

## RESEARCH ARTICLE

## Association of interleukin-10 (A1082G) gene polymorphism with oral squamous cell carcinoma in north Indian population

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### Abstract

The functional polymorphism A1082G in the gene (*IL10*) for interleukin-10 associated with risk of oral squamous cell carcinoma (OSCC). The present case-control study was to evaluate the possible association between *IL10* A1082G gene and OSCC in north Indian population. Analysis of *IL10* A1082G genotype in 232 OSCC cases and 221 healthy controls of comparable age, gender, smokers, tobacco chewing and alcohol consumption. *IL10* A1082G status in cases and controls were evaluated by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The frequencies of *IL10* A1082G polymorphism AA, AG, GG genotypes were 29.74, 68.10 and 2.15% in OSCC cases and 57.46, 42.08 and 0.45% in healthy controls. The average frequency of G mutant allele was 36.20% in OSCC cases compared with 21.50% among the controls and this allele was associated with increased risk for OSCC cases. Heterozygous AG genotype was found statistically significant in OSCC cases than in controls (OR = 1.6, 95% CI = 1.1–2.2, *P* = 0.003), whereas homozygous mutant GG genotype was not found significant (OR = 4.7, 95% CI = 0.55–41.1, *P* = 0.2). Moreover, we found that G allele was significant in OSCC cases of tobacco chewing. The frequency of *IL10* A1082G polymorphism G allele and AG genotype is associated with OSCC cases as compared with controls; this may be due to smoking and tobacco chewing. Our findings showed that in *IL10* A1082G gene polymorphism AG genotype and G allele may participate in the progression of OSCC.

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### Introduction

Oral squamous cell carcinoma (OSCC) is the worldwide eighth common human malignancy with more than 4,260,000 patients and ~128,000 deaths each year (Petersen 2005; Ferlay *et al.* 2010). The incidence of oral cancer in India is 12.8 per 100,000 in males and 7.5 per 100,000 in females. Among all the oral malignancies, OSCC accounts for more than 90% cases (Neville and Day 2002). Previous studies reveal that OSCC is the most commonly occurring malignancy which is responsible for morbidity and mortality (Williams 2000; Massano *et al.* 2006). Oral cancer is a multistep process which occurs due to factors like smoking, tobacco chewing, alcohol consumption, nutrition deficiency, genetic and environmental factors (Neville and Day 2002; McDowell 2006).

Interleukin-10 (*IL-10*) have been associated with increased risk for the development of OSCC, which is an

immunosuppressant cytokine that inhibits activation and function of T cells; responsible for angiogenesis, thrombophilia autoimmune disease, inflammation and determines termination of inflammatory responses (de Vries 1995; Fortis *et al.* 1996). The encoding gene of *IL10* is located on chromosome 1 (1q31-1q32). *IL10* inhibits synthesis of cytokines and tumour necrosis factor-alpha (TNF- $\alpha$ ) in activated macrophage and interferon gamma (IFN $\gamma$ ) by T cells, thus it plays major role in antiinflammatory responses (Vairaktaris *et al.* 2007a). The synthesis of cytokines is controlled genetically that differs among individuals as functions of polymorphisms within the regulatory region of various genes determine the transcriptional activation (Cantagrel *et al.* 1999; Vairaktaris *et al.* 2007b, c; Yapijakis *et al.* 2009).

The *IL10* promoter is composed of three biallelic polymorphisms at position –1082 from the transcription start site which affect the production of *IL10* (Rasmussen *et al.* 2000). *IL10* polymorphisms are found to be associated with breast cancer (McCarron *et al.* 2002), cervical cancer (Trabace *et al.* 2002), multiple myeloma (Savage *et al.* 2004), cutaneous

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malignant melanoma (Palli *et al.* 2005), oral squamous cell carcinoma (Rhodus *et al.* 2005), gastric carcinoma (Hatanaka *et al.* 2001) and hepatocellular carcinoma (Nagata *et al.* 2002). High expression levels of *IL10* have been observed in OSCC cases and were associated with poor prognosis (Karcher *et al.* 1999; Fujieda *et al.* 1999). Genetic factors influence or regulate the level of expression of *IL10* gene-like polymorphisms at location A1082G in the promoter region of the gene (Lin *et al.* 2003). Due to relation disequilibrium of polymorphic alleles, high or low expression of *IL10* may be examined by studying single polymorphic position (Lin *et al.* 2003).

Thus, the presence of *G* allele of the *IL10* A1082G polymorphism is associated with higher production of *IL10* (Lin *et al.* 2003; Yilmaz *et al.* 2005). The allele *G*-transmitting genotypes have been found to be significantly frequent in the cases of lung squamous cell carcinoma (Seifart *et al.* 2005). *IL10* A1082G polymorphism in *AA* genotype was significantly frequent in prostate cancer and cutaneous malignant melanoma (Howell *et al.* 2001; McCarron *et al.* 2002). *IL10* A1082G was not found to be associated with oesophageal squamous cell carcinoma and gastroesophageal junction adenocarcinoma (Savage *et al.* 2004; Guo *et al.* 2005). In oriental Asians genotypes, high frequency of *G* allele ranges between 21–84% and 20–52% in Caucasians population (Meenagh *et al.* 2002; Savage *et al.* 2004; Guo *et al.* 2005). The functional polymorphisms *IL10* A1082G have influence on the gene expression and associated with risk for OSCC. The present case–control study was to evaluate the possible association between *IL10* A1082G and OSCC in north Indian population.

## Materials and methods

### Samples collection

In this study the population consisted of 232 cases of OSCC and 221 healthy controls. Samples were collected from Department of Oral Pathology and Microbiology, King George's Medical University, Lucknow, India. This study was carried out between March 2012 and June 2014. Ethical approval was obtained from the institutional ethical committee of King George's Medical University, Lucknow, India. Informed written consent was obtained from all subjects.

### Inclusion and exclusion criteria

All cases were divided into two subgroups according to OSCC status: (i) patients with early cancer stage (stages I and II) and (ii) patients with advanced cancer stage (stages III and IV), according to the American Joint Committee for Cancer staging and end-results reporting (AJCC 1992, Cancer staging manual, [http://seer.cancer.gov/manuals/historic/comp\\_stage1.1.pdf](http://seer.cancer.gov/manuals/historic/comp_stage1.1.pdf)). Clinicopathologic information on each case, including age, gender, alcohol and tobacco usage, tumour location, treatment, clinical and pathologic stages, presence or absence of tumoural recurrence and survival of the patients

were obtained from medical records and tumour registries. All cases included presented with primary squamous cell carcinoma of the oral cavity. Cases with a former positive history of other type of cancer or with oral histological lesions other than OSCC were excluded. The control group included healthy blood donors who were recruited by health care professionals and volunteers. Controls were matched to cases with regard to ethnicity, gender, age and a low-risk working environment. Healthy individuals with a positive history of cancer were also excluded from serving as controls as well as with a former positive history of other type of cancer or with oral histological lesions other than OSCC were also excluded.

### DNA isolation

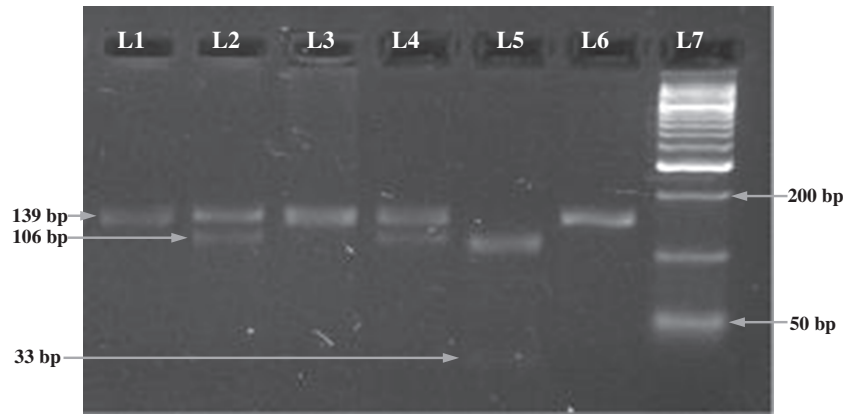
Genomic DNA extraction from 232 cases of OSCC and 221 healthy controls was done using a commercially available DNA extraction kit (Medox, Chennai, India) and was stored at  $-20^{\circ}\text{C}$ .

### *IL10* gene A1082G polymorphism

The *IL10* A1082G polymorphism was analysed by PCR followed by RFLP. Genomic DNA was amplified (Applied Biosystems, Veriti, Singapore) using the following PCR conditions:  $94^{\circ}\text{C}$  for 4 min, 35 cycles at  $94^{\circ}\text{C}$  for 50 s,  $54^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 55 s, and finally  $72^{\circ}\text{C}$  for 5 min. The primers used for amplification of the *IL10* (A1082G) gene polymorphisms were as follows: forward primer 5'-CTCGCTGCAACCCCACTGGC-3' and reverse primer 5'-TCTTACCTATCCCTACTTCC-3' (Rhodus *et al.* 2005). Amplification was performed with 20  $\mu\text{L}$  PCR reaction mixture containing 200 ng template DNA, 10 pmol of each primer and 2 $\times$  PCR master mixes (Fermentas, Sankt Leon-Rot, Germany). Amplification products were identified in all samples using 2% agarose gel electrophoresis. Subsequently the PCR products were subjected to digestion by *MnII* enzyme (NEB, Hertfordshire, UK) to screen for the *IL10* A1082G. The enzymatic mixture contained 1  $\mu\text{L}$  restriction enzyme, 1  $\mu\text{L}$  10 $\times$  buffer, 6  $\mu\text{L}$  PCR products and 2  $\mu\text{L}$  distilled water; the mixture was incubated for 5 h at  $37^{\circ}\text{C}$  for digestion. The digested product was electrophoresed on 3% agarose gel electrophoresis at 80 volts for 1 h. In the case of A1082G, an undigested 139 bp band showed wild-type *AA* genotype, while two bands of 106 and 33 bp confirmed mutant *GG* genotype and three bands of 139, 106 and 33 bp were detected in the heterozygous *AG* genotype (Manoochehr *et al.* 2008) (figure 1).

### Statistical analysis

The significance of this study was evaluated by chi-square test. Odds ratio (OR) was calculated as an estimate of relative risk of having disease according to the relative frequency of different genotypes among the cases as well as the controls. ORs are given with 95% confidence interval (CI). *P* value



**Figure 1.** PCR-RFLP was 3% agarose gel electrophoresis analysis of *IL10* A1082G. Lanes 1, 3 and 6 are *AA* genotype (139 bp), lanes 2 and 4 are *AG* genotype (139, 106, 33 bp), lane 5 is *GG* genotype (106, 33 bp) and lane 7 (50 bp) ladder.

was considered significant at  $<0.05$ . The value was expressed in mean  $\pm$  (Standard deviation) SD.

### Results

In our study, we recruited 232 OSCC cases, including 137 males and 95 females, age ranging from 26 to 60 years. Calculated mean age of cases were  $37.11 \pm 6.10$ . The mean age of 221 healthy controls (133 males and 88 females) were  $28.28 \pm 5.32$ . All the cases and controls were successfully genotyped by PCR-RFLP. The average *IL10* A1082G *AA*, *AG* and *GG* genotype frequencies in total OSCC cases were 29.7, 68.1, 2.15%, and 57.4, 42.0, 0.45% in healthy controls. The frequency of *IL10* A1082G and statistical analysis of the cases and controls are shown in tables 1 and 2. The *IL10* A1082G high expression mutant *G* allele frequency observed

was 36.20% in OSCC cases and frequency was 21.50% in healthy controls (table 2).

We correlated the *IL10* genotypes of OSCC patients under different groups, i.e. age, gender, smoking, tobacco chewing, alcohol consumption, clinical tumour stage (I + II and III + IV), tumour grade (grade 1/2 and grade 3), lymph node (yes and no) and metastasis (M0 and M1) are shown in tables 3 and 4. It is observed that smoking, tobacco chewing and alcohol consumption are risk factors for OSCC, we included these factors in our study. The distribution of patient biological characteristics and selected risk factors are shown in tables 1, 3 and 4.

In this study among 232 cases and 221 controls, we found that *IL10* A1082G genotype was present among 158 cases and 93 controls, the *GG* genotype was present in five OSCC cases and one in healthy controls and the *AA* genotype was present among 69 OSCC cases and 127 in healthy controls

**Table 1.** Clinicopathologic characteristics for demographic data in OSCC patients and healthy controls.

Variables	Cases ( $n = 232$ )	Controls ( $n = 221$ )	<i>P</i>
Age	$37.11 \pm 6.10$	$28.28 \pm 5.32$	0.466
Male	59.05% ( $n = 137$ )	60.19% ( $n = 133$ )	0.963
Female	40.95% ( $n = 95$ )	39.81% ( $n = 88$ )	0.942
Smoking	46.98% ( $n = 109$ )	26.69% ( $n = 59$ )	0.003*
Tobacco chewing	74.13% ( $n = 172$ )	51.13% ( $n = 113$ )	0.018*
Alcohol consumption	29.31% ( $n = 68$ )	18.09% ( $n = 40$ )	0.036*
Clinical tumour stage			
I+II	59.05% ( $n = 137$ )		
III + IV	40.95% ( $n = 95$ )		
Tumour grade			
Grade 1/2	69.40% ( $n = 161$ )		
Grade 3	30.60% ( $n = 71$ )		
Lymph node			
Yes	37.50% ( $n = 87$ )		
No	62.50% ( $n = 145$ )		
Metastasis			
M0	90.09% ( $n = 209$ )		
M1	9.91% ( $n = 23$ )		

\*Significant value.

**Table 2.** Genotype and allele frequencies of *IL10* A1082G in OSCC patients and healthy controls.

<i>IL10</i> A1082G genotyping	Cases (n = 232)	Controls (n = 221)	P value	Odds ratio	95% CI	Chi-square
AA	69 (29.74%)	127 (57.46%)	–	–	–	–
AG	158 (68.10%)	93 (42.08%)	0.003*	1.6	1.1–2.2	8.5
GG	05 (2.15%)	01 (0.45%)	0.2	4.7	0.55–41.1	1.3
A	296 (63.80%)	347 (78.50%)	–	–	–	–
G	168 (36.20%)	95 (21.50%)	0.0004*	1.6	1.2–2.2	12.6

\*Significant value.

(table 2). The heterozygous *AG* genotypes were more prevalent in OSCC cases than healthy controls and difference between OSCC cases and controls was statistically significant (OR = 1.6, 95% CI = 1.1–2.2, *P* = 0.003), whereas homozygous *GG* genotype was not significant. The frequency of mutant *G* allele in *IL10* A1082G was significantly higher in OSCC cases than in healthy controls (OR = 1.6, 95% CI = 1.2–2.2, *P* = 0.0004); suggesting that individual alleles were associated with OSCC cases (table 2).

This case–control study signifies the frequency of mutant *G* allele and heterozygous *AG* genotype are extremely associated with risk for tobacco chewing in OSCC patients (*P* = <0.0001 and *P* = <0.0001) and heterozygous *AG* genotype statistically significant for smokers in OSCC patients (*P* = <0.019) but it was not significant in alcohol consumption (table 3). Further, on grouping them under clinical tumour stage (I + II and III + IV), it was evident that heterozygous *AG* genotype was significant in OSCC patients (*P* = 0.035). Similarly, in case of metastasis (M0 and M1), we found that homozygous *GG* genotype was highly significant (*P* = 0.003) but we did not find any correlation in tumour grade and lymph node with *IL10* in OSCC cases (table 4).

## Discussion

Previous studies revealed that increased levels of *IL10* have been reported in patients with OSCC, suggesting that this pleiotropic cytokine might play imperative role in malignancy (Fortis et al. 1996; Fujieda et al. 1999; Karcher et al. 1999). The gene expression of inflammation causing factors is affected by functional DNA polymorphisms which may confer susceptibility, progression and severity of disease (Savage et al. 2004; Tsai et al. 2005; Xu et al. 2005; Vairaktaris et al. 2005, 2007d). The combined effects in a series of gene polymorphisms of factors participating in the same biological pathway may provide study which may illustrate the relative significance of each polymorphic site in the final clinical result. Previously few studies have reported increased serum/saliva levels of *IL10* in OSCC cases (Jablonska et al. 1997; St John et al. 2004; Palli et al. 2005). It has been reported that there are highly significant contributions of cytokines, specifically *IL6*, *IL10* and *TNFα*, which have an imperative role in the occurrence of OSCC cases (Fortis et al. 1996; Petersen 2005). *IL10* A1082G has been found to promote tumour proliferation by suppressing the immune and

**Table 3.** Prevalence of genotype and allele frequencies in *IL10* A1082G in OSCC patients and healthy controls with individuals under smoking, tobacco chewing and alcohol consumption criteria.

<i>IL10</i> A1082G genotyping	Cases	Controls	P value	Odds ratio	95% CI
Smoking	(n = 109)	(n = 59)			
AA	37 (33.94%)	32 (54.23%)	–	–	–
AG	71 (65.13%)	27 (45.76%)	0.019*	2.27	1.18–4.35
GG	01 (0.91%)	00 (0.00%)	0.355	2.60	0.10–66.10
A	145 (66.51%)	91 (77.11%)	–	–	–
G	73 (33.49%)	27 (22.89%)	0.056	1.69	1.01–2.83
Tobacco chewing	(n = 172)	(n = 113)			
AA	53 (30.81%)	66 (58.40%)	–	–	–
AG	117 (68.02%)	46 (40.70%)	<0.0001*	3.16	1.92–5.20
GG	02 (1.16%)	01 (0.88%)	0.862	2.49	0.21–28.23
A	223 (64.84%)	178 (78.75%)	–	–	–
G	121 (35.16%)	48 (21.25%)	<0.0001*	3.67	2.54–5.31
Alcohol consumption	(n = 68)	(n = 40)			
AA	25 (36.76%)	23 (57.50%)	–	–	–
AG	42 (61.76%)	17 (42.50%)	0.067	2.27	1.02–5.05
GG	01 (1.47%)	00 (0.00%)	0.342	2.76	0.10–71.31
A	92 (67.65%)	63 (78.75%)	–	–	–
G	44 (32.35%)	17 (21.25%)	0.110	1.77	0.92–3.37

\*Significant value.

**Table 4.** Prevalence of genotype and allele frequencies in *IL10* A1082G gene polymorphism with clinical tumour stage, tumour grade, lymph node and metastasis in OSCC patients.

Clinical tumour stage	<i>IL10</i> A1082G genotyping		<i>P</i> value	Odds ratio	95% CI
	I + II ( <i>n</i> = 137)	III + IV ( <i>n</i> = 95)			
<i>AA</i>	49 (35.76%)	20 (21.05%)	–	–	–
<i>AG</i>	87 (63.50%)	71 (74.73%)	0.035*	1.99	1.08–3.67
<i>GG</i>	01 (0.72%)	04 (4.21%)	0.063	9.80	1.03–93.2
<i>A</i>	185 (67.51%)	111 (58.42%)	–	–	–
<i>G</i>	89 (32.49%)	79 (41.58%)	0.056	1.47	1.00–2.17
Tumour grade	Grade 1/2 ( <i>n</i> = 161)	Grade 3 ( <i>n</i> = 71)			
<i>AA</i>	55 (34.16%)	14 (19.71%)	–	–	–
<i>AG</i>	104 (64.59%)	54 (76.05%)	0.052	2.04	1.04–3.99
<i>GG</i>	02 (1.24%)	03 (4.22%)	0.136	5.89	0.89–38.7
<i>A</i>	214 (66.45%)	82 (57.75%)	–	–	–
<i>G</i>	108 (33.55%)	60 (42.25%)	0.090	1.45	0.96–2.17
Lymph node	Yes ( <i>n</i> = 87)	No ( <i>n</i> = 145)			
<i>AA</i>	22 (25.28%)	47 (32.41%)	–	–	–
<i>AG</i>	61 (70.11%)	97 (66.90%)	0.413	1.34	0.73–2.44
<i>GG</i>	04 (4.59%)	01 (0.68%)	0.162	6.36	0.69–58.2
<i>A</i>	105 (60.35%)	191 (65.86%)	–	–	–
<i>G</i>	69 (39.65%)	99 (34.14%)	0.272	1.26	0.85–1.87
Metastasis	M0 ( <i>n</i> = 209)	M1 ( <i>n</i> = 23)			
<i>AA</i>	62 (29.66%)	07 (30.43%)	–	–	–
<i>AG</i>	146 (69.85%)	12 (52.17%)	0.705	1.37	0.51–3.65
<i>GG</i>	01 (0.47%)	04 (17.39%)	0.003*	35.4	3.45–363.0
<i>A</i>	270 (64.60%)	26 (56.52%)	–	–	–
<i>G</i>	148 (35.40%)	20 (43.48%)	0.357	1.40	0.75–2.60

\*Significant value.

inflammatory responses (Fortis *et al.* 1996; Hatanaka *et al.* 2001; Nagata *et al.* 2002).

The expression of *IL10* A1082G is affected by SNP in its promoter region. In the present study, *IL10* A1082G in heterozygous *AG* genotype was significantly frequent in OSCC cases, regardless of their family history of cancer, thrombophilia and cancer stages. The high expression of *G* allele behaves as a dominant trait. Based on such results, this may be concluded that high levels of *IL10* A1082G gene expression may have an important role in increasing susceptibility for development of oral cancer (Vairaktaris *et al.* 2008). This is in accordance with the previously observed increase of *IL10* A1082G gene expression in OSCC cases (Fujieda *et al.* 1999; Karcher *et al.* 1999).

In India, studies have been done on *IL10* and found it to play an important role in instigating different types of diseases like atopic asthma, asthma, type 2 diabetes mellitus, chronic hepatitis and gastric cancer (Chatterjee *et al.* 2005; Chand-Bhayal *et al.* 2012; Saxena *et al.* 2013; Srivastava *et al.* 2014; Raeiszadeh *et al.* 2015). Previous studies reported that *IL10* genotypes were associated with increased risk of gastric cancer, type 2 diabetes mellitus, asthma and chronic hepatitis B (Chand-Bhayal *et al.* 2012; Saxena *et al.* 2013; Srivastava *et al.* 2014; Raeiszadeh *et al.* 2015).

In our study *IL10* gene polymorphism is found to be associated with OSCC in north Indian population. The present findings are in accordance with previously reported observed increase of *IL10* genotype in OSCC in Greek and German populations (Vairaktaris *et al.* 2008). Previous studies infer

that the mutant *G* allele association was studied on the OSCC in early stages rather than advanced stages (Vairaktaris *et al.* 2008) and showed that *IL10* may increase susceptibility to oral cancer in a synergistic manner with other cytokines. *IL10* inhibits some cytokines, it may act as a cooperative growth factor. Such a role of *IL10* in OSCC might be extremely potent (Vairaktaris *et al.* 2008).

In the studied individuals, a significant difference was observed in the total group and subgroups of OSCC patients. *IL10* A1082G genotype *AG* heterozygous was significantly more frequent in patients with OSCC, in spite of their family history of either cancer and regardless of their cancer stage. Similar pattern was also observed in OSCC patients with smoking, tobacco chewing and clinical tumour stage. We found that effect of the high expression *G* allele is more pronounced in tobacco chewing and metastasis.

According to the above finding, this study showed that the significant contributions of cytokines *IL10* A1082G the occurrence of OSCC and for the first time, we report *IL10* in north Indian population. In conclusion, the most important finding of our study implies that *IL10* A1082G polymorphisms *AG* genotype and mutant *G* allele may participate in the progression of OSCC in north Indian population.

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