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Genetic variations of TLRs and their association with HPV/EBV, co-infection along with nicotine exposure in the development of premalignant/ malignant lesions of the oral cavity in Indian population

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ABSTRACT

Background: Despite being most preventable malignancies associated with smoked and smokeless tobacco products, squamous cell carcinoma of oral cavity is one of the most common malignancy in India. The aim of the present study was to evaluate the role of TLRs in oral pre-cancerous, cancerous cases and their genotypic correlation with HPV/EBV, co-infection & lifestyle habits in Indian population.

Methods: The present study was conducted on 300 subjects (100 OSCC, 50 pre-cancer & 150 controls). The amplification of TLRs gene and HPV/EBV co-infection was assessed by Nested PCR, PCR–RFLP and further confirmation by direct sequencing.

Results: The TLR 9(−1486 T/C), revealed that the TT vs. CT + CC genotype had a ˜5-fold increased risk for the development of pre-cancerous lesions as compared to controls ($p = 0.0001$). Further analysis showed that the risk of cancer was extremely pronounced in HPV/EBV, co-infection ($p = 0.0141$), implicating the possible interaction between TLR 9(−1486T/C) genotype and HPV infection in increasing cancer/pre-cancer risk. The 'G' allele of TLR 4(+896A/G) was also a higher risk of developing pre-cancerous lesions with 4.5 fold and statistically significant ($p = 0.0001$). The genotypic association of TLR 9(-1486T/C) in OSMF cases showed ˜8 fold increased risk and TLR 4(+896A/G) showed fourteen fold higher risk for leukoplakia ($p < 0.0001$, OR = 14.000).

Conclusion: Genetic polymorphism of TLR 9(−1486 T/C) and TLR 4(+896A/G) may influence the effects of HPV/EBV, co-infection and play the significant role in development of the disease. The significance of these TLRs seemed to be enhanced by tobacco chewing and smoking habits also, which act as an important etiological risk factor for OSCC.

1. Introduction

Cancer of the oral cavity is presently most commoving cancer worldwide which is ranked on 16th position with an estimated 354,864 new cases in the year of 2018 globally [1]. In India, the second most common cancer and age-standardized incidence rate is 9.1 per 100,000 population which accounts for 11.4% of all cancers in the country (GLOBOCON 2018).). The most prevailing type which is frequently found in this cancer is oral squamous cell carcinoma (OSCC).In developing countries like India people are highly influenced by western

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lifestyle practices like cigarette smoking, alcohol consumption and chewing of smokeless tobacco products (Khaini, Gutkha, supari, paan) which are possible causers for oral cancer (OC) [2], [3,4]. Tobacco used in any form either smoked/smokeless can cause a wide spectrum of oral pre-cancerous conditions like leukoplakia, erythroplakia, and oral submucous fibrosis (OSMF) that significantly enhance the incidence of OSCC [5–7].

Various molecular and epidemiological studies showed that the oncogenic viruses might play an important role in development of OSCC. Human papillomavirus (HPV), as well as some members of Herpes family, such as Epstein Bar virus (EBV) found to play the potential oncogenic role in the progression oral carcinoma [8–10]. There is some report indicate that first infection of HPV in the oral cavity might increase the capacity of epithelial cells to support the EBV life cycle, which could in turn increase EBV-mediated pathogenesis in the oral cavity [11].

Toll like receptors (TLRs) play an important role in innate immunity and involved in the regulation of inflammatory reactions which lead to the activation of adaptive immune response to eliminate the pathogenic microorganisms. TLRs act as the first defence against pathogenic microorganisms. Many polymorphic variants exit among TLRs that regulate the pattern of innate immune response [12]. Many studies reveal that the risk of cancer has been increased by the infection mediated chronic inflammation [13,14]. During viral infection, the role and response of TLR is very crucial in the transformation to malignancy [15]. TLRs can recognize the pathogen which enters into the cell after breaching the physical barrier formed by the epithelial lining. When the integrity of oral epithelium is breached by invasive pathogen or by the use of alcohol/cigarettes, may bind to the TLRs on the oral basal epithelium, resulting in overt inflammation [16]. TLRs recognized different damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) and bind to a wide range of exogenous and endogenous antigens [14] [15].There are various studies showed the important role of TLRs with infectious diseases and their signalling cascade in tumour development and progression [13].

A number of studies reported the interaction between TLRs and HPV, which showed the role of HPV in inhibiting the activity of TLR downstream components involved in the interferon pathway [17]. Besides HPV, EBV is linked not only with malignancies derived from B cells but also with epithelial malignancies, such as nasopharyngeal carcinoma. Subsequently, the expression of pro-inflammatory genes is the result of activation of downstream signalling molecules complex by TLRs nasopharyngeal carcinoma. Antigen-presenting cells (APCs) carry TLRs for optimal T-cell priming, probably through direct recognition of EBV-associated molecular patterns. TLR9 is involved in the detection of EBV and might complement each other for the identification of this virus [12]. Presence of single-nucleotide polymorphisms (SNPs) in TLRs genes could compromise TLRs functionality and shift the balance between pro- and anti-inflammatory cytokines and modulate risk of chronic infection and cancer development [10,18,19].

In the present study, we conducted a case-control study to find out the relevance of TLR9 -1486C/T (rs187084), TLR6 C745 T (Ser249Pro) rs5743810, TLR4 + 896A/G (Asp229Gly) rs4986790 and TLR1 T1805 G (I602S) rs5743618 polymorphisms in oral pre-cancer, cancer patients compared to the controls and their genotypic association with HPV/EBV, co-infection. Furthermore, we also investigated the correlation of these SNPs with clinicopathological variables & lifestyle habits and their role in the development of oral cancer. To the best our knowledge, this is the first study in Indian population which showed the genotypic association between TLRs and lifestyle habits with HPV/EBV, co-infection in pre-cancerous and cancerous patients of the oral cavity.

2. Material and methods

2.1. Clinical specimens

A total of 300 subjects comprises 150 (50 pre-cancerous + 100 OSCC) histopathologically confirmed cases and the equal number of healthy volunteers whose samples were selected from regular physical check-up unrelated to any malignancy or oral-related problems. The samples were collected from the Rajiv Gandhi Research Centre and Hospital, New Delhi and NICPR, Noida, India. The classification of the pre-malignant and malignant lesions was done in accordance with WHO criteria and the tumor/node/metastasis (TNM) classification. The demographic characteristics like 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) of the subjected included in the study were taken personally. 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 participant and the study was carried out in accordance with the principles of Helsinki Declaration. This study has been approved by the ethics committee of the institute (Ethical no.-ICPO/IEC/P-003/2011).

2.2. Extraction of genomic DNA and detection of HPV and EBV

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 [20]. Detection of HPV and EBV was done as follows:

2.2.1. Detection of HPV

Extracted DNA was proceeding for the HPV and EBV infection by Polymerase chain reaction (PCR). Detection of HPV was carried out by using consensus primers MY09 and MY011 located within the conserved L1 region of HPV genome [20,21]. A total volume of 25 µl reaction mixture containing 50–100 ng of genomic DNA, 12.5 pmol of each primer, 0.1 mM dNTPs, 1 × PCR buffer and 5U of Taq polymerase. The PCR reaction consisted of an initial melting step of 95 °C for 5 min; 35 cycles at 95 °C for 30 s, at 55 °C for 35 s and at 72 °C for 30 s and a final elongation step of 72 °C for 7 min. PCR product was then visualized on 2% ethidium-bromide stained agarose gel.

2.2.2. Detection of EBV

EBV was amplified by two steps i.e. Nested PCR by using EBV-specific outer and inner primers [22]. The first round of the PCR was carried out in a mixture with a total volume of 25 µl that included 5 µl of the template, 1X PCR buffer and 5U Taq DNA polymerase, 0.2 mM each dNTPs, 1.5 mM MgCl2, and 1 µM outer primers. PCR amplification, included an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. The second round of amplification was performed in a new tube with 2 µl of the first-round PCR product; 25 pmol of inner primers; and 1X PCR buffer, Taq DNA polymerase, dNTPs and MgCl2 at the concentrations described above. The program for the second PCR was 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1.5 min, with a final extension step at 72 °C for 7 min. Amplicon were detected by electrophoresis of 10 µl of sample in a 1.5% agarose gel containing 1 µg/ml of ethidium bromide. The expected sizes of the target fragment were 497 bp.

2.3. Identification of TLRs SNPs by polymerase chain reaction

The nonsynonymous SNPs of TLR9 -1486C/T (rs187084), TLR6 C745T (rs5743810), TLR4 + 896A/G (rs4986790) and TLR1 T1805G (rs5743618) were studied in the present study. PCR was performed with 1.5 µL of DNA extracts (100 ng/µL) in a 25 µL reaction mixture containing standard PCR buffer, 1.5 mM MgCl2, 200 µM dNTP, 0.5 µM of each primer, and 1.0 U Taq DNA polymerase [23]. PCR products were analyzed via electrophoresis in a 2% agarose gel. After electrophoresis, the gel was stained with ethidium bromide and photographed under the UV light transilluminator.

2.4. Enzymatic digestion of PCR products

The amplicon of TLR-9 (-1486C/T), TLR-6 (C745T), TLR-4 (+896A/G) and TLR-1 (I602S) were further identified by restriction fragment length polymorphism (RFLP) and digested with AluI, NcoI, AvaII and AflII, restriction enzymes, respectively [23]. A total of 10 µl of PCR product was digested with 5 U of respective restriction endonuclease from New England Biolabs in a final volume of 20 µl that contained 2 µl of 10X enzyme buffer according to the manufacturer instructions. The resulting fragments were separated by electrophoresis on 12% polyacrylamide gel and were visualized under UV light by staining with ethidium bromide. The further confirmation of homo- and hetero-duplexes was done by sequencing using Big Dye Terminator and automated DNA Sequencer ABI Prism 3100 (Applied Biosystems, Foster City, CA) with forward and reverse primers.

2.5. Statistical analysis

Statistical analysis was performed using Graph Pad Instant version 3.2. Allele and genotype frequencies were calculated by direct counting. Associations between disease groups and a specific allele, as well as between disease groups and genotypes, were analyzed using the X^2 test. In addition, odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. The Hardy-Weinberg expectation (HWE) was determined by comparing the observed number of different genotypes with those expected under the HWE for the estimated allele frequency. p-values < 0.05 were considered statistically significant.

3. Results

3.1. Studied individual characteristics

The demographic and clinicoepidemiological characteristics of the studied population are shown in Table 1. The population of male was found significantly higher in studied subjects. The mean age of studied subjects was 42.8 ± 14.37 in the pre-cancer cases, 49.75 ± 12.63 in cancer cases and 40.68 ± 14.95 in controls. The most prevailing lifestyle habits in the studied population were cigarette smoking, alcohol consumption and tobacco chewing. We observed that the majority of cases were smokers compared to non-smokers. The frequency of alcohol users was found highest in pre-cancerous cases as compared to cancer and controls. On the other hand, the frequency of tobacco chewer was very much higher (82%) in cancer cases as compared to the controls (25%). These associations were found to remain statistically significant and even slightly stronger after allowing for confounders in univariate analysis. We have studied both rural and urban population; the frequency of pre-cancerous cases was higher in those who belong to rural areas. The characterization of cancer cases according to histopathological grades; 79% were well differentiated squamous cell carcinoma (WDSCC), 17% moderately differentiated squamous cell carcinoma (MDSCC), and 4% poorly differentiated squamous cell carcinoma (PDSCC).

3.2. HPV and EBV infection in oral premalignant and malignant patients

The prevalence of HPV and EBV in the studied population has been presented in Table 2. HPV positive was detected 24% (12/50) in pre-cancerous cases, 22% (22/100) in cancer and 7% of the healthy individuals while the EBV was detected 42% (21/50), 42% (42/100), 9% (14/150) in the three groups of subjects cancer, pre-cancer & controls, respectively. Single infection (HPV^+/EBV^-, HPV^-/EBV^+) was detected 57% in pre-cancer cases, 75% in cancer, and 59% in controls. The frequency of co-infection ($HPV^+ + EBV^+$) was observed 43% in pre-cancerous cases, 25% in cancerous cases and 41% in controls. The frequency of co-infection was higher in precancerous lesions as compare to rest other groups. On the other hand, the presence of any of the infection was higher in cancer cases ($p < 0.0001$).

3.3. Genotypic distribution of TLR9 −1486T/C(rs187084) and TLR-6 C745T(rs5743810) polymorphism

The TLR 9(−1486 T/C) genotype frequency of TT, CT, and CC was 19%, 61%, and 20% in cases and 44%, 41%, and 15% in controls, respectively (Table 3 a). In the dominant model (TT vs. CT + CC), pre-cancerous cases vs. control analysis was found to be significant and at ˜5 fold higher risk of developing premalignant conditions ($p = 0.0001$, OR = 5.762, 95% CI = 2.314 to 14.344) and ˜2 fold increased risk of cancerous cases ($p = 0.0006$, OR = 2.786, 95% CI = 1.571–4.939). Furthermore, on studied recessive (TT + CT vs CC) and co-dominant model (TT vs. CT & TT vs. CC) between cases & controls, it was noticed that the precancerous cases of co-dominant model showed ˜7 fold higher risk which means that the homozygous dominant allele is more prone to develop disease ($p = 0.0003$, OR = 7.000, 95%CI = 2.398 to 20.437). Considering the allelic frequencies, our data indicate that the variant 'C' allele was associated with the increased risk of oral premalignant and malignant conditions ($p = 0.0001$, OR = 2.527, 95% CI = 1.592 to 4.013).

On the evaluation of TLR-6(C745 T), the frequency of carrier genotype (CT + TT) was higher in controls as compared to pre-cancer and cancer. In genetic models, the dominant (CC vs. CT + TT) and co-dominant (CC vs. CT & CC vs. TT) model showed the significant association between cases and controls ($p = 0.0001$). T allele was also observed to be significantly higher in controls as compared to cases. This observation indicates the possible protective role of TLR-6(C745T) in the development of the disease /oral carcinoma Table 3 a.

3.4. Genotypic distribution of TLR4 + 896A/G(rs4986790) and TLR-1 I602S(rs5743618) polymorphism

The genotypic distribution of TLR 4(+896A/G) and TLR-1(I602S) in pre-cancer, cancer, and the control group has been shown in the Table 3 b. The frequency of TLR 4(+896A/G) genotype (AA, AG & GG) was 32%, 66%, 2% in pre-cancer, 57%, 41%, 2% in cancer and 80%, 19% and 1% in controls. We observed approximately eight-fold increased risk (OR = 8.500, 95% CI = 4.152–17.402) of developing pre-cancerous lesions in the presence of AG + GG genotype as compared to controls while it was found at ˜3 fold higher risk for cancer development ($p = 0.0002$ OR = 3.018, 95% CI = 1.719–5.299). The risk was also shown to be significantly increased with hetero & homozygous mutant genotype in a co-dominant model (AA vs. AG and AA vs. GG) in pre-cancer & cancer cases. Similarly, pre-cancer patients carrying the heterozygous variant genotype showed ˜8 fold higher risk for the development of oral carcinoma ($p = 0.0001$ OR = 8.839, 95% CI = 4.280 to 18.255). Considering the allelic frequencies, it can be commented that the presence of 'G' allele was increased the risk of pre-cancerous lesions by 4.5 fold (OR = 4.510, 95% CI = 2.600 to 7.823) while for cancer it is only 2.4 fold and statistically significant ($p = 0.0001$). This finding suggests the strong association of this 'G' allele in the development of pre-cancerous oral lesions which can be used as a Biomarker

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)	42.8 ± 14.37	49.75 ± 12.63	47.43 ± 13.59	40.68 ± 14.95
Sex				
Male	33 (66)	68 (68)	101(67)	129 (86)
Female	17 (34)	32 (32)	49 (33)	21 (14)
***p*-value**	***0.0036**	***0.0011**	***0.0002**	**Reference**
OR (95%CI)	3.165 (1.565–6.667)	2.891 (1.549–5.396)	2.980 (1.679–5.290)	
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)	109 (73)
Alcoholic	39 (78)	63 (63)	102 (68)	41 (27)
***p*-value**	***< 0.0001**	***0.0002**	***< 0.0001**	**Reference**
OR (95%CI)	9.426 (4.410–20.146)	4.527 (2.632-7.784)	5.649 (3.438–9.284)	
Non Tobacco Chewers	12 (24)	18 (18)	30 (20)	113 (75)
Tobacco Chewers	38 (76)	82 (82)	120 (80)	37 (25)
***p*-value**	***< 0.0001**	***0.0005**	***< 0.0001**	**Reference**
OR (95%CI)	9.671 (4.578–20.430)	3.733 (1.807–7.710)	12.216 (7.077–21.088)	
Habitat				
Rural	35 (70)	64 (64)	99 (66)	114(76)
Urban	15 (30)	36 (36)	51 (34)	36(24)
***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	0	79 (79)	79(53)	–
MDSCC	0	17 (17)	17(11)	
PDSCC	0	4 (4)	4(3)	

*$p \leq 0.05$ 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.

for the management of oral pre-cancerous lesions. On the analysis of TLR-1 (T1805G) SNP, the frequency of TT, TG & GG genotypes were observed 80%, 20% & 0% for controls while 89%, 11% & 0% for cases. The frequency of carrier genotype was noticed to be slightly higher in controls but the difference could not be found significant in any of the genetic model.

3.5. Association between HPV/EBV, Co- infection with different genotypes of TLRs

The association of HPV/ EBV, co-infection with TLRs genotype in oral pre-cancer, cancer and control group is shown in Table 4. The genotype TT, CT & CC of TLR9 (−1486 T/C) in HPV infected patients was 17% (2/12), 33% (4/12) & 50% (6/12) in pre-cancerous cases, 18% (4/22),59% (13/22) & 23% (5/22) in cancerous and 80%,10% & 10% in controls. It was observed that the frequency of hetero and

Table 2
Prevalence of Single and co- infection of Human Papillomavirus (HPV) and Epstein - Barr virus (EBV) in oral pre-cancer, cancer patients and controls.

	Pre-cancern (%)	Cancern (%)	Casesn (%)	Controlsn (%)
HPV^+	12/50 (24)	22/100 (22)	34/150 (23)	10/150 (7)
HPV^-	38/50 (76)	78/100 (78)	116/150 (77)	140/150(93)
***p*-value**	***0.0017**	***0.0008**	***0.0002**	Reference
OR (95%CI)	4.421 (1.775–11.014)	3.949 (1.779–8.765)	4.103 (1.944–8.661)	
EBV^+	21/50 (42)	42/100 (42)	63/150 (42)	14/150 (9)
EBV^-	29/50 (58)	58/100 (58)	87/150 (58)	136/150 (91)
***p*-value**	***< 0.0001**	***< 0.0001**	***< 0.0001**	Reference
OR (95%CI)	7.034 (3.204–15.443)	7.034 (3.569-13.866)	7.034 (3.714–13.324)	
[a]**$HPV^+/EBV^- + HPV^-/EBV^+$**	13/23(57)	38/51 (75)	51/74 (69)	10/17(59)
[b]**$HPV^+ + EBV^+$**	10/23 (43)	13/51 (25)	23/74 (31)	7/17 (41)
***p*-value (a vs b)**	0.8843	0.3566	0.6084	Reference
OR (95%CI)	1.099 (0.3085–3.914)	0.4887 (0.1542-1.549)	0.6443 (0.2178–1.905)	
[c]**$HPV^- + EBV^-$**	27/50 (54)	49/100 (49)	76/150 (51)	133/150 (87)
***p*-value (a + bvs c)**	* < **0.0001**	* < **0.0001**	* < **0.0001**	Reference
OR (95%CI)	0.0001 (0.1500–0.3181)	0.1228 (0.06480–0.2328)	0.1313 (0.07220–0.2387)	

$p \leq 0.05$ is considered as significant; OR, odds ratio; CI, confidence interval; n, number; *p*-value, probability from chi-square test comparing with infected/Non-infected between cases and controls.

[a] $HPV^+/EBV^- + EBV^+$/ HPV- (Single infected either HPV^+/HPV^-, EBV^+/EBV^-).

[b] $HPV^+ + EBV^+$ (Co-infection, same sample infected with HPV/EBV both).

[c] $HPV^- + EBV^-$ (Non-infected).

Table 3a
TLR 9(−1486 T/C) & TLR 6(C745 T) genotypes among oral pre-cancer, cancer and equal number of control subjects.

TLR 9(−1486 T/C) rs187084

Samples	Genotyping n (%)				Dominant model(TT vs. CT + CC)		Recessive Model(TT + CT vs. CC)		Co-dominant Model				Allelic Association			
									TT vs. CT		TT vs. CC					
	TT	CT	CC	CT + CC	*p*-value	OR (95%CI)	*p*-value	OR(95%CI)	*p*-value	OR (95%CI)	*p*-value	OR(95%CI)	T	C	*p* -Value	OR (95%CI)
CONTROL (n = 150)	66 (44)	62 (41)	22 (15)	84 (56)	Reference		Reference		Reference		Reference		194	106	Reference	
PRE-CANCER (n = 50)	6 (12)	30 (60)	14 (28)	44 (88)	*0.0001	5.762 (2.314–14.344)	0.0558	2.263 (1.052-4.865)	*0.0004	5.323 (2.073–13.664)	*0.0003	7.000 (2.398–20.437)	42	58	*0.0001	2.527(1.592–4.013)
CANCER (n = 100)	22 (22)	62 (62)	16 (16)	78 (78)	*0.0006	2.786 (1.571–4.939)	0.9141	1.108 (0.5501-2.233)	*0.0004	3.000 (1.651–5.452)	0.0875	2.182 (0.9756–4.880)	106	94	*0.0119	1.623 (1.127–2.228)
CASES (n = 150)	28 (19)	92 (61)	30 (20)	122 (81)	* < 0.0001	3.423 (2.031–5.771)	0.2857	1.455 (0.7951-2.661)	* < 0.0001	3.498 (2.024–6.045)	*0.0018	3.214 (1.587–6.511)	148	152	*0.0002	1.880 (1.355–2.608)

TLR 6(C745 T) rs5743810

Samples	Genotyping n (%)				Dominant model (CC vs. CT + TT)		Recessive Model (CC + CT vs. CC)		Co-dominant Model				Allelic Association			
									CC vs. CT		CC vs. TT					
	CC	CT	TT	CT + TT	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	*p*-value	OR (95%CI)	C	T	*p*-Value	OR (95%CI)
CONTROL (n = 150)	102 (68)	45 (30)	3 (2)	48 (32)	Reference		Reference		Reference		Reference		249	51	Reference	
PRE-CANCER (50)	48 (96)	2 (4)	0 (0)	2 (4)	*0.0002	0.0885 (0.02065-0.3797)	0.7370	0.4173 (0.02117-8.224)	*0.0003	0.09444 (0.02198-0.4057)	0.5793	0.3019 (0.01528-5.965)	98	2	*0.0003	0.09964 (0.02379-0.4174)
CANCER (100)	89 (89)	10 (10)	1 (1)	11 (11)	*0.0002	0.2626 (0.1286-0.5366)	0.9181	0.4949 (0.05073-4.829)	*0.0003	0.2547 (0.1213-0.5349)	0.7257	0.3820 (0.03902-3.741)	188	12	*0.0005	0.3116 (0.1616-0.6011)
CASES (150)	137 (91)	12 (8)	1 (1)	13 (9)	* < 0.0001	0.2016 (0.1038-0.3919)	0.6147	0.3289 (0.03380-3.200)	* < 0.0001	0.1985 (0.09992-0.3945)	0.4323	0.2482 (0.02543-2.422)	286	14	* < 0.0001	0.2390 (0.1292-0.4423)

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

Table 3b
Distribution of TLR 4(+896A/G) & TLR 1(T1805 G) genotypes among oral pre- cancer, cancer and equal number of Control subjects.

TLR 4(+896A/G) rs4986790																
Samples	Genotyping n (%)				Dominant model (AA vs. AG + GG)		Recessive Model (AA + AG vs. GG)		Co-dominant Model				Allelic Association			
									AA vs. AG		AA vs. GG					
CONTROL (n = 150)	AA 120 (80)	AG 28 (19)	GG 2 (1)	AG + GG 30 (20)	*p*-value Reference	OR (95%CI)	*p*-value Reference	OR (95%CI)	*p*-value Reference	OR (95%CI)	*p*-value Reference	OR (95%CI)	A 268	G 32	*p*-Value Reference	OR (95%CI)
PRE-CANCER (n = 50)	16 (32)	33 (66)	1 (2)	34 (68)	* < **0.0001**	8.500 (4.152-17.402)	0.7370	1.510 (0.1339–17.029)	* < **0.0001**	8.839 (4.280 –18.255)	0.8126	3.750 (0.3213–43.765)	65	35	* < **0.0001**	4.510 (2.600–7.823)
CANCER (n = 100)	57 (57)	41 (41)	2 (2)	43 (43)	***0.0002**	3.018 (1.719-5.299)	0.6807	1.510 (0.2091–10.905)	***0.0002**	3.083(1.735–5.478)	0.8324	2.105 (0.2890–15.334)	155	45	***0.0005**	2.431 (1.483–3.987)
CASES (n = 150)	73 (49)	74 (49)	3 (2)	77 (51)	* < **0.0001**	4.219 (2.527-7.044)	0.6520	1.510 (0.2486–9.174)	***0.0010**	4.344 (2.574–to 7.332)	0.5885	2.466 (0.4023–15.114)	220	80	* < **0.0001**	3.045 (1.947–4.763)

TLR 1(T1805G) rs5743618																
Samples	Genotyping n (%)				Dominant model (TT vs. TG + GG)		Recessive Model (TT + TG vs. GG)		Co-dominant Model				Allelic Association			
									TT vs. TG		TT vs. GG					
CONTROL (n = 150)	TT 120 (80)	TG 30 (20)	GG 0 (0)	TG + GG 30 (20)	*p*-value Reference	OR (95%CI)	*p*-value Reference	OR (95%CI)	*p*–value Reference	OR (95%CI)	*p*–value Reference	OR (95%CI)	T 270	G 30	*p*–value Reference	OR (95%CI)
PRE-CANCER (50)	46 (92)	4 (8)	0 (0)	4 (8)	0.0820	0.3478 (0.1161–1.042)	0	0	0.0820	0.3478 (0.1161–1.042)	0	0	96	4	0.0977	0.3750(0.1287–1.092)
CANCER (100)	87 (87)	12 (12)	1 (1)	13 (13)	0.2056	0.5977 (0.2947–1.212)	0.8379	4.538 (0.1829–112.60)	0.1465	0.5517 (0.2674–1.138)	0.8760	4.131 (0.1662-102.70)	186	14	0.3178	0.6774 (0.3496–1.313)
CASES (150)	133 (88)	16 (11)	1 (1)	17 (12)	0.0566	0.5113 (0.2684–0.9738)	0.3165	3.020 (0.1220–74.789)	0.0395	0.4812 (0.2499–0.9265)	0.3430	2.708 (0.1092-67.156)	282	18	0.0979	0.5745 (0.3128–1.055)

*$p \leq 0.05$ 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 4
TLRs genotype association with single and co- infection (HPV + EBV) in oral pre-cancer & cancer patients.

Samples	TLR 9(−1486 T/C)									
	HPV					Samples		EBV		
	TT	CT	CC	CT + CC	*p*-Value	OR(95% CI)		TT	CT	CC
Control(n = 10)	8 (80)	1(10)	1(10)	2(20)	Reference		**Control(n = 14)**	9 (64)	2 (14)	3 (22)
Pre- Cancer(n = 12)	2 (17)	4 (33)	6 (50)	10 (83)	***0.0111**	20.000 (2.284–175.13)	**Pre- Cancer (n = 21)**	3 (14)	12 (57)	6 (29)
Cancer (n = 22)	4 (18)	13 (59)	5 (23)	18 (82)	***0.0031**	18.000(2.716–119.29)	**Cancer (n = 42)**	8(19)	27(64)	7(17)
Cases (n = 34)	6 (18)	17 (50)	11 (32)	28 (82)	***0.0009**	18.667(3.138–111.05)	**Cases (n = 63)**	11(17)	39(62)	13(21)
	TLR 6(C745 T)									
Samples	**CC**	**CT**	**TT**	**CC + TT**	***p*-Value**	**OR (95% CI)**	**Samples**	**CC**	**CT**	**TT**
Control (n = 10)	8 (80)	2 (20)	0 (0)	2 (20)	Reference		**Control (n = 14)**	10 (71)	4 (29)	0 (0)
Pre- Cancer (n = 12)	12 (100)	0 (0)	0 (0)	0 (0)	0.3788	0.1360 (0.00577–3.205)	**Pre- Cancer (n = 21)**	21 (100)	0 (0)	0 (0)
Cancer (n = 22)	19 (86)	2 (9)	1 (5)	3 (10)	0.6458	0.6316(0.08798–4.534)	**Cancer (n = 42)**	39 (93)	3 (7)	0 (0)
Cases (n = 34)	31(91)	2 (6)	1 (3)	3 (9)	0.6802	0.3871(0.05501–2.724)	**Cases (n = 63)**	60 (95)	3 (5)	0 (0)
	TLR 4(+896A/G)									
Samples	**AA**	**AG**	**GG**	**AG + GG**	***p*-Value**	**OR (95% CI)**	**Samples**	**AA**	**AG**	**GG**
Control (n = 10)	8(80)	2 (20)	0 (0)	2 (20)	Reference		**Control (n = 14)**	12 (86)	2 (14)	0 (0)
Pre- Cancer (n = 12)	3 (25)	9 (75)	0 (0)	9 (75)	***0.0323**	**12.000 (1.580-91.130)**	**Pre- Cancer (n = 21)**	7 (33)	14 (67)	0 (0)
Cancer (n = 22)	6 (27)	15 (68)	1 (5)	16 (73)	***0.0163**	10.667(1.742–65.300)	**Cancer (n = 42)**	23 (55)	18 (43)	1 (2)
Cases (n = 34)	9(26)	24(71)	1(3)	25 (74)	***0.0072**	11.111(1.976–62492)	**Cases (n = 63)**	30 (48)	32 (51)	1 (1)
	TLR 1(T1805 G)									
Samples	**TT**	**TG**	**GG**	**TG + GG**	***p*-Value**	**OR (95% CI)**	**Samples**	**TT**	**TG**	**GG**
Control(n = 10)	9 (90)	1 (10)	0 (0)	1 (10)	Reference		**Control (n = 14)**	12 (86)	2 (14)	0 (0)
Pre- Cancer (n = 12)	11 (92)	1 (8)	0 (0)	1 (8)	0.8923	0.8182(0.0446-15.006)	**Pre- Cancer (n = 21)**	19 (90)	2 (10)	0 (0)
Cancer (n = 22)	21 (95)	0 (0)	1 (5)	1 (5)	0.5546	0.4286 (0.02405–7.638)	**Cancer (n = 42)**	37(88)	5 (12)	0 (0)
Cases (n = 34)	32 (94)	1 (3)	1 (3)	2 (6)	0.6497	0.5625 (0.04560–6.938)	**Cases (n = 63)**	56 (89)	7 (11)	0 (0)

Samples	TLR 9(−1486 T/C)									
	EBV		Samples		Co-infection HPV + EBV					
	CT + CC	*p*-Value	OR(95% CI)		TT	CT	CC	CT + CC	*p*-Value	OR(95% CI)
Control(n = 10)	5 (36)	Reference		**Control (n = 7)**	5 (72)	1 (14)	1 (14)	2 (28)	Reference	
Pre- Cancer(n = 12)	18 (86)	***0.0072**	10.800 (2.095-55.689)	**Pre- Cancer (n = 10)**	1 (10)	4 (40)	5 (50)	9 (90)	***0.0364**	22.500 (1.608-314.77)
Cancer (n = 22)	34(81)	***0.0043**	7.650(2.008-29.146)	**Cancer (n = 13)**	1 (8)	10 (77)	2 (15)	12 (92)	***0.0141**	30.00 (2.188-411.25)
Cases (n = 34)	52(83)	***0.0010**	8.509(2.384-30.373)	**Cases (n = 23)**	2 (9)	14 (61)	7 (30)	21 (91)	***0.0034**	26.250 (2.939-234.49)
	TLR 6(C745 T)									
Samples	**CC + TT**	***p*-Value**	**OR (95% CI)**	**Samples**	**CC**	**CT**	**TT**	**CC + TT**	***p*-Value**	**OR (95% CI)**
Control (n = 10)	4 (29)	Reference		**Control (n = 7)**	6(86)	1(14)	0 (0)	1 (14)	Reference	
Pre- Cancer (n = 12)	0 (0)	***0.0394**	0.05426(0.00266–1.106)	**Pre-Cancer(n = 10)**	10(100)	0(0)	0(0)	0 (0)	0.8534	0.2063 (0.007257)
Cancer (n = 22)	3 (7)	0.1025	0.1923(0.03691–1.002)	**Cancer (n = 13)**	12(92)	1(8)	0(0)	1(8)	0.6392	5.000 (0.02642–9.464)
Cases (n = 34)	3 (5)	***0.0221**	0.1250(0.02424–0.6445)	**Cases (n = 23)**	22(96)	1(4)	0(0)	1(4)	0.9540	0.2727 (0.01477–5.036)
	TLR 4(+896A/G)									
Samples	**AG + GG**	***p*-Value**	**OR (95% CI)**	**Samples**	**AA**	**AG**	**GG**	**AG + GG**	p-Value	OR (95% CI)
Control (n = 10)	2 (14)	Reference		**Control (n = 7)**	6 (86)	1 (14)	0 (0)	1 (14)	Reference	

(*continued on next page*)

Table 4 (*continued*)

Samples	TLR 9(−1486 T/C)							
	EBV		Samples		Co-infection HPV + EBV			
	CT + CC	*p*-Value	OR(95% CI)		TT	CT	CC	CT + CC	*p*-Value	OR(95% CI)
Pre- Cancer (n = 12)	14 (67)	***0.0069**	12.000 (2.083-69.115)	**Pre- Cancer (n = 10)**	2 (20)	8 (80)	0 (0)	8 (80)	***0.0294**	24.000 (1.740 –331.02)
Cancer (n = 22)	19 (45)	***0.0796**	4.957 (0.9849–24.945)	**Cancer (n = 13)**	3 (23)	10 (77)	0 (0)	10 (77)	***0.0268**	20.000 (1.675–238.78)
Cases (n = 34)	33 (52)	***0.0219**	6.600 (1.364–31.943)	**Cases (n = 23)**	5 (22)	18 (78)	0 (0)	18 (78)	***0.0086**	21.600 (2.085–223.78)
	TLR 1(T1805 G)									
Samples	**TG + GG**	***p*-Value**	**OR (95% CI)**	**Samples**	**TT**	**TG**	**GG**	**TG + GG**	***p*-Value**	**OR (95% CI)**
Control(n = 10)	2 (14)	Reference		**Control (n = 7)**	6 (86)	1 (14)	0 (0)	1 (14)	Reference	
Pre- Cancer (n = 12)	2 (10)	0.6644	0.6316 (0.7816–5.104)	**Pre- Cancer (n = 10)**	9 (90)	1 (10)	0 (0)	1 (10)	0.7872	0.6667 (0.03459–12.850)
Cancer (n = 22)	5 (12)	0.8155	0.8108 (0.1388–4.735)	**Cancer (n = 13)**	13 (100)	0 (0)	0 (0)	0 (0)	0.7872	0.6667 (0.03459–12.850)
Cases (n = 34)	7 (11)	0.7381	0.7500 (0.1382–4.069)	**Cases (n = 23)**	22(96)	1 (4)	0 (0)	1 (4)	0.9540	0.2727 (0.01477–5.036)

$p \leq 0.05$ is considered as significant; OR, odds ratio; CI, confidence interval; n, number; *p*-value, probability from chi-square test in HPV/EBV, co-infected patients. Significant p-values are shown in bold.

homozygous genotype (CT + CC) was higher in HPV infected pre-cancerous cases as compared to cancer & control group and this symbiosis of these two (SNP + infection) ($p = 0.0111$ OR = 20.000, 95% CI = 2.284–175.13) play the important role for the initiation of diseases. In EBV infected cases, the frequency of TLR 9(−1486 T/C) genotype of wild (TT), hetero (CT) & homozygous (CC) allele was 14%(3/21), 57%(12/21) & 29%(6/21) in pre-cancer, 19% (8/42), 64% (27/42) & 17%(7/42) in cancer and 64% (9/14), 14%(2/14) & 22%(3/14) in control group. The frequency of EBV infected subjects carrying the heterozygous variant genotype 'CT' of TLR 9(−1486T/C) was higher in cancer cases as compared to the control with odd ratio 7.6 (95% CI 2.008–29.146) and the difference was statistically significant ($p = 0.0043$). The risk was also shown to be significantly increased with homozygous variant 'CC' genotype in pre-cancerous cases and the differences were statistically significant ($p = 0.0072$ OR = 10.800, 95% CI = 2.095–55.689). Therefore data demonstrated that TLR-9(−1486 T/C) allele 'C' was significantly associated with oral pre-cancerous and cancerous cases with development of disease but not associated with the progression of the disease. When studied the co-infection HPV& EBV, the frequency of TLR 9(−1486 T/C) wild type genotype (TT) was 10% (1/10) in pre-cancer, 8% (1/13) in cancer and 72% (5/7) in controls. The hetero & homozygous variant genotype (CT + CC) frequency was 90% (9/10) in precancerous cases, 92% (12/13) in cancer and 28% (2/7) in controls. After analyzed the co-infection (HPV + EBV) between cases & controls, CT + CC genotype frequency of TLR 9(−1486 T/C) was found in ascending order in pre-cancerous & cancerous cases. In co-infected cases, the TLR 9(−1486 T/C) hetero & homozygous (CT + CC) genotype showed approximately twenty two fold increased risk of precancerous patients ($p = 0.0364$ OR = 22.500, 95% CI = 1.608 to 314.77) and approximately thirty fold higher risk ($p = 0.0141$ OR = 30.00, 95% CI = 2.188–411.25) of developing oral cancer.

The evaluation of TLR 4 (+896A/G) genotype in HPV infected cases, the frequency was 25%(3/12), 75%(9/12), 0%(0/9) in pre-cancer, 27%(6/22),68%(15/22) & 5%(1/22) in cancer and 80%(8/10),20%(2/10) & 0%(0/10) in controls. The allelic frequency of hetero and homozygous (AG + GG) genotype was higher in cancer cases as compared to control and approximately ten fold higher increased risk for the progression of the oral cancer ($p = 0.0163$ OR = 10.667, 95% CI = 1.742–65.300). In contrast AA,AG & GG TLR 4(+896A/G) genotype frequency in EBV infected pre-cancerous cases was 33%(7/21),67%(14/21) & 0%(0/21), 55%(23/42),43%(18/42) & 2%(1/42) in cancer and 86%(12/14),14%(2/14) & 0%(0/14) in controls. We observed approximately twelve fold increased risk of disease ($p = 0.0069$ OR = 12.000, 95% CI = 2.083–69.115) as 67% frequency of heterozygous variant (AG) genotype in pre-cancerous cases as compared to 14% of control. Surprisingly this found that the minor allele 'G' exit only in EBV infected cancerous cases not in pre-cancerous cases. This genotypic correlation with EBV infection may suggest that the 'G' allele of TLR 4(+896A/G) play the significant role in progression of disease. In contrast, the TLR 4 (+896A/G) genotype in co-infected patients and they showed the significant association between cases and controls. The frequency of TLR 4(+896A/G) genotype AA, AG & GG was 20% (2/10), 80% (8/10) &0% (0/10) in pre-cancer, 23% (3/13), 77% (10/13) & 0% (0/13) in cancer and 86% (6/7), 14% (1/7) & 0% (0/7) in controls. In co-infected cases the frequency of heterozygous variant genotype 'AG' was higher only in pre-cancerous cases as compare to cancer & control group with relative risk of 22 (95% CI = 0.0214–2511.0) and the difference was statistically significant ($p = 0.0294$). This analysis showed that the TLR 4(+896A/G) polymorphism in co-infected patients may play the important role for developing precancerous conditions.

Moreover, we also studied TLR 6(C745T) and TLR 1(T1805=G) genotype in HPV/EBV, co-infected patients, the frequency of carrier genotype (CC + TT) of TLR 6 (C745T) was higher in controls as compare to cases including both viruses infection. This may suggest that the minor allele 'T' may play the protective role in oral pre malignant and

malignant cases. The TLR 1(T1805G) did not show any significant association in HPV, EBV or in co-infection.

3.6. TLRs genotype correlation with life style habits with progression of the disease

The etiology of oral cancer involves multiple factors, most of which are related to lifestyle habits. After analysis of genotypic data in the association with lifestyle habits, it can be strongly stated that lifestyle habits have a vast impact on the risk of oral carcinoma. Analyzed the genotypic association of above mentioned TLRs with life style habits such as cigarette smoking, alcohol consumption, and tobacco chewing are shown in Supplementary table (1A–D). When correlate the TLR 9 genotype of (−1486T/C) with smokers, the frequency of CT + CC genotype was substantially higher in cases as compared to controls and difference was statistically significant ($p < 0.0492$, OR = 2.260, 95% CI = 1.069–4.777). When comparison was made between alcoholic users between cases and controls, it was observed significant association of TLR 9(−1486T/C) genotype ($p < 0.052$, OR = 2.601, 95% CI = 1.081–6.259). We also analyzed the tobacco habits with genotypic data, it was noticed that tobacco users were at ˜8 fold higher risk of having pre-cancerous lesions ($p < 0.0024$, OR = 7.955, 95% CI = 2.063 to 30.677) while ˜3 fold higher risk of developing oral cancer ($p < 0.0183$, OR = 3.045, 95% CI = 1.285 to 7.217) (Supplementary Table 1A). In TLR 6(C745T), the frequency of carrier genotype (CC + TT) in smokers was higher in controls 31%(17/34) as compare to cases 12%(11/92) which showed significant association and same pattern was observed when analyzed the alcohol and tobacco habits ($p < 0.0001$). This observation concludes that these life style habits may not influence the risk for oral cancer in individuals presenting these genetic profiles (Supplementary Table 1B). We also examined the TLR 4(+896A/G) with lifestyle habits, showed ˜16 fold higher risk for pre-cancer ($p < 0.0001$, OR = 16.867, 95% CI = 5.443 to 52.270) and ˜4 fold increased risk ($p < 0.0019$, OR = 4.000, 95% CI = 1.716 to 9.324) in cancer group with the presence of AG + GG genotype along with cigarette smoker. On considering alcohol habits, carrier genotype (AG + GG) had a higher risk of initiation of disease in heavy drinkers and showed ˜6 fold increased risk in precancerous cases with the comparison of controls ($p < 0.0003$, OR = 6.246, 95% CI = 2.357 to 16.552). The stratification of results with respect to tobacco chewers showed ˜4-fold higher risk in pre-cancer and ˜2 fold increased risk in cancer patients having GG genotype ($p < 0.0001$) (Supplementary Tables 1C). We also studied TLR 1(T1805G) but could not find any significant association with genotype and life style habits (Supplementary Tables 1D).

3.7. Genotypic association of TLRs with clinical parameters

We further stratified our results with respect to pre-cancerous conditions and histological grades of oral cancer mentioned in Fig. 1. We also tried to observe the difference in TLRs genotypic allelic frequencies for all known variations in pre-cancerous cases categorized by leukoplakia & OSMF and cancerous cases categorized by histopathological conditions like well differentiated squamous cell carcinoma (WDSCC), moderately differentiated squamous cell carcinoma (MDSCC) & poorly differentiated squamous cell carcinoma (PDSCC). The frequency of variant allele 'C' of TLR 9(−1486 T/C), variant allele 'T' of TLR 6(C745 T and 'G' allele of TLR 4(+896A/G) showed higher significant association with the increased risk of OSCC. As most of the study also found that the frequency of TLR 9(−1486 T/C) homo & heterozygous variant genotype (CT + CC) showed ˜8 fold higher risk in OSMF cases as compare to controls ($p < 0.0027$, OR = 8.250, 95% CI = 1.866 to 34.466) and ˜4 fold increased risk for the development of leukoplakia ($p < 0.0083$, OR = 4.518, 95% CI = 1.489 to 13.708). When analyzed the genotype frequencies in histopathological grade, interestingly we could find only WDSCC showed higher frequency and significant association ($p < 0.0001$, OR = 4.387, 95% CI = 2.192–8.781) as compare to moderate & poorly differentiated squamous cell carcinoma. In concern of TLR 4(+896A/G), it also showed the significant association in OSMF, Leukoplakia and WDSSC, the higher frequency of carrier genotype 'G' was seen only in leukoplakia and the difference was found statistically significant ($p < 0.0001$, OR = 14.000, 95% CI = 5.193 to 37.746). From these findings we can predict that these polymorphisms can serve as a marker for high risk group. We also studied could not find any change of the allelic frequencies of TLR 1(T1805G) and advance stages of oral carcinoma for any of the known variations.

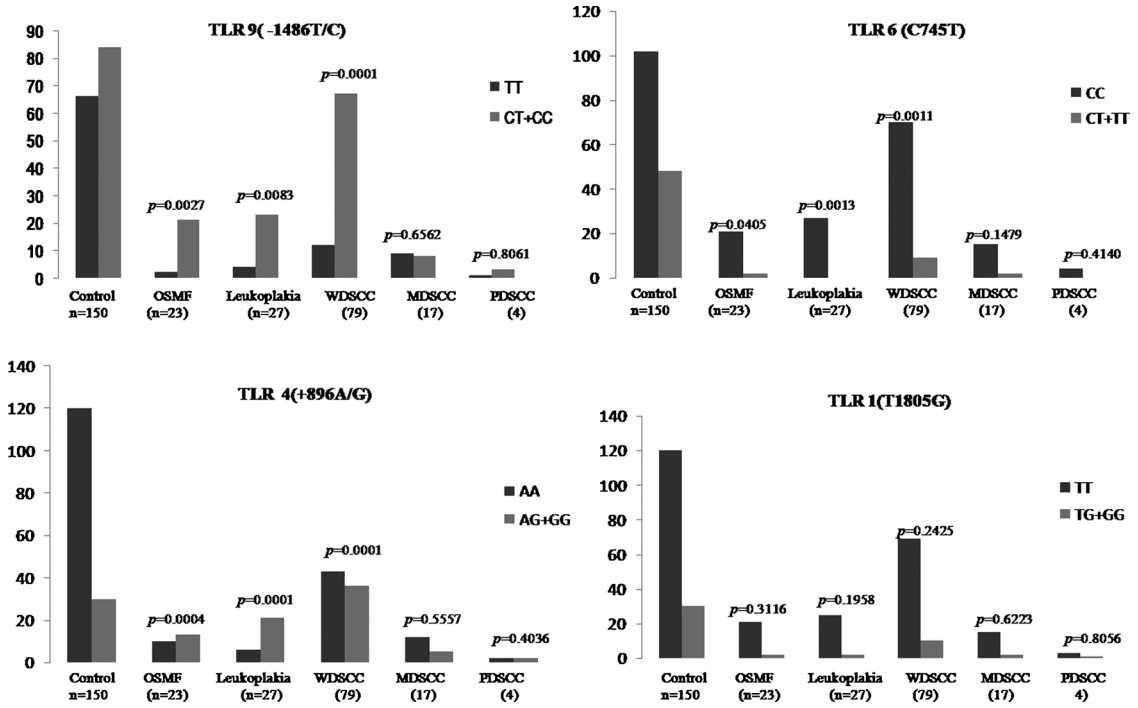

Fig. 1. Correlation of genotypic frequency of TLRs (TLR 9 − 1486 T/C, TLR-6(C745 T), TLR 4(+896A/G) & TLR-1(T1805 G) locus with Histological grades.

4. Discussion

Analysis of candidate gene polymorphisms has emerged as a powerful approach in understanding the host genetic disease association, which underscores the contribution of specific gene variants to the susceptibility to various cancers, including OC. The most prevailing type of this cancer is OSCC has a remarkably oppressive prognosis, revealing the significance of factors that modify the host immune response. Several studies shows patients with bacterial/viral infections are at high risk for OC. Various viruses such as HPV, EBV, cytomegalovirus and herpes simplex virus type 1 are known to reside in the oral cavity [24,25]. However, recent studies have revealed that HPV & EBV is much more commonly associated with cancers of the pharynx, larynx, esophageal and peritonitis [10,26–28]. The present study also showed the significance of HPV, EBV and their co-infection in oral pre-cancerous and cancerous cases. Patients infected with one virus, secondary co-infection with another virus may serve as an important co-factor that may cause initiation and/or progression of tumors. In present study, 24% prevalence of HPV DNA was found in pre-cancer and 22% in OSCC cases. HPV was detected more frequently in pre-cancerous lesions such as leukoplakia and OSMF than in controls. In previous studies one of the authors showed the prevalence of HPV positive cases was 91% in OSMF and 24% in OSCC. Various studies showed this type of similar figures and our results consistent with these findings [8,27,29–31]. Apart from HPV, the prevalence of EBV DNA was found 42% in cases of our studied individuals. Jalouli et al 2010 detected the presence of EBV in 25% OSMF and 29% in OSCC cases. Number of authors also emphasizes the prevalence of EBV DNA and their role in the development of OSCC [9,27,32]. The prevalence of EBV in OSCC reported from different parts of the world range from 15 to 82% which is in good agreement with our study. Several studies have been also point to co-infection with HPV & EBV in oral carcinoma [10,33,34]. Co-infections occur much more frequently in the areas of high prevalence of infectious agents, especially in developing countries [35]. The presence of HPV/EBV, co-infection in the present study was found 43% in pre-cancer and 25% in cancer patients. Bartłomiej et al showed the HPV/EBV, co-infection in oral cavity cancer and also through the light how these infection play the important role in initiating neoplastic transformation of human oral epithelial cells [26,33]. Jalouli et al proposed the frequency of HPV/EBV single and co-infection in OSCC in eight different countries including India and study enlightened Sudan has highest frequency of HPV /EBV, co-infection [9]. Infections by pathogens are very common cause of inflammation of tissues/organs which is one of the major factors for carcinogenesis. The possible explanations has been given by Makielski et al 2016 indicate that first infection of HPV in oral cavity may increase the capacity of epithelial cells to support the EBV life cycle, which could in turn increase EBV-mediated pathogenesis in the oral cavity [11]. There is sufficient evidence to suggest that human nasopharyngeal, oropharyngeal and oral cavity cancer have been infected by HPV and EBV together [9,26].

Ample of growing evidence that single nucleotide polymorphism (SNPs), due to the formation of specific gene alleles, makes an important contribution to the phenotypic differences among humans, including the individual features of the protective reactions development, as well as the susceptibility to a number of diseases. Regarding TLRs-polymorphism, it has been found that it can lead to a disruption of the infectious agent's recognition, an imbalance in the functioning of the innate immunity system, increased sensitivity to infections and the development of chronic inflammatory processes. Various studies have been focused on the TLRs polymorphism and their correlation with infection increased with risk of malignancy in different type of cancers including cervical, gastric, prostate and periodontal diseases [12]. During infection, epithelial lining of cells serve as the first line of defense, when the pathogens disrupt the physical barrier formed by the epithelial lining, they are recognized by toll-like receptors (TLRs) [36–38].The present study also found that the TLR 9(−1486T/C) polymorphism is associated with an increased risk of oral pre-cancerous and cancerous cases when compared with controls. In TLR 9(−1486T/C), the frequency of carrier genotype CC was higher in all genetic models and showed ˜5-fold higher risk in pre-cancerous cases and ˜3 fold increased risk of OC. Several studies showed homo and heterozygote variant was associated with an insignificant increased risk of Gastric, Prostate & cervical cancer and TC/CC genotypes contributed to the risk of in the dominant genetic models [12,39–42]. TLR 9(−1486T/C) is a potentially functional variant located in the promoter region, which is close to the region that interacts with HPV16 E6 and E7 oncoproteins. Th erefore, this SNP may regulate not only TLR9 basal transcript level but also transcription during HPV infection [39]. Several studies have investigated the effect TLR 9(−1486 T/C) on human diseases however this study also emphasizes the joint effects and significance of TLRs genotype with HPV, EBV single and co-infection in oral pre-cancerous & cancerous cases. It was observed that the single infection of HPV or EBV showed the significant association with TLR9 (−1486T/C) homo and heterozygous genotype (CT + CC) in pre-cancer & cancer cases ($p = 0.0001$). In his finding also observed that the TLR 9(−1486T/C) carrier genotype frequency (CT + CC) was higher in those patients who's infected with co-infection (HPV + EBV) and showed approximately twenty two fold increased risk in pre-cancerous patients (p = 0.0364 OR = 22.500) and approximately thirty fold higher risk ($p = 0.0141$ OR = 30.00) in cancer cases when compared with controls.

The TLR 4(+896A/G) polymorphism has been studied in different cancers and in different ethnic population, but many discrepancies noted in different studies due to environmental factors and ethnic backgrounds. In genetic model studies it showed significant association between cases and controls. The carrier genotype (AG + GG) showed the approximately eight fold higher risk of developing oral pre-cancerous lesions and 3 fold increased risk of cancerous cases. However, various studies indicated that the all genetic models of TLR 4(+896A/G), the variant genotype GG were strongly associated with moderately increased risk of many cancers [13,19,43–45]. Number of studies showed no polymorphism observed in dissimilar populations and reason may have different geographic regions & ethnicities [46–49]. TLR 4 is expressed on the cell surface and thus it is capable of recognizing viral proteins on the virion. In the studied population we observed the higher frequency of TLR 4(+896A/G) GG genotypes in HPV infected cancer patients and showed ˜10 fold increased risk of OC development. ($p = 0.0163$, OR = 10.667). This explains the possible role of 'G' minor allele in oral carcinogenesis in the studied population. One of the previous study found that TLR4 trended upward with increased disease severity after HPV infection, and the difference was statistically significant [17,50]. In present study variant allele 'G' of TLR 4 gene in EBV infected patients showed the higher frequency in cancer cases as compared to controls. Many previous studies showed, EBV is involved in many malignancies & infection including Burkett's lymphoma, Hodgkin lymphoma, and nasopharyngeal carcinoma, and human herpes virus 8, is a causal factor in Kaposi sarcoma [15,36,51]. Our finding also consistent with the previous reported studies in case of EBV infection.

Apart from infection, this study also enlightens the role of TLRs SNPs in pre-cancerous lesions and clinicopathological grades of OC. We found significant association of TLR 9(−1486 T/C) genotype with OSMF ($p = 0.0027$), leukoplakia ($p = 0.0083$) & WDSCC ($p = 0.0001$). Patients carrying the TLR 9(−1486T/C) variant genotype GG in OPMDs are more prone to developing the precancerous conditions. Moreover in TLR 4(+896A/G) the variant allele G showed the higher significance in leukoplakia and ˜14 fold higher risk for the development of disease ($p = 0.0001$ OR 14.00). These findings conclude that TLR 9(−1486T/C) and TLR 4(+896A/G) polymorphisms may serve as potential biomarker of OC progression. Omrane et al has been reported the significant association with clinicopathological parameters and TLR 4(+896A/G) polymorphism [52]. The present study has also through

the light on genetic and life style factors. We observed the significant association of TLRs genotype with well known risk factors associated with oral carcinogenesis i.e. tobacco chewing, cigarette smoking and alcohol consumption [53–55]. Fabiana Vargas –Ferreira et al reported the association of viral infection and environmental factor has been referred to the development of OSCC [56]. Number of studies TLRs polymorphism depends upon ethnicity and it is 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 smoking, alcohol drinking and tobacco chewing that increases the risk of OSCC. In this study, it was interesting to note that the impact of above mentioned habits together with risk genotypes of TLRs showed an increased risk of oral cancer. On analyzing TLR 9(−1486 T/C), we found that the homozygous variant C allele frequency was higher in those who smoked and drank alcohol who developed the pre-cancerous conditions whereas tobacco chewers were also associated increased risk of OC ($p < 0.0024$, OR ˜8). The TLRs genotype association with life style habits adapted from western culture (smoking, alcohol drinking & tobacco chewing) increased the risk of disease in habitual persons and these findings are consistent with previous studies [41,57,58]. This study shows the genetic architecture of TLRs gene with the combination of well known risk factors which are strongly associated with initiation and progression of OC.

In conclusion, our study indicates that the gene polymorphism of TLR 9(−1486T/C) and TLR 4(+896A/G) may play the significant role in developing premalignant/malignant lesions in the oral cavity. This study also provides the evidence, HPV/EBV; co-infection may influence the TLRs polymorphism and this affects the TLRs -mediated signaling pathway which enhances the pathogenesis of the OC. Our study revealed that the genotypic association of TLR 9(−1486T/C) with HPV/EBV, co-infection may play a significant role in the progression of Oral carcinoma together with tobacco chewing. In this study our observation showed, the tobacco users carrying the homozygous variant allele CC of TLR 9(−1486T/C) and smokers carrying the allele GG in TLR 4(+896A/G) play the significant role in developing oral pre-cancerous lesions. Our study also reports how the lifestyle habits and clinical parameters help to increase the risk of oral cancer in the Indian population. To the best our knowledge, this is the first study in Indian population with the combination of TLRs polymorphism and HPV/EBV, co-infection in oral pre-cancerous & cancerous cases that may suggest the presence of the variation at least one allele may lead to the development of oral cancer. The major limitation of this study is its smaller sample size. Future studies with a larger sample size to validate these findings of SNPs and evaluate their functional effects in oral carcinogenesis are warranted.

Authorship contribution statement

Ms Upma Sharma (First Author):Performed Experiments, Drafting of the manuscript, Data Analysis and interpretation.

Dr. Pallavi Singhal (Co-Author): Data Analysis

Dr. Kapil Bandil (Co-Author): Data Analysis

Mr. Rajeshwar Patle (Co-Author): Help in sample collection & experiments.

Dr. Anoop kumar (Co-Author): Data Analysis.

Dr. Kausar Neyaz (Co-Author): Clinical samples

Dr. Surojit Bose (Co-Author): Clinical samples

Dr. Ajay Kumar Dewan (Co-Author): Clinical samples

Dr. Ravi Mehrotra (Co-Author): Study Design

Dr. Veena Sharma (Co-Author) : Study Design

Dr. Mausumi Bharadwaj (Corresponding author): Study Design, article corrections or revising it critically for important intellectual content, approval of the final version.

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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.

Competing interests

The authors declare that they have no conflicts of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.canep.2019.05.003.

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