of freedom to compare observed genotype frequencies with expected genotype frequencies among the subjects. Independent-sample t test and Mann–Whitney U test were used for analyzing the viral burden data. All statistical analyses were performed using SPSS version 10 software (SPSS, Chicago, USA).

Acknowledgments We thank Dr. Hongyu Deng of the Institute of Biophysics, CAS for providing the pGL-3- promoter and pRL-CMV vectors and Ms. Darla Kroft for editorial assistance.

Author contributions SW and YL were responsible for the overall design and conduct and provided supervision; LZ, FSW, YL, and SW provided intellectual input and contributed to the experimental design; LZ, DL, and SW performed experiments; JF, HW, and FSW provided clinical samples; LZ, JF, HW, and FSW contributed to collection of clinical samples and retrieval of clinical data; XL provided technical assistance; and LZ, YL, and SW wrote the paper.

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