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Original article

Association of polymorphisms in inflammatory cytokine genes with the development of head and neck cancer in Pakistani population

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ABSTRACT

Head and neck cancer (HNC) is the most common type of carcinoma and represents a major health problem in developing countries. HNC is a multifactorial condition associated with a variety of risk factors comprising genetic, environmental and life style. Genetic polymorphisms of interleukin genes might contribute to the development of HNC. Present study was conducted to investigate a possible association of HNC with single nucleotide polymorphisms (SNPs) in interleukin genes (IL-8 rs4073 A/T, IL-4 rs2070874 C/T, IL-10 rs1800896 T/C, IL-10 rs180072 T/G, IL-6 rs1800796 C/G, IL-6 rs1800795 C/G, and IL-1-α rs17561 A/C) and oral hygiene in Pakistani Population. For this purpose, 231 cases and 219 controls were recruited. The SNPs were assessed through QuantStudio real time PCR system. Analysis of the data showed a significant association of IL-10 *rs1800896* with HNC while other SNPs were not associated was HNC pathogenicity. Further, no significant association was observed between hygiene associated factors and HNC in the studied population. This study presents useful aspects of HNC in Pakistani cohort.

1. Introduction

Head and neck cancer (HNC) comprising the malignancies of oral cavity, pharynx and larynx, is 5th most common cancer type in developing world, and accounts for>650,000 cases and 330,000 deaths per year. Alcohol and tobacco use are the two most important risk factors for HNCs, especially carcinomas of the oral cavity, oropharynx, hypopharynx, and larynx (Gandini et al., 2012). Moreover, some studies suggest the association of HNC with poor oral hygiene (Guha et al., 2007; Gandini et al., 2012; Eliot et al., 2013). Additionally, genetic risk factors of HNC have also been reported extensively (Sturgis and Wei, 2002).

Cytokines are the peptides released in response to inflammation and infection and can function to inhibit carcinogenesis. However, cytokines can cause defects in apoptosis, promote growth and facilitate metastasis. Studies show that disrupted serum level of interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and members of interleukin-1 family are associated with oncogenesis via various pathways. Interleukin-1α (IL-1α) (member of IL-1 family), IL-6 and L-10 are involved in differentiation, proliferation, B-cell activation and periodontitis lesion (Yamazaki et al., 1994). IL-4 has an inhibitory effect on inflammation, thrombosis, angiogenesis, growth, or invasion of some types

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of cancer (Vairaktaris et al., 2007). IL-8 is a pro-inflammatory, which promotes neutrophils chemotaxis and degranulation. The high expression of *IL-8* has been reported in endothelial cells, infiltrating neutrophils, and tumor-associated macrophages. IL-8 through its receptor on tumor cells, autocrine pathway, can support tumor migration and invasion, and through its receptors on endothelial cells, paracrine pathway, contributes to angiogenesis (Mojtahedi et al., 2014).

Present study was performed to find out the possible association of inflammation-related genes (IL-1α, IL-4, IL-6, IL-8 and IL-10) SNPs with HNC in Pakistani population. To the best of our knowledge, these genes polymorphisms have not been studied in Pakistani population.

2. Materials and methods

2.1. Study subjects

Present study was approved by the Ethics Committee of the Institute of Radiology and Nuclear Medicine (IRNUM) hospital, Peshawar, Pakistan. Written informed consent was taken from all the subjects. Histopathologically confirmed individuals with HNC including malignant neoplasm of the oral cavity, oropharynx, hypopharynx and larynx were recruited from IRNUM Peshawar, from November 2015 to August 2016. From all the participants including 231 patients and 219 age and sex control individuals, 3–5 ml blood samples were collected in EDTA containing vacutainer tubes.

2.2. Data collection

Data was collected by using a standard questionnaire that included questions regarding demographic variables (age, sex, family income, education level), oral hygiene (self-report of gum bleeding, use of denture, number of missing teeth and periodontal diseases), dental health care (tooth brushing, material used for cleaning teeth, dental floss use, mouthwash) and other risk factors (use of *naswar*, smoking, *paan* chewing). To assess the overall oral hygiene, visual inspection of each participant was performed with the help of dentist.

2.3. DNA extraction and genotyping

DNA was isolated from whole blood using phenol chloroform method. Extracted DNA from both cases and controls were used for genotyping. Seven SNPs (IL-8 rs4073 A/T, IL-4 rs2070874 C/T, IL-10 rs1800896 T/C, IL-10 rs180072 T/G, IL-6 rs1800796 C/G, IL-6 rs1800795 C/G, and IL-1-α rs17561 A/C) of five inflammation-related genes were genotyped using Taqman-based allelic discrimination method on a QuantStudio 5 Real Time PCR system (Thermo Fischer). The PCR conditions were as follows: 40 cycles of 60 °C for 30 sec, 95 °C for 5 min, 95 °C for 15 sec, and 60 °C for 60 sec. For each sample reactions were performed in duplicate and included negative control in every reaction.

2.4. Statistical analysis

Patient characteristics (age, gender, socioeconomic status, oral hygiene, alcohol consumption, and use of tobacco or *Paan chewing*) were evaluated to see if differences existed between cases and controls. These comparisons were examined using medians and interquartile range (25th and 75th percentiles) for age and frequency, percentage, and $\chi 2$ or Fisher's Exact Tests where applicable. Hardy-Weinberg Equilibrium was assessed using $\chi 2$ tests. The main focus of the study was to assess the relationship between case-control status and SNPs. This was performed using univariable and multivariable logistic regression to test for any difference in the genotypes for each SNP. In the multivariable model, age and gender were included as adjusters. The relationship between *naswar*, socioeconomic status variables, and oral hygiene variables were examined to check if one could be used as an adjuster to represent all of them, because of the sample size limit, the number of adjusters and to avoid multicollinearity. This was done using $\chi 2$ or Fisher's exact tests where appropriate.

3. Results

During the study period, 231 HNC cases (147 oral cancers, 29 laryngeal cancers, 24 Hypopharyngeal cancer, 23 Oropharyngeal cancers and 8 Pharyngeal cancers) and 219 controls were recruited.

3.1. Characteristics of the studied subjects

Cases and controls included in the current investigation were considerably different in age. The number of males were higher than females in both cases (55% males and 45% females) and control (72.2% males and 27.9% females) groups. Both cases and control varied in demographic distribution. Resident of rural area found to be high proportion in both cases and control (79.2% cases and 64.8% control) as compared to urban area (20.8% cases and 35.2% control). Highest frequency of cases was found to be illiterate as compared to controls (84.4% cases and 25.1% control) and majority of cases belong to underprivileged socioeconomic families as compared to controls (93.5% cases and 47% control). Also, a significantly high number of cases had poor oral hygiene compared to controls (90.5% cases and 23.7% control). Majority of cases never brush the teeth compared to controls (89.6% cases and 49.3% control). Moreover, the percentag of missing teeth and the use of naswar were significantly greater among cases compared to control. However, very less proportion subjects were found to be smokers. Only 1.7% of cases contrasting to 0.9% controls used to chew *paan*. Similarly, 1.3% of used to drink alcohol compared to 0.5% of controls (Table 1).

3.2. Genetic analysis

For genotyping, allele specific PCR was performed. Allele specific PCR revealed that the frequency of IL-8 rs4073 wild homozygous allele AA was 0.13 (15% cases and 11% controls), heterozygous AT alleles were 0.46 (45.1% cases and 48.6% controls), homozygous minor allele was AA 0.4 (39.8% cases and 40.5% control). However, no significant statistical difference was observed for rs4073 between the two groups (Table 2).

For IL-4 rs2070874, the frequency of wild type allele CC homozygous was found to be highest (0.7) among both groups (70.4% cases and 70.8% controls) and heterozygous CT alleles frequency was 0.26 (26% cases and 26.9% controls). The homozygous minor allele TT showed frequency of 0.3 (3.6% cases and 2.4% controls) was less frequent. However, the association between case-control was statistically insignificant (Table 2).

For IL-10 rs1800896, the wild type homozygous TT allele frequency was 0.45 (50% cases and 38.9% controls), the heterozygous TC alleles was found with almost similar of 0.46 (39% and 53.2%). While, the minor allele CC frequency was 0.09 (11% and 7.9%). When compared between the two groups, TC genotype was less prevalent in cases as compared to controls, the difference between two groups was statistically significant (P = 0.01) (Table 2).

For IL-10 rs180072, the frequency of homozygous wild type allele TT was 0.2 (22.7% cases and 17.9 controls), heterozygous TG alleles were 0.47 (45.3% cases and 49.1% controls) and homozy-

Table1
Demographic and lifestyle characteristics of cases and control subjects.

Characteristic	Cases		Controls		
	N	Median (IQR*) or Frequency (%)	N	Median (IQR*) or Frequency (%)	P-value
Age	231	56.00 (47.00, 62.00)	219	27.00 (22.00, 36.00)	<0.01 *
Gender	231		219		<0.01 *
Female	–	104 (45.0)	–	61 (27.9)	–
Male		127 (55.0)		158 (72.2)	
Residence Type	231	–	219	–	<0.01 *
Rural	–	183 (79.2)	–	142 (64.8)	–
Urban		48 (20.8)		77 (35.2)	
Literate	231		219		<0.01 *
No		195 (84.4)		55 (25.1)	–
Yes		36 (15.6)		164 (74.9)	
Family Income	231	–	219	–	<0.01 *
<20,000	–	216 (93.5)	–	103 (47.0)	–
20,000–50,000		13 (5.6)		78 (35.6)	
>50,000		2 (0.9)		38 (17.4)	
Oral Hygiene	231		219		<0.01 *
Poor	–	209 (90.5)	–	52 (23.7)	–
Fair		22 (9.5)		93 (42.5)	
Good		0 (0.00)		74 (33.)	
Brush teeth/day	231	–	219	–	<0.01 *
No		207 (89.6)	–	108 (49.3)	
Yes		24 (10.4)		111 (50.7)	
Missing Teeth	231	–	219	–	<0.01 *
None	–	42 (18.2)	–	139 (63.5)	–
1–10		93 (40.3)		71 (32.4)	
>10		96 (41.6)		9 (4.1)	
Ever Smoke	231	–	219	–	0.58
No	–	194 (84.0)	–	188 (85.8)	–
Yes		37 (16.0)		31 (14.1)	
Ever Use *Naswar*	231		219	–	<0.01 *
No	–	145 (62.8)		182 (83.1)	
Yes		86 (37.2)		37 (16.9)	
Ever Chew *Paan*	231	–	219	–	0.69
No	–	227 (98.3)	–	217 (99.1)	–
Yes		4 (1.7)		2 (0.9)	
Ever Drink Alcohol	231	–	219	–	0.62
No	–	228 (98.7)	–	218 (99.5)	–
Yes		3 (1.3)		1 (0.5)	

Table 2
Distribution and genotype frequencies of studied SNPs.

SNP	Genotype	CasesN (%)	ControlsN (%)	UnivariableP-value	MultivariableP-value
IL8 rs4073 A/T	AA	34 (15.0)	23 (11.0)	0.43	0.68
	AT	102 (45.1)	102 (48.6)	.	.
	TT	90 (39.8)	85 (40.5)	.	.
IL4 rs2070874 C/T	CC	157 (70.4)	150 (70.8)	0.75	0.19
	CT	58 (26.0)	57 (26.9)	.	.
	TT	8 (3.6)	5 (2.4)	.	.
IL10 rs1800896 T/C	TT	114 (50.0)	84 (38.9)	0.01	0.01
	TC	89 (39.0)	115 (53.2)	.	.
	CC	25 (11.0)	17 (7.9)	.	.
IL10 rs180072 T/G	TT	51 (22.7)	38 (17.9)	0.46	0.98
	TG	102 (45.3)	104 (49.1)	.	.
	GG	72 (32.0)	70 (33.0)	.	.
IL6 rs1800796 C/G	CC	18 (8.0)	15 (7.0)	0.22	0.99
	CG	81 (36.0)	95 (44.2)	.	.
	GG	126 (56.0)	105 (48.8)	.	.
IL6 rs1800795 C/G	CC	9 (4.0)	7 (3.3)	0.28	0.70
	CG	60 (26.7)	72 (33.6)	.	.
	GG	156 (69.3)	135 (63.1)	.	.
IL1-α rs17561 A/C	AA	20 (8.9)	14 (6.5)	0.63	0.90
	AC	102 (45.5)	99 (46.0)	.	.
	CC	102 (45.5)	102 (47.4)	.	.

gous minor allele GG was 0.32 (32% cases and 33% controls) genotype was less frequent (22.7% cases vs 17.9% controls). However, the difference among the two groups was statistically insignificant (Table 2).

For IL-6 rs1800796, the CC wild allele was least with frequency of 0.075 (7% cases and 8% controls), the heterozygous CG alleles have a frequency of 0.4 (36% and 44.2%), while, the homozygous minor allele GG has a frequency of 0.52 (56% cases and 48.8% control). But the association between the two subject groups was statistically insignificant (Table 2).

For IL-6 rs1800795, the homozygous wild allele CC was found less frequent with 0.035 frequency (4% and 3.3%), heterozygous

CG was 0.3 (26.7% cases and 33.6% controls) and GG homozygous allele was 0.66 (69.3% cases and 63.1% control). However, no significant association between rs1800795 and disease was found (Table 2).

For IL-1α rs17561, the wild type allele AA frequency was 0.077 (8.9% cases and 6.5% controls), the heterozygous AC was 0.455 (45.5% cases and 46% controls) and CC homozygous allele was 0.46 (45.5% vs 47.4%). Statistical analysis did not revealed any significant association of rs17561 and HNC in the studied population (Table 2).

Findings of present study show that frequencies of *IL8* rs4073, *IL4* rs2070874, *IL10* rs1800896, IL-10 rs180072, IL-6 rs1800796, IL-6 rs1800795 and IL-1α rs17561 genotypes were in line with the Hardy-Weinberg Equilibrium in controls ($P < 0.05$) except IL-10 rs1800896 T/C (Table 3).

In the present study logistic regression analysis (Univariate model) shows that when the *IL10* rs1800896 T/C TT homozygous genotype was used as reference the CC genotype was not associated with a significantly increased risk for oral cancer. While TC genotype was associated with a significantly increased risk for oral cancer (Fig. 1). However, multivariate model revealed that when the *IL10* rs1800896 T/C TT homozygous genotype was used as reference the TC genotype and CC genotype were not associated with a significantly increased risk for oral cancer (Fig. 2).

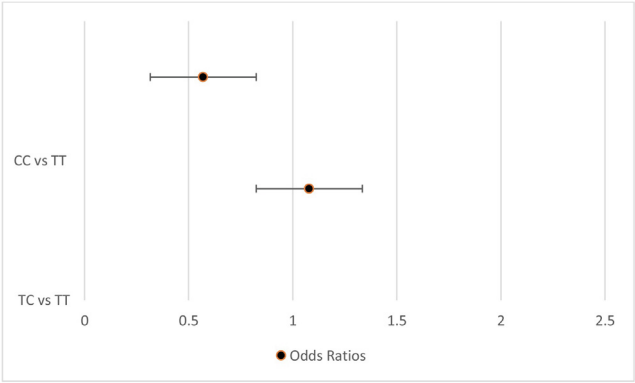

Fig. 1. Logistic regression analysis of association among significant SNP (*IL10* rs1800896 T/C) and risk of oral cancer (univariate model).

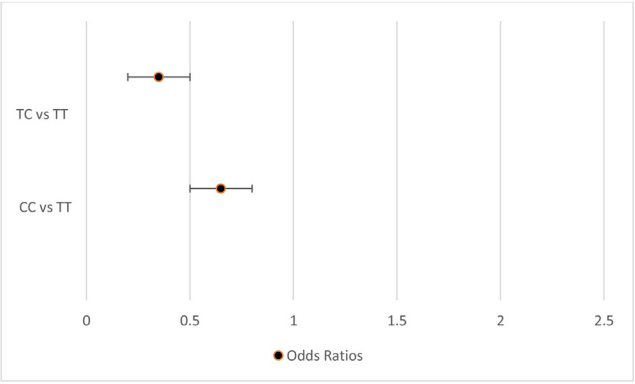

Fig. 2. Logistic regression analysis of association among significant SNP (IL10 rs1800896 T/C) and risk of oral cancer (multivariate model).

4. Discussion

HNC is becoming a serious health threat worldwide and accounts for>550,000 cases and 380,000 deaths per year (Fitzmaurice et al., 2017). In Pakistan, HNC is most prevailing (32.6%) in male and second most prevalent (15.1%) malignancy after breast cancer (38.2%) in female (Hanif et al., 2009). It has been revealed that use of tobacco, alcohol intake, infection with human papillomavirus (HPV) and Epstein-Barr virus (EBV), and exposure to environment are the main etiologic factors contributing to HNC. In relation to oral health, the polymicrobial supragingival plaque has a pertinent mutagenic association with saliva, it may be contemplated as a potential independent factor, and individual oral hygiene may be a co-factor in the progression of oral cancer by excessive secretion of inflammatory mediators like cytokines (Gaudet et al., 2010). In addition to exogenous risk factors, development of cancer is also dependent on individual genetic makeup such variations in genes related to growth, development, differentiation, immune system, apoptotic pathways, alcohol metabolism, DNA repair, and activation of proto oncogenes (Lin and Karin, 2007; Ma et al., 2011; Bediaga et al., 2015). Single nucleotide polymorphisms (SNPs) are the most common genetic variation in humans, which may affect gene expression, protein function, and disease predisposition (Nachman, 2001; Hsu et al., 2014).

This study was conducted to find out the association of oral cancer with oral hygiene, use of tobacco and SNPs in interleukin genes. Oral squamous cell carcinoma (OSCC) was found to the most frequent (63.6%) followed by tumors of larynx, hypopharynx, and oropharynx, which is different from previous studies, where cancer of buccal mucosa (32%) and cancer of tongue (50%) were reported the most frequent (Tahir et al., 2013; Gul et al., 2017). The highest frequency of oral cancer patients was observed in the age group of >40–60 consistent to previous studies (Divaris et al., 2010). The proportion of affected males was higher than female which is consistent to previous report (Teofil et al., 2007). In Pakistan, one reason for increased incidence may be the use of tobacco in males. Data indicated that oral cancer are more common in individuals with poor oral health, low socioeconomic background and deprived education, consistent with previous reports across the globe (Zheng et al., 1990; Marshall et al., 1992; Talamini et al., 2000). Also, a significant association of missing teeth and risk of oral cancer was observed, which is consistent with previous studies (Marshall et al., 1992; Balaram et al., 2002; Lissowska et al., 2003).

Smoking is always supposed to be a vital risk factor in the progression of different malignancies particularly oral carcinoma (Tai et al., 2010; Wyss et al., 2013). Though, in this study there was an insignificant association with smoking. However, a significant

Table 3
Testing deviation from HW (Controls).

SNP	Homozygous Major Allele	HeterozygousAlleles	Homozygous Minor Allele	Controls N	P value
IL8 rs4073 A/T	23	102	85	210	0.35
IL4 rs2070874 C/T	150	57	5	212	0.88
IL10 rs1800896 T/C	84	115	17	216	0.01
IL10 rs180072 T/G	38	104	70	212	0.95
IL6 rs1800796 C/G	15	95	105	215	0.29
IL6 rs1800795 C/G	7	72	135	214	0.48
IL1-α rs17561 A/C	14	99	102	215	0.12

association between *naswar* use and oral cancer was found (Table 1). Use of *naswar* is common practice in Pakistan, particularly in Khyber Pakhtunkhwa (KP) province, and it is particularly limited to population low socioeconomic strata (Imam et al., 2007). The association of alcohol and *Paan* is not clear, due to their rare use.

In present study, association of rs4073, rs2070874, rs1800896, rs180072, rs1800796, rs1800795 and rs17561 polymorphisms was studied with risk of HNC in Pakistani population. Among the studied SNPs, of rs4073, rs2070874, rs180072, rs1800796, rs1800795 and rs17561 didn't showed association with the oral carcinoma in the studied population, which is in contrast to previous reports. The different results can be explained by difference in genetic backgrounds, ethnicities, geographical area, environmental factors, and also a correlation of the SNP with different risk factors.

However, only rs1800896 was found associated with the disease, consistent with Vairaktaris et al. (2008) study of European population. The SNP rs1800896 is located upstream of *IL-10* gene, which is known to be involved in angiogenesis, inflammation, autoimmune diseases and many types of malignancies (including oral carcinoma). IL-10 is produced by lymphoid cells, monocytes and macrophages, a multifunctional immunosuppressant that not only interfere with the function of T-cells but also associated with the cessation of inflammatory responses (Rojas et al., 2017). Moreover, it regulates growth and differentiation of

B-cells, NK, cytotoxic and T helper cells, mast cells, granulocytes, dendritic cells, keratinocytes and endothelial cells (Iyer and Cheng, 2012).

Increased levels of IL-10 have been reported in cases with solid tumors, including oral squamous cell carcinoma (SCC), indicating that this pleiotropic cytokine may have a vital role in carcinoma (10–12). IL-10 has been observed to suppressing the immune and inflammatory responses and ultimately promote tumor proliferation (Ma et al., 2011; Hsu et al., 2014; Mojtahedi et al., 2014). Variations in promoters region of IL-10, including rs1800896, upregulate IL-10, which consequence in tumor proliferation by suppressing the immune and inflammatory responses.

One of the main limitation of the study is that sample size was not large enough. Further investigations with large sample size are required to disentangle and explain the observed link between polymorphism in IL10 rs1800896 and HNC.

5. Conclusion

In conclusion, this is the first attempt to study the association of interleukins with HNC in addition to oral-hygiene. The study shows that rs1800896 is a risk factor of HNC in Pakistani population.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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