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Research Article

Genetic Variation of PD-1 is Associated with The Development of Hepatocellular Carcinoma in Patients with Chronic Hepatitis C Infection

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Abstract

Introduction & Objectives: Hepatitis C virus persistence and pathobiology result from the interplay between viral replication and host immune responses. Programmed cell death-1 (PD-1) is an important immune effector with co-inhibitory activity involved in the progression of chronic viral infections. Our aim was to investigate the influence of the functional single nucleotide polymorphism (rs10204525) in the 3'-UTR of PD-1 and PD-1 mRNA expression on the outcomes of hepatitis C virus infection including disease progression in Moroccan patients.

Patients & Methods: A total of 200 healthy controls, 101 spontaneous resolved HCV patients and 300 chronic HCV subjects (95 patients with mild liver disease, 131 individuals with advanced liver disease and 74 patients with hepatocellular carcinoma, HCC) were enrolled in this study and genotyped for rs10204525 using TaqMan allelic discrimination. PD-1 mRNA expression in peripheral blood nuclear cells was determined by qRT-PCR. PD-1 mRNA expression in peripheral blood nuclear cells was determined by qRT-PCR.

Results: Multivariate logistic regression analysis showed the significant association of rs10204525 with progression liver disease (OR= 1.748, 95% CI = 1.034–2.955, P = 0.036). In addition, the T allele was related to an increased risk of HCC among patients with chronic HCV infection (OR= 1.528, 95% CI = 1.022–3.284, P = 0.038). In addition, PD-1 mRNA was overexpressed in chronic HCV infected patients with cirrhosis and hepatocellular carcinoma when compared to mild fibrosis group (P=0.0002) and the expression was even more pronounced when compared to HCV resolved group (P<0.0001).

Conclusion: These findings underline the importance of the functional polymorphism in PD-1 in the installation of HCV infection and its subsequent contribution to disease progression including the development of HCC.

Keywords: *Chronic HCV Infection, Programmed Cell Death-1, HCC, PD-1 Polymorphism*

Abbreviations

PD-1: Programmed Cell Death-1, IgSF: Immunoglobulin Super Family, HCV: Hepatitis C Virus, HCC: Hepatocellular Carcinoma, CTL: Cytotoxic T Lymphocytes, AdLD: Advanced Liver Diseases, mLD: Mild Liver Diseases, PBMCs: Peripheral Blood Mononuclear Cells, GAPDH: Human Glyceraldehyde-3-Phosphate Dehydrogenase, MAF: Minor Allele Frequency

Introduction

Chronic hepatitis C (CHC) represents a serious global health burden with 170 million people chronically infected worldwide [1] and leads to over 350,000 deaths each year [2]. Hepatitis C virus (HCV) exhibits a remarkable propensity to cause hepatic fibrosis, cirrhosis, hepatocellular carcinoma (HCC) and liver-related mortality [3]. The nucleocapsid core protein is the first protein synthesized following HCV viral infection, and is well conserved in different HCV genotypes. It has been demonstrated that HCV core protein inhibits T-cell proliferation and promotes B-cell activation through differential regulation of PD-1 signaling in vitro [4]. Additionally, HCV infection is associated with T-cell dysfunction and increased expression of PD-1 in T cell dysfunction [5].

Programmed cell death 1 (PD-1) is one of the latest immune regulatory genes that belongs to the immunoglobulin superfamily (IgSF). PD-1 gene is located on 2q37.3 and expressed on activated T and B lymphocytes, natural killer cells and monocytes. PD-1 protein is a transmembrane glycoprotein of the CD28 immunoglobulin superfamily and contains an immunologic receptor tyrosine-based inhibitory motif [6, 7]. With engagement to its ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC), PD-1 can inhibit cytokine secretion by downregulate T cells proliferation activity, enhance peripheral tolerance and induce T cell apoptosis [8, 9]. PD-1 can be induced by viral infection and its expression decreased after efficient antiviral therapies [10]. PD-1 is involved in immune disruption and viral persistence during chronic HCV infection [11] and is an important inhibitory receptor that downregulates T-cell function [12]. Studies focusing on CHC have shown that upregulation of PD-1 affects HCV specific CD8+ T cells in the liver and peripheral blood during chronic HCV infection [11, 13]. Moreover, several lines of evidence have been

reported on the importance of CD8+ T cells during HCC [14, 15]. Furthermore, Shi et al demonstrated that PD-1 and PD-L1 upregulation promotes CD8+ T cell apoptosis recurrence in patients with HCC [16].

In addition to the viral and immune factors, individual susceptibility factor has been identified to be one of the most important factors affecting the prognosis of the disease related to the HCV chronic infection. Recently, PD-1 polymorphisms were shown to be associated with the susceptibility and outcomes of HCV infection [17] and also with the susceptibility and disease progression in chronic hepatitis B virus (HBV) infection [18].

Likewise, recent reports have highlighted that PD-1 variants are associated with susceptibility to several types of cancer, such as breast cancer [19, 20], colon cancer [21], gastric cancer [22, 23], esophageal cancer [24] and hepatocellular carcinoma [18]. However, the association between PD-1 polymorphisms and progression of chronic hepatitis C and HCC development was unclear. So far, the role played by rs10204525 in chronic infection with HCV has been studied only in patients of Han ethnicity. To extend our knowledge about this potentially crucial polymorphism in North Africa, we investigated the influence of rs10204525 and mRNA expression on the outcome of chronic HCV infection in a Moroccan population.

Patients and Methods

Study Subjects

A cohort of 601 Moroccan subjects including 226 patients with chronic HCV infection, 101 patients spontaneously resolved from HCV infection and 200 healthy controls were enrolled in this study at the Medical Center of Biology at Pasteur Institute of Morocco and Service of Medicine B CHU Ibn Rochd Hospital, Casablanca, from September 2013 to September 2016. All individuals have given informed consent and the study was approved by the Ethics Committee of the Faculty of Medicine of Casablanca.

The persistent infection group was positive for anti-hepatitis C virus (anti-HCV) antibodies and HCV RNA determined by a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for at least six months. Among them, 95 had mild chronic hepatitis C (mCHC, patients with minimal fibrosis score F0 and F1-F2), 131 with HCV-related-AdLD (patients with advanced fibrosis F3-F4) and 74 with HCC. Spontaneous viral clearance was defined as anti-HCV antibodies positive and undetectable HCV RNA. The healthy controls had no medical history of any liver disease and they were negative for viral hepatitis markers and had normal serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Subjects co-infected with any other virus were excluded.

Serological markers for HBsAg, anti-HCV and anti-HIV were tested with commercially available kits (Abbott Architect i2000SR Analyzer (Abbott Laboratories, Abbott Park, IL, USA). All participants of this study gave their informed consent and in person interview was conducted using a structured questionnaire to get information about demographic data, medical history, lifestyle and other characteristics. The peripheral blood from the study subjects was collected into EDTA-containing tubes.

DNA extraction and PD-1 polymorphism genotyping

Peripheral blood samples were collected and stored at -20°C. The genomic DNA was carefully extracted from Peripheral Blood Mononuclear Cells (PBMC) of all subjects. The PD-1 rs10204525 A>G polymorphism was genotyped with primers and fluorescence dual color hybridization probes specific for PD-1 polymorphism (Applied

Biosystems; assay ID C_172862_10) using the LightCycler® 480 Real-Time PCR System (Roche diagnostics, Manheim, Germany).

RNA extraction and PD-1 mRNA expression

Total RNA was isolated from PBMCs using the TRIzol reagent (Invitrogen; USA). Total RNA was stored at −80°C until use. cDNA was synthesized from 10µl of extracted total genomic RNA by using MMLV Reverse Transcriptase (Bioline, London, UK) and Random Hexamer primer Mix, according to manufacturer's instructions. Quantitative real-time PCR on cDNA were performed in triplicates using SYBR Green PCR Master Mix (Applied Biosystems) and PD-1 primers sequences described previously [25]. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control and Ct was calculated from the differences in the mean Ct between the PD-1 gene and the internal controls. Primers used for GAPDH have been published previously [26].

Statistical Analysis

Statistical analysis was carried out using SPSS 21.0 software (SPSS, Chicago, Illinois, USA). Age was presented as the mean ± standard deviation (SD) and we used Student's t-test to determine the differences for continuous variables between groups. Chi-square test (X2) was used to compare the categorical variables and the genotypes distributions between groups. Hardy-Weinberg equilibrium was assessed by a goodness-of-fit $\chi 2$ test with 1 degree of freedom to compare observed and expected genotype frequencies. Multivariate logistic was conducted to obtain the odds ratios (OR) and their 95% confidence intervals (CI) for the relationship of PD-1 rs10204525 T>C polymorphism with hepatic fibrosis progression and hepatocellular carcinoma risk. The statistical significance was defined as ($P<0.05$).

Results

Demographic and clinical characteristics of the study subjects

The baseline of the general characteristics of the 300 chronic HCV patients, 101 spontaneous resolved patients and 200 healthy controls enrolled in this study are summarized in (Table 1). The age and gender between groups were not statistically different ($P>0.05$). Although, significant differences were observed in the distribution of ALT and AST between CHC group and healthy controls ($P<0.0001$). Likewise, total cholesterol, LDL-cholesterol and triglyceride levels were very significantly decreased in the CHC group as compared to the healthy subjects ($P<0.0001$). There was no difference in the distribution of parameters between CHC group and spontaneous resolved patients.

Effect of PD-1 polymorphism on the HCV infection and resolution

The genotype distributions of rs10204525 among chronic HCV patients, spontaneous resolved patients and healthy controls are shown in Table 2. Multivariate logistic regression analysis showed no significant associations of PD-1 polymorphism with HCV spontaneous clearance were observed between HCV chronically infected patients and subjects who spontaneously resolved the infection.

The impact of PD-1 polymorphism on progression liver disease and hepatocellular carcinoma

In order to analyze the effect of PD-1 polymorphism on liver disease progression and hepatocellular carcinoma in the Moroccan population, we compared genotypes distributions for rs10204525 in 95 patients with mCHC, 131 individuals with AdLD and 74 subjects with HCC. Among 95 of individuals with mild fibrosis, 59 (62.29%) were

homozygous for the wild type CC, 30 (31.58%) were heterozygous and 6 (6.31%) were homozygous for TT. In Advanced fibrosis and HCC group, genotype distribution was identified as, CC homozygous in 99 (48.29%) subjects, heterozygous in 88 (42.93%) and homozygous TT in18 (8.78%). The genotype distribution of this SNP was in accordance with the Hardy-Weinberg equilibrium (P>0.05). Multivariate logistic regression analysis showed the significant association of rs10204525 with progression liver disease (OR= 1.748, 95% CI = 1.034–2.955, P = 0.036). In addition, the T allele was related to an increased risk of HCC among patients with chronic HCV infection (OR= 1.528, 95% CI = 1.022–3.284, P = 0.038) (Table 3). These findings underline the importance of the functional polymorphism in PD-1 in the installation of HCV infection and its subsequent contribution to disease progression including the development of HCC.

Table 1: Demographic, clinical and biochemical characteristics of the subjects included.

Characteristics	**Persistently infected patients (n=300)**	**Spontaneous clearance subjects (n=101)**	**Healthy controls (n=200)**
Mean age ± SD, y	63.12 ± 12.11 [a*]	59.14 ± 13.75	56.11 ± 13.77
Gender N (%)			
Male	109 (36.33) [ns]	42 (41.58)	67 (33.5)
Female	191 (63.66)	59 (58.42)	133 (66.5)
Mean ± SD ALT (IU/L)	80.01 ± 55.87 [a*]	46.44 ± 56.24	35.33 ± 21.52
AST (IU/L)	68.28 ± 49.19 [a*]	34.98 ± 21.58	29.35 ± 16.39
Mean bilirubin (µmol/L)	15.19 ± 6.08	15.16 ± 11.17	Na
Mean creatinine (mmol/L)	108.54 ± 199.72	82.34 ± 34.55	Na
Fasting serum glucose (g/L)	1.06 ± 0.41 [a]	1.23 ± 0.54	0.95 ± 0.19
Total cholesterol (g/L)	1.53 ± 0.34 [a*]	1.72 ± 0.37	1.91 ± 0.38
Triglycerides (g/L)	1.05 ± 0.38 [a*]	1.37 ± 1.33	1.31 ± 0.68
HDL-cholesterol (g/L)	0.52 ± 0.17	0.48 ± 0.10	0.54 ± 0.37
LDL-cholesterol (g/L)	0.82 ± 0.53 [a*]	0.94 ± 0.35	1.12 ± 0.36
Median viral load (IU/ml)	2.8 E+06	-	-
	[0.9 E+03-64.5 E+06]		
Viral genotypes (%)			
Genotype 1	59.90	-	-
Genotype 2	39.06	-	-
Genotype 3	0.52	-	-
Genotype 4	0.52	-	-
mCHC	95		
AdLD	131		
HCC	74		

ALT: Alanine aminotransferase
AST: Aspartate aminotransferase
SD: Standard deviation

[a]Significant values (P < 0.05) when compared to the healthy controls
[a*]Significant values (P < 0.0001) when compared to the healthy controls
Na: Non applicable

Table 2: Effect of PD-1 polymorphism on the HCV infection susceptibility and resolution.

	Healthy controls (n=200)	**Persistent infection (n=300)**	**Subjects with spontaneous clearance (SpC) (n = 101)**	**Healthy controls vs. Subjects Persistent infection OR (95% CI)**	**P-value**	**Subjects with SpC vs. Subjects with persistent infection OR (95% CI)**	**P-value**
PD (rs1020452 5) CC	88 (44%)	158 (52.67%)	56 (55.45%)	1 (Reference)	-	1 (Reference)	-
CT	89 (44.5%)	118 (39.33%)	32 (31.68%)	0.738 (0.505-1.079)	0.11653	1.307 (0.796-2.145)	0.28894
TT	23 (11.5%)	24 (8%)	13 (12.87%)	0.581 (0.310-1.090)	0.08826	0.654 (0.312-1.372)	0.25930
C allele	0.662 ± 0.023	0.723 ± 0.018	0.713 ± 0.035	1 (Reference)	-	1 (Reference)	-
T allele	0.338 ± 0.023	0.277 ± 0.018	0.287 ± 0.035	0.751 (0.571-0.987)	0.03992	0.950 (0.667-1.352)	0.77437
Dominant		158/142	56/45	0.706 (0.493-1.012)	0.05756	1.118 (0.711-1.760)	0.62824
model Recessive model		276/24	88/13	1.494 (0.818-2.729)	0.18891	1.699 (0.830-3.477)	0.14343

Table 3: The impact of PD-1 polymorphism on progression liver disease and hepatocellular carcinoma.

Healthy controls (n = 200)	**Mild fibrosis group (n = 95)**	**Advanced fibrosis and HCC group (n = 205)**	**Healthy controls vs. Advanced fibrosis and HCC group OR (95% CI)**	**P-value**	**Mild fibrosis group vs. Advanced HCC Patients OR (95% CI)**	**P-value**
PD-1 (rs1020452) CC 88 (44%)	59 (62.11%)	99 (48.29%)	1 (Reference)	-	1 (Reference)	-
CT 89 (44.5%)	30 (31.58%)	88 (42.93%)	0.879 (0.582-1.326)	0.53853	1.748 (1.034-2.955)	0.03612
TT 23 (11.5%)	6 (6.31%)	18 (8.78%)	0.696 (0.352-1.374)	0.29434	1.788 (0.672-4.757)	0.23971
C allele 0.662 ± 0.023	0.779 ± 0.031	0.698 ± 0.022	1 (Reference)	-	1 (Reference)	-
T allele 0.338 ± 0.023	0.221 ± 0.031	0.302 ± 0.022	0.851 (0.633-1.144)	0.28475	1.528 (1.022-2.284)	0.03817
Dominant	59/36	99/106	0.841 (0.569-1.244)	0.38629	1.755 (1.068-2.884)	0.02582
model Recessive	89/6	187/18	1.350 (0.705-2.586)	0.36434	0.700 (0.269-1.825)	0.46418

PD-1 rs10204525 polymorphism and clinical markers in patients with chronic HCV infection

Demographic, biochemical and viral data were analyzed according to PD-1 rs10204525 polymorphism (Table 4). Individuals with CT genotype have higher AST levels compared to subjects with CC and TT genotype (P<0.005). Though, other clinic-pathological markers did not reveal any significant difference (P>0.05, Table 4).

Table 4: PD-1 rs10204525 polymorphism and clinical markers in patients with chronic HCV infection.

	CC genotype (n=125)	**CT genotype (n=82)**	**TT genotype (n=19)**
Mean age ± SD, y	60.94 ± 12.26	61.44 ± 11.38 ^{ns}	61.89 ± 14.65 ^{ns}
Gender (%) Male	39 (31.2)	21 (25.61) ^{ns}	7 (36.84) ^{ns}
Female	86 (68.8)	61 (74.39)	12 (63.16)
Mean ± SD			
ALT (IU/L)	74.01 ± 51.21	83.50 ± 62.51 ^{ns}	87.00 ± 55.82 ^{ns}
AST (IU/L)	57.40 ± 33.98	**78.54 ± 62.62** **	59.11 ± 23.05 ^{ns}
Mean bilirubin (µmol/L)	15.53 ± 6.28	15.00 ± 6.17 ^{ns}	14.27 ± 4.25 ^{ns}
Mean creatinine (mmol/L)	105 ± 192.71	100.50 ± 221.52 ^{ns}	192.92 ± 314.05
HDL-cholesterol (g/L)	0.54 ± 0.18	0.50 ± 0.16 ^{ns}	0.54 ± 0.13 ^{ns}
LDL-cholesterol (g/L)	0.82 ± 0.65	0.85 ± 0.31 ^{ns}	0.73 ± 0.19 ^{ns}
Median viral load (IU/ml)	2.4 E+06	3.2 E+06 ^{ns}	2.8 E+06 ^{ns}
	[0.9 E+03-31.8 E+06]	[0.9 E+03-64.5 E+06]	[9.6 E+03-9.4 E+06]
Viral genotypes n (%) Genotype 1	85 (68)	51 (62.2) ^{ns}	14 (73.7) ^{ns}
Genotype 2	38 (30.4)	31 (37.8)	5 (26.3)
Genotype 3	1 (0.8)	-	-
Genotype 4	1 (0.8)	-	-
mCHC	59	30 ^{ns}	6 ^{ns}
AdLD	66	52	13

ALT: Alanine aminotransferase

AST: Aspartate aminotransferase

SD: Standard deviation

*Significant values (P < 0.05) when compared to the CC genotype

** Significant values (P < 0.005) when compared to the CC genotype

*** Significant values (P < 0.0001) when compared to the CC genotype

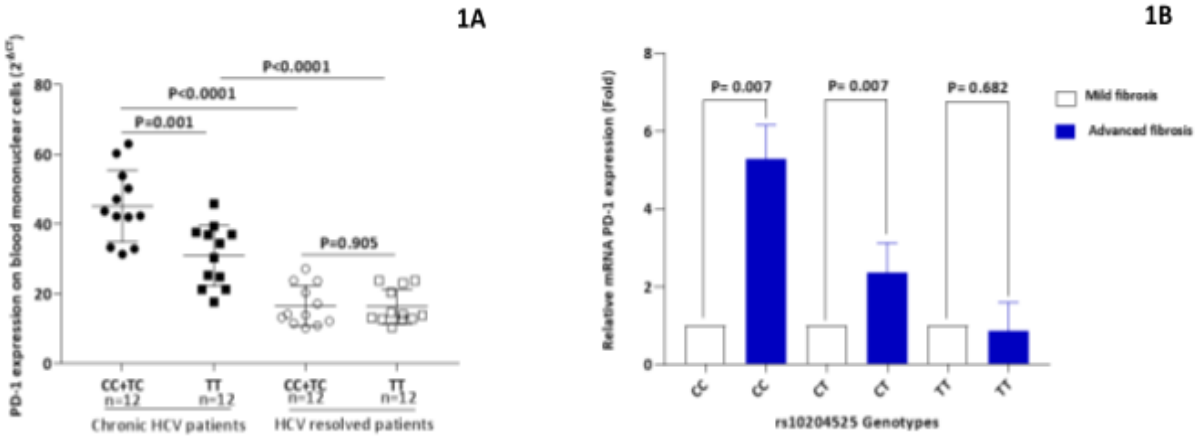
Na: Non applicable

Analysis of PD-1 mRNA expression according to rs10204525 genotypes and clinical parameters

The PD-1 mRNA expression levels were detected in peripheral mononuclear cells of chronic HCV patients and spontaneous resolved individuals according to rs10204525 genotypes. As shown in Figure 1A, the expression of PD-1 mRNA in chronic HCV patients was significantly higher than that in HCV resolved patients (P<0.0001). Among chronic HCV patients, the expression of PD-1 mRNA was significantly higher in patients with PD-1 rs10204525 CC or TC genotype than those carrying the TT genotype (P=0.001). No significant difference was observed in PD-1 rs10204525 genotypes among HCV resolved patients (Figure 1A). To evaluate the effect of PD-1 gene expression in HCV-infected patients at different stages of liver disease. Taking as baseline expression levels in mCHC patients, a

statistically significant 6-fold upregulation of PD-1 expression was observed in the AdLD group carrying CC genotype (P=0.007, Figure 1B).

Figure 1: PD-1 expression on blood mononuclear cells in chronic HCV patients and HCV resolved patients according to rs10204525 genotypes. Mean ± SD in different groups are shown. The horizontal bars among the symbols indicate mean and 95% CI values in each group. P-values were determined by independent t-test (1A). PD-1 relative expression levels according to rs10204525 genotypes in HCV infected patients with advanced fibrosis and mild fibrosis patients (1B).



In addition, PD-1 mRNA was overexpressed in chronic HCV infected patients with cirrhosis and hepatocellular carcinoma when compared to mild fibrosis group (P=0.0002) and the expression was even more pronounced when compared to HCV resolved group (P<0.0001, Figure 2). Overall, differences in PD-1 expression were found to be associated with liver disease progression. Likewise, similar analysis according to HCV RNA was shown that PD-1 mRNA relative expression levels was significantly different among patients with HCV RNA levels (IU/mL) of <103, 104~, 105~ and ≥106 (P<0.0001), (Figure 3). The analysis of PD-1 gene expression according to age, sex and metabolic parameters of the population did not show any significant differences (data not shown).

Figure 2: PD-1 mRNA levels in patients with different clinical diseases of chronic hepatitis C infection and HCV resolved patients.

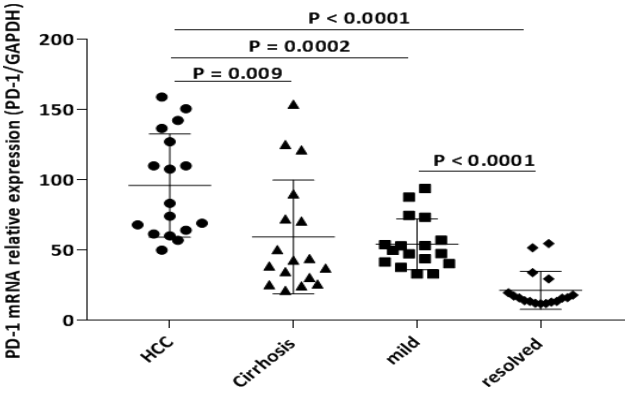
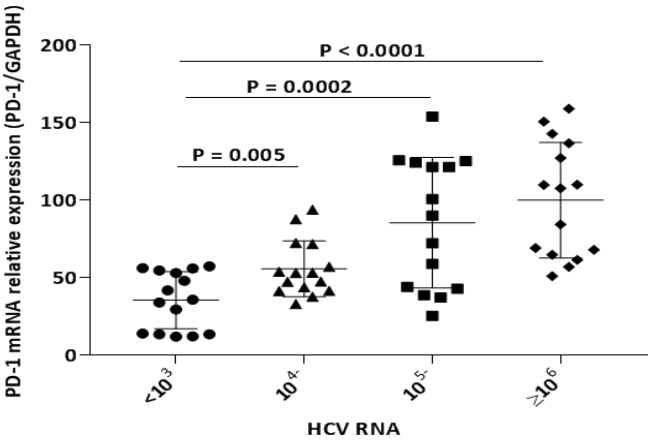


Figure 3: PD-1 mRNA expression in patients with chronic HCV infection of different HCV RNA levels.



Discussion

Chronic HCV infection is a major cause of cirrhosis and HCC [27, 28]. It is known that genetic variation of the host, viral and environmental factors are modulating disease progression [29]. In this study, we analyzed the impact of rs10204525 and mRNA expression on and outcome of chronic HCV infection. The PD-1 protein plays a role in the progression to chronic viral infection [30]. It has been reported in various studies that an elevated expression of PD-1 gene on peripheral lymphocytes is strongly associated with the dysfunction of immune response in chronic HCV infection and HCV-related HCC [31, 32]. Additionally, it has described that PD-1 attenuated the immunoregulation of T cells, which finally resulted in chronic viral infection [9].

In the present study, a significant relationship between PD-1 expression and HCV infection progression was found. PD-1 mRNA was highly expressed in patients with cirrhosis and HCC and this expression was significantly associated with the CC genotype of rs10204525 polymorphism. In addition, a significant association between increased PD-1 expression on PBMCs and high HCV RNA load levels of HCV viral replication was revealed in this study. This finding supports previous studies that the increased PD-1 mRNA levels were correlated with higher HCV RNA levels, which represents an indicator of active HCV viral replication [31, 33]. With respect to the association of PD-1 mRNA levels with rs10204525 variant, this study showed that PD-1 mRNA expressions were sequentially increased from PD-1 rs10204525 genotypes TT, CT to CC ($p=0.001$). This result seems in line with recent studies conducted in the Moroccan HBV infected subjects [34] and in treated HIV-1-infected Moroccan subjects ($p< 0.005$) indicating the same observations [35]. However, at odds with our findings, Xiao and colleagues found a decrease in PD-1 mRNA levels from PD1 rs10204525 TT, CT, to CC genotype in patients with CHC [17]. The same result was observed also in CHB [25]. Interestingly, the significant increase of PD-1 mRNA levels in patients carrying TT genotype was the hallmark of chronic hepatitis in Eastern Asian populations [17, 25]. In the current study, we found that T allele was associated with progression of liver fibrosis and related to HCC among patients with chronic HCV infection. This result was in opposite with earlier studies showing that T allele was associated with spontaneous clearance [17]. This discrepancy between results found in studies conducted on subjects with different ethnicity may be related to the genetic background or to allele frequency differences between populations.

In conclusion, this study showed that patients with chronic infection had significantly elevated levels of PD-1 mRNA expression that were correlated with clinical diseases and HCV viral replication as well as PD-1 rs10204525 polymorphism, suggesting that increased PD-1 expression may affect the disease course of chronic HCV infection

by facilitating HCV viral replication via suppressing host antiviral immune response, and this may at least partially relate to rs10204525 in PD-1 3'-UTR. These data may provide immunogenetic information for monitoring disease prognosis and designing immunotherapeutic approaches to HCV-associated diseases.

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Experimental Ethics

All individuals gave informed consent and the study was approved by the Ethics Committee of The Faculty of Medicine of Casablanca in accordance with the ethical guidelines of the Declaration of Helsinki

Disclosure of Conflicts of Interest

The authors declare no conflicts of interest

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