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Evaluation of the Association between *Programmed Cell Death-1* Gene Polymorphisms and Hepatocellular Carcinoma Susceptibility in Turkish Subjects. A Pilot Study

Abdullah Fatih Demirci[1], Coskun Ozer Demirtas[2], Fatih Eren[3,4], Demet Yılmaz[5], Caglayan Keklikkiran[2], Osman Cavit Ozdogan[2], Feyza Gunduz[2,4]

1) Marmara University, School of Medicine, Department of Internal Medicine, Istanbul;
2) Marmara University, School of Medicine, Department of Gastroenterology, Istanbul;
3) Marmara University, School of Medicine, Department of Medical Biology, Istanbul;
4) Marmara University, Institute of Gastroenterology, Istanbul;
5) Marmara University, Institute of Health Sciences, Medical Biology and Genetics, Istanbul, Turkey

ABSTRACT

Background & Aims: Programmed cell death-1 (PD-1) has a vital role in regulating T-cell function, and immune escape mechanism of cancer cells. It was shown that there could be a relationship between single nucleotide polymorphisms (SNPs) in the *PD-1* gene and susceptibility to hepatocellular carcinoma (HCC) based on various studies. We aimed to investigate the role of three SNPs within the *PD-1* gene in susceptibility to HCC in the Turkish population.

Methods: Single nucleotide polymorphisms of *PD-1.1, 1.5*, and *1.6* were genotyped by using TaqMan Allelic Discrimination Assays in blood samples of 137 HCC and 136 control subjects, matched for age and gender. The genotype, allele and haplotype frequencies were compared in HCC and control groups using logistic regression analysis.

Results: Genotype distributions of P*D-1.1, PD-1.5* and *PD-1.6* SNPs were in Hardy-Weinberg equilibrium. No significant difference was observed in the genotype distribution of *PD-1.1, PD-1.5* and *PD-1.6* polymorphisms among gender and age-matched HCC (M/F: 96/41; mean age: 61.4 ±11.7 years) and control group (M/F: 94/42; mean age: 61.4±10.1). In the haplotype analysis of *PD-1.1/PD-1.5/PD-1.6*, no significant difference was found among HCC and control group adjusted for sex and age (all p values>0.1).

Conclusion: Our findings, firstly reporting the association of *PD-1.5* polymorphism with HCC, and *PD-1.1* and *PD-1.6* with HCC in the Turkish population, suggest that *PD-1* polymorphisms are not predisposing factors for HCC development. Future studies with larger sample sizes and different ethnic populations are required to validate our findings.

Address for correspondence:
Feyza Gunduz, M.D.
Marmara University School of Medicine
Department of Internal Medicine, Section of Gastroenterology
drfgunduz@yahoo.com

Key words: hepatocellular carcinoma – *programmed cell death-1* – single nucleotide polymorphisms - susceptibility.

Abbreviations: BCLC: Barcelona Clinic Liver Cancer; BMI: body mass index; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; NAFLD: non-alcoholic fatty liver disease; PCR: polymerase chain reaction; *PD-1: programmed cell death-1*; SNP: single nucleotide polymorphisms.

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INTRODUCTION

Hepatocellular carcinoma (HCC) has an etiology involving many genetic and environmental factors and considering that it is the most commonly seen malignancy of the liver, the fifth most prominent diagnosed cancer worldwide, it follows that the incident and death numbers are on the rise [1, 2]. Although commonly brought about by chronic hepatitis B, chronic hepatitis C, non-alcoholic fatty liver disease (NAFLD) and alcoholic liver disease, cirrhosis as well as HCC can be caused by any chronic inflammation of the liver. However, it is important to note possible genetic factors contributing to HCC, as only a small percentage of patients living with hepatitis B virus (HBV) or hepatitis C virus (HCV) are ultimately diagnosed with HCC [3].

Programmed cell death-1 (*PD-1*; CD279) is a member of CD28/B7 superfamily costimulatory molecules that balance T cell tolerance. PD-1 is a type 1 transmembrane receptor protein encoded by a human gene located on chromosome 2q37.3 [4, 5]. PD-1 is expressed on activated T cells, B cells, and monocytes. PD-1 ligands PD-L1 and PD-L2 are able to maintain peripheral tolerance by binding with PD-1 which triggers an inhibitory signal against T-lymphocyte activation and proliferation and suppresses cytokine secretion, leading

to apoptosis [6, 7]. It has been shown that the increase of the expression of PD-L1 along cell lines or tissues has been linked to tumor evasion and aggressivity, poor HCC prognosis, and ultimately increased mortality [8, 9].

Polymorphisms of *PD-1 gene*, PDCD1, may affect the PD-1 expression or function. Based on the inhibitory role of PD-1 in anti-tumor responses, we considered the *PD-1* gene as a candidate to test for genetic susceptibility of individuals to HCC. It was shown that it could be a relationship between single nucleotide polymorphisms (SNPs) in the *PD-1* gene and susceptibility to HCC based on various studies [10, 11]. To test the hypothesis that the polymorphisms of *PD-1.1*, *PD-1.5* and *PD-1.6* might be associated with the risk of developing HCC in the Turkish population, we performed a genotyping analysis using allelic discrimination in a hospital-based case–control study.

METHODS

The study population comprised 137 patients with HCC and 136 healthy control participants. Blood samples were taken from all 137 patients seen in the Department of Gastroenterology spanning the period between September 2017 and June 2019. Simultaneously, 136 community residents entering the hospital for routine check-ups were enrolled as the control group. The control group members had no evidence of hepatocellular or other cancers, history of liver disease, serological evidence of hepatitis B or C infection, nor any kin relationship to the experimental group. Each control was pair-matched by sex, age (±3 years). All participants were of Turkish ancestry. The HCC diagnostic criteria were based on the guidelines proposed by the European Association for the Study of the Liver.

The participants recruitment was approved by the Committee for Ethics of Medical Experiments on Human Subjects, the Faculty of Medicine in Marmara University. The study was performed in accordance with the principles of the Helsinki Declaration. All participants provided written informed consent.

The genotyping was performed at the Marmara University Institute of Gastroenterology. Venous blood samples (2 mL) were collected from participants into EDTA-containing tubes and stored at -80 °C until analysis. Genomic DNA was isolated from peripheral blood leukocytes using the "Pure Link Genomic DNA Purification Kit" (Invitrogen, California, USA) and used as a template for genotyping procedures. *PD-1.1* (rs36084323), *PD-1.5* (rs2227981) and *PD-1.6* (rs10204525) SNP variants genotyping was performed by allelic discrimination using specific C__57931321_10, C__57931286_20 and C__172862 TaqManTM SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA), respectively. Polymerase chain reaction (PCR) conditions were as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturing at 95°C for 15 seconds, annealing/extension at 60°C for 1 minute. All assays were performed in 96-well plates, including negative template controls.

The sample size of the study was derived by using QUANTO 1.1. Statistical Package for Social Sciences for Windows was used in data analysis. Experimental and control groups were analyzed using the Student's t-test and Chi-square test for instances of disparity in demographic characteristics. Using the Hardy Weinberg equilibrium theory, expected and actual genotype frequencies among cases and controls were compared. The preset power of the study was 80%. Statistical analysis of genotypes and haplotype estimation were analyzed using the website for SNP statistics: https://www.snpstats.net. Logistic regression analysis was used to analyze the association of genotypes in inheritance models (codominant, dominant, recessive, overdominant and log-additive) in the case and control groups. Results are expressed as odds ratios with 95% confidence interval (CI). All tests were two-sided and p value < 0.05 was considered significant. The Pearson's Chi-square test was used to investigate results for a significant difference in allele and genotype frequency in the case and control group. Multivariate logistic regression analysis was performed with diagnosis as a dependent variable and independent variables, including age and gender. Also each genotype was considered. A p value <0.05 was considered statistically significant.

RESULTS

Table I summarizes general characteristics of the participants, without significant difference found in regard to age and gender between the HCC and control group. While

Table I. Characteristics of patients with hepatocellular carcinoma and controls

	HCC (n=137)	Control (n=136)	p
Age (years), mean ± SD	61.47 ± 11.77	61.47 ± 10.06	1.0
Gender, n (%)			0.864
Female	41 (29.9)	42 (30.9)	
Male	96 (70.1)	94 (69.1)	
BMI, median (min- max)	28.0 (16.8-45.7)	28.7 (18.4-43.3)	0.3
Smoker, n (%)	36 (34.3)	46 (33.8)	0.940
Alcohol drinker, n (%)	17 (12.4)	6 (4.4)	0.017
Etiology, n (%)		-	-
HBV	77 (56.1)		
HCV	24 (17.5)		
NAFLD	20 (14.6)		
Other	16 (11.6)		
BCLC, n (%)		-	-
0	17 (12.4)		
A	43 (31.4)		
B	51 (37.2)		
C	20 (14.6)		
D	6 (4.4)		
Child-Pugh classification, n (%)			
A	92 (67.2)		
B+C	45 (32.8)		
Cirrhosis, n (%)	117 (85.4)	-	-
Portal vein thrombosis, n (%)	20 (14.6)	-	-
Vascular invasion, n (%)	12 (8.8)	-	-
Metastasis, n (%)	4 (2.9)	-	-
Lymph node involvement, n (%)	16 (11.7)	-	-
First year survival, n (%)	80 (58.4)	-	-
Fifth year survival, n (%)	11 (8.0)	-	-
Survival (months), mean ± SD	42.3 ± 25.1	-	-

BCLC: Barcelona Clinic Liver Cancer, BMI: body mass index, HCC: hepatocellular carcinoma

there was no significant difference in the smoking status of two groups, the results suggested that alcohol consumption was significantly higher in the HCC group (p=0.017). Other demographic variables such as etiology, cirrhosis status and Child-Pugh class of cases are also outlined in Table I.

The genotype and allele frequencies of *PD-1.1, PD-1.5* and *PD-1.6* in HCC cases and control group are listed in Table II. The genotype frequencies of the patients and controls were in Hardy–Weinberg equilibrium, suggesting that there was no population stratification and no sampling bias. The analysis of *PD-1.1, PD-1.5* and *PD-1.6* genotype distribution found no significant differences between HCC patients and controls. *PD-1.1* GG and GA genotype frequencies were very similar between cases and controls with 90.4% vs. 90.5% (p=1) and 9.6% vs. 9.5% (p=1) respectively. No *PD-1.1* AA genotype was observed among the case or control group. *PD-1.1* allele frequency analysis also showed very similar results between case and control groups with 95.2% vs. 95.3% (p=0.99) respectively for G allele. Frequencies of *PD-1.5* CC, CT and TT genotypes were 66 (48.2%), 55 (40.1%) and 16 (11.7%) in HCC patients and 56 (41.2%), 67 (49.3%), 13 (9.6%) in the control group, respectively (p=0.32). *PD-1.5* T and C allele

Table II. The PD-1 genotypes and allele frequencies of HCC and control group

	Polymorphism	Control n=136, (%)	HCC n=137, (%)	OR	95%CI	p
	PD-1.1					
	Genotype					
Codominant	GG	123 (90.4)	124 (90.5)	1.00		1
	GA	13 (9.6)	13 (9.5)			
	AA	-	-			
	Allele					
	G	259 (95.2)	261 (95.3)	1.00		0.99
	A	13 (4.8)	13 (4.7)	1.00	0.45-2.19	
	PD-1.5					
	Genotype					
Codominant	CC	56 (41.2)	66 (48.2)	1.00		0.32
	CT	67 (49.3)	55 (40.1)	0.69	0.42-1.16	
	TT	13 (9.6)	16 (11.7)	1.05	0.46-2.36	
Dominant	TT+CT	80 (58.8)	71 (51.8)	0.75	0.47-1.22	0.25
	CC	56 (41.2)	66 (48.2)	1.00		
Recessive	CC+CT	123 (90.4)	121 (88.3)	1.00		0.57
	TT	13 (9.6)	16 (11.7)	1.25	0.58-2.71	
Overdominant	TT+CC	69 (50.7)	82 (59.9)	1.00		0.13
	CT	67 (49.3)	55 (40.1)	0.69	0.42-1.12	
	Allele					
	T	93 (34.2)	87 (31.8)	0.90	0.63-1.28	0.55
	C	179 (65.8)	187 (68.2)	1.00		
	PD-1.6					
	Genotype					
Codominant	GG	93 (68.4)	96 (70.1)	1.00		0.28
	GA	35 (25.7)	38 (27.7)	1.06	0.61-1.82	
	AA	8 (5.9)	3 (2.2)	0.36	0.09-1.41	
Dominant	AA+GA	93 (68.4)	96 (70.1)	1.00		0.77
	GG	43 (31.6)	41 (29.9)	0.93	0.55-1.55	
Recessive	GG+GA	128 (94.1)	134 (97.8)	1.00		0.11
	AA	8 (5.9)	3 (2.2)	0.36	0.09-1.38	
Overdominant	GG+AA	93 (68.4)	96 (70.1)	1.00		0.7
	GA	35 (25.7)	38 (27.7)	1.06	0.61-1.82	
	Allele					
	G	221 (81.2)	230 (83.9)	1.00		0.41
	A	51 (18.8)	44 (16.1)	0.83	0.53-1.29	

CI: confidence interval; HCC: hepatocellular carcinoma; OR: odds ratio, *PD: programmed cell death*.

frequencies were 87 (31.8%), 187 (68.2%) in HCC patients and 93 (34.2%), 179 (65.8%) in control group, respectively (p=0.55). Frequencies of *PD-1.6* GG, GA and AA genotypes were 96 (70.1%), 38 (27.7%) and 3 (2.2%) in HCC patients and 93 (68.4%), 35 (25.7%), 8 (5.9%) in the control group, respectively (p=0.28). *PD-1.6* G and A allele frequencies were 230 (83.9%), 44 (16.1%) in HCC patients and 221 (81.2%), 51 (18.8%) in the control group, respectively (p=0.55).

Patients with HBV related HCC comprised 56.1% of our patient group. Subgroup analysis of HBV related patients also did not show any statistically significant results against the control group (Table III). Furthermore, we did not find any significant association between the different genotypes and alleles of *PD-1.1, PD-1.5* and *PD-1.6* polymorphisms and clinical prognostic parameters such as liver cirrhosis, Child–Pugh grade, metastasis, vascular invasion and portal vein thrombosis status at the time of diagnosis (data not given).

The haplotypes of *PD-1.1, PD-1.5* and *PD-1.6* were constructed and compared between patients and controls. No haplotype was found to be significantly different from HCC and control groups (Table IV).

Table III. The *PD-1* genotypes and allele frequencies of HBV related HCC and control group

	Polymorphism	Control n=136, (%)	HCC n=77, (%)	OR	95%CI	p
	PD-1.1					
	Genotype					
Codominant	GG	123 (90.4)	71 (92.2)	1.00		0.79
	GA	13 (9.6)	6 (7.8)	0.87	0.31-2.44	
	AA	-	-			
	Allele					
	G	259 (95.2)	148 (96.1)	1.00		0.79
	A	13 (4.8)	6 (3.9)	0.87	0.32-2.40	
	PD-1.5					
	Genotype					
Codominant	CC	56 (41.2)	39 (50.6)	1.00		0.28
	CT	67 (49.3)	29 (37.7)	0.63	0.34-1.16	
	TT	13 (9.6)	9 (11.7)	1.04	0.40-2.75	
Dominant	TT+CT	80 (58.8)	38 (49.4)	0.69	0.39-1.24	0.22
	CC	56 (41.2)	39 (50.6)	1.00		
Recessive	CC+CT	123 (90.4)	68 (88.3)	1.00		0.57
	TT	13 (9.6)	9 (11.7)	1.31	0.52-3.29	
Overdominant	TT+CC	69 (50.7)	48 (62.3)	1.00		0.11
	CT	67 (49.3)	29 (37.7)	0.62	0.35-1.12	
	Allele					
	T	93 (34.2)	47 (30.5)	0.86	0.56-1.33	0.5
	C	179 (65.8)	107 (69.5)	1.00		
	PD-1.6					
	Genotype					
Codominant	GG	93 (68.4)	56 (72.7)	1.00		0.76
	GA	35 (25.7)	18 (23.4)	0.92	0.47-1.80	
	AA	8 (5.9)	3 (3.9)	0.60	0.15-2.45	
Dominant	AA+GA	43 (31.6)	21 (27.3)	0.85	0.45-1.61	0.63
	GG	93 (68.4)	56 (72.7)	1.00		
Recessive	GG+GA	128 (94.1)	74 (96.1)	1.00		0.48
	AA	8 (5.9)	3 (3.9)	0.61	0.15-2.48	
Overdominant	GG+AA	101 (74.3)	59 (76.6)	1.00		0.87
	GA	35 (25.7)	18 (23.4)	0.95	0.48-1.85	
	Allele					
	G	128 (71.5)	74 (75.5)	1.00		0.54
	A	51 (28.5)	24 (24.5)	0.84	0.47-1.49	

CI: confidence interval; HBV: hepatitis B virus; HCC: hepatocellular carcinoma; OR: odds ratio, *PD: programmed cell death;*

Table IV. Haplotype analysis of PD-1 polymorphisms

	PD.1.1	*PD.1.5*	*PD.1.6*	Freq	Control n=136, (%)	HCC n=137, (%)	OR (95% CI)	p
1	G	C	G	0.6227	166 (61)	174 (63.5)	1	---
2	G	T	G	0.2033	55 (20.2)	56 (20.4)	0.97 (0.64 - 1.48)	0.89
3	G	T	A	0.1264	38 (14)	31 (11.3)	0.77 (0.45 - 1.31)	0.34
4	A	C	A	0.0476	13 (4.8)	13 (4.8)	1.00 (0.44 - 2.28)	0.99

CI: confidence interval; HCC: hepatocellular carcinoma, OR: odds ratio, *PD: programmed cell death.*

DISCUSSION

The occurrence of HCC is affected by the immune system's ability to ward off and eliminate cancer cells. Consequently, insufficient tumor maintenance and detection by the immune system is commonly seen in HCC patients. Anti-tumor immunity and anti-tumor suppressive milieu is caused by the interaction of PD-1 and PD-L1 as it has been shown to negatively impact T-cell activation. Findings indicate that HCC outcomes are more likely to be poor when there is an over expression of PD-1 and or PD-L [9]. It has also been shown that in Chinese populations, *PD-1.1* and *PD-1.6* polymorphisms combined with chronic HBV infection increase one's risk of HCC [10, 12]. Therefore, the identification of SNPs that affect *PD-1* gene expression and contribute to an increased risk of HCC are noteworthy areas of inquiry as they may provide a method for identifying at-risk individuals and clarify pathophysiologic mechanisms relevant to HCC. The relationship between the risk of HCC and *PD-1* polymorphism in the Turkish population has only been previously investigated by means of *PD-1.3* and no association has been established [13]. To the best of our knowledge, our study is the first to investigate the association of functional *PD-1* allele polymorphisms (*PD-1.1, 1.5, 1.6*) and HCC development in Turkish population. Moreover, this is the first report to declare the role of *PD-1.5* and genetic susceptibility to HCC. Our results indicate that none of the studied *PD-1* polymorphisms are associated with HCC development.

PD-1.1 polymorphism was found to be associated with various cancers such as breast, lung, gastric and colon cancer [14, 15]. In a study conducted in China, *PD-1.1* A allele frequency was found to be significantly higher among patients with p53 mutation, a known mutation associated with aggressive disease progression. The same study also revealed that the *PD-1.1* GG genotype was associated with a decreased risk of breast cancer [15]. The *PD-1.1* GA genotype was found to be associated with a decreased HCC risk compared with hepatitis B patients in a study among a Chinese population (p=0.001, OR=0.314). The same study also reported that *PD-1.1* A allele is found to be associated with decreased cirrhosis and HCC risk in chronic hepatitis B patients [11]. In this study, *PD-1.1* GG and GA genotype frequencies were reported as 37.3% and 30.2% respectively. However, in an Italian study conducted with 189 Caucasian HCV-associated HCC patients, all HCC patients were found with a *PD-1.1* GG homozygous genotype [16]. These aforementioned results from the Italian study together with our *PD-1.1* genotype frequencies (GG 90.5% and GA 9.5% in HCC patients, similar to levels seen in control group) supports the major *PD-1.1* polymorphism frequency difference between Asian and Caucasian populations. Therefore, this *PD-1.1* polymorphism difference could be a contributing factor to increased HCC incidence in Asian patients compared with Western countries. Notwithstanding, our study did not report any association between *PD-1.1* polymorphism and HCC risk.

There are many studies that have investigated *PD-1.5* polymorphism in association with different cancers. In a meta-analysis of 7 studies assessing cancer risk of *PD-1.5* polymorphism in the development of various cancers, *PD-1.5* T allele and TT genotype is found to be related with a decreased cancer risk [17]. Another meta-analysis also confirmed that the *PD-1.5* TT genotype decreases the cancer risk [18]. As far as can be determined, there has been no study on *PD-1.5* polymorphism and its effect on the development of HCC. Our study did not report a statistically significant association of the *PD-1.5* polymorphism and HCC.

PD-1.6 GG genotype and G allele were reported as an HCC risk reducing factor among hepatitis B patients in a Chinese study [12]. Other research has previously reported increased survival rates among HBV related HCC patients carrying *PD-1.6* G allele and G carrying genotypes [19]. It also has been found that the *PD-1.6* AA genotype increases HCC risk as predicted [10]. Contrary to Asian studies, our results demonstrated a slightly decreased HCC risk with AA genotype (OR=0.36, 95%CI: 0.09-1.38, p=0.11); however, it was unable to reach statistical significance. The allele distribution of the *PD-1.6* genotype in this study was not similar with the distribution of the previously studied Asian population. Our samples included a *PD-1.6* AA genotype frequency of 5.9% and 2.2% in the control and patient groups respectively, while the *PD-1.6* AA genotype frequency was 66% in the HCC group in a Chinese study and similar frequencies were seen among other Asian studies [10, 12, 19]. These results could suggest the need for a further inquiry into the mechanism of how *PD-1.6* is associated with HCC. Furthermore, the *PD-1.6* polymorphism may be increasing the cancer risk with its linkage to the disequilibrium of other genes, downplaying the role of the miR-4717 binding disruption as previously suggested based on our study's conflicting results against Asian studies [20].

There are a number of limitations of the present study. Firstly, due to the fact that this study was a hospital-based case-control study, and cases were selected from a single tertiary institution, it is possible that our relatively small sample was unrepresentative of HCC patients in the general population. However, control subjects were also recruited from the same hospital and observed distributions of *PD-1* polymorphism genotype frequencies suggested no selection bias according to the Hardy–Weinberg equilibrium model. Moreover, addition of

intermediate control groups bearing chronic hepatitis and/or cirrhosis could add value to our study, particularly if the results indicated no association between selected polymorphisms and HCC. Furthermore, this study only focused on three loci on a single gene without taking into consideration gene–environment interactions and gene–gene interactions which may affect the risk of HCC. Finally, although the power of the study was set at 80%, the study is limited by the relatively small number in the subgroup analysis.

CONCLUSION

No relationship between *PD-1* polymorphisms and HCC was found in this study. However, this study highlighted the role of ethnicity in the observed frequencies of *PD-1* polymorphisms in relation to HCC susceptibility. Even though the results were not significant in terms of *PD-1.1* and *PD-1.6* in their relationship with HCC, our results of *PD-1.6* were considerably different than those seen in Asian studies. For the first time in literature, *PD-1.5* polymorphism was investigated for a potential role in genetic susceptibility to HCC and no significant relationship was found. Further studies, including patients of different ethnic origins, are required to validate these findings in a significant larger series in order to better understand the implications of *PD-1* polymorphisms and one's susceptibility to HCC.

Conflicts of interest: None to declare.

Authors' contribution: A.F.D., C.O.D., F.G. and O.C.O. contributed to the conception and design of the study. A.F.D., C.O.D., F.E., D.Y., C.K. participated in data collection and performed the experiments. A.F.D. and C.O.D. wrote the manuscript. C.O.D. performed detailed statistical analyses of the data. F.G. reviewed and refined the manuscript.

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