Egyptian Journal of Medical Microbiology Volume 29 / No.2 / April 2020 55-62 Online ISSN: 2537-0979

ORIGINAL ARTICLE

Polymorphism of programmed death-1 (PD-1) gene in relation to susceptibility and progression of hepatitis B virus infection in Egyptian patients

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Key words:
PD-1polymorphism, HBV, HCC

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ABSTRACT

Background: *chronic infection with hepatitis B virus (HBV) is a major risk factor for liver cirrhosis and hepatocellular carcinoma (HCC). Programmed death-1 (PD-1) has been involved in regulating immune responses to viral infections and tumors and has a critical role in HBV infection.* ***Objectives:*** *to investigate the effect of PD1 (+8669 G/A) single-nucleotide polymorphism (SNP) on disease susceptibility and progression of chronic HBV infection in Egyptian patients.* ***Methodology:*** *Blood samples were taken from 70 patients with confirmed chronic hepatitis B (CHB), HBV-related liver cirrhosis (LC) or HCC on top of HBV infection and 25 healthy controls. Genotyping of PD1 (+8669 G/A) polymorphism was studied using bidirectional PCR amplification of specific alleles (Bi-PASA).* ***Results:*** *A significantly higher frequency of PD-1 (+8669 G/A) AA genotype and A allele was found in CHB group (36.8 %) and LC group (21.7%) compared to control group (0.0%) (P< 0.05). On the other hand, the GG genotype and G allele were over represented in healthy controls (72.7%) than CHB group (52.6%) and LC group (47.8%). In assessing the risk of susceptibility to infection with HBV, The G allele was significantly predominant in healthy subjects compared to hepatitis B patients (89.4% versus 68.5%) (P< 0.05). G allele was markedly increased with disease progression to HCC (P = 0.006).* ***Conclusion:*** *Our study suggested that PD1 (+8669) polymorphism has significantly implicated in the disease susceptibility with AA genotype and A allele as a predisposing and the GG genotype and G allele as a preventive factors for chronic HBV infection. Also this study revealed a significant effect of the PD1 (+8669) polymorphism in the progression to HCC in hepatitis B patients.*

INTRODUCTION

Despite the existence of prophylactic vaccines, HBV remains a major global health problem, with approximately 257 million people infected with the virus worldwide. CHB cannot be cured and is considered a major risk factor for hepatic cirrhosis and HCC [1].

The outcome of HBV infection depends on the interaction between the virus and the host immunity [2]. Four phases of liver diseases may occur during the natural course of HBV infection with variable severity including; asymptomatic carrier status, CHB, LC and HCC [3].

Immunologic responses together with genetic background play an important role in the immunopathogenesis of HBV infection due to the noncytopathic nature of the virus to the hepatocytes [4]. The T cell exhaustion which is a deterioration of T cell function is a major characteristic of CHB which contributes to the immunopathogenesis of liver diseases associated with HBV as cirrhotic changes of the liver and HBV carcinogenesis [5].

PD-1, an inhibitory modulator of T cell activity, is playing a crucial role in the regulation of immune responses against viral infections and tumors [6]. PD-1 is expressed on T-cells, B-cells and myeloid cells as an inhibitory immunereceptor. It is a member of the immunoglobulin super family B7/CD28 which involved in T cells and B cells activation [7].

In chronic viral hepatitis, PD-1 is involved in the down-regulation of immune responses [3], because it is significantly up-regulated on the virus specific T cells leading to inhibition of T cell proliferation and cytokine production[8]. Thus positive correlation is suggested between expression of immune inhibitory factors and viral disease chronicity [9].

More than 30 SNPs within the PD-1 gene have been identified. Many studies have highlighted the effect of some SNPs in PD-1 on the gene expression and

transcription[10], such as polymorphisms in the promoter or intron region of the gene; these studied attempted to study the pathogenesis of many immune related-diseases as rheumatoid arthritis (RA) [11], systemic lupus erythematosus (SLE) [12], and type1 diabetes [13].

SNP PD1 +8669 G/A in the 3'-untranslated region (3'-UTR) of PD-1 has also received much attention in HBV infection [14], and considering the possible effects of this genetic polymorphism on the host immune response to the virus, the current study aimed to study the impact of PD1 (+8669 G/A) polymorphism on the genetic susceptibility and progression of chronic HBV infection in Egyptian patients.

METHODOLOGY

Study participants:

This study was conducted in the period from November 2017 to December 2019, in National Liver Institute, Menoufia University, Egypt. A total of 95 study participants included 70 patients with confirmed HBV infection, and 25 healthy controls. The patients were classified into three groups: 25patients with CHB (Group A), 25 patients with LC (Group B), and 20 patients with HCC on top of HBV (Group C). Group D included 20 healthy controls with both HBsAg and HBV DNA negative and no history of liver disease or any other viral diseases.

Diagnosis of chronic HBV infection was based on positive serological findings for HBsAg, HBeAg or anti- HBc and anti-HBe for more than six months and chronic hepatitis was defined as chronic infection with HBV with persistent or intermittent elevation of liver functions parameters or with evidence of liver tissue lesions, without the evidence of liver cirrhosis or HCC [15]. HBV-related liver cirrhosis was diagnosed by liver biopsy or imaging criteria of cirrhosis in ultrasound and computerized tomography (CT) with positive HBsAg, and elevated liver function parameters. The diagnosis of HBV-related HCC was based on positive CT and magnetic resonance imaging, or positive findings on pathological or cytological examination of liver biopsy [16]. The institutional review board of the National Liver Institute approved the study and written informed consents were taken from all participants before their enrollment in the study.

Exclusion criteria:

Individuals who were infected with other viral infections or those received antiviral treatment or immunotherapy during the past 6 months, and patients report any other type of liver disease (for example, autoimmune hepatitis, and so on), or other cancers were excluded from the study.

Laboratory investigations and detection of HBV:

Demographic data were collected from all subjects. Liver functions tests were done on Integra 800 Auto analyzer (Roche-Germany Catalogue number; M, 87432). HBV markers were measured by DIA.PRO Diagnostic Bioprobes kit (Milano, Italy), based on Enzyme Linked Immunosorbent Assay (ELISA) technique according to manufacturer's instructions. Detection and quantification of HBV-DNA was quantified using the COBAS TaqMan HBV version 2.0 assay (Roche Diagnostics, Tokyo, Japan).

Molecular detection of PD1 (+8669 G/A) polymorphism:

Blood samples were collected from all participants in the morning after an overnight fast. Genomic DNA was extracted from 3 ml of EDTA-treated peripheral blood using QIAGEN DNA extraction kit following the manufacturer's instructions.

Genotyping of PD-1 polymorphism was performed by bidirectional polymerase chain reaction amplification of specific alleles (Bi-PASA). Four primers were used, 2 outer primers (P and Q) and 2 inner allele-specific primers (A and B) (Table 1). The Bi-PASA amplification reaction was performed on a final volume of 25 µl containing 12.5 µl of 2×Master Mix (2×Taq PCR Mix, 1.0 µl (10 µmol/l) of each of P primer and Q primer, 0.5 µl (10 µmol/l) of each of A primer and B primer, and 3.5 µl of double-distilled H2O. The thermocycler conditions were 3 minutes at 94˚C, followed by amplification for 30 cycles by denaturation at 94˚C for 30 seconds, annealing at 65˚C for 30 seconds and extension at 72˚C for 1 minute in each cycle and a final extension at 72˚C for 10 minutes. PCR products were analyzed by 2% agarose gels after ethidium bromide staining and revealed under UV light [17]. Genotypes were determined for all samples and the products size of each genotype were mentioned in table 1. Figure.1 shows a representing sample of gel electrophoresis.

Table 1: primers used in the study.

Target SNP	Primers sequences (3`-5`)	Product size (bp)	Reference
P-D1(+8669 G/A)	P: 5'-GAAGTTTCAGGGAAGGTCAG-3' Q: 5'-CAGTGTGTGGATGTGAGGAG-3' A: 5'-GGGCGGGCGGACCTAGGGCCCCCCATA-3'(variant-specific) B: 5'-GGGCGGGCGGAGCTCCCAGGGTGGGCAC-3'(wild-type specific)	GG genotype: 2 fragments of 490 and 353 bp AA genotype: 2 fragments of 490 and 171 bp GA genotype: 3 fragments of 490, 353 and 171 bp	**(17)**

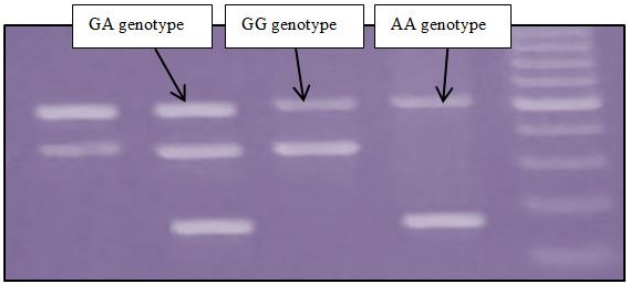

Fig. 1: Agarose gel electrophoresis demonstrated the PD-1 genotypes using 100 bp ladder

Statistical analysis:

Analysis of data was done using SPSS (Statistical Package for Social Science) program, (version 20; SPSS Inc., Chicago, IL). Fisher's exact test or $\chi 2$ was used to compare qualitative variables. Calculation of odds ratios and 95% confidence intervals was done using logistic regression analysis for risk estimation. P values less than 0.05 were considered significant.

RESULTS

This study included 70 patients chronically infected with HBV and 25 healthy volunteers as control group. They were categorized into four groups. Baseline demographic and laboratory data of the studied groups are shown in table 2. There was highly significant difference between the studied groups as regard age. Also there were significant higher levels of serum AST, and bilirubin in HCC, LC and CHB patients compared to the control group. But albumin levels were decreased significantly in HCC and LC groups when compared with the control and CHB groups.

Table 2: Demographic and laboratory data of the studied groups.

Characteristics	Group A (CHB) N=25	Group B (LC) N=25	Group C (HCC) N=20	Group C (Controls) N=25	Test of significance	P-value
Age (years): Mean±SD	37.90±9.914	54.33±9.299	57.89 ± 9.719	44.56±9.891	ANOVA= 16.6	P <0.001
Gender					$\chi 2$	.4
Male	16(64%)	14(65%)	16(80%)	18(68%)	=2.96	NS
Female	9(36%)	11(44%)	4(20%)	8(32%)		
ALT Mean±SD	55.17±68.01	49.00±24.42	46.50±12.20	30.84±5.19	Kruskal-wallis 8.662	.034
AST Mean±SD	51.96±89.71	43.45±16.92	34.25±19.34	25.20±5.59	Kruskal-wallis 12.800	.005
Albumin Mean±SD	4.28±.41	3.41±.58	2.94±.85	4.36±.24	Kruskal-wallis 33.807	P <0.001
DBIL (µmol/L) Mean±SD	.57±1.04	.33±.16	1.43±1.51	.13±.03	Kruskal-wallis 31.056	P <0.001
TBIL (µmol/L) Mean±SD	1.55±1.68	1.46±1.21	2.25±1.83	.66±.11	Kruskal-wallis 21.093	P <0.001
HBV DNA level) (10^3IU/ml) Mean±SD	14035±47480	11151±33861	2772±3306	_	ANOVA 0.249	.781 NS

*:Kruskal-Wallis test, $\chi 2$ = Chi-square test, AST**:** aspartate aminotransferase, ALT: alanine aminotransferase, DBIL: Direct bilirubin, TBIL: Total bilirubin.

Hepatitis B markers of the studied patients are presented in table (3), All of the patients were positive for HBsAg and anti-HBc antibodies. There was no significant difference between the studied groups.

Table 3: Hepatitis B markers among hepatitis B patients

		Group A (CHB)		Group B (LC)		Group C (HCC)		χ^2	P- value
		No.	%	No.	%	No.	%		
HBs Ag	**Positive**	25	100.0%	25	100.0%	20	100.0%	-	-
	Negative	0	0.0%	0	0.0%	0	0.0%		
HBs Ab	**Positive**	0	0.0%	0	0.0%	0	0.0%	-	-
	Negative	25	100.0%	25	100.0%	20	100.0%		
HBe Ag	**Positive**	4	16.0%	1	4.0%	5	25%	5.194	.075
	Negative	21	84.0%	24	96.0%	15	75%		
HBe Ab	**Positive**	4	16.0%	1	4.0%	2	10.0%	2.000	.368
	Negative	21	84.0%	24	96.0%	18	90.0%		
antiHBc Ab	**Positive**	25	100%	25	100%	20	100%		
	Negative	0	0.0%	0	0.0%	0	0.0%		

The AA genotype and the A allele were significantly predominant in CHB group **(**36.8 %) and LC group (21.7%) compared to the control group (0.0%) (P< 0.05). On the other hand, the genotype GG and G allele were over represented in healthy controls (72.7%) than CHB group (52.6%) and LC group (47.8%) (Table 4).

Table 4: Distribution of PD1 (+8669 G/A) genotypes and alleles among the studied groups.

Genotypes and allele frequencies		Group A (CHB)		Group B (LC)		Group C (HCC)		Group D (Controls)		Test of significance	P-value
		No.	%	No.	%	No.	%	No.	%		
PD1	**AA**	7	36.8	5	21.7	2	10	0	0	χ^2= 15.245	0.018 S
	GA	2	10.5	7	30.4	2	10	6	27.3		
	GG	10	52.6	11	47.8	16	80	16	72.7		
PD1 Allele	**A**	16	42.1	17	37	6	15	6	13.6	χ^2= 13.687	.003 S
	G	22	57.9	29	63	34	85	38	86		

χ^2 = Chi-square test

The frequencies of PD-1 (+8669 G/A) genotypes and alleles in HBV-infected patients (combined) and controls are presented in table (5). The G allele was significantly predominant in healthy controls compared to cases (89.4% versus 68.5%) (P< 0.05). The AA genotype and the A allele were over represented in cases than controls but this difference is of no statistical importance (P> 0.05).

Table 5: The genotypes and allele frequencies of PD-1 (+8669 G/A) in patients with CHB, LC and HCC combined (cases) and controls.

Genotypes and alleles frequencies		Cases		controls		P - value	OR	95% CI
		No.	%	No.	%			
PD1	**AA**	14	22.6	0	0	0.925	#	
	GA	11	17.7	6	27.3	0.998	#	
	GG	37	59.7	16	72.7	0.998	#	
PD1 Allele	**A**	39	31.5	6	13.6		1®	
	G	85	68.5	38	86.4	0.022	0.34	0.13 - 0.88

®: Reference value for odds ratio OR: Odds ratio, CI: Confidence interval

The association of PD1 polymorphism with the risk of progression of CHB into LC was estimated by Logistic regression analysis of PD1 genotypes between CHB and LC. No significant difference was noticed between the genotypes and alleles frequencies of PD-1 (+8669 G/A) and the progression risk to LC (Table 6).

Table 6: Association of PD1 (+8669 G/A) polymorphism with the risk of progression of CHB to LC

Genotypes and alleles frequencies		Group A (CHB)		Group B (LC)		Test of significance	P-value	OR	95% CI
		No.	%	No.	%				
PD1	AA	7	36.8	5	21.7	$\chi 2$= 2.8	.273	1®	
	GA	2	10.5	7	30.5		.109	4.900	0.7 -34.3
	GG	10	52.6	11	47.8		.555	1.540	0.368-6.448
PD1 Allele	A	16	42.1	17	37	$\chi 2$= 0.23		1®	
	G	22	57.9	29	63		0.63	1.24	0.51- 2.99

®: Reference value for odds ratio OR: Odds ratio, CI: Confidence interval, $\chi 2$ = Chi-square test

The association of PD1 polymorphism with the risk of progression to HCC in hepatitis B patients was estimated by Logistic regression analysis of PD1 genotypes between (CHB + LC) versus HCC. GG genotype was predominant in HCC patients (80%) compared to non HCC patients (50%). Also the G allele is significantly overpresented in HCC patients (85%) compared to non HCC patients (60.7%) with P< 0.05. (Table 6)

Table 6: Association of PD1 (+8669 G/A) polymorphism with the risk of progression to HCC

Genotypes and alleles frequencies		CHB +LC		HCC		Test of significance	P-value	OR	95% CI
		No.	%	No	%				
PD-1 genotypes	**AA**	12	28.6	2	10	$\chi 2$= 5.11	.094	1®	
	GA	9	21.4	2	10		.792	1.333	.157-11.36
	GG	21	50	16	80		.068	4.571	.894-23.38
PD-1 alleles	**A**	33	39.3	6	15	7.41		1®	
	G	51	60.7	34	85		0.006	3.67	1.39- 9.69

®: Reference value for odds ratio OR: Odds ratio, CI: Confidence interval, $\chi 2$ = Chi-square test

DISCUSSION

One of the regulatory functions of the human immune system is to maintain the balance between eradicating pathogen and avoiding tissue damage from excessive immune response. This achieved by complex interactions between antigen-presenting cells (APCs) and T cells, and the expression of immune checkpoint receptor PD-1 on T cell is important for immune homeostasis [18].

PD-1, an important inhibitory receptor on T cell, has been noticed to be involved in several diseases such as chronic viral infections [19]. Increasing evidence showed that the level of this gene expression in the liver and the degree of hepatocytes inflammation were positively correlated, suggesting that the PD-1 pathway has an important role in protecting the liver from immune-mediated destruction [20].

Based on the important role of PD-1 pathway in the HBV infection, we aimed to evaluate the associations between PD1 (+8669 G/A) and HBV susceptibility and progression in Egyptian patients.

In the present study, The LC and HCC patients were significantly older compared to CHB patients and healthy controls. Nearly similar results were reported by Ji et al. [21], and Zhengwen et al., [22] . These findings can be explained by the the natural course of HBV infection as patients with chronic infection can remain asymptomatic for long periods, but by the time some of the carriers may develop cirrhosis or HCC as they become older.

While Li et al., [23] , Hou et at., [18] and Han et al., [24] reported no statistical significant age difference between HBV patients with different clinical diagnosis.

In the current study, a statistically highly significant difference was noticed between the studied groups as regard serum albumin, total and direct bilirubin. Also there was a significant difference between groups as regard AST and ALT. Similar findings were reported by Zhang et al., [17]and Hou et al., [18].

Our study showed that there was no significant difference between patients groups regarding viral load (P> 0.05). In contrast, Hou et al., [18] found that there was highly significant difference between groups regarding to viral load. While Ji et al., [21] found that circulating HBV DNA was significantly lower in HCC patients than in HBV-infected patients without HCC.

In the current study, all patients were positive for HBs Ag and antHBc Ab. Also there was no significant difference between different patients groups regarding

HBe Ag and HBe Ab. In contrast, Li et al., [23] found positive correlation between HBeAg positivity and cirrhosis and HCC in HBV infected subjects.

Regarding the distribution of the PD1 (+8669) genotypes and alleles in the studied groups, The AA genotype and the A allele were significantly predominant in CHB group (36.8 %) and LC group (21.7%) but not detected in control group (0.0%) ($P< 0.05$).While GG genotype and G allele were over represented in healthy controls (72.7%) than CHB group (52.6%) and LC group (47.8%). These findings suggested that the PD1 (+8669) polymorphism may play an important role in HBV infection in the Egyptian population.

Our results agreed with Zhang et al., [17] , Li et al., [23] and Hou et al., [18] who reported that PD1 (+8669) genotype AA and A allele may have a predisposing role in HBV infection. They suggested that the genetic variation of PD-1 gene may affect the gene expression and function,hence affecting T-cell activation and immune responses to HBV via PD-1: PD-L pathway.

However a study in China disagreed with our results, reported that PD1 gene polymorphism has no correlation with genetic susceptibility to HBV infection in Chinese patients [6].

PD-1 (+8669 G/A) Polymorphism and susceptibility to HBV infection was investigated in the present study. Interestingly we found the genotype GG was overpresented in controls than patients (72.7% versus 59.7%) and the G allele was significantly predominant in healthy subjects compared to patients (89.4% versus 68.5%) ($P< 0.05$). On the other hand the frequency of genotype AA and the allele A were higher in patients than controls. These results agreed with that reported by Zhang et al., [17] Li et al., [23] and Hou et al., [18].

According to these findings the genotype AA and A allele might be considered a predisposing factor for infection with HBV, while genotype GG or G allele might be preventive factor, as PD1 (+8669) polymorphism with G allele may be implicated in activation of T-cell and production of interferon-γ, tumor necrosis factor and other antiviral cytokines associated with the immune responses through down regulation of PD-1 expression in the T cells, conferring a protective effect [17].

Regarding the role of PD-1 (+8669 G/A) in the progression of CHB to LC or HCC, the current study revealed that the frequencies of PD-1(+8669) G allele was 60.7% in non HCC patients (combined CHB and LC) compared to 85% in HCC patients. These results were significantly different ($p= 0.006$), suggesting that the PD1 (+8669) polymorphism may also have predisposing role in the disease progression to HCC. But the relation between the genotypes and alleles frequencies of PD-1 (+8669 G/A) and the progression risk to LC was insignificant.

The results of this study confirmed the previous reports which suggested that PD1 (+8669) polymorphisms may be involved in the progression of CHB to cirrhosis and HCC [17, 23].

Our results can be explained by the fact that PD-1 upregulation on HBV-specific CD8+ T cells and PD-1/PD-L1 interaction are involved in the dysfunction and exhaustion of T cells in CHB infection and increased expressions of PD-1 on T cells from the circulation, tumor and the liver in HCC patients may relate to T-cell exhaustion in tumor genesis. Notably, PD-1/PD-L1 pathway blockade could improve T-cell functions in persistent infection with HBV and increase the frequency of tumor-specific T cells in HCC patients [23].

Our study has some limitations, including the relatively small sample size also HBV was only genotyped in 76% of CHB patients, 92% of LC patients, 100% of HCC patients and 88% of control enrolled in this study, The explanation for our findings may also be precluded by the multiple factors involved in the course of the disease, and therefore more investigations are needed to clarify the implications.

CONCLUSION

In conclusion, our study on HBV patients in Egypt suggests that PD1 (+8669) polymorphism may be involved in the disease susceptibility with AA genotype and A allele as a predisposing and the genotype GG and G allele might be preventive factors for chronic HBV infection. Also this study revealed a significant effect of the PD1 (+8669) polymorphism in the progression to HCC in hepatitis B patients. However, prospective longitudinal studies in large populations of the susceptible polymorphisms are needed to replicate and enrich the multiple genetic factors associated with HBV infection including SNPs of PD-1.

Authors' Contributions

All authors contributed to conception, design, analysis of data and the manuscript writing.

Conflict of interest:

- The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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