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D-1 Gene Polymorphism in Salivary Gland Tumors

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Objectives This study aimed to assess PD-1gene polymorphism in salivary gland tumors in patients referred to Khalili Hospital in Shiraz.

Methods This case-control study evaluated 48 patients with salivary gland tumors and 100 age- and sex-matched healthy controls. First, 5cc blood samples were obtained from patients and transferred to vials containing anti-coagulated EDTA. DNA was extracted, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed on the samples. The PD-1 gene genotype was determined using the Fermentas kit. After 24 hours of incubation, all the samples were electrophoresed. The genotypes were reported based on the size of bands, and the chi-square test was applied. To compare the alleles, the Fisher's Exact test was applied. The Yates correction was used to compare the genotype and genotypic alleles based on the tumor grade.

Results The mean age was 44.81±15.69 years in patients and 46.54± 13.86 years in controls. Statistical analysis did not show any significant difference in PD1 gene polymorphism between the two groups (P=0.098). No significant correlation was found between the genotype frequency and lymph node involvement (P=0.06), tumor genotype (P=0.12), side (right or left) (P=0.22), tumor location (P=0.27), and size or invasion of the tumor to the surrounding tissue (P=0.14). PD1.3 genotype frequency did not differ significantly between malignant and benign tumors (P=0.6).

Conclusion This study did not reveal any significant difference in genotype frequency of PD1.3 in the patient and control groups; however, further studies are needed with a larger sample size to obtain more accurate results.

Keywords Polymorphism; Genetic; Programmed Cell Death 1 Receptor; Salivary Gland Neoplasms

Introduction

In mammals, the salivary glands are exocrine glands producing saliva through a system of ducts. Salivary gland tumors are uncommon, and often involve the parotid, submandibular, and sublingual glands.[1] In the United States, less than 3% of all malignancies and less than 6% of the head and neck cancerous tumors are formed in the salivary glands.[2] The global prevalence of salivary gland tumors is 4.7% (4.0% to 5.4%) for benign tumors and 0.9% (0.5% to 1.3%) for malignant tumors in 100,000 people, annually.[3-5] PD1, also called the CD279, is a protein that induces peripheral tolerance in T lymphocytes via negative regulation. It belongs to the immunoglobulin superfamily and CTLA4/CD28. This molecule induces a negative signal to the T cells during the reaction with its ligands, leading to regulation of the immune system and prevention of autoimmunity.[6] Several studies have suggested a relationship between the polymorphism of this gene and the emergence of various autoimmune diseases as well as some cancers. Some studies have been conducted in Iran on salivary gland tumors. In one study conducted in Shiraz, the most common benign tumors were pleomorphic adenoma, Warthin's tumor, myoepithelioma, basal cell adenoma, and oncocytoma in order of importance.[7, 8]

There is much evidence that the immune system affects the function of the salivary glands. Cytokines play a significant role in the onset and continuation of inflammation in the secretory glands, and any imbalance between the pro-inflammatory and anti-inflammatory cytokines can lead to further damage to the glands and adversely affect their secretion function.[9] Furthermore, it has been shown that increased expression of TLR3 in salivary glands results in rapid loss of its activity. This phenomenon is associated with the production of type 1 interferon and other inflammatory cytokines in the salivary glands.[10] Kobayashi (2005) studied the expression of PD-1/PD-L1 gene in the salivary glands of patients with Sjogren's syndrome and showed that PD-1 had been expressed by T lymphocytes and PD-L1 had been expressed by the epithelial cells of the salivary glands, which had not been expressed at the onset of Sjogren's syndrome.[11] PD-1 has two ligands: PD-Ls on cell lines and tissues expressed in epithelial cancers such as pancreatic, lung, esophagus and cervical cancers as well as squamous cell carcinoma.[12-17] A number of single nucleotide polymorphisms (SNPs) have been identified in the PD-1 gene, which may be associated with cancer potency or various autoimmune diseases. Some of these polymorphisms may affect the copy process and gene expression.[14-16] Since the relationship between this gene and salivary gland tumors has not been determined, this study was conducted to assess PD-1gene polymorphism in

salivary gland tumors in patients referred to Khalili Hospital in Shiraz.

Materials and Methods

In this case-control study, 48 patients with salivary gland tumors (mucoepidermoid carcinoma, adenoid cystic carcinoma, squamous cell carcinoma, epithelial myoepithelial carcinoma, and salivary duct carcinoma), and 100 healthy age- and sex-matched controls were evaluated who did not have any history of cancer or autoimmune diseases. Approval was obtained from the Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.DENTAl.REC N: 8695183).

The sample size was calculated to be 48.

$$n=\frac{(Z_{(1-\alpha_2 z)}+Z_{(z-\beta)})^2\ \delta^2+\delta^2}{d^2}=48$$

$Z_{(1-\alpha_1)}$= 1.96 d=0.1, $Z_{(1-\beta)}$= 1.28 power test=90%

To increase the study precision, we doubled the sample size for the control group. The patients were selected by a physician after confirming their diagnosis based on clinical findings and the pathology reports. Patients who were reluctant to participate in the study and those who had a history of malignancy, autoimmune or genetic diseases and those whose first-degree relatives had the aforementioned conditions were excluded from the study. After obtaining informed consent, approximately 5 cc of blood was obtained from the patients and transferred into vials containing EDTA. Then, DNA was extracted using the DNA extraction kit, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was performed on the samples. Using the enzyme limiting Fermentas kit (USA) to determine the genotype of the PD-1 gene and after 24 hours of incubation, the product was electrophoresed on agarose gel, and the genotypes were reported based on the size of the bands.

DNA extraction:

Using the salting out technique, DNA was extracted from 5 mL of peripheral blood in both the patient and control groups. After determining the concentration of DNA using a Bio Photometer device, 300 ng concentration of DNA was prepared using the following formula:

N1V1=N2V2

Where N1 is the known DNA concentration, N2 is the required concentration, V1 is the required volume and V2 is the final volume.

PCR:

PCR-RFLP was used to determine the genotype of individuals at position +7146 G/A in PD-1 gene (PD-1.3). For this purpose, the DNA fragment was amplified with a length of 456 open pairs using specific primers amplified by PCR. The presence and size of the desired part were confirmed by running on 2% agarose gel. The amplified fragment was then subjected to the enzymatic fragmentation of the Pst I restriction enzyme and determined based on the pattern of the parts of the individuals' genotype. In the duplicate piece, there were 2 fragmentation sites for the PstI enzyme, which were added to another cutting location in the mutation position in the presence of the A allele. Therefore, individuals with GG genotype after fracture with enzymes had fragments of 310, 127 and 19 open pairs, and individuals with GA genotype had fragments of 310, 212, 127, 98, 19 open pairs. Individuals with AA genotype with 127, 212 and 98 open pairs were also detected. The 19-pair piece was not detected, since it was short on the gel. In addition, the pieces of 98 and 127 open pairs were not distinguishable due to slight differences in the gel but were considered a single-band. The data were analyzed by the SPSS software version 18. To compare the frequency of genotypes and alleles between the two groups, the chi-square test was applied. To compare the alleles, the Fisher's Exact test was used. Furthermore, the Yates Correction was used to compare the genotype and genotypic alleles based on the tumor grade. The algorithms in Arlequin (version 3.1) were used to determine whether the studied groups had SNPs in the Hardy-Weinberg Equilibrium.

Results

Our study population included 48 patients with salivary gland tumors with a mean age of 44.81 ± 15.69 years. Of which, 23 (48%) were males with an average age of 47.47 ± 16.39 years, and 25 (52%) were females with an average age of 42.46 ± 14.98 years. The control group consisted of 100 healthy subjects with a mean age of 46.54 ± 13.86 years (47%), 47 males with a mean age of 51.12 ± 11.48 years and 53 females with a mean age of 42.41 ± 14.61 years. Of the 48 patients, 16 (33%) had malignant tumors and 32 (67%) had benign tumors. The most common type of malignant tumors was mucoepidermoid carcinoma (n=9, 56.3%), and the most common type of benign tumors was pleomorphic adenoma (n=28, 87.5%). The tumor stage was determined based on the TNM staging system, and it was determined that the tumors were mainly in stages II and III (31.3%) (Tables 1 and 2). Data analysis revealed that the most frequent genotype was GG in 43 (89.6%) and 73 (73%) subjects in the patient and control groups, respectively. The lowest frequency was related to AA genotype with a frequency of zero in the patient group and 1(1%) in the control group. We used the sub-group analysis for genotype analysis, since in both case and control group, the frequency of AA genotype was extremely low (Table 3). The results indicated that homozygote GG genotype was more frequent in both the patient and control groups, but it did not have a statistically significant difference in terms of SNP+7146G/A gene PD-1 between the two groups (P=0.098). The results demonstrated that the G allele, the

wild allele and no mutation, were the dominant allele in the case and the control groups, respectively and there was no significant difference between the case and control groups.

Table 1- Clinical and Pathological Characteristics of Malignant Salivary Gland Tumors

	Clinicopathologic characteristic	Frequency (%)
Gender	Male	8 (50%)
	Female	8 (50%)
Histological tumor type	Mucoepidermoid carcinoma	9 (56.3)
	Adenoid cystic carcinoma	5 (31.3%)
	Squamous cell carcinoma	1 (6.3%)
	Epithelial myoepethelial carcinoma	1 (6.3%)
Tumor location	Parotid gland	13 (92.9%)
	Sublingual gland	1 (7.1%)
	Missing	2
Tumor side	Right	4 (33.3%)
	Left	8 (66.7%)
	Missing	4
TNM stage	I	1 (7.1%)
	II	5 (35.7%)
	III	5 (35.7%)
	IV	3 (21.4%)
	Missing	2
Histological grade	1	4 (33.3%)
	2	6 (50%)
	3	2 (16.7%)
	Missing	4
Lymph node (LN) status	Free	10 (71.4%)
	Involved	4 (28.6%)
	Missing	2
Tumor size category	T1	1 (7.1%)
	T2	6 (42.9%)
	T3	7 (50%)
	Missing	2
Metastasis	No	14 (100%)
	Missing	2

Table 2- Clinical and Pathological Characteristics of Benign Tumors of Salivary Glands

	Clinicopathologic characteristic	Frequency (%)
Gender	Male	14(43.7%)
	Female	18(56.3%)
Histological tumor type	Pleomorphic adenoma	28(87.5%)
	Warthins tumor	3(9.4%)
	Parotid gland	1(3.1%)
Tumor location	Parotid gland	12(92.3%)
	Sublingual gland	1(7.7%)
	Missing	19
Tumor side	Right	5(38.5%)
	Left	8(61.5%)
	Missing	19
Tumor size category	T1	3(23.1%)
	T2	7(53.8%)
	T3	3(23.1%)
	Missing	19

G allele had the highest frequency in both the patient and control groups, and A allele was the least frequent. Statistical analysis showed no significant difference between the frequency of G and other alleles in the PD-1.3 position between the patient and control groups (P = 0.07, P = 0.09, respectively). Analysis of the data suggested a close but not significant relationship between genotypes PD-1.3 position and lymph nodes involvement by the tumor. (P = 0.06).

Table 3- Frequency of Genotypes and Alleles in the Patient and Control Groups

Position			Case n=48 2n=96	Control n=100 2n=200	p-value
PD-1.3 (+7146G/A)	Genotype	GG	43 (89.6%)	73 (73%)	
		GA	5 (10.4%)	26 (26%)	
		AA	0	1 (1%)	
	Allele	G	93 (96.9%)	172 (86%)	
		A	3 (3.1%)	28 (14%)	
PD-1.3 (+7146G/A)	Genotype Group	GG	41 (86%)	73 (73%)*	0.098
		AG or AA	7 (14%)	27 (27%)	

No significant correlation was found between the genotype frequency and lymph node involvement (P=0.06), tumor genotype (P=0.12), side (right or left) (P=0.22), tumor location (P=0.27), and size or invasion of the tumor to the surrounding tissues (P=0.14). Also, PD1.3 genotype frequency did not differ significantly between the malignant and benign tumors (P=0.6).

The frequency of PD-1.3 alleles in the patient and control groups showed that there was no significant difference in PD-1.3 (+7146 G/A) position, and G and A alleles. The frequency of G allele was 93% and 86%, and the frequency of A allele was 7% and 14% in the patient and control groups, respectively (P=0.1).

Discussion

The study results indicated that polymorphism of PD1.3 gene was not significantly different in the patient and control groups. Furthermore, there was no significant relationship between the frequency of PD1.3 genotypes and lymph node involvement, tumor location, tumor position, tumor size, invasion to the surrounding tissues, and malignant and benign tumors. During the immune response, the T cells activated in the secondary lymphoid tissue are prepared to migrate to infected tissues. The binding of the TCR-CD3 complex to the MHC-complex is essential for the first stage of T cell activity. The second signal, known as the stimulant, is required to propagate cloning and various activities of the T cells' specific antigen. The balance between the stimulatory and inhibitory sequences maintains the immunological balance and prevents the formation of autoimmunity.[18] The PD-1 gene polymorphism is a determining factor in regulation of immune responses to various diseases, including autoimmune diseases. The response of the receptor to its peptides, PD-L1 and PD-L2, generates a preventive message for the activity of the cell.[18] Many studies have been conducted to investigate the

association between PD-1 gene polymorphism and autoimmune diseases and tumors. Mojtahedi et al. (2012) studied 200 patients with colon cancer and compared them to 200 healthy individuals at the Cancer Research Center of Shiraz. The polymorphism of PD-1.5 C/T gene was significantly observed at position 1/5.[19] The present study did not report the same results, since the results did not show significant differences between the patient and control groups. In this regard, Haghshenas et al. (2011) conducted a study in Shiraz on 443 women with breast cancer and compared them to healthy controls in terms of PD-1.5 gene polymorphism. The results indicated that there was no significant relationship between the two groups.[20] In addition, they did not find a significant relationship regarding the development of tumor, metastasis to the lymph nodes, or distant metastasis, which was consistent with our results. In this area, Ghaderi et al. (2011) conducted a study on PD-1 gene polymorphism and the incidence of thyroid cancer in 105 patients, whose results did not show a significant relationship, but it was significant concerning haplotype and allele type.[21] Dehghani et al, (2009) in Fars Province conducted a study on the polymorphism of PDCD1 with CTLA-4 and p53 in women with the gestational trophoblastic disease, whose results revealed a relationship between PDCD1, CTLA-4 and p53 gene polymorphism in these cases.[22] Lin et al. (2004) studied 98 patients with lupus and 84 patients with rheumatoid arthritis in terms of PD-1 gene polymorphism. There was a significant relationship between PD-1 polymorphism and rheumatoid arthritis, but this relationship was not significant in lupus disease.[23] The results of the study conducted by Lin et al. are in line with the results obtained in the current study. Savabkar et al. (2013) studied the polymorphism of PD-1.5 C/T in 166 cases with metastatic stomach cancer in Tehran. Although they did not draw definite conclusions about the polymorphism of PD-1.5 C/T gene and gastric cancer, disruptions in the gene with the developing and progressing the disease were not irrelevant due to occurrence of some alleles in cases with gastric cancer.[24] Using immunohistochemical methods and PCR, Bolstad et al. (2003) studied mRNA of salivary glands in patients with Sjogren's syndrome. The expression of mRNA for Fas and FasL and CTLA-4 PD-1 in cases was significantly higher than in healthy controls.[25] In this study, we focused on PD-1 gene polymorphism and did not study other genes. Using flow cytometry, Kobayashi et al. (2005) showed the occurrence of PD-1 and PD-L1. In cases with Sjogren, the average fluorescence intensity was significantly higher than that in healthy subjects.[11] We did not use this method in our study. Differences regarding the autoimmune and cancer incidence rates in different populations may be due to different mechanisms of these diseases, as well as the different races of the subjects. It may also be due to the number of SNPs or the involvement of several genetic, immunological, environmental and other factors in these diseases. Although there was no significant association between the frequency of PD1.3 genotypes with lymph node involvement based on the type of tumor, more studies on larger populations are needed to confirm or reject this finding. Due to the small sample size of this study, findings should be interpreted with caution. This was a novel study conducted in Iran and can be replicated in other contexts.

Conclusion

This study did not reveal any significant difference in genotype frequency of PD1.3 in the patient and control groups; however, further studies are needed with a larger sample size to obtain more accurate results.

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Conflict of Interest

Non Declared ■

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