



Association of -330 interleukin-2 gene polymorphism with oral cancer

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Background & objectives: Cytokines play an important role in the development of cancer. Several single-nucleotide polymorphisms (SNPs) of cytokine genes have been reported to be associated with the development and severity of inflammatory diseases and cancer predisposition. This study was undertaken to evaluate a possible association of interleukin 2 (IL-2) (-330A>C) gene polymorphisms with the susceptibility to oral cancer.

Methods: The SNP in IL-2 (-330A>C) gene was genotyped in 300 oral cancer patients and in similar number of healthy volunteers by polymerase chain reaction (PCR)-restriction fragment length polymorphism and the association of the gene with the disease was evaluated.

Results: IL-2 (-330A>C) gene polymorphism was significantly associated with oral cancer whereas it was neither associated with clinicopathological status nor with cancer pain. The AC heterozygous genotype was significantly associated with oral cancer patients as compared to controls [odds ratio (OR): 3.0; confidence interval (CI): 2.14-4.20; $P < 0.001$]. The C allele frequency was also significantly associated with oral cancer (OR: 1.80; CI: 1.39-2.33; $P < 0.001$). IL-2 (-330A>C) gene polymorphism was also associated with oral cancer in tobacco smokers and chewers.

Interpretation & conclusions: Our results showed that oral cancer patients had significantly higher frequency of AA genotype but significantly lower frequency of AC genotype and C allele compared to controls. The IL-2 AC genotype and C allele of IL-2 (-330A>C) gene polymorphisms could be potential protective factors and might reduce the risk of oral cancer in Indian population.

Key words Cancer pain - inflammation - interleukin-2 - oral cancer - single-nucleotide polymorphism

Oral cancer is the eighth most frequent cancer worldwide¹. Of all the annually diagnosed cancer patients, approximately 20-30 per cent suffer from oral cancer. It is one of the most common malignancies among Indian males². In India, oral cancer is one of the major causes of cancer-related deaths¹. Oral cancer includes all malignancies originating in the region of

head and neck and has a high incidence, with a poor prognosis^{3,4}. This may be due to the absence of effective diagnostic and prognostic methods which can guide suitable management at early stages. Oral cancer pain directly affects patients' quality of life, daily activities, eating, speech and psychological status⁵. Significant pain has been reported in up to 25 per cent of patients

undergoing active treatment and in up to 90 per cent of patients with progressive cancer⁶. Pain in oral cancer is multifactorial, encompassing a variety of phenotypes. Each phenotype is a result of an underlying oral disease and superimposing environmental factors, such as tobacco chewing, smoking, alcohol consumption and genetic factors⁷. Cytokines are small glycoproteins released by many different cell types which play a complex role in cancer immunity and carcinogenesis⁸. Several pro-inflammatory cytokine gene products have been recognized that mediate a critical role in the suppression of apoptosis, proliferation, angiogenesis, invasion and metastasis. Some previous studies have suggested that the common polymorphisms in angiogenesis, thrombosis and inflammation-related genes are associated with increased risk for oral cancer and cancer-related pain⁹⁻¹².

Interleukin 2 (IL-2), a 15-kDa α -helical cytokine of the Th1 type produced by activated T-cells, promotes the proliferation of lymphocytes, natural killer (NK) cells and macrophages¹³. IL-2 effectively regulates the immune response and plays important roles in the differentiation of CD41-positive T-cells into Th1 and Th2 effector subsets, while inhibiting T-helper 17 differentiation¹⁴. IL-2 is located on chromosome 4q26-q27 and has two single-nucleotide polymorphisms at -330 and -384 promoter regions, which affect IL-2 expression¹⁵. The production of IL-2 protein was greater in CC genotype of IL-2 (-330 A>C) as compared to the AA and AC genotypes in healthy individuals¹⁶. Several studies have reported that the IL-2 (-330) gene polymorphism is significantly associated with various cancers, such as peptic ulcer or gastric cancer^{17,18}. Cytokines also play an important role in the pathogenesis of cancer-related pain. Cytokines are released by activated glial cells in response to any inflammatory process or tissue damage (as in cancer). These cytokines alter the perception of cancer pain, either by the changed activity of nociceptors or due to hyperexcitability of pain-transmitting neurons¹². The present study was carried out to investigate the role of IL-2 (-A330C) genetic polymorphism (rs 2069762) in the pathogenesis of oral cancer in patients attending a tertiary care hospital in north India.

Material & Methods

Study design

Patient selection and clinical evaluation: This population-based case-control study was conducted in the department of Anaesthesiology, King George's

Medical University (KGMU), Lucknow, India. The sample size was calculated with 80 per cent of power using the n-Masters 1.0 sample size calculation software developed by Christian Medical College, Vellore, Tamil Nadu, India¹⁹. Three hundred oral cancer patients and similar number of healthy volunteers were included in this study, on the basis of well-defined inclusion and exclusion criteria. The histopathologically confirmed oral cancer patients were included in the case group, at the time of baseline treatment from the department of Surgical Oncology of KGMU, Lucknow, between December 2013 and July 2015. The healthy volunteers (controls) comprised those who visited KGMU outdoor clinic as patients to different departments for minor trauma or health check-up, teaching/non-teaching staff and self-willing persons. The participants with a past positive history of any type of cancer or with oral lesions were excluded from the study. Ethical clearance was obtained from the Institutional Ethical Committee. Informed written consent was obtained from all the participants.

Collection of socio-demographic and clinical data:

Clinical information, including age, sex, tobacco chewing, smoking, alcohol consumption and disease status, was obtained from patients' medical records. Oral cancer patients were categorized on the basis of TNM (tumour node metastasis) staging into low risk group (stage I+II, N0 and M0) and high-risk group (stage III+IV, N1+N2 and M1). Clinical data as well as a history of comorbid conditions (heart disease, diabetes, stroke, kidney disease and liver disease) and stage of oral cancer were also extracted from the patients' records. Self-reported pain 'during the past week' was assessed using an 11-point numeric scale (0, 'no pain' and 10, 'worst pain or earlier'), and a visual analogue scale was used for pain scores²⁰.

Sample collection and molecular analyses: Blood samples (5 ml) were collected in ethylene diamine tetraacetic acid (EDTA) tubes and stored at -80°C until DNA extraction. High molecular weight DNA was extracted using high salting-out method²¹.

PCR-RFLP analysis of interleukin 2 (-330A>C) gene polymorphism: The final volume of the PCR reaction mixture included 25 μ l volume containing 40 ng genomic DNA, 10 pico mole each of forward and reverse primers (F -5'-TAT TCA CAT GTT CAG TGT AGT TCT-3' and R -5'-CTC TTT GTT ACA TTA GCC CA-3') at a concentration of 1 \times , 1 \times PCR master mixture (Applied Biosystems, USA). The cycling

conditions were as follows: an initial denaturation at 95°C for 4 min, followed by 35 cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 40 sec, with a final extension step of 7 min at 72°C in the last cycle. The human β -globin was used as positive control during PCR amplification in genotyping. The PCR products were digested at 37°C with *MaeI* restriction enzyme to detect the IL-2 alleles and then subjected to three per cent agarose gel electrophoresis (Bio-Rad, USA). Three genotypes were found in our study: genotype A/A, with one fragment, 159 bp; genotype A/C, with two fragments, 159 and 136 bp; and genotype C/C, with one fragment, 136 bp (Figure).

Statistical analysis: The Chi-square test was used to compare the dichotomous/categorical variables. The unpaired Student's *t* test was used to compare discrete variables between oral cancer patients and controls. Allele and genotype frequencies were analyzed by Chi-square test and Fisher's exact test. The odds ratios (OR) with 95 per cent confidence interval (CI) were calculated to estimate the strength of association between IL-2 (-330A>C) gene polymorphisms and oral cancer.

Results

The demographic profile included age, gender, body mass index (BMI), relative environmental risk factors and tumour staging. In this study, the age of the participants (47.67 ± 12.67 and 43.03 ± 8.49 yr) was significantly different between oral cancer patients and controls, respectively. However, BMI in the two groups was similar (Table I). The influence of tobacco smoking, chewing and alcohol consumption was also monitored in oral cancer patients and controls, as shown in Table I. The tobacco smoking and chewing were significantly associated with oral cancer. Frequency

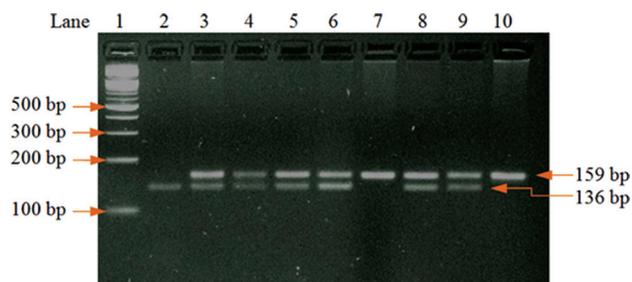


Figure. Gel picture showing the polymerase chain reaction-restriction fragment length polymorphism analysis; after digestion of PCR product with *MaeI* restriction enzymes (lane 1: 100 bp ladder; lane 2: CC homozygous genotype; lane 3, 4, 5, 6, 8, 9: heterozygous AC genotype; lane 7, 10: homozygous AA genotype).

of smoking was significantly different between oral cancer patients and controls. Alcohol consumption was not significantly different in oral cancer patients and controls. Distribution of oral cancers according to the positions *viz.* buccal mucosa (44%), tongue (27%), alveolar ridge (13%), lip and gingivobuccal sulcus (5%), hard pallet, retromolar trigone and others (2%) was also observed.

The occurrence of tumour Stages I, II, III and IV was 8, 56.3, 33.3 and 2.3 per cent, respectively. Lymph node status (N0: 70.7%, N1+N2: 29.3%) and metastasis (M0: 98.7%, M1: 1.3%) were also analyzed. The occurrence of pain according to tumour Stages I, II, III and IV was 37.5, 40.2, 43.0 and 71.4 per cent, respectively. Severity of oral cancer pain according to stage was: Stage I (mild: 50%, moderate: 50% and severe pain: 0%), Stage II (mild: 22.1%, moderate: 73.5% and severe pain: 4.4%), Stage III (mild: 25.6%, moderate: 65.1% and severe pain: 9.3%) and Stage IV (mild: 20%, moderate: 40% and severe pain: 40%).

The distribution of genotype and allele at locus was compared between oral cancer patients and controls. The frequencies of the AA, AC and CC genotypes of IL-2 (-330A>C) were 58.3, 38.7 and 3.0 per cent in cases and 33.0, 65.7 and 1.3 per cent in controls, respectively, whereas the allele frequencies of A and C were found to be 77.7 and 22.3 per cent of the study cases and 65.8 and 34.2 per cent in controls, respectively. There were significant differences in the homozygous AA and AC genotype frequencies of the IL-2 (-330A>C) gene polymorphism between oral cancer patients and controls (OR: 3.0; CI: 2.14-4.20; $P < 0.001$), whereas homozygous AA and homozygous CC genotypes did not show any significant difference between the groups. Alleles A and C were also significantly (OR: 1.80; CI: 1.39-2.33; $P < 0.001$) associated with oral cancer (Table II).

Oral cancer patients were categorized into two groups: low risk and high risk. Low-risk group patients had Stage I+II, N0 and M0 and high-risk group had Stage III+IV, N1+N2 and M1. There was no significant difference between the genotype distribution of the IL-2 (-330A>C) gene polymorphism and tumour stage, lymph node status and metastasis between these two groups (Table III).

The frequencies of AA, AC and CC genotypes of IL-2 (-330A>C) were 60.0, 35.2 and 4.8 per cent in oral cancer patients with pain and 57.14, 41.14 and 1.72 per cent in oral cancer patients without pain.

Table I. Distribution of demographics, tobacco (chewing and smoking) and alcohol habits in oral cancer patients and controls

Parameters	Oral cancer patients (n=300), n (%)	Controls (n=300), n (%)
Age (yr)	47.67±12.67***	43.03±8.49
BMI (kg/m ²)	21.37±3.19	21.75±2.38
Gender (male)	202 (67.33)	193 (64.33)
<i>Exclusive smoking habit</i>		
Exclusive smokers	94 (31.3)*	62 (20.7)
<i>Smoking duration</i>		
>20 yr	29 (30.9)	19 (30.6)
In between 10 and 20 yr	26 (27.6)	24 (38.7)
≤10 yr	39 (41.5)	19 (30.6)
<i>Individuals with smoking frequencies per day</i>		
>10	13 (13.8)**	23 (37.1)
≤10	81 (86.2)**	39 (62.9)
<i>Exclusive tobacco chewing habit</i>		
Exclusive tobacco chewers	182 (60.7)***	139 (46.3)
<i>Tobacco chewing duration</i>		
>20 yr	60 (33.0)*	32 (23.0)
In between 10 and 20 yr	61 (33.5)	50 (36.0)
≤10 yr	61 (33.5)	57 (41.0)
<i>Individuals with frequencies per day</i>		
>10	70 (38.5)	56 (40.3)
≤10	112 (61.5)	83 (59.7)
<i>Exclusive alcohol habit</i>		
Exclusive alcoholic	44 (14.7)	32 (10.7)
<i>Alcohol consumption duration</i>		
>20 yr	13 (29.5)	7 (21.9)
In between 10 and 20 yr	9 (20.5)	9 (28.1)
≤10 yr	22 (50.0)	16 (50.0)
<i>Individuals with alcohol consumption frequencies (/wk)</i>		
≤7	44 (100)	32 (100)
<i>Distribution of oral cancer</i>		
Buccal mucosa	133 (44)	-
Tongue	81 (27)	-
Alveolar ridge	38 (13)	-
Lip	16 (5)	-
Gingivobuccal sulcus	16 (5)	-
Hard pallet	6 (2)	-
Retromolar trigone	4 (2)	-
Other	6 (2)	-

P *<0.05, **<0.01, ***<0.001 compared to controls

Table II. The genotype and allele frequencies of interleukin-2 (-330A>C) in oral cancer patients and control group

Allele frequency	Oral cancer patients (n=300), n (%)	Controls (n=300), n (%)	OR (95% CI)	P
Genotype frequency				
AA	175 (58.3)	99 (33.0)	1.00 (reference)	
AC	116 (38.7)	197 (65.7)	3.0 (2.14-4.20)	<0.001
CC	9 (3.0)	4 (1.3)	0.79 (0.24-2.62)	0.776
Allele frequency				
A	466 (77.7)	395 (65.8)	1.00 (reference)	
C	134 (22.3)	205 (34.2)	1.80 (1.39-2.33)	<0.001

OR, odds ratio; CI, confidence interval

Table III. Interleukin-2 (-330A>C) gene polymorphism and tumour stage, lymph node and metastasis in oral cancer patients

Genotype frequency	Stage		OR (95% CI)	P
	Low risk, n (%)	High risk, n (%)		
	I + II (n=193)	III + IV (n=107)		
AA	109 (56.5)	66 (61.7)	1.00 (reference)	
AC	77 (39.9)	39 (36.4)	0.84 (0.51-1.37)	0.56
CC	7 (3.6)	2 (1.9)	0.47 (0.10-2.34)	0.49
Genotype frequency	Lymph node		OR (95% CI)	P
	N0 (n=225)	N1 + N2 (n=75)		
AA	129 (57.3)	46 (61.3)	1.00 (reference)	
AC	88 (39.1)	28 (37.3)	0.89 (0.52-1.54)	0.78
CC	8 (3.6)	1 (1.3)	0.35 (0.04-2.88)	0.45
Genotype frequency	Metastasis		OR (95% CI)	P
	M0 (n=296)	M1 (n=4)		
AA	172 (58.1)	3 (75.0)	1.00 (reference)	
AC	115 (38.9)	1 (25.0)	0.50 (0.05-4.85)	1.00
CC	9 (3.0)	0 (0.0)	-	-

Low risk (Stage I + II, N0 and M0); High risk (Stage III + IV, N1 + N2 and M1). OR, odds ratio; CI, confidence interval

However, frequencies of A and C alleles were 77.6 and 22.4 per cent in oral cancer patients with pain and 77.7 and 22.3 per cent in oral cancer patients without pain. The frequencies of AA, AC and CC genotypes and A and C alleles of the IL-2 (-330A>C) were not significantly associated with oral cancer pain (Table IV).

Associations between IL-2 (-330A>C) promoter region genetic variations and exposure to related environmental factors on oral cancer susceptibility are shown in Table V. There were significant differences in the homozygous AA and heterozygous AC genotype frequencies of the IL-2 (-330A>C) gene polymorphism between oral cancer patients and controls, who were known smokers [OR: 3.18 (1.62-6.28); $P<0.001$] and in tobacco chewers

[OR: 2.95 (1.86-4.68); $P<0.001$]. Allele frequencies of A and C were significantly different in smoking (OR: 1.96; CI: 1.18-3.26; $P=0.01$) and tobacco chewing individuals (OR: 1.75; CI: 1.21-2.45; $P=0.003$) among oral cancer patients and controls. The frequencies of AA, AC and CC genotypes and A and C alleles of the IL-2 (-330A>C) were not significantly associated with alcohol consumption in oral cancer patients and controls (Table V).

Discussion

Pro-inflammatory cytokines play a significant role in the pathogenesis of a tumour¹⁵. Genetic modification also plays a significant role in the inflammatory response, which may be a contributory factor in the risk of oral cancer¹⁵. IL-2 is an immune regulatory cytokine with

Table IV. Analysis of genotype and allele frequencies of interleukin-2 (-330A>C) gene polymorphism in oral cancer patients having pain

Frequency	Pain		P
	Yes (n=125)	No (n=175)	
Genotype			
AA	75 (60.0)	100 (57.14)	1.00 (reference)
AC	44 (35.2)	72 (41.14)	0.40
CC	6 (4.8)	3 (1.71)	0.17
Allele			
A	194 (77.6)	272 (77.7)	1.00 (reference)
C	56 (22.4)	78 (22.3)	0.974

biological functions of pro- and anti-inflammation. It has been demonstrated that IL-2 influences the T-cell proliferation, differentiation and survival of effectors²². An increasing number of evidences indicate that IL-2 may affect the development and the destructive biological nature of different tumors²³. In the current study, we investigated the role of IL-2 (-330A>C) polymorphisms in susceptibility to oral cancer in Indian population and observed that the IL-2 (-330A>C) gene polymorphism could be an anti-tumour factor for oral cancer.

Our results showed that oral cancer patients had a significantly higher frequency of AA genotype, as compared to control group. On the other hand, the frequencies of C allele and heterozygous AC genotype of IL-2 (-330A>C) were significantly lower in oral cancer patients as compared to controls. A previous study has reported that tumour growth is associated with a downregulation of Th1-type activity and upregulation of Th2-type activity. IL-2 is an important inflammatory cytokine which is produced by helper T-cells and activated natural killer (NK) cells and cytotoxic T-cells²³. Furthermore, IL-2 also influences the role of cytotoxic T-cells, induced by IL-2 receptor expression, endorsing cell migration, improving the interaction between the cells and inducing cytokine secretion²³. Pei *et al*²⁴ demonstrated that the abnormal level of IL-2 was significantly associated with tumour. Other groups^{23,25} found that the IL-2 (-330) promoter region gene polymorphism was a risk factor and a prognostic marker for cancer development. Some studies have reported the IL-2 gene polymorphism to be associated with decreased expression of IL-2 gene^{17,26}. Shin *et al*²⁷ did not find any significant association between IL-2 genetic polymorphism and gastric cancer. In our study also no association was

Table V. Analysis of genotype and allele frequencies of interleukin-2 (-330A>C) gene polymorphisms in oral cancer patients and controls among individuals exposed to tobacco chewing, smoking and alcohol

Genotype/ allele	Smoking			Chewing			Alcohol		
	Oral cancer patients (n=94) n (%)	Controls (n=62) n (%)	OR (CI), P	Oral cancer patients (n=182) n (%)	Controls (n=141) n (%)	OR (CI), P	Oral cancer patients (n=44) n (%)	Controls (n=34) n (%)	OR (CI), P
AA	56 (59.6)	20 (32.3)	1.00 (reference)	108 (59.3)	49 (35.3)	1.00 (reference)	25 (56.8)	15 (44.12)	1.00 (reference)
AC	36 (38.3)	41 (66.1)	3.18 (1.62-6.28), 0.001	68 (37.4)	91 (64.0)	2.95 (1.86-4.68), 0.0001	18 (40.9)	19 (56.88)	1.759 (0.71-4.37), 0.321
CC	2 (2.1)	1 (1.6)	1.40 (0.12-16.30), 1.00	6 (3.3)	1 (0.7)	0.37 (0.043-3.14), 0.677	1 (2.3)	0 (0.0)	-
A	148 (78.7)	81 (65.3)	1.00 (reference)	284 (78.0)	189 (67.3)	1.00 (reference)	68 (77.3)	49 (72.06)	1.00 (reference)
C	40 (21.3)	43 (34.7)	1.96 (1.18-3.26), 0.01	80 (22.0)	93 (32.7)	1.75 (1.21-2.45), 0.003	20 (22.7)	19 (27.94)	1.24 (0.60-2.58), 0.694

OR, odds ratio; CI, confidence interval

found between IL-2 (-330A>C) gene polymorphism and progression of oral cancer. Similarly, Savage *et al*¹⁸ also showed no significant association of IL-2 gene polymorphism with the progression of gastric cancer.

The actual molecular mechanism, by which cytokines influence pain, has not yet been established. In our study, the genotype of IL-2 was not significantly associated with oral cancer pain; however, some studies suggested that cytokines released during tissue damage or inflammation change the activity of nociceptors, contributing to pain hypersensitivity²⁸. IL-2 levels have been implicated in pain response²⁸ and in complex regional pain syndrome²⁹. Our study also showed that the oral cancer pain severity increased with increasing tumour stages. There was a strong association of AC genotype and C allele of IL-2 (-330A>C) with oral cancer and tobacco use (smoking and chewing) in our study. Seyedroudbari and Khan³⁰ have also reported that the smokeless tobacco upregulates pro-inflammatory cytokines.

In conclusion, the AC genotype and C allele of IL-2 (-330A>C) gene polymorphisms were significantly different among oral cancer patients and controls. The AC genotype and C allele of IL-2 (-330A>C) might be protective factors against the promotion of oral cancer. Our results did not show any significant association between IL-2 (-330A>C) gene polymorphisms with progression of oral cancer or its associated pain. Further studies with large sample sizes will be necessary to confirm our findings.

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Conflicts of Interest: None.

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