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Association of programmed death-1 gene polymorphisms with the risk of basal cell carcinoma

Farshid Fathi[1] | Milad Ebrahimi[2] | Asma Eslami[1] | Hossein Hafezi[3] | Nahid Eskandari[1] | Hossein Motedayyen[4]

[1]Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

[2]Faculty of Medicine, Department of Immunology, Shahed University, Tehran, Iran

[3]Department of Dermatology, Isfahan University of Medical Sciences, Isfahan, Iran

[4]Autoimmune Diseases Research Center, Kashan University of Medical Sciences, Kashan, Iran

Correspondence
Hossein Motedayyen, Autoimmune Diseases Research Center, Shahid Beheshti Hospital, Kashan University of Medical Sciences, 5th kilometer of Ravand Road, Kashan, Iran.
Email: hmotedayyen@gmail.com

Funding information
Isfahan University of Medical Sciences (IUMS)

Abstract

Environmental and genetic factors play a fundamental role in the pathogenesis of basal cell carcinoma (BCC) defined as the most common cancer of skin. Programmed death-1 (PD-1), encoded by programmed cell death-1 (*PDCD1*) gene, serves as an inhibitory molecule in the suppression of immune responses and a risk factor in the development of different cancers. In this study, we investigated the role of two single nucleotide polymorphisms (SNPs) within *PDCD1* gene, and haplotypes defined by these SNPs, in the development of BCC in an Iranian population. Whole blood samples were obtained from 210 BCC and 320 healthy subjects. Genomic DNA was extracted from whole blood samples, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype determinations of PD1.3 (rs11568821) and PD1.5 (rs2227981) SNPs, and 4 haplotypes were constructed by PDCD1 SNPs. The frequency of G allele of PD1.3 was significantly higher in BCC patients than healthy subjects ($p < 0.02$), while these significant differences were not observed in the frequencies of PD1.5 alleles between BCC and healthy subjects. Moreover, we found that there were no statistically significant differences in PD1.3 and PD1.5 genotypes between BCC and control groups. Of all estimated haplotypes for *PDCD1*, only AC haplotype was associated with BCC (OR = 0.22, 95% CI = 0.06–0.79, $p < 0.01$). These findings suggest that PD1.3G allele and AC haplotype of *PDCD1* contribute to BCC in the Iranian population. However, further studies in different populations with larger sample size are required to confirm this study.

KEYWORDS
basal cell carcinoma, polymorphism, programmed death-1

1 | INTRODUCTION

Basal cell carcinoma (BCC) is known as the most common cancer of skin with a good prognosis, mainly caused by photogenic mutations in patched homolog 1 (PTCH1) gene (Bukhardt Pérez, Ruiz-Villaverde, Naranjo Díaz, Blasco Melguizo, & Naranjo, 2007; Fernandes et al., 2017; Helsing, Gjersvik, & Tarstedt, 2015; Lear, Harvey, Berker, Strange, & Fryer, 1998). BCC is followed by clinical manifestations in middle adulthood such as telangiectasia, pigmented areas, rodent ulcer and red patch (Elghissassi et al., 2009; Marzuka & Book, 2015). The aetiology of BCC remains unknown, but it is suggested that a combination of environmental and genetic factors is involved in susceptibility to BCC (Trakatelli et al., 2007, Carucci et al. (2008), Hogan, To, Gran, Wong, & Lane, 1989). Previous studies have shown that exposure to ultraviolet (UV) radiation as the most important environmental factor can contribute to genetic factors to induce

pathologic changes in keratinocytes and immune cells, thereby leads to skin cancers (Linos, Swetter, Cockburn, Colditz, & Clarke, 2009, Carucci et al. (2008)).

Programmed death-1 (PD-1), a member of the CD28 family, is encoded by *programmed cell death-1*(*PDCD1*) gene located at 2q37.3 locus (Zak et al., 2015). PD-1 expresses on a variety of immune cells such as T, B lymphocytes and myeloid cells (Brahmer et al., 2010; Gettinger et al., 2015; Zak et al., 2015). Under normal condition, PD-1 binding to its ligand (PD-L1) triggers a crucial signalling pathway that results in the negative regulation of activated lymphocytes and tolerance to self-antigens by limiting the initiation and duration of immune responses (Nielsen, Hansen, Husby, Jacobsen, & Lillevang, 2003; Riella, Paterson, Sharpe, & Chandraker, 2012). PD-1 serves as a crucial immune checkpoint blockade that is well studied in different cancers. This inhibitory molecule balances the pro- and anti-immune responses to avoid autoimmune diseases and malignancies. Cancer cells exert some immunosuppressive responses to evade the attack from the immune system via upregulation of *PDCD1* (Deng et al., 2015). Extensive data from the literature have reported that PD-L1 is over-expressed in many solid tumours, including lung, breast, melanoma, lymphoma, kidney, bladder, brain, thymus, oesophagus, gastrointestinal tract and pancreas (Gatalica et al., 2014; Majzner et al., 2017; Velcheti et al., 2014). PD-1 binding to PD-L1 on different immune cells leads to inhibit immune responses against various tumours (Brahmer et al., 2010; Salmaninejad et al., 2018). Recently, genome-wide association studies (GWAS) have suggested genetic single nucleotide polymorphisms (SNPs) in genes encoding immunosuppressive molecules participate in the failure of immune responses against tumour progression and thereby contribute to the risk of developing different tumours (Chahal et al., 2016; Li, Liang, Vivo, Tang, & Han, 2016b). To date, several SNPs have been identified in *PDCD1* gene such as PD1.3 (rs11568821) and PD1.5 (rs2227981), which act as the risk factor for the development of different cancer (Li, Liang, et al., 2016b; Salmaninejad et al., 2018). Previous reports have demonstrated that PD1.3 SNP as a regulatory SNP is associated with the increased risk of colon cancer (Salmaninejad et al., 2018). In addition, polymorphism studies have indicated that PD1.5 (rs2227981) may increase susceptibility to several types of cancers including cervical, colon and gastric cancers (Li, Jiang, Zhang, & Jia, 2016a; Mojtahedi et al., 2012; Savabkar, Azimzadeh, Chaleshi, Mojarad, & Aghdaei, 2013). In our knowledge, the role of PD-1 SNPs in susceptibility to BCC has not yet been investigated. Therefore, this study aimed to investigate the role of *PDCD1* gene polymorphisms at 2 positions (PD1.3 and PD1.5), and haplotypes constructed by these SNPs, in the risk of developing BCC in the Iranian population.

2 | MATERIALS AND METHODS

2.1 | Study population

The study population comprised of 210 unrelated patients who were referred to a dermatology clinic in Al Zahra Hospital, Isfahan, Iran, and 320 age-matched healthy volunteers without history of autoimmune, asthma, allergy, chronic infectious diseases and malignancy. The study was preformed from February 2018 to September 2018. Study participants were Persian ethnic and selected from Isfahan province of Iran. The disease was diagnosed by National Comprehensive Cancer Network (NCCN) criteria (Bichakjian et al., 2016). The study protocol was approved by the Ethical Committee of Isfahan University of Medical Sciences, and informed consent was obtained from all individuals before entering the study.

2.2 | Sample collection and DNA extraction

EDTA-treated peripheral blood (5 ml) was collected from participants, and white blood cells were isolated from whole blood using red blood cell (RBC) lysis buffer. Genomic DNA was extracted by a human DNA extraction kit (Bioteke Corporation) according to the manufacturer's instructions. The yield and purity of extracted DNAs were assessed by a spectrophotometer (NanoDrop 8000 spectrophotometer, Thermo Scientific).

2.3 | Polymerase chain reaction (PCR) and PD-1 genotyping

To determine PD-1 genotypes, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to evaluate PD1.3 and PD1.5 SNPs. PCR-RFLP was performed on thermocycler (Bio-Rad, Hercules) in a 15 μl mixture containing 1 μl (300 ng) of genomic DNA, 0.45 μl of $MgCl_2$ (50 mM), 0.3 μl of each primer (10 p.m.), 1.5 μl of PCR buffer (10X), 0.45 μl of dNTP (10 mM), 1 μl of Taq DNA polymerase (5 U/μl) and 10.9 μl of nuclease-free water (all from SinaClone). The temperatures and times in the cycling parameters were as follows: initial 5 min incubation at 94°C followed by 35 cycles, including 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. All assays were carried out in duplicate, and the investigators were blinded to sample information while performing the experiment. Specific primers were designed using Allele ID 7.5 software (Premier Biosoft) and checked by Primer-BLAST (NCBI).

The amplified PCR products were incubated at 37°C for overnight and then digested, respectively, by the Pst1 and PvuII restriction endonucleases for PD1.3 and PD1.5 according to the manufacturer's guideline (Thermo Scientific). The digested PCR products were then stained with KBC solution (Kowsar Biotech Company) and runned on 3% agarose gel in order to visualize the DNA bands with ultraviolet light. All information related to PCR conditions (primers, fragment lengths, annealing temperatures and restriction enzymes) were shown in Table 1.

2.4 | Statistical analysis

The results were analysed using SPSS (v18; SPSS Inc.) and Epi info programs (Epi info v6.04a—July 1996, CDC, Atlanta, GA, and World Health Organization). Kolmogorov–Smirnov Z test was used to determine the normal distribution of the data. Independent *t* test and

TABLE 1 Restriction enzymes and primer sequences used to genotype programmed cell death 1.3 and PD1.5 polymorphisms in the *PDCD1* gene

Position	Primer sequences	Annealing temperatures	Restriction enzymes	Fragment lengths
PD1.3 (rs11568821,+7146 G/A)	Forward (5′–3′) GCCTGGAGGACTCACATTCT	58	PST 1	G: 381bp
	Reverse (5′–3′) GTCCCCCTCTGAAATGTCC			A: 277bp,104bp
PD1.5 (rs2227981,+7785 C/T)	Forward (5′–3′) AGACGGAGTATGCCACCATTGTC	57	PVUII	C: 196bp
	Reverse (5′–3′) AAATGCGCTGACCCGGGCTCAT			T: 125bp, 71bp

Abbreviation: PD, programmed cell death.

Mann–Whitney U test were used to compare 2 groups with normal and non-normal distribution, respectively. Comparisons of the allele and genotype frequencies of PD-1 were performed using chi-square ($\chi 2$) and Fisher's exact tests. Arlequin software package (CMPG, University of Berne, 2006) was used to check the consistency of genotype frequencies with Hardy–Weinberg equilibrium and to determine haplotypes. p Values <0.05 were considered statistically significant. Bonferroni correction, as a post hoc analysis, was used to the pairwise comparisons with significant level of 0.0125.

3 | RESULTS

3.1 | Description of subjects

In this study, 210 patients (109 males and 101 females, mean age: 62.67 ± 12.14, mean ± *SD*, range: 18–91 years) and healthy subjects (170 males and 150 females, mean age: 62.63 ± 10.3, mean ± *SD*, range: 18–92 years) were participated. Of all participants, 86 BCC patients and 112 healthy voluntaries had history of smoking (Table 2). Of the 210 BCC patients, 183 had lesions on head and neck, 11 had occupational exposure to mutagens (Table 2). The size and number of lesions varied from a range of <1 cm^2–>2 cm^2 and 1–>5, respectively (Table 2). A total of 146 patients had one lesion, 60 patients had lesion sizes <1 cm^2, 121 had lesion sizes between 1 and 2 cm^2, and 29 had lesion sizes >2 cm^2 (Table 2). The investigations of PD1.3 and PD1.5 polymorphism associations with these characteristics demonstrated that the clinicopathological factors of BCC patients were not associated with *PDCD1* polymorphisms ($p > 0.05$). The demographic and clinicopathological characteristics of BCC and healthy subjects were showed in Table 2.

TABLE 2 The demographic and clinicopathological features of BCC and healthy individuals

	Patients (n = 210)	Healthy control (n = 320)
Age (mean ± *SD*)	62.67 ± 12.14	62.63 ± 10.3
Gender	Male: 109 (51.9%)	Male: 170 (53.1%)
	Female: 101 (48.1%)	Female: 150 (46.9%)
History of smoking	Yes: 86 (41%)	Yes: 112 (35%)
	No: 124 (59%)	No: 208 (65%)
Location of lesions	Head and neck: 183 (87.1%)	–
	Trunk: 16 (7.6%)	
	Extremity: 11 (5.2%)	
Number of lesions	1: 146 (69.5%)	–
	2: 31 (14.8%)	
	3–5: 19 (9%)	
	>5: 14 (6.7%)	
Size of lesions	<1 cm^2: 60 (28.6%)	–
	1–2 cm^2: 121 (57.6.6%)	
	>2 cm^2: 29 (13.8%	
Sun exposed lesions	Yes: 194 (92.3%)	–
	No: 16 (7.7%)	
Occupational exposure to mutagens	Yes: 11 (5.2%)	–
	No: 199 (94.8%)	

Note: The reported percentages in the table are valid percentages, not considering the missing cases.

3.2 | The associations of PD-1 genotype and allele frequencies with BCC

The frequencies of all genotypes of patient and control subjects were agreed with Hardy–Weinberg equilibrium ($p > 0.05$). As shown in Table 3, GG genotype of PD1.3 was the most frequent genotype in both BCC (87.1%) and control (79.7%) groups, while CC genotype of PD1.5 had the highest frequency in BCC patients (44.3%) and healthy (46.9%) subjects. Our results showed that there were no significant differences in the frequencies of PD1.3 and PD1.5 genotypes between patient and control groups ($p > 0.05$, Table 3).

Other results demonstrated that the frequency of PD1.3G allele was significantly higher in BCC patients than the control group ($p = 0.02$, Table 3). However, no significant differences were observed in PD1.5 allele frequencies between BCC patients and

TABLE 3 Genotype and allele frequencies of programmed cell death 1.3 and PD 1.5 polymorphisms in BCC and healthy individuals

Position	Genotypes/alleles	Patients *n* = 210	Controls *n* = 320	Odds ratio	*p* Value
PD1.3 (rs11568821, +7146 G/A)	GG	183 (87.1%)	255 (79.7%)		0.08
	GA	24 (11.4%)	58 (18.1%)		
	AA	3 (1.4%)	7 (2.2%)		
	GG versus others	183 (87.1%) versus 27 (12.9%)	255 (79.7%) versus 65 (20.3%)		0.03
	GA versus others	24 (11.4%) versus 186 (88.6%)	58 (18.1%) versus 262 (81.9%)		0.04
	AA versus others	3 (1.4%) versus 207 (88.6%)	7 (2.2%) versus 313 (97.8%)		0.76
	Alleles	Patient (2*n* = 420)	Control (2*n* = 640)		
	G	390 (92.8%)	568 (88.8%)	1.05–2.57	0.02
	A	30 (7.2%)	72 (112%)		
PD1.5 (rs2227981, +7785 C/T)	CC	93 (44.3%)	150 (46.9%)		0.56
	CT	87 (41.4%)	134 (41.9%)		
	TT	30 (14.3%)	36 (11.2%)		
	CC versus others	93 (44.3%) versus 117 (55.7%)	150 (46.9%) versus 170 (53.1%)		0.61 0.99
	CT versus others	87 (41.4%) versus 123 (58.6%)	134 (41.9%) versus 186 (58.1%)		0.36
	TT versus others	30 (14.3%) versus 180 (85.7%)	36 (11.2%) versus 284 (88.8%)		
	Alleles	patient (2*n* = 420)	Control (2*n* = 640)		
	C	273 (65%)	434 (67.8%)	0.67–1.14	0.34
	T	147 (35%)	206 (32.2%)		

Note: After applying Bonferroni correction, *p*-Values <0.0125 were considered significant.
Abbreviation: PD, programmed cell death.

TABLE 4 Programmed cell death-1 (PDCD1) haplotype frequencies in BCC patients and healthy controls

Haplotypes		Frequencies				
PD1.3 (rs1156882, +7146 G/A)	PD1.5 (rs2227981, +7785 C/T)	Patient *n* = 210%	Control *n* = 320%	OR (95% CI)	$\chi 2$	*p* Value
G	C	247 (58.8)	370 (57.9)	1.04 (0.81–1.33)	0.1	0.79
G	T	143 (34)	198 (30.1)	1.15 (0.88–1.49)	1.12	0.32
A	T	4 (1)	8 (2)	0.75 (0.22–2.53)	0.2	0.87
A	C	26 (6.2)	64 (10)	0.59 (0.36–0.95)	4.73	0.03

Note: After applying Bonferroni correction, *p*-Values less than 0.0125 were considered significant.
Abbreviation: BCC, basal cell carcinoma; PDCD1, Programmed cell death-1.

healthy subjects (Table 3). Genotype and allele frequencies in PD1.3 and PD1.5 positions of *PDCD1* gene were shown in Table 3.

In the next step, the patients were divided into 3 groups according to size of lesions, including the following: (a) patients with lesions <1 cm^2; (b) patients with lesions between 1 and 2 cm^2; (c) patients with lesions >2 cm^2. Our data showed that there were not associations between *PDCD1* polymorphisms and lesion sizes in BCC patients ($p > 0.05$).

3.3 | Evaluations of PD-1 haplotype frequencies in BCC patients and control group

Four haplotypes were constructed by PD-1 SNPs. GC haplotype was the most common haplotype in both BCC patients (58.8%) and control group (57.9%) (Table 4). The frequencies of AC haplotype in BCC patients and healthy subjects were 6.2% and 10%, respectively (Table 4). The reduction in AC haplotype frequency was associated

with BCC (OR = 0.59, 95% CI = 0.36–0.95, p = 0.03). This haplotype had a protective role in development of the disease (OR = 1.68, 95% CI = 1.04–2.7, p = 0.03). After Bonferroni correction, the results demonstrated that GC, GT, and AT haplotype frequencies were not associated with BCC (p > 0.0125). Haplotype frequencies were shown in Table 4.

4 | DISCUSSION

The binding of PD-1 to PD-L1 triggers an important signalling pathway which leads to negative regulation of immune responses. Overexpression of PD-1 is well demonstrated in cancers, whereas low expression of this molecule may cause intolerance to self-tissue in autoimmune diseases (Chodon, Koya, & Odunsi, 2015; Dai, Jia, Zhang, Fang, & Huang, 2014). The genetic risk factors have the strong association with increased risk of cancers such as BCC (Chahal et al., 2016; Soheilifar et al., 2018; Stacey et al., 2015). SNP, as the most common genetic variation, contributes to the molecular pathogenesis of complex diseases via different functional mechanisms including effect on RNA splicing, transcription factor binding, DNA methylation at promoters, miRNAs binding to its target mRNA and long noncoding RNAs (Salmaninejad et al., 2018). *PDCD1* polymorphisms are associated with different human diseases (Zhou et al., 2016). In the present study, we investigated the *PDCD1* gene polymorphisms at 2 loci in BCC patients and compared to healthy control subjects in an Iranian population.

The rs11568821 (+7146 A/G, PD-1.3) polymorphism is located in the intron 4, while rs2227981 (+7785 C/T, PD-1.5) affects the exon 5 of the *PDCD1* gene that is mapped on chromosome 2q37.3 (Haghshenas, Naeimi, Talei, Ghaderi, & Erfani, 2011). The rs11568821 polymorphism may alter *PDCD1* gene expression due to a substitution of A for G, which can lead to impairment of PD-1 inhibitory functions in subjects who carry PD-1.3 A allele (Prokunina et al., 2002). Dysregulation in the inhibitory effect of PD-1 due to presence A allele in the PD-1.3 polymorphism leads to higher lymphocyte activity that increases the anti-tumour responses and diminishes liability of cancer cells (Dong et al., 2016). In the present study, PD1.3 G allele showed higher frequency in BCC patients than healthy subjects. It is thought that PD1.3 G allele contributes to BCC development in the Iranian population. However, other results suggest the possible absence of clinical relevance of the association of PD1.3 G with BCC, regarding the fact that more than 85% of the study population carrying the wild allele. In line with the role of PD-1.3 polymorphism in development of various cancers, other studies have demonstrated that there are the associations between PD-1.3 polymorphism and different types of cancer regard to both genotypes and alleles (Bayram et al., 2012, Ma et al., 2015, Haghshenas et al., 2011, Namavar Jahromi et al., 2017). A meta-analysis study conducted by Dong et al in 2016 revealed that PD-1.3 genotypes were associated with the decreased risk of cancer, our findings also indicated a certain trend towards an association of PD1.3 genotypes with BCC, although this association was not statistically significant.

PD1.5 polymorphism is known as a synonymous variation which does not exert any change in the final amino acid structure of the protein. Linkage imbalance between PD1.5 variation and other *PDCD1* gene polymorphisms may conduce to modify *PDCD1* expression at mRNA and protein levels (Salmaninejad et al., 2018). Polymorphism studies have indicated the strong association of PD1.5 polymorphism with several types of cancer (Salmaninejad et al., 2018). In contrast to our results, Li et al demonstrated the association of PD1.5 polymorphism with susceptibility to cervical cancer (Li, Jiang, et al., 2016a). In their study, authors revealed that the frequencies of PD1.5 genotypes in patients significantly differed from healthy controls. However, these differences in distributions of PD1.5 alleles were not significant. Recently, Haghshenas et al. showed the association of PD-1.5 polymorphism with susceptibility to thyroid cancer in an Iranian population (Haghshenas, Dabbaghmanesh, Miri, Ghaderi, & Erfani, 2017). This study demonstrated the significant differences in the distribution frequencies of PD1.5 genotypes and alleles between patients with thyroid cancer and healthy controls. In consistent with our results, Li et al indicated that there was not significant association between the PD1.5 genotype frequencies and risk of epithelial ovarian cancer (EOC) (Li, Zhang, Kang, Zhou, & Wang, 2017). However, the authors found statistically significant differences in the distribution frequencies of PD1.5 alleles between patients and controls, whereas our results did not show these significant differences between 2 groups of our study.

Cancer is a multifactorial disease, and different environmental and genetic factors are involved in the risk of developing it. SNPs combination, which is produced different haplotypes, can change the expressions and functions of various proteins, thereby influences susceptibility to different diseases (Fathi et al., 2018). In line with this notion, although there are some reports showing no association between haplotypes and susceptibility to various disorders (Fawwaz et al., 2007), numerous studies have shown that PD-1 haplotypes affect the development of various diseases in different populations, including brain tumour in Iranian (Namavar Jahromi et al., 2017), Type 1 diabetes mellitus (T1DM) in Japanese (Ni et al., 2007), rheumatoid arthritis in Hong Kong Chinese (Kong, Prokunina-Olsson, Wong, LAU, Chan, 2005), and ankylosing spondylitis in Korean population (Lee et al., 2006). In this study, we showed AC haplotype of PD1.3 G/A and PD1.5 C/T polymorphisms decreased the risk of BCC. Other results indicated that there was a certain trend towards an association of GT haplotype with the higher risk of BCC, although this association was no statistically significant. This observation suggests that GT haplotype may act as a genetic risk factor for the developing BCC in the Iranian population.

Although this study revealed the associations of *PDCD1* polymorphisms with BCC, there are several limitations such as low sample size, the possibility of spurious associations due to population substructure/admixture, and no possibility for doing functional tests on *PDCD1* gene variants. Furthermore, in addition to clinicopathological characteristics mentioned in this study, it is better that the associations of other clinicopathological characteristics with BCC were evaluated.

Taken together, our study for the first time provides evidence to indicate that PD1.3 G allele may participate in BCC development. Furthermore, the frequency of AC haplotype emerged from PD1.3 and PD1.5 positions showed a significant association with BCC. Although there was a certain trend towards association of PD1.3 genotypes and GT haplotype with BCC, it is likely that other *PDCD1* gene polymorphisms at 2 PD1.3 and PD1.5 loci do not be involved as genetic markers for BCC development in the Iranian population. Nevertheless, it should be noted that more robust studies in different populations with larger sample size are required to confirm this study.

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CONFLICT OF INTEREST

The authors report no conflict of interest.

ORCID

Hossein Motedayyen https://orcid.org/0000-0002-4863-1771

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