Investigation of Programmed Cell Death-1 (PD-1) Gene Variations at Positions PD1.3 and PD1.5 in Iranian Patients with Non-small Cell Lung Cancer

Zahra Pirdelkhosh*,, Tohid Kazemi*, Mohammad Reza Haghshenas***, Mohammad Ali Ghayumi****, Nasrollah Erfani****,*****♦**

Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
**Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran*
***Cancer Immunology Group, Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
****Department of Internal Medicine, Faghihi Hospital, Shiraz University of Medical Sciences, Shiraz, Iran*
*****Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*

Abstract

Background: Tumor cells express PD-1 ligands to bind PD-1 on immune cells and escape immune responses. In the present study, we aimed to investigate whether single nucleotide polymorphisms at positions PD1.3 (+7146, rs11568821) G/A, and PD1.5 (+7785 C/T, rs2227981) may be considered risk factors for susceptibility to non-small cell lung cancer in the Iranian population.

Methods: This study enrolled 206 histopathologically confirmed lung cancer patients and 173 age/sex matched healthy controls. We performed PCR-RFLP to determine the genotypes of the extracted genomic DNA.

Results: The frequencies of PD1.3 GG, GA and AA genotypes were 171 (83%), 31 (15%) and 4 (1.9%) out of 206 patients, and 144 (83.2%), 26 (15%), and 3 (1.7%) out of 173 controls, respectively. The frequencies of PD1.5 CC, CT and TT genotypes were 78 (37.9%), 100 (48.5%), and 28 (13.6%) in patients, and 60 (34.7%), 89 (51.4%), and 24 (13.9%) in controls. There were no significant differences in genotype analysis between patients and controls at positions PD1.3 (*P*=0.98) or PD1.5 (*P*=0.80). No significant differences existed in the frequencies of alleles and haplotypes between the two groups (*P*>0.05).

Conclusion: Our data have indicated no association between PD1.3 (+7146) G/A and PD1.5 (+7785) C/T with susceptibility to non-small cell lung cancer. Investigation of other PD1 genetic variations and emerged haplotypes are required to completely define the role of PD1 genetic variations in susceptibility to lung cancer.

Keywords: NSCLC, PD-1, Gene variation, Single nucleotide polymorphisms (SNP)

♦Corresponding Author:
Nasrollah Erfani, PhD
Professor of Immunology, Department of Immunology and Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
Tel/Fax: +98 711 230 3687
Email: erfanin@sums.ac.ir

Introduction

Among all types of cancers, lung cancer has the highest prevalence and mortality in men, and ranks third in prevalence and second in mortality for women. Small cell lung carcinoma includes 15% and non-small cell lung cancer (NSCLC) comprises 85% of lung cancer patients. Multiple risk factors under consideration include genetics, smoking, diet and alcohol, chronic inflammation, radiation, occupational exposures, and air pollution.[1-3] Novel therapeutic approaches are under development with the concept that cancer cells express ligands for programmed cell death-1 (PD-1) on immune cells and therefore escape anti-cancer immune responses.[4,5]

Programmed cell death-1 expressed on various immune cells which include innate immune cells [natural killer (NK) cells, monocytes, and dendritic cells] as well as adaptive immunity cells (B and T lymphocytes). Ligation of PD-1 by its ligands, PD-L1 and PD-L2, triggers inhibitory signaling and dampens immune effector function of the cell.[6] Interestingly, such an interaction could take place with regulatory T (Treg) cells and give rise to the activation and proliferation of Treg cells.[7] Expressions of ligands for PD-1 immune checkpoint molecule have been shown in NSCLC.[8,9] Several single nucleotide polymorphisms (SNPs) at different locations of the PD-1 gene have been identified and investigated for possible association with malignancies.[10,11] We reported a significant association of the PD1.5 C/T polymorphism and GT haplotype (PD1.3 G, PD1.5 T) with genetic susceptibility to thyroid cancer.[12] In the present study, we aimed to investigate a possible association of PD-1.3 (+7146 G/A, rs11568821) and PD-1.5 (+7785 C/T, rs2227981) SNPs with susceptibility to NSCLC in the Iranian population.

Patients and Methods

A total of 206 patients with clinically and histopathologically diagnosed NSCLC and 173 age- and sex-matched control subjects with no history of cancer, autoimmune or inflammatory diseases enrolled in this study. The patient group included 174 men with a mean age of 65.7 years and age range of 30-90 years, and 32 women with a mean age of 61.3 years and age range of 37-83 years. The control group comprised 144 men with a mean age of 64.97 years and age range of 30-86 years as well as 29 women with a mean age of 60.93 years and age range of 37-79 years. The Ethics Committee at Shiraz University of Medical Science, Shiraz, Iran approved this study. Participants provided informed consent before sample collection.

Genomic DNA was extracted from 5 ml vein peripheral blood that contained EDTA anti-coagulant using the standard salting out method.[13] Genotypes were determined by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). Briefly flanking regions of both SNPs were amplified by conventional PCR that used the specific primers listed in Table 1. Amplification of flanking regions of PD-1.3 and PD-1.5 SNPs gave rise to specific bands of 381 and 196 bp. After verification of amplification on agarose gel, these PCR products were subjected to digestion by 10 units Pst1 and PvuII restriction enzymes (Fermentas, Lithuania) for determining the genotypes of the PD-1.3 and PD-1.5 SNPs, respectively. After agarose gel electrophoresis and according to the digestion pattern for each SNP (Table 1), we determined the genotypes of the patients and control subjects.

Statistical analysis

Haplotype analyses as well as deviation from Hardy-Weinberg equilibrium were evaluated by the algorithms in the Arlequin 3.1 software package.[14] Data was analyzed using SPSS software package (version 11.5; SPSS Inc., Chicago, IL, USA). The differences in the genotypes and allele frequencies were evaluated using Pearson's chi-square test with Yates correction. The significant level was set at $P<0.05$.

Results

Hardy-Weinberg equilibrium analysis showed no deviation in distribution of the genotypes from the equation, or in the patient or control groups.

Table 1. Primer sequences and restriction enzymes used to genotype programmed cell death (PD)-1.3 and PD-1.5 polymorphisms in the PDCD1 gene.

SNP	Primer sequence	Restriction enzyme	Annealing temperature	Digestion pattern of RFLP products	Ref.
PD-1.3 (+7146G/A, rs11568821)	F:GCCTGGAGGACTCACATTCT R: GTCCCCCTCTGAAATGTCC	Pst1	58°C	G: 381 bp A:277 bp, 104 bp	Homemade primer
PD-1.5 (+7785 C/T, rs2227981)	F:AGACGGAGTATGCCACCATTGTC R: AAATGCGCTGACCCGGGCTCAT	PvuII	58°C	C: 196 bp T: 125 bp, 71 bp	(15)

SNP: Single nucleotide polymorphism; F: Forward; R: Reverse

Table 2 shows the frequencies of the genotypes and alleles in the NSCLC patient and control groups. As indicated, the frequencies of PD1.3 GG, GA, and AA genotypes were 171 (83%) for GG, 31 (15%) for GA, and 4 (1.9%) for AA out of 206 patients and in controls, there were 144 (83.2%) GG, 26 (15%) GA, and 3 (1.7%) AA out of 173 participants, which showed no significant difference (P=0.98). The frequencies of PD1.5 CC, CT and TT genotypes were 78 (37.9%), 100 (48.5%), and 28 (13.6%) in patients, respectively. In controls, it was 60 (34.7%) CC, 89 (51.4%) CT, and 24 (13.9%) TT with no statistically significant differences between the two groups (P=0.80). Analysis of allele frequencies revealed a higher frequency of PD-1.3 G [373 (90.5%)] and PD-1.5 C [256 (62.1%)] alleles in both studied groups. There were no significant differences in allele frequencies between patients (P=0.98) and controls (P=0.67). GC haplotype was the most frequent haplotype in patients [219 (53.9%)] and controls [180 (52%)]. Statistical analyses revealed no significant differences in the frequency of haplotypes between patients and controls (Table 3).

Discussion

The PD-1/PD-1L interaction is one of the immune checkpoints employed in the tumor microenvironment to overcome or modulate anti-tumor immune mechanisms.[5] In the present study, we have aimed to investigate whether SNPs at

Table 2. Genotypes and allele frequencies of programmed cell death-1 (PD-1) gene variations in non-small cell lung cancer (NSCLC) and control groups.

Single nucleotide polymorphism (SNP)	Genotype/allele	Patients n = 206 (%) 2n = 412 (%)	Healthy controls n = 173 (%) 2n = 346 (%)	P-value
PD-1.3 (+7146G/A, rs11568821)	Genotype			
	GG	171 (83)	144 (83.2)	
	GA	31 (15)	26 (15)	
	AA	4 (1.9)	3 (1.7)	0.98
	Allele			
	G	373 (90.5)	314 (90.7)	
	A	39 (9.5)	32 (9.3)	0.98
PD-1.5 (+7785 C/T, rs2227981)	Genotype			
	CC	78 (37.9)	60 (34.7)	
	CT	100 (48.5)	89 (51.4)	
	TT	28 (13.6)	24 (13.9)	0.80
	Allele			
	C	256 (62.1)	209 (60.4)	
	T	156 (37.9)	137 (39.6)	0.67

Table 3. Programmed cell death-1 (PD-1) haplotype distribution in patients with non-small cell lung (NSCLC) cancer and healthy controls.

Haplotypes		Haplotype frequencies			P-value
PD1.3 Allele	PD1.5 Allele	Patients n = 206 (%)	Healthy controls	n = 173 (%)	
G	T	154 (37.9)	134 (38.7)	0.75	
A	C	37 (9.1)	29 (8.3)	0.87	
G	C	219(53.9)	180 (52)	0.81	
A	T	2 (0.4)	3 (0.8)	0.84	

positions PD1.3 (+7146 G/A) and PD1.5 (+7785 C/T) might be considered risk factors for susceptibility to NSCLC in the Iranian population. Results indicated the frequencies of PD1.3 genotypes and alleles did not significantly differ between patients with NSCLC and healthy control subjects. We observed approximately the same results for the PD1.5 polymorphism. Statistical analyses revealed no significant differences in the frequency of haplotypes between patients and controls.

Programmed cell death-1.3 and PD-1.5 SNPs have been suggested to be involved in PDCD1 gene regulation and alter expression of the PD-1 protein. PD-1.3 (+7146 G/A, rs11568821) is a substitution of A for the G nucleotide in intron 4 which leads to interference with binding of RUNX1 transcription factor and lower expression of PD-1.[11] The PD-1.5 polymorphism (+7785 C/T, rs2227981) is located in an exon location and leads to substitution of T for the C nucleotide with no change in amino acid sequence. Occurrence of this allele has been suggested to be in linkage disequilibrium with another polymorphism in the PDCD1 gene that is associated with a different expression level of the PD-1 protein.[15] A possible association of the PDCD1 gene polymorphism with susceptibility to NSCLC in some ethnic groups has been studied by a limited number of investigators. Sasaki et al.[16] reported that the PD1.1 GG genotype (rs36084323) located in the promoter region of PD-1 gene had an association with lower survival and worse prognosis in NSCLC patients. However, they observed no significant differences in genotype frequencies between patients and controls. In another study, Yin et al.[17] investigated only PD-1.5 C/T in Chinese Han patients diagnosed with NSCLC. In contrast to the current study results, this group showed a higher frequency of the CC genotype among patients compared to controls. They reported a significant association of the C allele with risk for NSCLC. Of interest, they found the CC genotype to be the most frequent genotype in the patient (61.1%) and control (54.8%) groups. The current study results indicated that the CT genotype had the highest frequency in 48.5% of patients and 51.4% of control subjects (Table 2). A similar study in a Chinese population with NSCLC[18] showed no significant association between PD-1.3 and PD-1.5 SNP with cancer risk. Similar to the previous group, these researchers also reported the CC genotype as the most frequent genotype of PD-1.5 in patients (46.2%) and normal subjects (42.7%). The pattern of distribution of the GG, GA, and AA PD-1.3 genotypes was in the same order as the current study population, which determined that GG was the most frequent and AA the less frequent genotype in both Chinese and Iranian NACLC patients and normal subjects (Table 2). Collectively, it seemed that the frequency of the PD-1.5 genotype differed between our population and the Chinese study. However PD-1.3 showed a similar pattern.

The current study results have failed to show any significant association between each of the PD-1.3 and PD-1.5 PDCD1 SNPs and risk for NSCLC in the Iranian population. More investigations that include genome-wide association studies (GWAS) on different PDCD1 SNPs, along with other genes considered important in tumor cell evasion in various ethnic groups, are needed to obtain a consensus about the association of such genetic variants in immune-

cancer cell cross-talk.

Acknowledgement

Authors' Contribution

The first and second authors had equal contribution in this work

Conflict of interest

No conflict of interest is declared.

References

1. Malhotra J, Malvezzi M, Negri E, La Vecchia C, Boffetta P. Risk factors for lung cancer worldwide. *Eur Respir J*. 2016;48(3):889-902.
2. Mao Y, Yang D, He J, Krasna MJ. Epidemiology of Lung Cancer. *Surg Oncol Clin N Am*. 2016;25(3):439-45.
3. Zappa C, Mousa SA. Non-small cell lung cancer: current treatment and future advances. *Transl Lung Cancer Res*. 2016;5(3):288-300.
4. He J, Hu Y, Hu M, Li B. Development of PD-1/PD-L1 pathway in tumor immune microenvironment and treatment for non-small cell lung cancer. *Sci Rep*. 2015;5:13110.
5. Kazemi T, Younesi V, Jadidi-Niaragh F, Yousefi M. Immunotherapeutic approaches for cancer therapy: An updated review. *Artif Cells Nanomed Biotechnol*. 2016;44(3):769-79.
6. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*. 2008;26:677-704.
7. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med*. 2009;206(13):3015-29.
8. Boland JM, Kwon ED, Harrington SM, Wampfler JA, Tang H, Yang P, et al. Tumor B7-H1 and B7-H3 expression in squamous cell carcinoma of the lung. *Clin Lung Cancer*. 2013;14(2):157-63.
9. Velcheti V, Schalper KA, Carvajal DE, Anagnostou VK, Syrigos KN, Sznol M, et al. Programmed death ligand-1 expression in non-small cell lung cancer. *Lab Invest*. 2014;94(1):107-16.
10. Dong W, Gong M, Shi Z, Xiao J, Zhang J, Peng J. Programmed cell death-1 polymorphisms decrease the cancer risk: A meta-analysis involving twelve case-control studies. *PLoS One*. 2016;11(3): e0152448.
11. Haghshenas MR, Naeimi S, Talei A, Ghaderi A, Erfani N. Program death 1 (PD1) haplotyping in patients with breast carcinoma. *Mol Biol Rep*. 2011;38(6):4205-10.
12. Haghshenas MR, Dabbaghmanesh MH, Miri A, Ghaderi A, Erfani N. Association of PDCD1 gene markers with susceptibility to thyroid cancer. *J Endocrinol Invest*. 2017;40(5):481-6.
13. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988;16(3):1215.
14. Excoffier L, Laval G, Schneider S. Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evol Bioinform Online*. 2007;1:47-50.
15. Lin SC, Yen JH, Tsai JJ, Tsai WC, Ou TT, Liu HW, et al. Association of a programmed death 1 gene polymorphism with the development of rheumatoid arthritis, but not systemic lupus erythematosus. *Arthritis Rheum*. 2004;50(3):770-5.
16. Sasaki H, Tatemaysu T, Okuda K, Moriyama S, Yano M, Fujii Y. PD-1 gene promoter polymorphisms correlate with a poor prognosis in non-small cell lung cancer. *Mol Clin Oncol*. 2014;2(6):1035-42.
17. Yin L, Guo H, Zhao L, Wang J. The programmed death-1 gene polymorphism (PD-1.5 C/T) is associated with non-small cell lung cancer risk in a Chinese Han population. *Int J Clin Exp Med*. 2014;7(12):5832-6.
18. Ma Y, Liu X, Zhu J, Li W, Guo L, Han X, et al. Polymorphisms of co-inhibitory molecules (CTLA-4/PD-1/PD-L1) and the risk of non-small cell lung cancer in a Chinese population. *Int J Clin Exp Med*. 2015;8(9):16585-91.