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Prevalence of Herpes Simplex Virus (HSV) and Cytomegalovirus (CMV) in Oral Squamous Cell Carcinoma patients with a history of Nicotine and Alcohol abuse.

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Abstract

Several risk factors cause Oral Squamous cell carcinoma such as alcohol and smoking. In considerable studies, patients without exposure to these habits develop OSCC which emphasizes the role of other factors like oncogenic viruses and genetic susceptibility. Several viruses are frequently associated with OSCC along with co-factors on which the viruses are dependent for their carcinogenic abilities. The accurate role of viruses in cancer development is yet to be studied to improve treatment and prognosis of OSCC. In this study, the prevalence of HSV-1, HSV-2, and CMV in the patients diagnosed with OSCC are reported with help of molecular and quantification techniques such as PCR and ELISA which recognize the DNA of the virus in the host and IgG, IgM antibodies of HSV-1, HSV-2 and CMV in the blood serum. The results are statistically analyzed, determining the significance of the results obtained. OSCC occurrence because of HSV infection with risk factors like smoking, alcohol consumption, tobacco chewing was less as compared to the cancer caused by infection of CMV with combination of risk factors. The genetic material of both the studied virus HSV (1 and 2) and CMV were observed to be in majority biopsy samples of the patients.

Keywords: Oral Squamous Cell Carcinoma, HSV, CMV, antibodies, quantification, EBV, Vaccines, oncogenic proteins, OSCC, HPV, oncogenic viruses, head and neck cancers, PCR, tobacco chewing, smoking, oral cancer.

Introduction

Oral cavity cancer is the sixth most recurrent cancer reported worldwide, of which almost 5% has been observed in developed countries. In the Indian subcontinent, due to its large population, it was always regarded as an epicentre for oral carcinomas and a major public health challenge by the WHO1. There is a vast variation in the global burden of this disease, which has vital incidents in India, Southeast Asia, and South Asia being the highest in the world. An increase in the incidences was observed in Latin America, Europe, and other Pacific parts. An enormous effect on the biological system by environment and lifestyle factors carried out a direct or indirect role in cancer development2. OSCC is primarily originating from mucosal epithelium that is non-keratinized and stratified. Some similarities to Squamous cell carcinomas occur on other body parts morphologically like the bronchi, anus, and cervix. It was typically assumed that alcohol, tobacco, and betel quid consumption caused the development of OSCC majorly3, 4.

Since the 20th century, studies on the impact of viruses on OSCC were reported, where the infectious agent was identified and isolated, and which was later observed to be able to induce a tumor in chicken5-8. OSCC etiology depends upon several factors which include environment, lifestyle, genetic alterations, and infectious agents. The chief role in etiology is played by viruses. The most typical viruses which cause OSCC are HPV9, Herpes group10, CMV11, adenovirus12 and HPV13. HSV includes eight viruses which are HSV-1, HSV-2, EBV, CMV, and VZV14. In the past few years, HSV has been investigated in association with human cancers. HSV-1 antibodies in patients with oral cancers were observed to be high15, 16. Mainly, IgA and IgM classes were observed in the patients, while anti-HSV IgM retained a transient life in the host. The more considerable risk was observed when risky habits like alcohol consumption, smoking, chewing tobacco along with infection with HSV and CMV caused high-risk cancer. Reports were observed where the genetic material of the viruses was found in the oral cancer patients17-19. It was deemed that the association of HSV and CMV with oral cancers is difficult to study as the transformed cells do not express the antigens or specific genes to viruses.

Recent years have seen little progress in the study of the malignant potential of HSV. The polymerase chain reaction (PCR) assay is a technique that has several advantages over other methods4, 20. It can detect viral presence in initial infections and requires only a limited quantity of biological material. PCR detection of HPV, Epstein–Barr virus (EBV), and HSV are exceptionally sensitive and specific and can supplement the clinical detection of virus-associated oral lesions18, 21. Because the herpes virus subtypes can vary in distinct parts of the world, there have been few studies in Iran about the prevalence of HSV in OSCC3, 11, 17. This study was conducted to determine the prevalence of HSV and CMV in OSCC (in isolation or in combination with tobacco and alcohol) by PCR technique. The genetic material of both HSV and CMV in the biopsy samples taken from various sources of the oral cavity of patients and healthy controls and the prevalence of IgG and IgM antibodies in the blood serum that were collected from patients as well as control subjects.

Methods

All the OSCC cases were diagnosed during the study which was carried out by following all the ethical committee standards. All procedures were followed in accordance with the standards of the liable hospital or institution committee. All personnel were interviewed with a planned questionnaire to collect information regarding their lifestyle and habits.

Criteria of selection

All patients have been included in the exclusion criteria as well as exclusion criteria with the help of their clinical data acquired. The required information like age, gender, alcohol intake, smoking, and chewing tobacco products was noted. The exclusion criteria included the patients who denied consent and patients who have to undergo radio or chemotherapy or have any pre-existing conditions like heart diseases. The inclusion criteria included the patients who were confirmed with oral carcinoma and who were AIDS and Hepatitis B negative.

Sample collection

Tumour tissue and blood were collected from OSCC patients and healthy patients at the time of the biopsy. The tissue samples were stored in saline and formalin solution till further use. Blood samples were collected in the EDTA coated tubes to prevent coagulation, and serum was separated which is used as a control in several experiments.

Molecular analysis

DNA was extracted from the biopsy samples of both healthy and OSCC patients. Both forward and reverse primers were designed for HSV :(5'-AGCCTGTACCCCAGCATCAT-3' for-

ward and reverse primer 5'-TGGGCCTTCAC-GAAGAACA-3' for HSV-1 DNA and 1 µl each of the forward primer 5'-AGGCCTACCAACAG-GGCG-3' and the reverse primer 5'-CTGGATC-GACGGGATGTGC-3' for HSV-2) and CMV (forward primer 5'-TTT GGA GAA AAC GCC GAC -3' and the reverse primer 5'-CGC GCG GCA ATC GGT TTG TTG TA -3').

The target DNA fragment was amplified using PCR using Bio-Rad kit maintaining appropriate temperature and time cycles for 40 repeats after which the PCR products were analyzed on 3% agarose gel stained with ethidium bromide and visualized under UV transilluminator.

Quantitative determination of Herpes simplex 1,2 and CMV Ig G and Ig M in the blood serum

This assay was performed with DIESSE Diagnostic Senese kit, Italy. This kit is based upon the ELISA technique where the antigen containing the partially purified or inactive virus is bound to the solid phase. Specific immunoglobulins to the selected viruses were bound to the antigen by incubating them together in human serum. Subsequent washes were performed to eliminate any loose proteins. The complex of the antigen and the specific immunoglobulin was incubated along with a conjugate made up of human IgG monoclonal antibodies in a complex with horseradish peroxidase. Peroxidase substrate is added after washing to remove any unconjugated complexes. Development of color was observed which is proportional to the antibody concentration of the serum sample. The reaction is interrupted by adding a stop solution which develops a yellowish color in the samples which can be read using a microplate reader.

Results and Discussion

Demographic characteristics of the population

The current study has employed 100 patient samples of Oral Squamous Cell Carcinoma (OSCC) as well as 100 healthy controls. The demographic characters and the involved risk factors were already described [Table 1]. The demographic profiles of all the subjects including age, sex, habits that can cause OSCC were noted. 45% of the 100 OSCC patients were male, while the rest 55% were females. In a similar fashion, in the control group, males were 55% while females were 45%. The age groups for patients range from 9-87 while for the control population, age recorded was 21-76. The average age of the patient samples, as well as healthy controls during diagnosis, were recorded as 50.53 and 55.27 respectively. The subjects with carcinomas were divided into four sub-groups which are < 25, 26-45, 46-65, and > 66 age groups. Habits such as tobacco chewing, alcohol, and tobacco chewing, alcohol and smoking, and alcohol, smoking, tobacco chewing in OSCC as well as controls were observed to be 32%, 30% and 17%, 13% and 12%, 6%, and 10%, 6% respectively. The biopsy for the cancer patients was categorized as tongue, buccal mucosa, mandible, oral cavity, retromolar trigone, the floor of the mouth, lip, tongue base, maxilla, and palate which showed incidence as 23%, 35%, 12%, 10%, 8%, 4%, 3%, 2%, 2% and 1% respectively. The tumor was categorized into four stages like stage 1 of 6%, stage 2 of 21%, stage 3 of 39% and stage 4 of 34% [Table 1].

Table 1: Demographic table of patient and healthy population:

Clinical Characteristics	n = 100 (Cases)	n = 100 (Controls)
Gender		
Males	45(45%)	55(55%)
Females	55 (55%)	45 (55%)
Mean age & Range Males	50.53/9-87	
Mean age & Range Females	55.27/30-75	
Age Distribution		
26-45	28 (28%)	22(22%)

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46-65	60 (60%)	70(70%)
66 and above	12 (12%)	8 (8%)
Habitual Risk		
Alcoholics	_	3 (3%)
Smokers	6(6%)	13 (13%)
Tobacco chewing	31(31%)	30 (30%)
Alcohol + Smoking	14 (14%)	6 (6%)
Alcohol + Tobacco chewing	26 (26%)	13(13%)
Smoking + Tobacco chewing	2 (2%)	4 (4%)
Alcohol + Smoking + Tobacco chewing	10(10%)	6 (6%)
No Habits	11%(11%)	25(25%)
Site of Diagnosis		
Tongue	23(23%)	
Buccal mucosa (BM)	35 (35%)	
Mandible	12 (12%)	
Oral Cavity	10 (10%)	
Retromolartrigone	8(8%)	
Floor of mouth	4(4%)	
Lip	3(3%)	
Base of tongue	2 (2%)	
Maxilla	2 (2%)	
Palate	1 (1%)	
Staging		
Stage 1	6 (6%)	
Stage 2	21 (21%)	
Stage 3	39 (39%)	
Stage 4	34(34%)	

HSV-1 and HSV-2 genotyping

Among the 100 selected patients, the DNA of Herpes Simplex Virus was found to represent 7% in OSCC and 2% in control samples when the molecular analysis was performed on the samples. 4% incidence of HSV-1 was observed while HSV-2 represented 1% while there was no incidence (0%) in control samples when HSV-2 was present. Both HSV-1 and the two were observed in 2% of the combined patient population and 0% in the control population. The results were statistically significant with the odd ratio of 2.04, CI of 0.36 - 11.4, and p-value of 0.005 when the patients and controls were compared. Differences between the cases and controls were observed to be not significant when the risk was for HSV-2, where the OR = 2.04; CI=0.00-1.04 P=0.4 and for the presence of HSV-1 and 2 were reported as OR=0.02; 95%CI=0.00-1.55; P=0.08. [Table 2, Figure 1]

Table 2 HSV genotyping in OSCC and control group

HSV typing	Cases N (%)	Controls N (%)	Odds Ratio	95% CI	P-Value
HSV-1					
Positive	4	2	2.04	0.36-11.4	0.05
Negative	96	98			
HSV-2					
Positive	1	0	0.01	0.00-1.04	0.4
Negative	99	0			
HSV-1&2					
Positive	2	0	0.02	0.00-1.55	0.08
Negative	98	0			

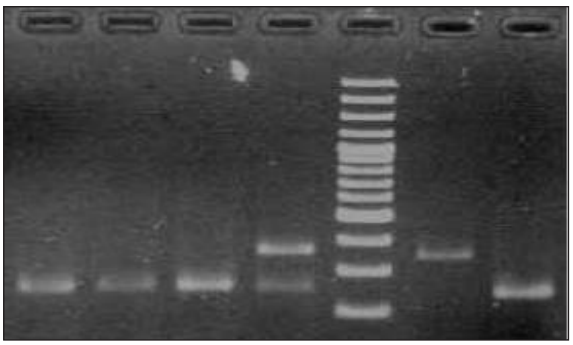

Figure 1: HSV infected OSCC Samples

1 – HSV negative
2 – HSV negative
3 – HSV negative
4 – HSV-1 positive
5 – Ladder
6 – HSV-2 positive
7- HSV negative

Genotyping of HSV in OSCC and control groups according to demographics

The presence of viral DNA in the patient and control population was categorized according to the demographics. Under the gender category, three males and 1 female patient have shown HSV-1, 1 male has shown HSV-2, and two females were observed to have both HSV-1 and 2 DNA. The results were not significant as the p-value = 0.232. Underage distribution, 2 falls under 26- 45, 2 under 46-65 for HSV-1. Only one patient was observed to have HSV-2 who falls under 46-65 group. Two patients under the group 46-65 were observed to have both HSV-1 and 2; the results were not significant as the p-value is 0.733. Under the category site of diagnosis, one patient each for the palate, RMT, the oral cavity, and BM samples revealed the presence of HSV-1 DNA. HSV-2 was observed in only one patient whose sample was mandible. Two patients whose tongue sample was taken have shown the presence of both HSV-1 and two. The results obtained were not significant as the p-value was reported as 0.175. HSV-1 was observed in three patients whose stage is 2 and one patient whose tumour is at stage 4. HSV-2 was observed in only one patient with stage three tumours. Both HSV-1,2 were observed in two patients whose tumour was in stages 3 and 4 respectively. The results when analyzed were not significant as their p-value is 0.595. Under the category of habits, one patient each has alcohol + smoking, alcohol+smoking+chewing, and patients with no habits contain HSV-1 DNA. HSV-2 was observed in the one patient each maintaining habits like alcohol consumption, smoking, and alcohol +smoking. Both HSV-1 and two were observed in one patient each retaining habits like alcohol consumption, smoking, and alcohol+chewing. The results were not statistically significant as their p-value is > 0.05. [Table 3]

Table 3 Correlation of HSV-1 and 2 genotypes with demographical factors of OSCC patients

Demographical Characteristics	HSV-1	HSV-2	HSV-1&2	F-value	P-value
Gender					
Males (n=45)	3	1	0	1.453	0.232
Females (n=55)	1	0	2		
Age Distribution					
<25	0	0	0	0.428	0.733
26-45	2	0	0		
46-65	2	1	2		
66 and above	0	0	0		
Site of Diagnosis					
BM	1	0	0		
Tongue	0	0	2		
BOT	0	0	0		
FOM	0	0	0		
LIP	0	0	0	1.689	0.175
Mandible	0	1	0		
Maxilla	0	0	0		
Palate	1	0	0		
RMT	1	0	0		
Oral Cavity	1	0	0		
Staging					

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Stage 1	0	0	0	0.633	0.595
Stage 2	3	0	0		
Stage 3	0	1	1		
Stage 4	1	0	1		
Habitual Risk					
Alcoholics	0	0	1	0.103	0.958
Smokers	0	0	1	0.753	0.523
Chewers	0	0	0		
Combination Risk Factors					
Alcohol+Smoking	1	1	0	0.673	0.571
Alcohol+Chewing	0	0	1		
Smoking+Chewing	0	0	0		
Alcohol+Smoking +Chewing	1	0	0		
No Habits	1	0	0		

Quantification of HSV-1, 2 IgG and IgM in serum

46% of the total OSCC patients and 5% of the control subjects were observed to have HSV-1 IgG antibodies in their serum which was greater than equal to 1.2 Mmol/L while the HSV-1 IgM antibodies were observed in 11% of OSCC patients and 4% of the control population. HSV-2 IgG antibodies were observed in 8% and 3% of patients and control populations respectively whereas 3% of patients and 2% of the control population were positive for HSV-2 IgM. Univariate analysis was performed which revealed that the results of HSV-1 and HSV-2 IgG, as well as IgM levels, were not significant when the patient samples were compared to controls. The levels were elevated which were significant in the case of HSV-1 IgG (o.001) in OSCC cases as compared to the control groups while there was no association between HSV-1 IgM, HSV-2 IgG, and HSV IgM. [Table 4]

Table 4: Levels of HSV IgG and Ig M 1 &2 in the serum of OSCC and controls

Serum HSV-1	Cases n(%)	Mean±SD	Controls n(%)	Mean±SD	F-value	P-Value
IgG levels (Mmol/L)						
< 1.2	54	0.58±0.31	95	0.50±0.22	111.2	0.001
≥1.2	46	3.35±1.85	5	3.18±2.32		
IgM levels (Mmol/L)						
< 1.2	89	0.59±0.3	96	0.43±0.19	5.12	0.005
≥1.2	11	3.13±3.3	4	1.86±0.43		
Serum HSV-2 IgG levels (Mmol/L)						
< 1.2	92	0.64±0.24	97	0.53±0.29	47.5	0.001
≥1.2	8	3.72±1.73	3	3.29±2.0		

Serum HSV-2 IgM levels (Mmol/L)						
< 1.2	97	0.45±0.21	98	0.47±0.23	2.69	0.91
≥1.2	3	1.29±0.00	2	1.49±0.14		

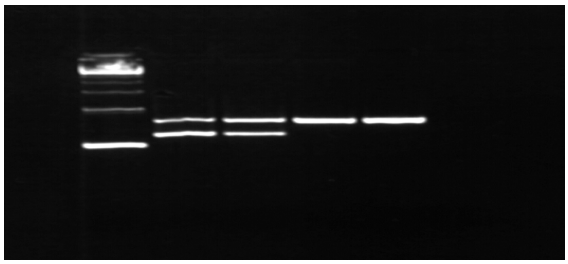
CMV genotyping

In the population of both OSSC patients and control, DNA of CMV was found in 21% of patients and 2% of control when the samples were analysed molecularly. The results obtained were significant statistically with an odd ratio of 13.02, 95%CI range of 2.96-57.2, and the P-value of 0.007. [Table 5, Figure 2]

Table 5 CMV genotyping in OSCC and control group

CMV Typing	Cases N (%)	Controls N (%)	Odds Ratio	95% CI	P-Value
Positive	21	2	13.02	2 . 9 6 - 57.2	0.0007
Negative	7	98			

Figure 2: CMV infected OSCC Samples



L - 123bp ladder 2 - CMV positive patient 2
1 - CMV positive patient 1 3 & 4 - CMV negative patient

CMV genotype in association with demographic features

CMV DNA presence in OSCC patients was categorized demographically. Samples of 13 males and 8 females were observed to have the viral DNA; the results obtained are not significant as the p-value obtained is 0.08. Underage groups, one patient below 25 years, eight patients in the range 26-45, 11 patients in the range 46-65, and one patient in the range 66 and above were reported to have CMV DNA in their samples; these results were not significant as p-value is 0.09. Under the diagnosis site category, five patients of BM, eight patients of the tongue, one patient of FOM, two patients of the mandible, one patient of the palate, one patient of RMT, and three patients of the oral cavity were reported to have the viral DNA. The results obtained were not significant as the p-value was calculated as 0.6. CMV DNA was observed in all 4 stages of tumor groups 3 in stage 1, eight in stage 2, nine in stage 3, and one in stage 4; the p-value was calculated as 0.003 which means the results were significant. Under the category of habits, one patient with a habit of smoking, 10 patients who chew tobacco, four patients who consume alcohol and smoke, two patients who consume alcohol and chew tobacco, one patient who smoke and chew tobacco, one patient who consumes alcohol, chew and smoke tobacco and two patients with no habits were reported to have CMV DNA in their biosamples. The p-value was 0.05 which shows that the results were significant. [Table 6]

Table 6 : Correlation of CMV genotypes with demographical factors of OSCC patients

Demographical Characteristics	CMV N=21 (%)	P-value
Gender		
Males (n=45)	13 (61.90)	0.08
Females (n=55)	8 (38.10)	
Age Distribution		
<25	1 (4.76)	0.09
26-45	8 (38.10)	
46-65	11 (52.38)	
66 and above	1 (4.76)	

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Site of Diagnosis		
BM	5 (23.81)	
Tongue	8 (38.10)	
BOT	0 (0.00)	
FOM	1 (4.76)	
LIP	0 (0.00)	
Mandible	2 (9.52)	
Maxilla	0 (0.00)	
Palate	1 (4.76)	
RMT	1 (4.76)	
Oral Cavity	3 (14.29)	0.6
Staging		
Stage 1	3 (14.29)	0.003
Stage 2	8 (38.10)	
Stage 3	9 (42.86)	
Stage 4	1 (4.76)	
Habitual Risk		
Alcoholics	0 (0)	
Smokers	1 (4.76)	
Chewers	10 (47.6)	
Combination Risk Factors		
Alcohol+Smoking	4 (19.05)	
Alcohol+Chewing	2 (9.52)	
Smoking+Chewing	1 (4.76)	
Alcohol+Smoking +Chewing	1 (4.76)	
No Habits	2 (9.52)	0.05

Quantification of CMV IgG and IgM antibodies in serum

28% of the total OSCC patients and 4% control serum samples were observed to be positive for CMV IgG at a concentration less than 1.2 Mmol/l while CMV IgM antibodies were observed in 9% of OSCC patients and 1% control samples. Univariate analysis was performed to the results obtained for IgM of CVM gave a p-value of 0.02 which is significant while for IgG of CVM gave a p-value of 0.45 which is not significant. [Table 7]

Squamous Cell Carcinoma malignancy is primarily associated with gender, geographical region, and location22, 23. Southern Asia was reported to be more prevalent amongst other countries. SCC is observed more in the male gender as compared to females. In India, SCC is more prevalent in the form of Oral and tongue cancers24 mandating focus on associated key genetic players that may contribute to OSCC pathogenesis, and the curative potential25. IgM antibody is important to act against HSV in the pretext of oral cancers26 and HSV enacts a crucial role in cancer incidence27. An association between mandibular OSCC and HSV infection where in the prevalence of HSV-1 was 18% and HSV-2 represented 6% was reported in a study28. HSV prevalence in worldwide patients suffering from OSCC was almost 15% while the highest was observed in the United Kingdom. This clarifies that geographical differences affect HSV prevalence29. 5% of the patients infected with HSV-1 are with OSCC30 and HSV-2 has a

Table 7: Levels of CMV IgG and IgM in the serum of OSCC and controls

Serum CMV	Cases n(%)	Mean±SD	Controls n(%)	Mean±SD	F- value	P-Value
IgG levels (Mmol/L)						
< 1.2	72	0.38±.0.22	96	0.40±0.25	1.32	0.25
≥1.2	28	1.37±0.18	4	1.37±0.11	2.67	0.45
IgM levels (Mmol/L)						
< 1.2	91	0.46±0.24	99	0.44±0.2	1.44	0.02
≥1.2	9	1.24±0.02	1	0	--	--

high prevalence in OSCC in the samples which are differentiated tissue with HSV-1 (7.5%) and HSV-2 (15%), while 5% were coinfected with both HSV-1 and HSV-2 31. Several studies have reported that HSV-1 and HSV-2 in combination with tobacco usage by chewing or smoking and concurrent alcohol consumption or any other consumption of chemical carcinogens can promote development of OSCC11, 27. CMV was observed to have a high prevalence in OSCC than in other cancers32. Several articles have discussed the presence of CMV in tumors and its involvement in the development of tumors33, 34. Studies have reported that age, gender, location, histological stage, grade, size and distribution of the lesions in patients are associated with CMV prevalence in OSCC patients. It was reported that the general prevalence of CMV in oral cancer patients was different in several areas ranging from 0 to 91.5% 35-38. In a study, CMV percentage is high in OSCC (42.5%) than in benign (25%) and control samples (7.5). Similarly, CMV DNA was found in OSCC which was nearly 28% 32. Many clinical and experimental results have recommended the partial contribution of CMV to malignancy and chemoresistance in tumor cells39, and may play a significant role in modulating the tumor microenvironment40. CMV lays dormant in the body after infection, due to the exposure of the squamous cells to tobacco and alcohol consumption. Cell damage and subsequent infection of the damaged cells by CMV was observed in several in vitro studies. These infected cells then transform to cancerous cells leading to OSSC in several individuals.

The current study predominantly focuses on the molecular and serological epidemiology of HSV-1, HSV-2, and CMV in patients with Oral Squamous Cell Carcinoma. The risk factors like alcohol consumption, smoking, chewing tobacco were considered individually or in combinations showed that in the combined patient population, HSV-1 (3%), HSV-2 (1%), HSV-1,2 (1%), and CMV (21%) prevalence was observed. Tumors in several stages were tested for the prevalence where HSV-1 (4%), HSV-2 (1%), HSV-1, 2 (2%), and CMV (21%) of the patient population were observed having viral prevalence. Although there was a reduced rate of HSV infection it could be due to the limited population, size of the sample amongst others. Our findings were in contrast to several studies which reported a majority of OSCC in the male gender. According to the results, patients infected with the HSV genome were less as compared to the patients infected with CMV which means HSV has a low infection rate. Our results are similar to studies 30 which confirmed that HSV has a low prevalence rate in patients with OSCC. Results obtained in this study are similar to several studies which have reported that the prevalence of CMV in the OSCC tissue samples was more than other viruses as they can increase the oncogenesis and infect the cancerous tissues opportunistically as monocytes and lymphocytes are the key focus sites of CMV.

Conclusion

Oral cancer is one of the significant reasons for mortality and morbidity mainly in developing countries which can be foreseen as a rise in the near future. Viruses associated with head and neck cancers were studied for several critical insights into the mechanism of oral cancer. Viral infection, as well as co-infections, could provide targets for diagnostic tests and therapy along with understanding the tumor development mechanisms. Diagnosis of Oral Squamous Cell Carcinoma is typically made by oral examination which is then followed by tissue biopsy. Due to this approach, most of the cases go unaffected and are diagnosed at more recent stages, which cause OSCC to metastasize leading to less survival rate in 50% of the diagnosed population. Even though new diagnostic techniques in the detection of oral cancer exist they are incapable of surpassing histopathological, molecular, and biopsy is considered gold in standards. In this study, the prevalence of HSV-1 and HSV-2 along with CMV viruses was studied among the selected patient and control population further associated with several risk

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factors observing that the genetic material of the virus is integrated into the host DNA. The quantification of IgG and IgM of both the selected viruses has shown that after the viral infection, the antibodies are present in the blood serum of the patients as well as control subjects.

Conflict of Interest

The authors declare no conflict of interest.

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