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Association between Interleukin 6 Gene Polymorphism and Human Papilloma Virus Infection in Oral Squamous Cell Carcinoma Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MZ, NIH and SB designed the study. Author MZ collected the samples, did the bench work, wrote the protocol, and wrote the first draft of the manuscript. Authors NIH and SB facilitated in literature search and finalization of the manuscript. Author NZ assisted in analyses of the data. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To find out an association between Human Papilloma Virus and IL 6 gene polymorphism in Oral Squamous Cell Carcinoma patients.
Study Design: Cross-sectional study.
Place and Duration of Study: Ziauddin Hospital (Dental OPD), Karachi, Pakistan. In between the period of January 2014 to May 2015.
Methodology: This cross-sectional study consisted of a total of 140 oral squamous cell carcinoma patients of 18 years and above (104 males and 36 females). Detailed questionnaire followed by sample collection from each patient was done. These samples were analyzed by polymerase chain reaction for human papilloma virus and IL 6 gene polymorphism was analyzed through restriction fragment length polymorphism.

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Results: Mean age of the patients was 43.5±11.84 years (range 31-40 years). Most of the patients (45; 32.1%) belonged to the Urdu speaking ethnic group. Pan (87; 62.1%) and Gutka (82; 58.6%) were used by most of the patients. (17;12.1%) patients had history of systemic disease (e.g hypertension, diabetes). And (3021.4%) patients had a positive family history of OSCC. The most common site of OSCC was buccal mucosa (86; 61.4%) in these patients. Majority of the patients (77; 55%) had histologically moderately differentiated OSCC, and more than half of these patients (78; 55.7%) had Group B (stage III & IV) of OSCC. (12;8.6%) out of 140 samples tested positive for human papilloma virus gene and the following pattern was observed for IL 6 gene polymorphism, GG: (46.4%), GC (39.3%), CC (14.3%). A positive association was observed for Group B (stages III & IV) of oral squamous cell carcinoma with IL 6 genotypes: GC heterozygote (OR=3.819, 95% CI=1.782-8.183, P=0.001) and CC homozygote (OR=6.833, 95% CI=2.046-22.822, P=0.002), and also a strong positive association was found between human papilloma virus and CC homozygote genotype (OR=21.333, 95% CI=2.318-196.311, P=0.007).
Conclusion: Human papilloma virus association with IL 6 gene polymorphism in oral squamous cell carcinoma patients suggests rapid and aggressive progress of oral carcinogenesis.

Keywords: OSCC; HPV; IL 6 gene polymorphism.

1. INTRODUCTION

Oral cancer is one of the most common malignancies occurring worldwide, with a very low survival rate despite its location which should make detection easier at an early stage [1]. Annually more than 500,000 new cases are diagnosed worldwide [2], having higher rates among the Southeast Asia, Latin America, Western and Eastern Europe, and the Caribbean as compared to the rest of the world [3]. About 90% of the oral cancer detected, are of squamous cell carcinoma (SCC) type, occurring mostly in the buccal mucosa, floor of the mouth, lateral side of the tongue and over the lips in the form of lump, white/red or mixed patch or like an ulcer [1]. Multiple factors are involved in the development of oral cancer including tobacco and alcohol abuse, changes in tumor suppressor genes and oncogenes, and infectious agents [4]. In Asian countries a huge percentage of OSCC have been reported, due to the use of smokeless tobacco (gutka and betel quid) [5], therefore variation is seen regarding incidence of OSCC in different geographic areas. Currently infectious agent such as, human papilloma virus (HPV) has been identified as one of the causative factor for the increasing incidence of oral cancers among young population and non-smokers [6]. HPV is a small, circular, double stranded DNA virus causing benign and malignant tumors of the oral and anogenital regions [7]. HPV and oral carcinogenesis association was first investigated by Syrjanen et al. in 1983 [8]. More than 100 genotypes of HPV have so far been identified and are divided as high risk and low risk types [9]. The HPV oncoproteins E6 and E7 causes inactivation of the host tumor suppressor genes (p53 and retinoblastoma gene) respectively, and leads to development of oral cancer [10].

Several genetic changes and abnormalities in the signaling pathways lead to the formation of OSCC [11]. Among these changes include uncontrolled cell growth, inhibition of apoptosis, neo-angiogenesis, invasion and metastasis, with the help of oncogenes activation and tumor suppressor genes inactivation [12]. The detection of oral cancer is usually late, because of the lack of early sign and symptoms, so researchers are now mainly focusing on salivary diagnostic techniques for early detection of oral squamous cell carcinoma. Saliva of patients with oral squamous cell carcinoma contains high levels of different enzymes, proteins and chemicals which serve as potential biomarkers [13]. Pathological change occurring in oral cavity can be predicted by these biomarkers with detection of the disease at an early stage [14]. These biomarkers may be helpful as a screening tool, irrespective of lesion localization, and can detect both the premalignant and malignant changes in the oral cavity [15].

IL 6 gene is an emerging; most studied and investigated biomarker. It is a pro-inflammatory cytokine produced by many different cells of the human body including, endothelial cells, keratinocytes, T and B lymphocytes, and mesangial cells both in normal and cancer tissues [16]. IL 6 is a T cell derived cytokine, inducing maturation of B cells into antibody producing cells, and has multiple functions, which differ in different kinds of tissues and cells of the body [17]. Some researchers have investigated the association between IL6 gene

polymorphism and development of oral cancer [18,19]. According to various studies high IL6 levels have been detected in saliva, serum and tumor biopsies of OSCC patient [20]. IL6 is expressed in high levels in patients with premalignant lesions, and OSCC patients, when compared to the normal subjects [21]. Increased levels of IL6 in serum samples of OSCC patients can predict tumor recurrence, tumor metastasis and poor prognosis [22].

Both HPV and IL 6 gene polymorphism have independently proven their association with OSCC, and their presence indicates that the patient is a high risk candidate for developing OSCC. So we assume that the positivity of both HPV and IL 6 in a patient with OSCC would raise the threshold of patient towards the aggressiveness of the disease. Therefore, the aim of our study was to find out an association between HPV and IL 6 gene polymorphism in OSCC patients, so we can rule out the patients with poor prognosis, and by finding any association between these two variables, we would be able to identify the patients with an aggressive nature of the disease, which may prove helpful in the therapeutic intervention of OSCC in future.

2. MATERIALS AND METHODS

This cross-sectional study was conducted from 13th Jan 2014 to 25th May 2015 at Ziauddin University Karachi, after approval by the Ethical Review Committee of Ziauddin University, Karachi. The study consisted of a total 140 pre-operative oral squamous cell carcinoma patients of age 18 years and above, recruited from the Ziauddin hospital. Exclusion criteria included patients with any pre-malignant oral lesions or any other type of oral cancers, and patients not willing to give written inform consent. Detailed history was taken from each patient and grade and stage of OSCC was assessed according to the CAP protocol [23]. Patients were graded into well differentiated, moderately differentiated, and poorly differentiated tumors and staged from I to IV. As reported in literature patients with stages I & II were categorized as (Group A) and stages III & IV as (Group B) [18]. Purposive sampling was done after a written informed consent. Prior to sample collection gentle brushing of the oral mucosa with the help of a brush or the other end of a dental floss was done and 40ml of oral rinse samples were obtained from all the patients and stored at 4ºC, followed by DNA extraction through a protocol previously reported [24]. Finally PCR for HPV, and (-174G/C) polymorphism in IL6 gene was carried out by restriction fragment length polymorphism.

2.1 Polymerase Chain Reaction for HPV

PCR was performed by using HPV consensus primers Gp5+/Gp6+. Primers sequence for HPV - Gp5+ was 5'-TTTGTTACTGTGGTAGATACTAC-3', and HPV-Gp6+ was 5'-GAAAAATAAACTGTAAATCATATTC-3' (Gene Link NY USA). DNA was amplified in a conventional thermo cycler (BIOFLUX). A HPV positive control, human ß-globin gene and a blank was used with every reaction. The PCR amplification was carried out in a volume of 25 µl containing 12.5 µl of master mix (Promega) containing as final concentrations of 10 mm Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates (dNTPs), and 1 unit of Taq DNA polymerase (Promega) and 10 µl DNA (1 µg). One µmol of primers Gp5+ and Gp6+. The first DNA denaturation was performed for 5 min at 94ºC; followed by 35 cycles of PCR consisting of denaturation for 30 sec at 94ºC, annealing for 30 sec at 55°C, and extension for 1 min at 72ºC, and a final extension for 5 min at 72ºC. The amplified product of 150 base pair for HPV was analyzed on 2% Agarose gel by electrophoresis [24].

2.2 RFLP for IL-6 Gene (-174G/C) Polymorphism

Molecular detection of the (-174G/C) polymorphism in the IL-6 gene was performed by restriction fragment length polymorphism typing. This involved a combination of PCR amplification and digestion with restriction endonuclease *Nla* III followed by gel electrophoretic analysis. The PCR conditions consisted of an initial denaturation step at 95ºC, followed by 35 cycles of 94ºC for 55 seconds, 61ºC for 1 minute, and 72ºC for 50 seconds, and a final elongation step at 72ºC for 5 minutes. The primers used were:

Forward: 5'-TGACGACCTAAGCTGCACTTTTC-3' and
Reverse: 5'-GGGCTGATTGGAAACCTTATTAAGA-3'.

A PCR product of 93 base pair was cleaved by restriction enzyme *Nla* III, if the C allele was present, into two fragments of 52 base pair and 41 base pair [18].

2.3 Statistical Analyses

Data was entered on SPSS version 20. Quantitative data was presented as mean and standard deviation. And for Qualitative data frequencies and percentages were calculated. Association between IL6 gene polymorphism and HPV infection in OSCC patients was seen through application of Chi-square. Odds ratio was calculated by logistic regression at 95% CI and P-value less than 0.05 was considered significant.

3. RESULTS

In total, 140 patients (104 males and 36 females), were analyzed in this cross-sectional study. The mean age of the patients was 43.5±11.84 years. The histological tumor type of all 140 patients was squamous cell carcinoma. They were further divided into six ethnic groups and most of the patients (45;32.1%) belonged to the Urdu speaking ethnic group. All the patients gave history of tobacco usage mostly in the form of Pan (87;62.1%) and Gutka (82;58.6%). History of systemic disease (e.g hypertension, diabetes) was present in (17;12.1%) patients. And (30; 21.4%) patients had a positive family history of OSCC. In this study we observed that the most common site of OSCC in the patients was buccal mucosa (86;61.4%). Grade and stage of OSCC were also evaluated in these patients, which showed majority of these tumors (77;55%) were histologically moderately differentiated OSCC, and more than half of these patients (78; 55.7%) were in (Group B) stage III & IV of OSCC. Human Papilloma virus was found in (12;8.6%) of these patients only. The genotype frequencies and the demographic details are given in Table 1.

Table 1. Demographic details, tobacco usage and clinical characteristics of OSCC patients

OSCC patients		Frequency	Percentage
Gender	Male / Female	104 / 36	73.4% / 25.7%
Mean age			43.5±11.84
Ethnicity	Urdu Speaking	45	32.1%
	Sindhi	23	16.4%
	Balochi	14	10%
	Pathan	28	20%
	Punjabi	20	14.3%
	Others	10	7.1%
Tobacco use	Naswar	49	35%
	Pan	87	62.1%
	Gutka	82	58.6%
	Betel Nut	66	47.1%
	Smoking	58	41.4%
Location of OSCC	Buccal Mucosa	86	61.4%
	Tongue	45	32.1%
	Tongue with floor	3	2.1%
	Palate	4	2.9%
	Submandibular Gland	2	1.4%
Stage of OSCC	Group A (Stage I & II)	62	44.2%
	Group B (Stage III & IV)	78	55.7%
Grade of OSCC	Well Differentiated	35	25%
	Moderately Differentiated	77	55%
	Poorly Differentiated	28	20%
IL 6 Genotype	GG	65	46.4%
	GC	55	39.3%
	CC	20	14.3%
HPV	Positive	12	8.6%
	Negative	128	91.4%
Any systemic disease		17	12.1%
Family history of OSCC		30	21.4%

The frequencies of genotypes in (Group A) stages I & II were GG (41;29.3%), GC (17;12.2%) and CC (4;2.8%), and in (Group B) stages III & IV were GG (24;17.1%), GC (38;27.1%), and CC (16;11.5%), and a statistically significant P value (P=<0.001) association was observed between stage of OSCC and IL 6 gene polymorphism. Additionally, GC heterozygote had (OR=0.262,95% CI=0.122-0.561,*P*=0.001) for developing oral cancer in (Group A) stages I & II and (OR=3.819, 95% CI=1.782-8.183, *P*=0.001) in (Group B) stages III & IV. And for CC homozygote the odds ratio was (OR=0.149,95% CI=0.044-0.489,*P*=0.002) for (Group A) stages I & II, while the odds ratio and confidence interval was even greater for CC homozygote (OR=6.833,95% CI=2.046-22.822, *P*=0.002) for (Group B) stages III & IV.

The genotype distribution among HPV positive patients were, GG (1;0.7%), GC (6;4.3%), and CC (5;3.6%), and a statistically significant (*P*=0.003) association was observed between HPV and IL 6 gene polymorphism. The odds ratio for disease advancement and aggressiveness of the disease in (Group B) stages I & II for GC heterozygotes was (OR=7.837,95% CI=0.913-67.240, *P*=0.060) and for the CC homozygotes was much higher (OR=21.333,95% CI=2.318-196.311, *P*=0.007) respectively (Tables 2 & 3).

4. DISCUSSION

Both HPV and IL 6 gene polymorphism has been associated with the risk of oral squamous cell carcinoma [18,25]. To the best of our knowledge the relationship between the HPV and IL 6 gene polymorphism in oral cancer has so far not been investigated. So in this study we observed the association between HPV and IL 6 gene polymorphism in OSCC patients.

The maximum number of patients in our study belonged to the younger age group (31-40 years). This was in similarity with a study conducted by Bhurgri et al. [26] showing prevalence of oral cancer in young adults. This early presentation of oral cancer is mainly attributed to the use of tobacco in various forms in our younger generation, compared to the

Table 2. Frequency of IL 6 gene polymorphism in patients with OSCC, along with odds ratio and 95% CI of developing aggressiveness of disease in regard to IL 6 gene polymorphism and cancer stage in these patients

Stage of OSCC	IL 6 Genotype			P-value
	GG n (%)	GC n (%)	CC n (%)	
Group A*	41 (29.3%)	17 (12.2%)	4 (2.8%)	< 0.001
Group B **	24 (17.1%)	38 (27.1%)	16 (11.5%)	
Stage of OSCC	**Genotype**	**OR**	**OR**	**OR**
Group A *	GC	0.262	0.122-0.561	0.001
	GG	1 (referent)	1 (referent)	1 (referent)
	CC	0.146	0.044-0.489	0.002
Group B **	GC	3.819	1.782-8.183	0.001
	GG	1 (referent)	1 (referent)	1 (referent)
	CC	6.833	2.046-22.822	0.002

Group A (Stage I & II); Group B (Stage III & IV)*

Table 3. Frequency of HPV in patients with OSCC, along with odds ratio and 95% CI of developing aggressiveness of disease in regard to HPV presence and IL 6 gene polymorphism in these patients

HPV	IL 6 Genotype			P-value
	GG n (%)	GC n (%)	CC n (%)	
Positive	1 (0.7%)	6 (4.3%)	5 (3.6%)	**0.003**
Negative	64 (45.7%)	49 (35%)	15 (10.7%)	
HPV	**Genotype**	**OR**	**95% CI**	**P-value**
	GC	7.837	0.913-67.240	0.060
	GG	1 (referent)	1 (referent)	1 (referent)
	CC	21.333	2.318-196.311	0.007

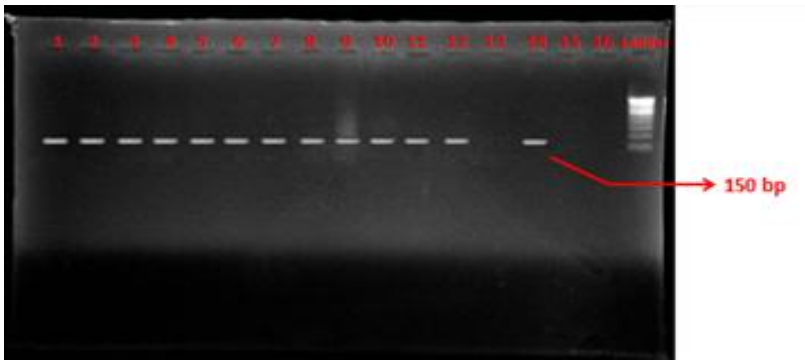

Fig. 1A. PCR analysis of Human papilloma virus. Lane (M): 100 bp DNA ladder
Lanes (1-12): Represents HPV. Lanes (13 and 16): Negative control. Lane (14): Positive control

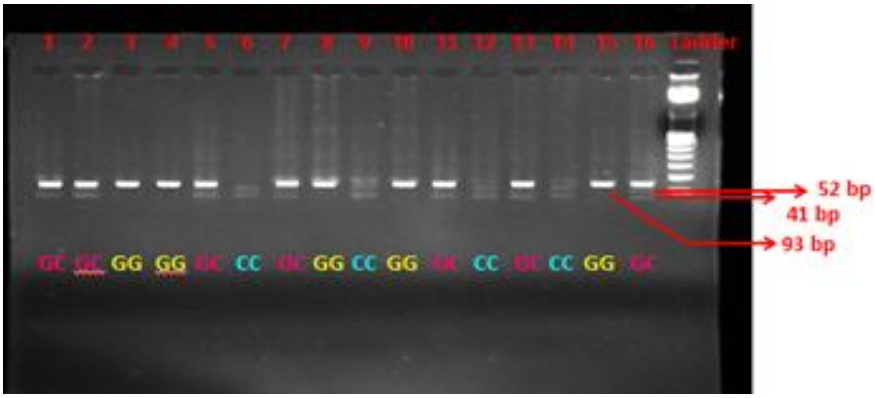

Fig. 1B. RFLP analysis of IL-6 (-174G/C) gene polymorphism
Lane (M): 50 bp DNA ladder. Lanes (3, 4, 8, 10 and 15): GG genotype
Lanes (1, 2, 5, 7, 11, 13, and 16): GC genotype. Lane (6, 9, 12 and 14): CC genotype

western countries where the cause is mainly the human papilloma virus infection Brown et al. [27]. Male predominance (104;74.3%) seen in our study, has also been reported by other studies [28,29]. This supports the fact that males are more prone to develop OSCC as compared to females due to exposure to the risk factors like tobacco and HPV. The ethnic group mostly affected in our study was Urdu speaking; this was in accordance to the finding of Akram et al. [30]. And the most commonly involved site by OSCC in this study was buccal mucosa followed by tongue, the same pattern of involvement observed by Rajkumar et al. [31] and Akram et al. [30], this appears to be mainly due to the prolonged exposure of such sites to the carcinogens present in chewable tobacco kept in the buccal pouches for longer duration Tanaka et al. [32]. Most of the patients in our study had (Group B) stage III & IV OSCC in similarity to other studies by Santarelli et al. and Chen et al. [33,29]. Histological grading of majority of our patients showed moderately differentiated OSCC (77;55%) and a similar pattern was also observed by Alamgir et al. [34] in a study on a section of Karachi population.

The association of stages of OSCC and HPV with IL 6 gene polymorphism was evaluated in this study, showing a positive association between stages of OSCC and IL 6 genotypes (*P*=<0.001). The odds ratio for developing OSCC was much higher for GC heterozygote (OR=3.819, 95% CI=1.782-8.183, *P*= 0.001) and CC homozygote (OR=6.833,95% CI=2.046-22.822, *P*=0.002) for stages III & IV band this finding of our study was in accordance with the study conducted by Vairaktaris et al. [18].

A statistically significant (*P*=0.003) association between HPV positive patients and IL 6 genotypes was observed in this study. Like all viruses, HPV binds to Toll like receptors (TLRs) which are expressed on immune cells such as macrophages etc. Through interaction with viral nucleic acids these receptors activate an

inflammatory response by releasing inflammatory cytokines which includes IL-6 [35]. IL 6 binds to its receptor gp130 on cell membrane and leads to activation of Janus kinases which further causes stimulation of STAT3 signaling pathway. STAT3 once activated has multiple functions leading to uncontrolled growth, anti-apoptosis, neo-vascularization and tumor metastasis [36].

Among the genotypes the relative risk for aggressiveness of the disease and poor prognosis was more for CC homozygote (OR= 21.333,95% CI=2.318-196.311,*P*=0.007) as compared to GC heterozygote (OR=7.837,95% CI=0.913-67.240,*P*= 0.060). These findings were in contrast to the finding of the study by Porto et al. [37], conducted a study to observe frequency of polymorphism of IL6 in HPV positive and negative women, and observed a similarity of IL 6 gene polymorphism in both the cases. While another study by Rosa et al. [38] over cervical specimens and blood, suggested that IL 6 levels were associated with the persistence of HPV infection among women. To the best of our knowledge this is the first study carried in this regard. The limitation of our study was small number of sample size, and to get even more accurate results a study with larger sample size should be conducted over this aspect. While a comparative study could also be conducted for association between IL 6 gene polymorphism and HPV genotypes among healthy subjects, oral pre-cancerous lesions patients and OSCC patients, to get more clear view of the relative risk of developing OSCC among healthy subjects and oral pre-cancerous lesion patients, and progression of the disease in OSCC patients.

5. CONCLUSION

Despite of the vast advancements in the diagnosis and treatment, the prevalence of OSCC is increasing worldwide. This situation can be better controlled if the disease and its risk factors are identified at early stages. Strong positive association between IL 6 gene polymorphism and HPV in OSCC patients may help us rule out the patients with poor prognosis and aggressive nature of disease. This association may be helpful for the therapeutic intervention of OSCC in future and in undertaking certain preventive measures to safeguard the health status and lives of individuals at risk among general population.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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