Genetic variations of IL-10: Identification of novel variations and evaluation of the impact of the SNPs/haplotype in the promoter region with the progression of Oral Squamous Cell Carcinoma in Indian population

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ABSTRACT

The correlation of interleukin 10 (IL-10) with the outbreak and progression of cancer has been well established as it contributes to tumor immune evasion. Convincing number of evidences has been accumulated to reflect the critical correlation between IL-10 polymorphism and tumorogenesis. Several polymorphic sites at promoter regions have been reported to be associated with cancer susceptibility. The purpose of this study was to examine the effect of modulated genotypes in the promoter region of IL-10 gene with life-style habits in oral squamous cell carcinoma (OSCC) in the Indian population. A total of 300 subjects (100 OSCC, 50 precancer and 150 healthy controls) were recruited in this study. The IL-10 promoter region was amplified in 14 overlapping fragments by PCR and further screened through the high throughput technique of denaturing high-performance liquid chromatography (dHPLC) followed by sequencing. We identified three novel variations at positions (-924, -1045 & -1066); we also found some known SNPs ($-592C/A$, $-657G/A$, $-851G/A$, $-819C/T$, $-1082A/G$). The identified novel variations were submitted to the NCBI Gene Bank (accession numbers **KT153594**, **KT291742** and **KT291743**). We also noticed a significant association of polymorphisms ($-592C/A$, $-819C/T$ and $-1082A/G$) individually as well as in combination (haplotypes) along with lifestyle habits for the risk of oral carcinoma ($p < 0.0001$). We have reported three novel SNPs in the Indian population for the first time, and these SNPs may be associated with OSCC. Besides, we showed the first evidence of IL-10 haplotypes, i.e., CC**G** and C**TG**, may act as a biomarker for early detection of oral pre-cancerous/cancerous lesions or treatment management of oral carcinoma.

1. Introduction

Cancer of oral cavity is a life-threatening disease with markedly increasing morbidity and mortality rates all over the world. It is ranked 11th globally, but it is the second-most disease causing mortality in developing nations like India [1]. In India, the majority cases of oral cancer consist of oral squamous cell carcinomas (OSCC), which arises from the epithelium of the oral mucosa [2,3]. The advent of Western lifestyle practices like cigarette smoking, alcohol consumption and chewing of smokeless tobacco products like khaini, gutka, supari, paan, etc. are possible causative factors of oral cancer in Indian population [4–6]. Any form of tobacco, either smoked or smokeless, can cause a wide spectrum of precancerous lesions or conditions like leukoplakia, erythroplakia, and oral submucous fibrosis (OSMF) that significantly

enhance the incidence of oral squamous cell carcinomas (OSCC) [7–9].

Various molecular and epidemiological studies have shown a strong association between environmental (tobacco, smoking and alcohol consumption) and genetic factors that increase the risk of OSCC [10,11]. Moreover, the same group of people when exposed to the same environmental factors showed different stages of disease development, signifying the role of genetic variations such as single nucleotide polymorphisms (SNPs) that may contribute to oral carcinogenesis [12]. Recently, it has been documented that SNPs in the promoter region of a cytokine can alter the cytokines level and lead to altered immunity, which increases the susceptibility of diseases [13,14].

Over the past decades, the role of leucocytes and their corresponding cytokines in inflammatory pathways and malignant transformation has been well established and documented by numerous studies. Interleukin-10 (IL-10) is a potent anti-inflammatory and immunosuppressive Th2 cytokine that promotes tumorogenesis by down regulating cell-mediated and cytotoxic inflammatory responses [15]. IL-10 also suppresses immune responses through individual actions on T cells, B cells, antigen presenting cells (APCs), and other cell types to distort the immune response from Th1 to Th2 [16]. Various other studies have reported the elevated level of IL-10 production in various cancers; this finding supports its role in tumor evasion of the host immune response [17].

The modulation in the transcription and expression patterns of IL-10 gene has been attributed to its polymorphic nature, which has been demonstrated by many studies. In the present study, we examined the promoter region of IL-10 gene for the identification of SNPs so as to investigate its role in oral cancer susceptibility in the Indian population. We have examined the cumulative effect of genotype and lifestyle habits, which may also play a crucial role in disease development. In addition, the present study also pin-points the link of genetic variation (genotypes/haplotypes) with lifestyle habits. To the best of our knowledge, in the Indian context, no study has reported the potent role of IL-10 genotypes and their interaction with lifestyle habits, which escalate mortality rate in oral oncology. Therefore, the present study has been designed to investigate the potential association of SNPs/ haplotype IL-10 gene promoter with the susceptibility to OSCC along with other etiological factors.

2. Material and methods

2.1. Clinical specimens

The present study comprised of 300 subjects. The oral tissue biopsy samples of 150 subjects were histopathologically confirmed to be associated with cancer (50 pre-cancerous + 100 OSCC), and the remaining subjects were healthy volunteers whose samples were selected from regular physical check-up unrelated to any malignancy or oral-related problems. All the samples were collected from the Rajiv Gandhi Research Centre and Hospital, New Delhi and I.T.S Dental College Hospital & Research Centre, Greater Noida (U.P.). The collected samples were histopathologically confirmed before inclusion in this study. The WHO criteria and the tumor/node/metastasis (TNM) classification were used to classify pre-malignant and malignant lesions. All the subjects were personally interrogated for their demographic characteristics that included ethnicity, socio-economic status, and lifestyle habits like smoking (> 4 cigarettes routinely per day), alcohol consumption (100–150 ml per day for 3–4 times per week), and smokeless tobacco chewing (4–6 packs per day). These criteria were decided after consulting with pathologists and studying the guidelines of Centre for Disease Control and Prevention, WHO, and previously studied reports. Written consent was obtained from each patient, and the study was carried out in accordance with the principles of Helsinki Declaration. This study has been approved by ethics committee of the institute (Ethical no. – ICPO/IEC/P-003/2011).

2.2. Extraction of genomic DNA

High quality genomic DNA was isolated from freshly collected oral pre-cancer/cancer tissue biopsies and oral scrapes (control) using the standard Proteinase K method followed by phenol/chloroform treatment [18].

2.3. Identification of IL-10 promoter region SNPs by dHPLC

The promoter region of the IL-10 gene was amplified in 14 different overlapping fragments by PCR using specific primer pairs. In brief, the reactions were carried out in the final volume of 25 µl containing 80–100 ng genomic DNA, 0.3 µM primers, 100 µM dNTPs, 3 U Taq DNA polymerase and 2.5 µl 10X PCR buffer at different temperature profiles [19]. The amplified promoter region was further screened by denaturing high-performance liquid chromatography (dHPLC) using WAVE DNA fragment analysis system (Trangenomic, Crewe, UK), which is based on ion-paired reversed phase HPLC [20]. Amplified PCR products were denatured at 95 °C for 5 min, followed by re-annealing at 25 °C over 60 min to allow heteroduplex formation. The products were loaded into the column and separated (at a flow rate of 1.5 ml/min) through a 5% linear acetonitrile gradient using commercially available Wave Optimised™ buffers A, B and D (Trangenomic, Crewe, UK). The homo- and hetero-duplexes were released in the column at different times at the critical denaturing temperature. DNA molecules having mutations or SNPs are largely dependent on the temperature and gradient selected for each PCR fragment (predicted using Wave Maker 4.1 software). The standard buffers were prepared from concentrated triethylammonium acetate (100 ml Trangenomic Part No. 553301). Amplified DNA was eluted from buffer A and buffer B: 60% A-40% B for 30 s; 50% A-50% B for 5.5 min; 25% A-75% B for 10 s; 5% A-95% B for 1 min; and 60% A-40% B for 1.33 min. Wash buffer was 8% acetonitrile. The analysis of each sample lasted 2.5 min. Any peak falling below 2 mV was not considered reliable and re-analyzed under dHPLC conditions.

2.4. Sequencing

The PCR products obtained by analyzing the homo- and heteroduplexes using Big Dye Terminator and automated DNA Sequencer ABI Prism 3100 (Applied Biosystems, Foster City, CA) were sequenced with forward and reverse primers. The SNPs were identified after comparing the sample sequence with IL-10 reference sequence from NCBI (Ref.ID-NG_012088.1).

2.5. Detection of IL-10 gene promoter region SNPs by allele specific PCR

After dHPLC analysis, we found some known sequence variations (−592C/A, −657G/A, −851G/A, −819C/T, −1082A/G), which were further confirmed by standard amplification refractory mutation system, i.e., ARMS-PCR. Two complementary reactions were established for each allele consisting of target DNA simultaneously: allele-specific ARMS primers and the common primer [21]. The standard conditions for this reaction consisted of 40 ng genomic DNA in each reaction volume of 15 µl containing 1X reaction buffer, 1.5 µM MgCl₂, 30 µM of each dNTP, 0.16 µM of each primer, and 0.3 U of Taq DNA polymerase. PCR products were loaded directly onto 2% agarose gels containing 0.5 mg/ml ethidium bromide, electrophoresed and visualized by photography under UV transilluminator.

2.6. Enzyme-linked immunosorbent assay (ELISA) for IL-10

ELISA was performed on 60% (180/300) of total subjects which included 90 controls, 30 pre-cancer and 60 cancerous cases. Selection of the samples was done on the basis of genotypic frequencies. The serum concentration of IL-10 in control and cases were analyzed by standard

sandwich ELISA using Booster's Human IL10- ELISA Kit as per the manufacturer protocol (Boster Biological Technology Co., Ltd, USA, Cat. No EK0416). Briefly, the human serum samples were diluted in 1:2 ratio, and then 100 μl of diluted human serum samples were added to ELISA plate wells along with diluted standard/controls and incubated for 90 min. Biotinylated anti-human IL-10 antibody were added and incubated at 37 °C for 60 min. The plate was washed thrice with 0.01 M PBS to remove any excess unbound antibody. ABC working solution was added to the plate and incubated for 30 min. The plate was then washed for 5 times. A chromogen substrate TMB solution was added to the plate, which resulted in the development of a blue-colored complex. This was followed by the addition of H_2SO_4 as a stop reagent, which produced the yellow-colored product. Absorbance was taken at 450 nm, and the concentration of IL-10 in samples, standards, and control was directly proportional to the intensity of the color complex.

2.7. Statistical analysis

Statistical analysis was performed by using statistical software PLINK v.1.07 (http://pngu.mgh.harvard.edu/Purcell/plink) and GraphPad InStat version 3.0. Chi-square test/Fisher's exact test (for smaller or subgroup analysis) was used to compare the genotypic distributions of IL-10 polymorphisms between cases and controls. Risk estimates were calculated for genetic models using most common homozygous genotype as a reference. Interactions between gene and subjects' environment and their combined effects were another aspect that was examined between the genotypes and lifestyle habits in all the subjects with haplotype analysis. This was done using PLINK v. 1.07 software, and its linkage equilibrium was determined by Haploview [22]. Genotypes were further confirmed by Hardy-Weinberg equilibrium. Statistical significance was considered as significant at the $p \leq 0.05$ level. The calculated power of the present study was > 80% using QUANTO software version 1.1 (http://hydra.usc.edu/gxe).

3. Results

3.1. Population characteristics

The basic characteristics of the studied population are listed in Table 1. The frequency of male cancer patients was 68% (n = 68/100) while there were 32% (32/100) females. In the pre-cancer group, 66% were male (33/50) and 34% were female (17/50) while in the controls, 83% were male (125/150) and 17% (25/150) were female. The age of the studied population ranged at 35–75 years. The mean age of the subjects was 49.75 ± 12.6 years in cancer group, 42.8 ± 14.4 years in pre-cancer group and 40.68 ± 14.9 years in controls. After careful observation and study of smoking habits of the subjects, it was found that most of the patients were smokers, whereas the majority of controls were non-smokers. Univariate analysis showed that the risk of developing oral cancer was higher among smokers as compared to non-smokers. The statistical analysis showed the smokers have a 3-fold higher risk of having oral cancer (p < 0.0001, OR 3.027, 95% CI = 1.790 to 5.119) when compared with the non-smokers. In terms of alcohol intake habit, alcoholics had a ~5-fold higher risk of having pre-cancerous lesions compared to non-users (p < 0.0001, OR = 5.785, 95% CI = 2.743–12.199). In the studied population, the frequency of tobacco chewers was very high in cancerous (82%) as well as pre-cancerous groups (72%) when compared with the controls (24%). From statistical analysis, it was revealed that tobacco chewers are ~14 times more prone to have oral carcinoma (p < 0.0001, OR 14.426, 95% CI = 7.659–27.171) while they are at a ~10-fold higher risk of having pre-cancerous lesions (p < 0.0001, OR = 10. 028, 95% CI = 4.739–21.220). These associations were found to remain statistically significant and even slightly stronger after allowing for confounders in univariate analysis.

3.2. Screening of IL-10 gene

Six out of 14 fragments exhibited 12 different types of peak patterns. Based on the different peak patterns, we made diverse groups, and 50% subjects of each group were sequenced for further validation. Interestingly, we found various SNPs in which most of them had been previously reported in other studies with the exception of three SNPs located at positions −1066, −1045, −924 (Figs. 1 and 2 and Supplementary Fig. 1). New identified SNPs are indicated on IL-10 gene SNP database map shown in Fig. 3. Variations that were reported and cited in NCBI data were designated as "known variations" while the others not found in SNP database were designated as "novel variations." The details of novel and known variations are showed in Tables 2 and 3.Fig.4

3.3. Novel variations

In our study, three novel variations were identified that were present in a small population. These three novel variants were deposited in the Gene Bank **(accession numbers – KT291743.1, KT153594.1 & KT291742.1)** Table 3.

3.4. Genotyping of IL-10 promoter region SNPs in oral cancer

The distribution analysis for the genotypic and allelic frequencies of the IL-10 gene promoter SNPs −592 C/A(rs1800872), −819 C/T(rs1800871) and −1082 A/G(rs1800896) in the cases (cancer + pre-cancer) and control groups are provided in Tables 4a–c.

3.4.1. Genotypic distribution of IL-10 (−592 C/A) polymorphism

The genotypic frequency of CC, CA, and AA were 20%, 58%, and 22% in cases and 29%, 59%, and 12% in controls, respectively (Table 4a). When the control group is compared with pre-cancer and cancer groups, there was no significant association found in carrier genotype in the dominant model (CC vs. CA + AA). However, we observed, in a recessive model (CC + CA vs. AA), there was a ~2-fold increase in the risk of cancer (p = 0.0125, OR = 2.444, 95% CI = 1.252–4.772) in comparison to controls. Considering the allele frequencies, our data indicates that the 'A' allele was associated with the increased risk of oral cancer (p = 0.0269, OR = 1.525, 95% CI = 1.064–2.2186).

3.4.2. Genotypic distribution of IL-10 (−819 C/T) polymorphism

For −819C/T locus, the genotypic allocation of CC, CT and TT was 19%, 45% and 36% of cases while 20%, 67% and 13% in controls, respectively (Table 4b). We observed an approximately 4-fold increase in the risk of oral cancer (p < 0.0001, OR = 4.791, 95% CI = 2.565–8.950) as compared to controls when the recessive model (CC + TT vs. TT) was taken into consideration. When the analysis was done in the co-dominant model (CC vs. TT), a ~3-fold higher risk was observed that was significantly associated with oral carcinoma (p = 0.0054, OR = 3.237, 95% CI = 1.476–7.097).

3.4.3. Genotypic distribution of IL-10 (−1082 A/G) polymorphism

The genotypic and allele frequency of −1082 A/G locus in cancer and pre-cancer cases and control population are presented in Table 4c. The frequency of AA, AG and GG genotype was 48%, 39%, and 13% in cases and 64%, 33%, and 0% in controls, respectively. When the dominant model (AA vs. AG + GG) was studied, we observed a 2-fold increased risk in cancer and pre-cancer cases as compared to controls (p = 0.0024, OR = 2.110, 95% CI = 1.323–3.364). However, the data from the recessive model (AA + AG Vs GG) showed that the cancer patients carrying GG genotype had a much higher risk (p < 0.0001, OR = 46.440, 95% CI = 2.725–791.40) for the development of oral cancer compared with those of AA + GG genotypes. Furthermore, when we considered the allelic frequencies, our data indicate that the

Table 1
Demographic and clinic-epidemiological characteristics of the studied population.

Characteristics	Precancer n (%) n = 50	Cancer n (%) n = 100	Cases n (%) n = 150	Controls n (%) n = 150
Age (mean ± SD)	49.75 ± 12.6	42.8 ± 14.4	47.43 ± 13.5	40.68 ± 14.9
Sex				
Male	33 (66)	68 (68)	101(67)	125 (83)
Female	17 (34)	32 (32)	49 (33)	25 (17)
p-value	*0.0161	*0.0074	*0.0021	**Reference**
OR (95%CI)	2.576 (1.246–5.323)	2.353 (1.290–4.291)	2.426 (1.401–4.199)	–
Habits				
Non Smoker	21 (42)	37 (37)	58 (39)	96 (64)
Smoker	29 (58)	63 (63)	92 (61)	54 (36)
p-value	*0.0102	* < 0.0001	* < 0.0001	**Reference**
OR (95%CI)	2.455 (1.277–4.718)	3.027 (1.790–5.119)	2.820 (1.766–4.504)	–
Non Alcoholic	11 (22)	37 (37)	48 (32)	93 (62)
Alcoholic	39 (78)	63 (63)	102 (68)	57 (38)
p-value	* < 0.0001	*0.0002	* < 0.0001	**Reference**
OR (95%CI)	5.785 (2.743–12.199)	2.778 (1.647–4.687)	3.467 (2.154–5.580)	–
Non Tobacco Chewers	12 (24)	18 (18)	30 (2)	114 (76)
Tobacco Chewers	38 (76)	82 (82)	120 (80)	36 (24)
p-value	* < 0.0001	* < 0.0001	* < 0.0001	**Reference**
OR (95%CI)	10.028 (4.739–21.220)	14.426 (7.659–27.171)	12.667 (7.321–21.917)	–
Habitat				
Rural	35 (70)	64 (64)	99 (66)	114
Urban	15 (30)	36 (36)	51 (34)	36
p-value	0.5120	0.0561	0.0749	**Reference**
OR (95%CI)	1.357 (0.6661–2.765)	1.781 (1.023–3.101)	1.631 (1.9849–2.702)	–
Histological grade				
WDSCC		79 (79)		
MDSCC		17 (17)		
PDSCC		4 (4)		

 * *p* ≤ 0.05 is considered as significant; OR, odds ratio; CI, confidence interval; n, number; P-value, probability from chi-square test comparing the genotype distribution for controls and cases. WDSCC, well-differentiated squamous cell carcinoma; MDSCC, moderately differentiated squamous cell carcinoma; PDSCC, poorly differentiated squamous cell carcinoma.

'G' allele was also found to be associated with increased risk of precancer (OR = 2.576, 95% CI = 1.542–4.304) and differences were found statistically significant (*p* = 0.0004).

Genotypic distribution of SNPs −851(A/G) and −657(A/G) were analyzed but were not statistically significant (data not shown).

3.5. Association of life style habits with progression of the disease

The etiology of oral cancer involves multiple factors, most of which are related to lifestyle. After analysis of genotypic data in the association of lifestyle habits, it can be strongly stated that lifestyle habits have a vast impact on the risk of oral carcinoma. We analyzed the association of IL-10 (−592C/A, −819C/T & −1082A/G) genotypes with different demographic characteristics such as cigarette smoking, alcohol consumption, and tobacco chewing. The AA genotype frequency of −592C/A loci was found to have a significant association to those who intake alcohol (*p* < 0.0001, OR = 11.786, 95% CI = 4.713–29.472). Present evidence shows that tobacco is a mixture of multipotent carcinogens, which may cause cancer in many different ways. Meanwhile, when we analyzed tobacco habits with genotypic data, we surprisingly found a significant association of AA genotype with tobacco habits in cancerous (*p* < 0.0001, OR = 22.333, 95% CI = 7.891–63.206) and pre-cancerous cases (*p* < 0.0001, OR = 16.111, 95% CI = 5.089–51.009).

When we studied the alliance of TT genotype (−819C/T) with its demographic characteristics, we observed that the subjects having TT genotypes together with high intake of alcohol and tobacco showed a significantly increased risk of oral carcinoma (*p* < 0.0001).

We also examined the combined impact of GG genotype (−1082A/G) and lifestyle habits on the risk of oral cancer. The risk was shown to be significantly increased when we compared the carrier genotype between alcoholic and non-alcoholic individuals. We observed a ~28-fold

higher risk for pre-cancer (*p* < 0.0001, OR = 28.800, 95% CI = 7.618 to 108.88) and ~21-fold increased risk (*p* < 0.0001, OR = 21.103, 95% CI = 5.962 to 74.702) in cancerous cases in the presence of GG genotype along with alcohol intake. The stratification of results with respect to tobacco chewers showed an ~8-fold increased risk in precancer and cancer patients having GG genotype (*p* < 0.0001) (Supplementary Table 1a–c).

3.6. Association of IL-10 genotype with clinical conditions plays a significant role in progression of oral carcinoma

We further stratified our results with respect to precancerous clinical conditions and histological grades of oral cancer (Supplementary Table 2). We observed the increased frequency of carrier genotype of IL-10 (−592C/A, −819C/T, −1082A/G) loci with the progression of oral precancerous and cancerous conditions. When we compared the IL-10 genotypes with pre-cancer (OSMF and leukoplakia) and cancerous cases and histological grades, we found the person having leukoplakia carrying genotype (AG + GG) of IL-10 (−1082) loci is at a 3-fold increased risk for pre-cancerous cases (*p* = 0.0068, OR = 3.400, 95% CI = 1.450–7.970) and ~2-fold increased risk of well-differentiated squamous cell carcinoma [*p* = 0.0262, OR = 1.950, 95% CI = 1.118–3.403). On the other hand, we could not find any trend in the frequency of carrier genotype (CA + AA and CT + TT) of −592C/A and −819C/T loci with the progression of oral cancer.

3.7. Association of IL-10 haplotypes with the progression of OSCC

Haplotype analysis was performed by using Haploview and PLINK, as shown in Table 5. For further analysis, we systematized our data between cases and controls. In our studied population, we have found eight haplotypes (CC**A**, **AT**A, **ATG**, CT**A**, **A**CA, C**TG**, and **ACG** & CC**G**),
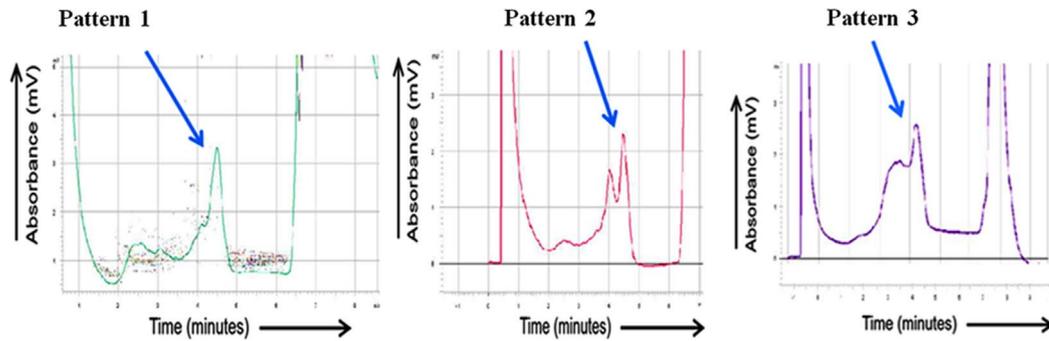
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Fig. 1. Novel variations showed three different heterozygous peak patterns of IL-10 gene obtained by dHPLC.
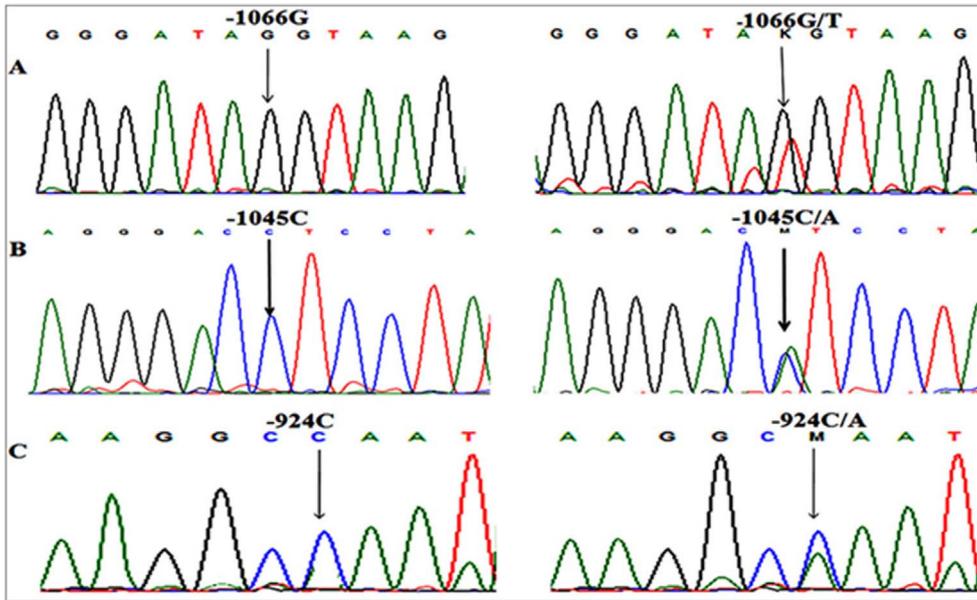

Fig. 2. DNA sequence electropherogram of novel variants in the promoter region of the IL-10 gene. The SNP change is indicated by an arrow. (A) −1066G Homozygous (wild) genotype change to Heterozygous genotype G/T, (B) −1045C Homozygous (wild) genotype change to Heterozygous genotype C/A, (C) −924C Homozygous (wild) genotype change to Heterozygous genotype C/A.

the following four haplotypes were the most prevalent CCA (39%), ATG (17.5%), C**TG** (3%) and CC**G** (2%). Our findings indicate that the CTG haplotype was only found in cancerous cases with significant association but not in pre-cancerous cases meanwhile CCG haplotype was only found in pre-cancerous cases but not in cancer cases. The association between minor alleles of SNPs −592A (**AT**A, **ATG**, **A**CA & **A**C**G**), −819T (**AT**A, **ATG**, C**T**A & C**TG**) and −1082G (**ATG**, C**TG** AC**G** & CC**G**) was observed in the overall population. Only **ATG** carried all three minor alleles and showed a significant association with pre-cancer ($p = 0.0143$ OR 2.25) and total cases ($p = 0.037$ OR 1.63), i.e., cancer + pre-cancer. The C**TG** minor allele of −819T and −1082G was highly

significant in cancer cases ($p = 0.0050$ OR 1.63e + 004) but not in pre-cancerous cases shown in Table 5. These risk haplotypes (C**TG** + CC**G**) have been reported for the first time in Indian population.

3.8. Association of the haplotype with lifestyle habitudes

Consistent with our findings in oral cancer, we analyzed how the lifestyle habits (smoking, alcohol and tobacco chewing) are associated with risk haplotypes (Table 6). We further scrutinized our results with major and minor alleles. The present study showed the risk haplotypes (**ATG** + C**TG** + CC**G**) were significantly and strongly associated with
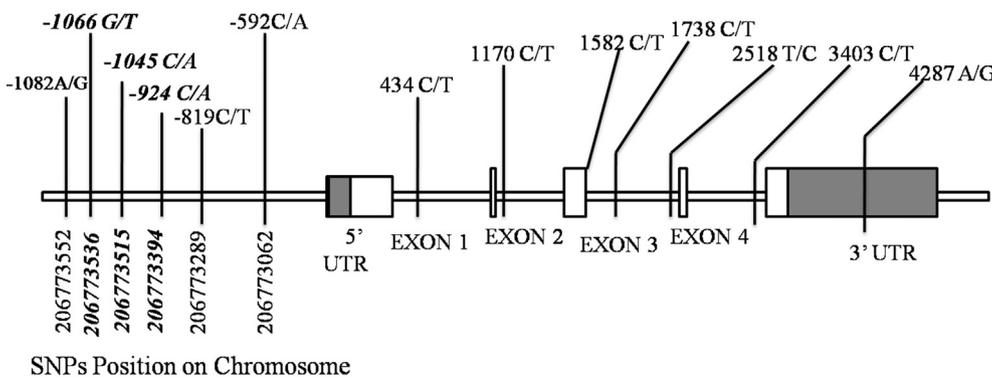

Fig. 3. The SNP database map shows the newly identified SNPs along with well established ones of IL-10 gene. Novel SNPs are highlighted with italic and bold font on SNPs database map.

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Table 2
List of known variants observed in the present study.

IL-10 gene	rs Numbers	Position on complete CDS	Position on promoter region	Nucleotide Change
	rs1800871	3289	−819	C/T
	rs1800872	3562	−592	A/C
	rs1800894	3321	−851	A/G
	rs1800895	3127	−657	A/G
	rs1800896	3552	−1082	A/G

lifestyle habits when compared between cases and control subjects ($p < 0.0001$). We also found, cigarette smokers in cases ($p < 0.0001$, OR = 28.125, 95% CI = 3.530–222.07) and alcohol drinkers ($p < 0.0001$, OR = 108.33, 95% CI = 6.106–1922.1) in cancer showed higher significant association with the increased risk of oral carcinoma.

3.9. Serum concentration of IL-10

Il-10 serum concentration was evaluated by ELISA and was found in higher concentrations in OSCC patients. The mean serum level of IL-10 was found in control (37.694 ± 27.6 pg/ml), pre-cancer (58.145 ± 21.7 pg/ml), and cancer (83.233 ± 22.8 pg/ml) cases. The statistical analysis was significant in control vs. cancer ($p = 0.0025$) and control vs. precancer vs. cancer ($p < 0.0001$) cases but not significant in control vs. precancer ($p = 0.1180$) cases. When we correlated these findings with the combination of haplotypes, we found minor allele A**TG** haplotype has a higher serum concentration of IL-10 as compared to major allele haplotype CC**A**, but they are not statistically significant ($p = 0.135$) as shown in Fig. 5.

4. Discussion

OSCC has a remarkably oppressive prognosis, revealing the significance of factors that modify the host immune response. Sufficient evidence proves the correlation of IL-10 with the risk for development of various types of malignant tumors including oral cancer. IL-10 is an immunomodulatory cytokine, and it has been acknowledged that the expression level of IL-10 may play an important part in the formation of cancers and polymorphism in the promoter region and can influence the expression level of IL-10 [23]. In patients with solid tumors, including OSCC, higher levels of IL-10 were observed. This infers that this pleiotropic cytokine may have a key role in malignancy.

In IL-10 promoter region, various SNPs have been reported across the world. Polymorphisms within the promoter region of the IL-10 gene are likely to affect IL-10 mRNA transcription and IL-10 expression levels [24–26]. Among the numerous numbers of polymorphisms, we could find −592C/A, 657G/A, −851G/A, −819C/T, −924C/A, −1045C/A, −1066G/T, and −1082A/G as some of the major SNPs in Indian population in association with oral cancer. IL-10 polymorphisms at −1082A/G, −819C/T and −592C/A loci are SNPs that were found to play a more prominent role in oral carcinogenesis in the presently studied population, which was in accordance with other reports. This finding is also similar to the findings of other previous studies [12,27–30].
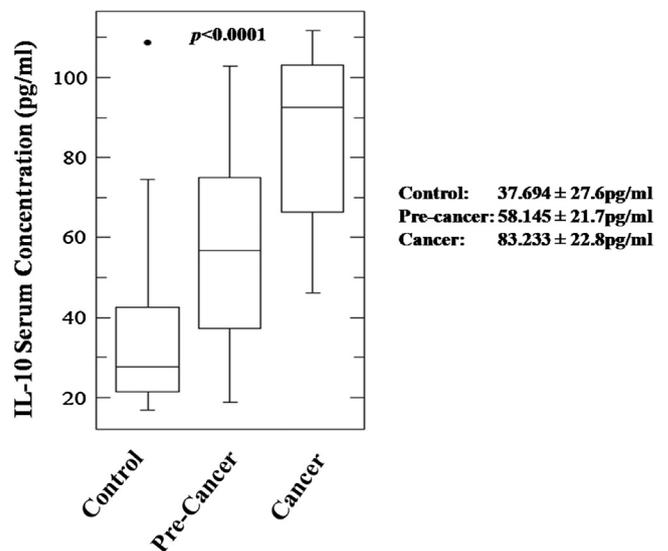

Fig. 4. Box plot showing the level of IL-10 serum concentration in controls (n = 90), pre-cancer (n = 30) and cancer (n = 60) cases. P-values were calculated by using one-way ANOVA: control vs. precancer (p = 0.1180), control vs. cancer (*p = 0.0025), control vs. precancer vs. cancer (*p < 0.0001). *p ≤ 0.05 is considered as significant.

In the present study, we noticed that the frequency of carrier genotype of −1082A/G showed a ~2-fold increased risk in the dominant model (AA vs. AA + GG, $p = 0.0188$, OR = 1.922, 95% CI = 1.144–3.228), but in the recessive model, it showed ~46 times higher risk of susceptibility of oral cancer in the Indian population ($p < 0.0001$, OR = 46.440, 95% CI = 2.725–791.40). This finding is consistent with the previous studies in Chinese, Taiwan, Greeks, and German population [28–30]. But in contrast, several other studies could not notice any significant association of −1082A/G with the progression of oral carcinoma [31,32]. In case of −819C/T, the recessive and co-dominant model, TT genotype showed a significant association with oral pre-cancerous (CC + TT vs. TT, $p = 0.0450$, OR = 2.422, 95% CI = 1.095–5.361) and cancerous cases (CC + TT vs. TT, $p < 0.0001$, OR = 4.791, 95% CI = 2.565–8.950; CC vs. TT, $p = 0.059$, OR = 2.940, 95% CI = 1.416–6.106) in the studied population. This explains the possible role of 'T' minor allele in oral carcinogenesis in the Indian population. This observation is in good agreement with a previous meta-analysis on −819C/T polymorphism in various human cancers in which −819 TT genotype in the IL-10 gene emerged as a protective factor for cancer in Asian populations, especially for gastric cancer [33].

In −592C/A, there was no significant association in the dominant model but in recessive and co-dominant models. The analysis showed some sort of association of this SNP in pre-cancerous and cancerous cases in our population. Recessive and co-dominant models (CC + CA vs. AA, $p = 0.0125$, OR = 2.444, 95% CI = 1.252–4.772; CC vs. AA, $p = 0.0134$, OR = 2.689, 95% CI = 1.285–5.628) revealed a ~2-fold increased risk of oral cancer. However, various studies indicated that the variant genotypes were strongly associated with moderately increased risk in Asians in all genetic models [23,27].

In developing countries like India, lifestyle habits are the main causes of developing oral cancer, especially in Southeast Asia. It is

Table 3
List of novel variants observed in the present study.

IL-10 gene	Accession No.	Position on promoter region	Nucleotide change	Pre-cancer n (%) n = 50	Cancer n (%) n = 100	Control n (%) n = 150
Pattern 1	KT291743.1	−1066	G/T	20 (40)	15 (15)	80 (53)
Pattern 2	KT153594.1	−1045	C/A	18 (28)	25 (25)	10 (7)
Pattern 3	KT291742.1	−924	C/A	22 (44)	60 (60)	10 (7)

Table 4a
Distribution of IL-10 (−592 C/A) genotypes among Oral Pre-cancer, Cancer and equal number of Control subjects.

−592 C/A (rs1800872)

Samples	Genotyping n (%)			Dominant model (CC vs. CA + AA)		Recessive model (CC + CA vs. AA)		Codominant model				Allelic association			
								CC vs. CA		CC vs. AA					
	CC	CA	AA	P-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	C	A	p-Value	OR (95%CI)
Control (n = 150)	44 (29)	88 (59)	18 (12)	Reference		Reference		Reference		Reference		176	125	Reference	
Pre-cancer (n = 50)	9 (18)	33 (66)	8 (16)	0.1653	1.891 (0.8474–4.220)	0.6273	1.397 (0.5665–3.444)	0.2049	1.833 (0.8064–4.168)	0.2670	2.173 (0.7238–6.523)	51	49	*0.0234	1.353 (0.8589–2.131)
Cancer (n = 100)	21 (21)	54 (54)	25 (25)	0.1854	1.562 (0.8604–2.834)	*0.0125	2.444 (1.252–4.772)	0.5229	1.286 (0.6912–2.392)	*0.0139	2.910 (1.310–6.467)	96	104	*0.0269	1.525 (1.064–2.2186)
Cases (n = 150)	30 (20)	87 (58)	33 (22)	0.0817	1.660 (0.9747–2.828)	*0.0314	2.068 (1.106–3.868)	0.2354	1.450 (0.8360–2.515)	*0.0134	2.689 (1.285–5.628)	147	120	0.4621	1.149 (0.8240–1.603)

* $p \leq 0.05$ is considered as significant; OR odds ratio; CI, confidence interval; p-value probability from chi-square test comparing the genotype distribution for controls and cases. Significant p-values are shown in bold.

Table 4b
Distribution of IL-10 (−819 C/T) genotypes among Oral Precancer, Cancer and equal number of Control subjects.

−819 C/T (rs1800871)

Samples	Genotyping n (%)			Dominant model (CC vs. CT + TT)		Recessive model (CC + TT vs. TT)		Codominant model				Allelic association			
								CC vs. CT		CC vs. TT					
	CC	CT	TT	P-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	C	A	p-Value	OR (95%CI)
Control (n = 150)	30 (20)	101 (67)	19 (13)	Reference		Reference		Reference		Reference		161	139	Reference	
Pre-cancer (n = 50)	9 (18)	28 (56)	13 (26)	0.9179	1.139 (0.4990–2.599)	*0.0450	2.422 (1.095–5.361)	0.8563	0.9241 (0.3931–2.172)	0.1825	2.281 (0.8173–6.634)	46	54	0.2251	1.360 (0.8634–2.141)
Cancer (n = 100)	20 (20)	39 (39)	41 (41)	1.0000	1.000 (0.5311–1.883)	* < 0.0001	4.791 (2.565–8.950)	0.1571	0.5792 (0.2946–1.139)	*0.0054	3.237 (1.476–7.097)	79	121	*0.0026	1.774 (1.234–2.551)
Cases (n = 150)	29 (19)	67 (45)	54 (36)	0.8845	1.043 (0.5901–1.844)	* < 0.0001	3.878 (2.160–6.965)	0.2770	0.6862 (0.3779–1.246)	*0.0059	2.940 (1.416–6.106)	125	175	*0.0042	1.622 (1.174–2.239)

* $p \leq 0.05$ is considered as significant; OR odds ratio; CI, confidence interval; p-value probability from chi-square test comparing the genotype distribution for controls and cases. Significant p-values are shown in bold.

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Table 4c
Distribution of IL-10 (−1082 A/G) genotypes among Oral Pre-cancer, Cancer and equal number of Control subjects.

−1082 A/G (rs1800870)

Samples	Genotyping n (%)			Dominant model (AA vs. AG + GG)		Recessive model (AA + AG vs. GG)		Codominant model				Allelic association			
								AA vs. AG		AA vs. GG					
	AA	AG	GG	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	p-value	OR (95%CI)	A	G	p-Value	OR (95%CI)
Control (n = 150)	100 (67)	50 (33)	0 (0)	Reference		Reference		Reference		Reference		250	50	Reference	Reference
Pre-cancer (50)	22 (44)	22 (44)	6 (12)	*0.0074	2.545 (1.324–4.894)	*0.0001	43.996 (2.428–796.29)	0.0665	2.000 (1.012–3.954)	*< 0.0001	58.067 (3.153–1069.4)	66	34	*0.0004	2.576 (1.542–4.304)
Cancer (100)	51 (51)	36 (36)	13 (13)	*0.0188	1.922 (1.144–3.228)	*< 0.0001	46.440 (2.725–791.40)	0.2706	1.412 (0.8183–2.436)	*< 0.0001	52.689 (3.068–904.79)	138	62	*0.0003	2.246 (1.466–3.442)
Cases (150)	73 (49)	58 (39)	19 (12)	*0.0024	2.110 (1.323–3.364)	*< 0.0001	44.635 (2.667–747.00)	0.0787	1.589 (0.9794–2.578)	*< 0.0001	53.327 (3.166–898.14)	204	96	*< 0.0001	2.353 (1.595–3.471)

* $p \leq 0.05$ is considered as significant; OR odds ratio; CI, confidence interval; p-value probability from chi-square test comparing the genotype distribution for controls and cases. Significant p-values are shown in bold.

Table 5
Distribution of haplotype frequencies of IL-10 genotypes among oral pre-cancer, cancer and controls groups.

S. No	Haplotype (IL-10) n = 300 (-592 C/A,-819 C/T,-1082 A/G)	Precancer n (%) n = 50	Cancer n (%) n = 100	Cases n (%) (Precancer + Cancer) n = 150	Control n (%) n = 150
1	CCA(117; 0.39)	17 (34)	28 (28)	45 (30)	72
	p-Value	*0.002	*< 0.0001	*< 0.0001	Reference
	OR	0.4	0.304	0.355	1
2	ATA(66; 0.223)	9 (18)	23 (23)	32 (21)	34
	p-Value	0.201	0.956	0.641	Reference
	OR	0.642	1.01	0.901	1
3	ATG(53; 0.175)	13 (26)	19 (19)	32 (21)	22
	p-Value	*0.014	*0.146	*0.037	Reference
	OR	2.25	1.45	1.63	1
4	CTA(30; 0.098)	5 (10)	12 (12)	17 (11)	13
	p-Value	0.788	0.188	0.296	Reference
	OR	1.12	1.56	1.38	1
5	ACA(13; 0.046)	3 (4)	6 (6)	9 (6)	5
	p-Value	0.465	0.256	0.292	Reference
	OR	1.48	1.63	1.54	1
6	CTG(7; 0.027)	(0)	6 (6)	6 (4)	1
	p-Value	–	*0.005	*0.005	Reference
	OR	–	1.63e + 004	5.97e + 004	1
7	ACG(4.3; 0.018)	(0)	4 (4)	4 (3)	1
	p-Value	–	0.215	0.173	Reference
	OR	–	157	1.37e + 004	1
8	CCG(9; 0.023)	3 (6)	2 (2)	5 (3)	2
	p-Value	*0.050	0.93	0.295	Reference
	OR	4.6	1.08	2.07	1

* **$p \leq 0.05$ is considered as significant**; OR, odds ratio; CI, confidence interval; Significant p-values are shown in bold.

noteworthy that the current study was carried out in North India, where people have different geographical predilection and ethnic backgrounds and are highly influenced by Western culture habits like different types of smoking, alcohol drinking and tobacco chewing that increases the risk of OSCC. The results of a case-control study established that people who smoked, chewed betel quid, and consumed alcohol had a 123-fold higher risk of oral cancer as compared to those who did not indulge in any such activities [34]. In our study, it was interesting to note that the impact of these habits together with risk genotypes showed an increased risk of oral carcinogenesis. On analyzing −1082A/G, we found that those who smoked and drank alcohol showed ~32- and ~28-folds higher risk of developing oral pre-cancer ($p < 0.001$, OR ~32; $p < 0.001$ OR ~28), whereas tobacco chewers were directly associated with cancer patients ($p < 0.001$, OR ~8). The association between consumption of alcohol along with tobacco and the incidence as well as the risk of developing oral cancer has been well established by various studies [35]. The observations of our study has validated, keeping in agreement with previous studies in oral cancer, that IL-10-1082A/G genotype is associated with several different types of cancer such as lung cancer [36,37], gastric cancer [38–40], cervical cancer [17], breast cancer [41,42], prostate cancer [43,44], and nasopharyngeal carcinoma [45,46].

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Table 6
Association of IL-10 haplotype with lifestyle habits.

	Samples	CCA n (%)	Risk Haplotypes ATG + CTG + CCG n (%)	p-values	OR (95% CI)
Tobacco Users	Control (n = 36/150)	26 (72)	2(5)	Reference	
	Precancer (n = 38/50)	13 (34%)	9 (24%)	*0.0118	9.00 (1.693–47.857)
	Cancer (n = 82/100)	21 (27%)	23 (28%)	*0.0002	14.238 (3.005–67.453)
	Cases (n = 120/150)	34 (28%)	32 (27%)	*0.0003	12.235 (2.683–55.800)
Smoker users	Control (n = 54/150)	25 (46%)	1 (2%)	Reference	
	Precancer (n = 29/50)	9 (31%)	10 (34%)	*0.0006	27.778 (3.100–248.90)
	Cancer (n = 63/100)	16 (25%)	18 (28%)	*0.0002	28.125 (3.411–231.91)
	Cases (n = 92/150)	25 (27%)	28 (30%)	* < 0.0001	28.000 (3.530–222.07)
Alcohol users	Control (n = 57/150)	37 (65%)	0(0)	Reference	
	Precancer (n = 39/50)	12 (31%)	13 (33%)	* < 0.0001	81.000 (4.479–1464)
	Cancer (n = 63/100)	13 (21%)	19 (30%)	* < 0.0001	108.33 (6.106–1922.1)
	Cases (n = 102/150)	25 (25%)	32 (31%)	* < 0.0001	77.542 (4.547–1322)

* $p \leq 0.05$ is considered as significant; OR odds ratio; CI, confidence interval; p-value probability against controls. Significance of p-value & OR is assessed by chi-square test/Fisher's
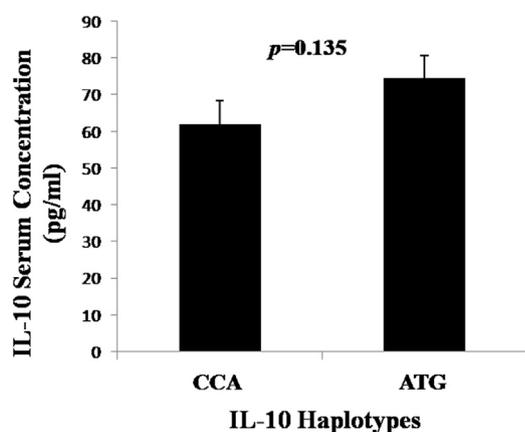

Fig. 5. Bar diagram showing the IL-10 serum concentration in major (CCA, n = 70/180) and minor (ATG, n = 33/180) allele haplotypes. *P-value* is calculated by using the one-way ANOVA. Horizontal line and error bars denote Mean ± SEM, *p ≤ 0.05 is considered as significant.

To complement further, three loci haplotypes were constructed, and their distribution was compared with cancer, pre-cancer and healthy control groups. Haplotype **ATG** was found to significantly increase the risk of oral pre-cancer and cancer cases with p-value < 0.0001 when compared with controls. The C**TG** haplotype emerged as the foremost risk haplotype found only in cancerous cases and showed a statistically significant association when compared with the controls (p = 0.005). Therefore, the C**TG** haplotype acts as a biomarker in invasive carcinoma as it is not present in pre-cancerous patients. Haplotype CC**G** showed statistically significant association in pre-cancerous cases and had a 4-fold increased risk of incidence for the development of disease (p = 0.05, OR = 4.6). The CC**G** haplotype of IL-10 promoter region may enhance transcription and IL-10 expression. These results down-regulated the gene expression of Th1 cytokines, thus, allowing the tumor cells to escape immune surveillance [46]. We have also analyzed the synergetic effects of polymorphisms or haplotypes with smoking, alcohol, and tobacco chewers and found a strong significant association than abstainers. As far as our knowledge is concerned, this is the first study showing the role of IL-10 (−1082A/G, −819C/T, 592C/A) haplotypes in susceptibility and development of OSCC in the Indian population.

IL-10 possesses two functions such as immuno suppression and anti-angiogenesis and is a multifunctional cytokine, which results in both tumor promoting and tumor inhibiting properties [47]. According to the study of Ahamarneh et al., in the patients with lymph node involvement and oral carcinoma, the IL-10 serum level was remarkably higher in advanced stages of the disease [48,49]. In the present study, we observed the serum concentration in controls, precancer and cancer samples with the correlation of risk haplotypes. In our findings, we observed higher IL-10 serum concentration in cancerous (83.233 + 22.8 pg/ml) and precancerous (58.145 + 21.7 pg/ml) cases as compared to controls (37.694 + 27.6 pg/ml). Our observations are similar to the findings reported by Kozlowski et al. [50] and Singhal et al. [17]. We further stratified our results and found that the haplotype **ATG** has higher serum concentration as compared to **CCA** haplotype.

In conclusion, we have identified three novel SNPs (KT153594, KT291742, and KT291743) that are associated with the disease. The prevalence of these novel SNPs were not as frequent as compared to the known SNPs (−592C/A, −819C/T & −1082A/G), but they play an important role in oral carcinoma. However, we will further try to determine the role of new SNPs and development of more efficient biomarkers in the diagnosis and treatment of oral cancer. We also found that the IL-10 (−1082A/G) and its haplotype CC**G** play an important role in the development of the disease. The haplotype (CC**G** & C**TG**) can be used for diagnosis of the early and late stage of oral cancer because one of the haplotype (CC**G**) is found only in pre-cancerous cases and the other haplotype (C**TG**) is found only in cancerous cases only. As far as our knowledge, this is the pilot study on oral cancer in the Indian population and has shown a significant association between genotype and lifestyle habits. Our study also reports how these habits help to increase the risk of oral cancer in the Indian population. The major limitation of this study is its small sample size. Future studies should increase the sample size to validate the findings of novel SNPs and evaluate their effects in oral carcinogenesis.

Ethical approval and informed consent

This study was approved by the Institutional Ethical Committee (ICPO/IEC/P-003/2011), Noida. Informed consent was obtained from all participating individuals.

Compliance with ethical standards

Competing interests: The authors declare that they have no conflicts of interests.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2017.09.016.

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