Association of *PD-1.9* polymorphism with the risk of HBV-related hepatocellular carcinoma

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Summary

Objective: This case-control study aimed to determine the association between *PD-1.9* polymorphism and the risk of HCC in the cohort of chronic HBV-infected patients. *Subject and method:* Genotyping of *PD-1.9* polymorphism was performed by direct sanger sequencing in 499 HBV-infected patients. Patients were assigned into two groups, including CHB (n = 193) and HCC (n=306) based on clinical manifestations. Binary logistic regression adjusted for age and gender was performed to analyze the association of *PD-1.9* variant with liver disease progression applying for different genetic models. *Result:* The frequencies of genotype *PD-1.9 TT* and minor allele T were significantly higher in HCC patients compared to CHB patients; genotype *PD-1.9 TT*: co-dominant model, OR = 2.1 (1.01-4.3), p_{adj}=0.047 and recessive model, OR = 1.8 (1.1-3.6), p_{adj}=0.042; allele *PD-1.9 T*: OR = 1.3 (1.1-1.8), p_{adj}=0.029. In addition, AFP levels were significantly higher in patients with genotype *PD-1.9 TT* compared to *CT/CC* genotype. *Conclusion*: This study, for the first time, reveals the association between *PD-1.9* variant and the risk of HCC development in chronic HBV infection.

Keywords: PD-1, PD-1.9 polymorphism, HBV infection, CHB, HCC.

1. Background

Hepatitis B virus (HBV) is one of the most prevalent viruses, circulating over the world. WHO estimates that 257 million people are chronically infected with 780,000 HBV-related deaths annually [1]. During HBV infection, liver damage and disease progression are mainly driven by both innate and adaptive immune responses. Several compelling evidences have shown that the cellular immune response plays a crucial role in disease pathogenesis mediated through activities of $CD8^+$ and $CD4^+$T cells, in particular, HBV-specific $CD8^+$T cells [2]. The HBV-specific $CD8^+$T cells response is relatively weak in patients with chronic HBV infection [3]. Importantly, the progressive HBV-specific $CD8^+$T cell exhaustion is a key mechanism and may associate with the activation of the inhibitory checkpoint PD-1/PD-L1 (Programmed cell death 1/Programmed cell death ligand 1) signaling pathway [4].

PD-1 is an immunoinhibitory receptor, a transmembrane protein especially expressed on the surface of T cells and also found in other

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immunological cells, such as B cells, natural killer T cells and activated monocytes. Several investigations have examined the association of genetic variants of *PDCD1* (encode PD-1) with various human disease conditions such as cancers, autoimmune and infectious diseases in different ethnic populations. Among several variants, *PD-1.9 (+7625C/T)* in the exon 5 has been shown to play a significant role in human diseases because this variation is a missense variant that alter the function of its encoded polypeptides. So far, there have not been studies investigating the functional role of *PD-1.9 variant* in chronic HBV infection and liver disease progression. Thus, to extend our knowledge, we conducted this case-control study to determine the association between PD-1.9 polymorphism and the risk of HCC development in the cohort of chronic HBV-infected patients.

2. Subject and method

2.1. Patients and blood specimens

Four hundred and ninety-nine (499) HBV-infected patients were recruited in a case-control design at 108 Military Central Hospital (108 MCH), Hanoi, Vietnam, between 2013 and 2016. Patients were assigned to subgroups based on clinical manifestations and final diagnosis: Chronic hepatitis (CHB, n = 193) and hepatocellular carcinoma (HCC, n = 306). All patients were confirmed negative for anti-HCV and anti-HIV by ELISA assays. Laboratory parameters including HBV-DNA loads and liver function tests including alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin and direct bilirubin, albumin, prothrombin were obtained from the patients' medical records. Plasma and blood cells were collected, separated and frozen at -80°C until use. The study was approved by the institutional review board of the 108 Military Central Hospital, Hanoi, Vietnam.

2.2. PD-1 genotyping

Genomic DNA was isolated from 200µl of whole blood using a DNA purification kit (Qiagen, Hilden, Germany). The amplicon containing the *PD-1.9* (*rs2227982*) was amplified by PCR (PCR1) using the specific primer pairs (Table 1). PCR amplification was carried out in 25µl reaction volumes containing: 1x PCR buffer, 0.2mM dNTPs, 1mM $MgCl_2$, 0.15mM of each primer, 1 unit of Taq polymerase and 50 ng of genomic DNA. Cycling conditions: Denaturation at 95°C for 5 min, followed by 35 cycles of three-step cycling with denaturation at 94°C for 40s, annealing at 66°C (PCR1) and 60°C (PCR2) for 40s, and extension at 72°C for 45s and a final extension at 72°C for 7min.

Direct sanger sequencing: PCR products were purified using the Exo-SAP-IT PCR product cleanup reagent (Affymetrix Santa Clara, USA) 5µl of purified PCR products were used as templates. Sequencing was performed using the BigDye terminator v.1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3130XL DNA sequencer according to the manufacturer's instructions.

Table 1. Characteristics of *PD-1.9* variant and primers used for amplification

db SNP rs # ID[$]	Location	Chromosome Position	Base change	Amino acid change	Primers	Sequence (5'-3')	Tm °C	Length
PD-1.9 (rs2227982)	Exon 5	chr2:241851281	C>T	Ala215Val	Forward	5'- GCA AGA ATG CCA GGG ACA TTT CAG AG -3'	66	618bp
					Reverse	5'- TGC CTG		

						GTG CAG GTG CAG -3'		
[$]: db = databases; rs # = reference SNP								

2.3. Statistical and genetic analysis

All statistical analysis was performed using R version 3.1.2 (http://www.r-project.org). Genotype and allelic frequencies were determined by simple gene counting. The deviations from Hardy-Weinberg equilibrium were calculated for each group. We used a binary logistic regression adjusted for age and gender to analyze association of PD-1 variants with HBV-related liver diseases applying for different genetic models. Adjusted odds ratios (aOR) with 95% confidence intervals (CI) were calculated. Chi-square tests were used to test for significant differences of categorical variables and Mann-Whitney-Wilcoxon tests applied to compare quantitative variables between groups. Significance was set at a value of $p<0.05$.

3. Result

3.1. Baseline characteristics of study participants

Table 2. Demographic and clinical characteristics of HBV patients and carriers

Clinical characteristics	CHB (n = 193)	HCC (n = 306)	p-values
Age (years)	40 (15 - 85)	57.5 (15 - 90)	<0.0001
Male (n, %)	166 (86%)	286 (92%)	0.006
HBsAg	Positive	Positive	NA
Anti-HCV	Negative	Negative	NA
Anti-HIV	Negative	Negative	NA
AFP (IU/mL)	3.7 (1.01 - 300)	109 (0.84 - 1660)	<0.0001
HBV DNA (copies/mL)	6.8×10^5 (1.83×10^2 - 4.01×10^{10})	1.64×10^5 (80 - 2.28×10^{10})	<0.0001
WBC ($x10^3$/mL)	6.25 (3.6 - 13.9)	6.15 (2.65 - 17.86)	0.5061
RBC ($x10^6$/mL)	4.8 (3.15 - 6.3)	4.51 (1.74 - 6.34)	<0.0001
PLT ($x10^3$/mL)	188.5 (19 - 356)	164 (35 - 479)	0.0009
AST (IU/mL)	139 (16 - 7700)	61.5 (17 - 2158)	<0.0001
ALT (IU/mL)	166 (9 - 4968)	44.5 (10 - 934)	<0.0001
Total Bilirubin (umol/mL)	19 (6 - 551)	16.9 (4.3 - 391.9)	0.0204
Direct Bilirubin (umol/mL)	6.25 (0.7 - 349)	5.25 (0.8 - 247.3)	0.0333
Albumin (g/L)	41 (23 - 50)	38.5 (18 - 49)	<0.0001
Prothrombin (%)	90 (17 - 267)	88 (19.6 - 269)	0.2236

Comments: Most of HBV patients were male (88.4% in CHB and 92% in HCC). ALT, AST and HBV-DNA loads, liver enzyme levels were significantly higher in CHB patients than in the HCC ($p<0.0001$). Albumin, red blood cells and platelets were lower in HCC patients than CHB ($p<0.05$). As evident, AFP levels were significantly higher in HCC patients than in CHB patients ($p<0.0001$). Data were presented as median and range or percentile where appropriate.

3.2. Genotyping of PD-1.9 variant and distribution of PD-1.9 genotypes in CHB and HCC patients

We successfully established the protocol for amplification of 618bp amplicon containing the variant *PD-1.9* (as described in the Method section). The Figure 1 illustrates the genotype of *PD-1.9* variant. (A): Genotype *CC* shows only one peak with C allele. (B): Genotype *CT* shows two peaks with *C* and *T* allele that are overlapping. (C) genotype *TT* shows one peak with *T* allele. The genotype and allele frequencies of *PD-1.9* (rs2227982) in clinically classified 499 HBV patients are described in the Table 3.

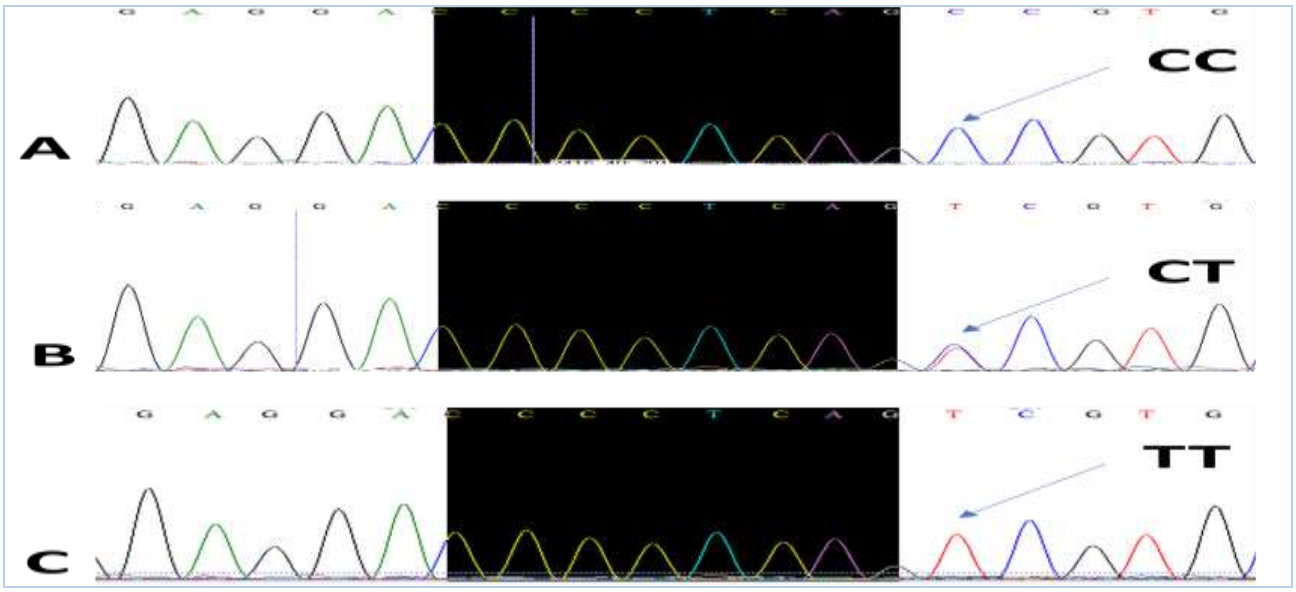

Figure 1. Representative sequences illustrate the distinct genotypes of *PD-1.9* polymorphism

3.3. Association between PD-1.9 variants and HBV-related liver disease

Table 3. Association of *PD-1* polymorphisms with liver disease progression

PD-1.9 polymorphism	CHB n (%)	HCC n (%)	HCC vs. CHB	
			aOR (95%CI)	p*
CC	74 (38.3)	94 (30.7)	Reference	
CT	103 (53.4)	156 (51)	1.3 (0.8 - 1.9)	NS
TT	16 (8.3)	56 (18.3)	2.1 (1.01 - 4.3)	0.047
Allele				
C	251 (65)	344 (56.2)	Reference	
T	135 (35)	268 (43.8)	1.3 (1.1 - 1.8)	0.029
Dominant				
CC	74 (38.3)	94 (30.7)	Reference	
CT&TT	119 (51.7)	212 (69.3)	1.4 (0.9 - 2.1)	NS
Recessive				

CC&CT	177 (91.7)	250 (81.7)	Reference	
TT	16 (8.3)	56 (18.3)	1.8 (1.1 - 3.6)	0.042

aOR: Adjusted Odds Ratio; ORs and (*) *P*-values were calculated by using binary logistic regression model adjusted for age and gender.

Comments: The frequencies of homozygous genotype *TT* were significant higher in HCC patients compared to CHB patients in the co-dominant model [HCC vs. CHB: OR = 2.1 (1.01 - 4.3), P_{adj} = 0.04 and recessive model [HCC vs. CHB: OR = 1.8 (1.1 - 3.6), P_{adj} = 0.042. The minor *allele PD-1.9 T* was also exhibited the similar trend [HCC vs. CHB: OR = 1.3 (1.1 - 1.8), P_{adj}=0.029] (Table 3).

Association of PD-1.9 genotype with laboratory parameters

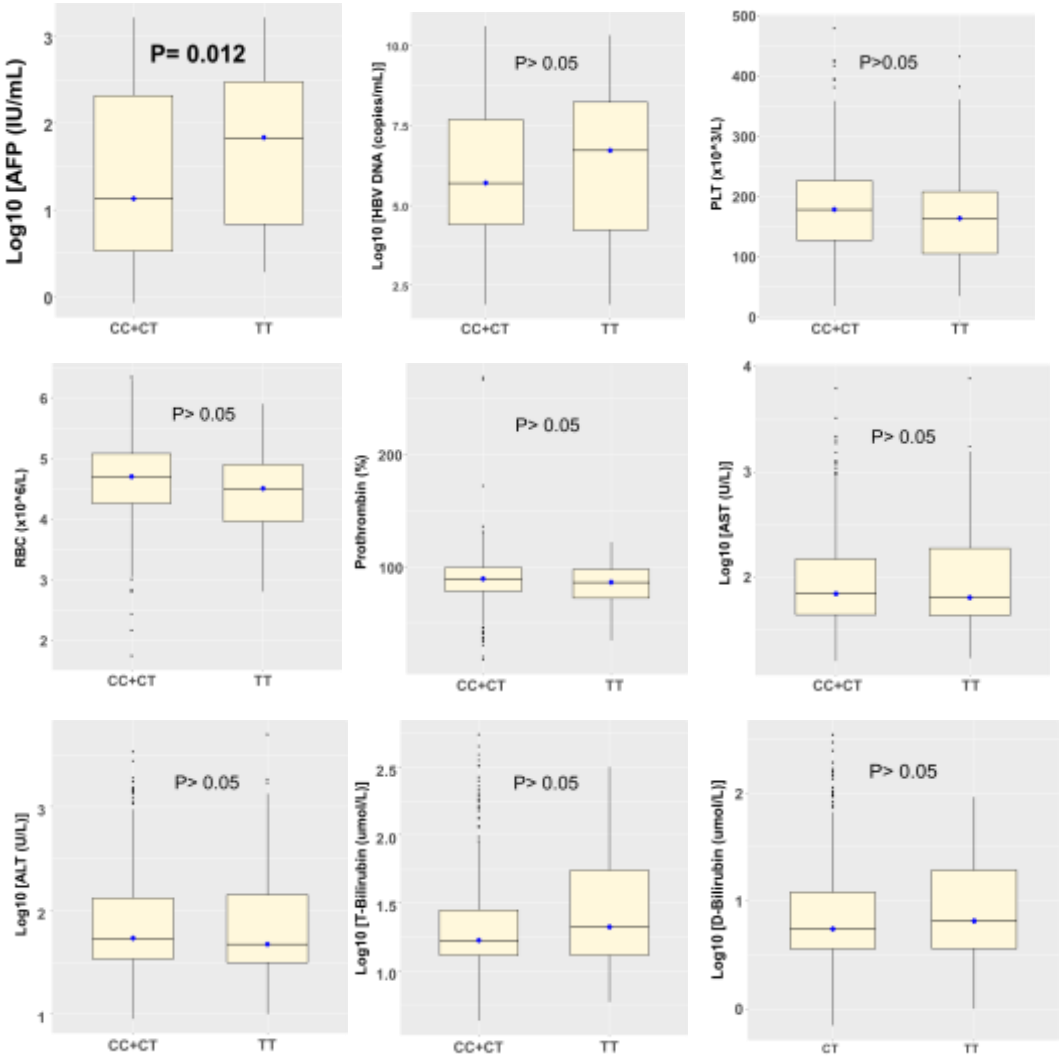

Figure 2. Association between *PD-1.9* genotypes and laboratory parameters in all HBV patients

Comments: Patients with the genotype *TT* had significantly higher AFP levels compared to those with genotype *CC+CT.* Although, patients carrying genotype *TT* had unfavorable clinical manifestations however the difference did not reach statistical significance (p>0,05). Box-plots illustrate median values with 25 and 75

percentiles and range; P values were calculated by Mann-Whitney test.

4. Discussion

The *PD-1.9* polymorphism has been identified to play an important role in various human diseases. This is a missense variant that causes the substitution of amino acids of valine (V) by alanine (A) during protein synthesis, which probably leads to a structural and functional variation of PD-1. Previous studies have shown significant associations between these two variants and several cancers types and infectious diseases, including HBV infection [5-7].

Associations of polymorphisms *PD-1.9* with susceptibility to HBV infection have been reported in previous studies [6]. Study showed that the *rs2227982 T* allele is a predisposing factor for HBV susceptibility (OR = 1.23, 95% CI = 1.04 - 1.46, p=0.018) [6]. In addition, the frequency of *TT* and *CT* genotypes in HBV patients was significantly higher than that of HC in several genetic models (dominant model: OR = 1.49, 95% CI = 1.12 - 1.98, p=0.006; co-dominant model (*TT:CC*): OR = 1.47, 95% CI = 1.08-1.99, p=0.014; co-dominant model (*CT:CC*): OR = 1.53, 95% CI = 1.09 - 2.15, p=0.014). Based on the reported results, Huang et al. pointed out that the CC genotype of rs2227982 and the C allele are protective factors against HBV infection [6]. In our study, we also reported that when analyzed with the age- and sex-adjusted logistic regression model, the frequency of *CT* genotype was significantly higher in HBV patients than in controls [co-dominant (*CT:CC*) model: OR = 1.4 (1.01 - 1.98), p=0.042].

PD-1 is a member of the immunoglobulin superfamily and its cytoplasmic domain contains an immunoreceptor tyrosine inhibitory motif associated with inhibitory signaling. The interaction between PD-1 and PD-L1 is known to contribute primarily to the depletion of T cells and subsequently play an important role in carcinogenesis. The properties of *PD-1.9* polymorphism on cancer risk have been studied. Few studies suggest that *PD-1* polymorphisms play a role as a convincing genetic indicator for different cancers in different ethnic populations. Nevertheless, the results of some studies were contradictory and ambiguous

To date, comprehensive meta-analyses have been conducted to investigate the association of the *PD-1.9* polymorphism with cancer risk [8-10]. In general, no significant association between this variant and overall cancer susceptibility was established in these meta-analyses [8-10]. However, Hashemi et al [10] performed stratified analyses and indicated that the *PD-1.9* polymorphism was associated with increased risk of general cancer in hospital-based studies (OR = 1.2, 95% CI = 1.05 - 1.37, p=0.008, *CT/TT* vs. *CC*). Additionally, stratified analyses found the association between *PD-1.9* and increased risk of gastrointestinal cancer (OR = 1.16, 95% CI = 1.03 - 1.30, p=0.017, *CT/TT* vs. *CC)* but decreased risk of breast cancer (OR = 0.73, 95% CI = 0.60 - 0.89, p=0.002, *CT/TT* vs. *CC*).

It should be noted that these above meta-analyses included several case-control studies but no studies have examined the association between *PD-1.9* polymorphisms and HCC risk. To our knowledge, this is the first study in which we have examined the link between *PD-1.9* polymorphism and the progression of liver disease including HCC. We have shown that the *PD-1.9* polymorphism is associated with the progression of HBV-related liver diseases. We have shown that, the variant *PD-1.9* shows a recessively homozygous advantage in disease progression and might be a risk factor for HCC. The results from our study indicate that *PD-1.9* polymorphism may influence liver disease progression by modulating the PD-1 protein expression and thus down-regulate the T cell signaling leading to disease progression and HCC development in chronic HBV infection. The basic for our speculation was supported by

convincing evidence that PD-1 overexpression in relation to T-cell dysfunction and exhaustion in chronic HBV infection and HCC development and prognosis of HCC [11, 12].

5. Conclusion

In this study, for the first time, we determined the genotypes and alleles of *PD-1.9* polymorphism in 499 Vietnamese chronic HBV-infected patients. We could conclude that there was a possible association between *PD-1.9* polymorphism and the risk of HCC development, in which *PD-1.9 TT* and minor allele *T* could be the risk factors.

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