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Epstein- Barr viral load in exfoliated cells of oral squamous cell carcinoma and oral potentially malignant disorders - A cross-sectional study

Kailash Kumar Gopalakrishnan Mahalingam[a], Leena Sankari Sankar[a], K.M.K. Masthan[a], Krishnan Mahalakshmi[b,*], Venkatesan Naveen Kumar[b,c]

[a] Department of Oral Pathology and Microbiology, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research, Chennai- 600100, Tamil Nadu, India

[b] Department of Microbiology/Research lab for Oral and Systemic health, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research, Chennai- 600100 Tamil Nadu, India

[c] ImmuGenix Biosciences Pvt Ltd, Perambur, Chennai-600011, Tamil Nadu, India

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ABSTRACT

Oral Squamous Cell Carcinoma (OSCC) has varied etiology. Among the etiological factors, genetic predisposition or the presence of oncogenic viruses can cause impairment in the physiological mechanisms of cellular proliferation control. The present study was chosen to determine the association of Epstein- Barr virus (EBV) with oral squamous cell carcinoma and oral potentially malignant disorders (OPMD). Oral exfoliated cells were collected from the subjects diagnosed with OSCC (n = 19), OPMD (n = 23) and healthy subjects without any deleterious condition (n = 39). The DNA extraction was performed with DNeasy Blood & Tissue Kits (Qiagen, Germany). Quantitative Real-Time PCR was then carried out with QuantiNova® SYBR® Green PCR Kit (Qiagen, Germany). Statistical analysis was performed using Chi-square test. EBV DNA was detected in all samples of all the three groups. Compared to healthy subjects, very high EBV load was observed in the oral exfoliated cells of OSCC and OPMD patients. The presence of EBV DNA in all the subjects of all the three groups reveals that EBV is an oral resident. Besides its presence, the abundance of EBV DNA among OSCC and OPMD suggests it's association to these pathologies. The high odds and risk ratio obtained for EBV copy number further supports a strong association of this virus to OSCC and OPMD. This may be the first study to quantify EBV DNA in oral exfoliated epithelial cells of OSCC and OPMD.

Background

Oral squamous cell carcinoma (OSCC) is on the rise in India and Indian subcontinent countries. The etiology of OSCC is varied and comprises many factors. Tobacco habit is one of the major risk factors for OSCC [1]. However, etiological factors such as familial inheritance or oncogenic viruses can damage the physiological mechanisms involved in the regulation of cellular proliferation [2]. The human viruses *viz.*, Human Papilloma Virus (HPV), Hepatitis C virus, Human T-cell lymphotropic virus, Epstein–Barr virus (EBV), Kaposi's sarcoma virus, Hepatitis B virus have gained importance in contributing to 10–15% of cancer burden worldwide. However the link of EBV to OSCC is still debatable [3,4].

EBV was the first human virus reported to be associated with the pathogenesis of many types of tumor [5]. Globally, EBV infection is reported in more than 90% population [6]. The EBV infection is initiated in the oropharyngeal epithelial cells with gradual migration to sub epithelial B-cells. Acquired immunity does not lead to elimination of EBV infection. Like all herpesviruses, the infection remains latent for the life of the person. The latency-associated viral proteins in EBV comprises three membrane proteins (Latent membrane protein (LMP) - 1, 2A and 2B) and six nuclear antigens (Epstein– Barr nuclear antigen [EBNA] - 1, 2, 3A, 3B, 3C, leader protein (LP). The EBV nuclear antigens (EBNA1, EBNA 3C and LMP 1) promote genomic instability, breaking of DNA and phosphorylation of histone H2AX [7]. It has been hypothesized that EBV interferes with diverse cellular activities leading to genomic instability, which is crucial in the events of viral oncogenesis [8]. Chronic usage of tobacco products can lead to recurrent EBV infection and deficiency in local immunity [2,9–11]. In conformity, this might result in the shedding of EBV from natural reservoirs and its replication [12]. Thus EBV may also play a synergistic role with tobacco in the malignant transformation of oral potentially malignant disorders (OPMD).

* Corresponding author at: Department of Microbiology/Research lab for Oral and Systemic health, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research, Chennai- 600100, Tamil Nadu, India.

E-mail address: mahalakshmi.sbdch@bharathuniv.ac.in (K. Mahalakshmi).

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Hardly any studies indicate the EBV load in the oral epithelial cells of patients with OSCC and OPMD in India. Hence the present study was designed to determine the EBV copy number in the oral epithelial cells of patients with OSCC and OPMD and its possible association.

Methods

This study was approved by the Institutional Ethics Committee of Sree Balaji Dental College & Hospital, Chennai. The study participants were recruited between September 2016 to June 2017. Written consent was obtained from the patients and healthy individuals after explaining the study proposal.

The study population included patients with OSCC (Group A, $n = 19$) and patients with OPMD (Group B, $n = 23$). Age and sex - matched individuals with no mucosal lesions and no history of deleterious habits comprised healthy controls (Group C, $n = 39$). Patients before treatment for OSCC/OPMD were included for the study. Patients who were terminally ill, patients on antibiotic and antifungal therapy for the past three months were excluded. All subjects in the study group (OSCC & OPMD) were diagnosed clinically. Incisional biopsy was performed for all cases of OSCC and suspicious cases of OPMD which included red lesions (Erythroplakia), red and white lesions (Erythroleukoplakia or speckled leukoplakia), and severe oral submucous fibrosis (OSMF) to rule out malignancy.

Sample collection

Oral epithelial cells were collected from Group A and Group B subjects with a sterile cytobrush by rotating it for 10 s on the lesion. The cytobrush with oral epithelial cells were transferred to a sterile 1.5 ml tube containing 1 ml of RNA *later* (Qiagen, Germany) by gentle rotary movement of cytobrush. Among healthy subjects oral epithelial cells were collected from normal buccal mucosa. The samples were stored at −20 ˚C until assay.

DNA extraction

The RNA later containing oral epithelial cells was centrifuged at 7500 rpm for 10 min and the supernatant was discarded. DNA was extracted from all the samples using DNeasy Blood & Tissue Kits (Qiagen, Germany) as per the manufacturer's instructions. The extracted DNA was stored at −20 ˚C until assay.The purity of the DNA was checked and quantified by Qubit 4.0 Fluorometer. (Thermo Fisher Scientific, US.)

Real- time PCR protocol

Quantitative Polymerase chain reaction (qPCR) targeting EBV EBNA-1 gene was performed using the primers (EBV-EbNA-1F:5′-CCGCTCCTACCTGCAATATCA-3′; and EBV-EbNA-1R:5′-GGAAACCAGGGAGGCAAATC-3′) previously described by Fellner et al. [13] Real-Time PCR amplification was performed in a 10 µl reaction volume consisting of 5 μl of QuantiNova® SYBR® Green PCR Master Mix (Qiagen, Germany), 0.1 μl of QN ROX Reference Dye, 0.5 μl of 10 pmol of each EBV-primer, 2 μl of DNA template and DNase and RNase-free PCR grade water. The amplification was performed in QuantStudio 5 Real-Time PCR system (Applied Biosystems, USA) with initial hold stage at 95 ˚C for 2 min, PCR stage at 95 ˚C for 10 s, 60 ˚C for 30 s for 40 cycles, reaction specificities were finally verified by melting curve analysis using melt curve stage at 95 ˚C for 1 s, 60 ˚C for 20 s and 95 ˚C for 1 s. Known positive (calibrator) and no template control (NTC) was also included. NTC contained all the components of the reaction except the template (DNA).The qPCR assay was performed for all the samples in duplicate and the mean of the two results were recorded.

Positive control (calibrator) comprised a fragment of EBNA-1 coding gene from the EBV genome produced by gene synthesis (BioServe, India). A standard curve was constructed with the C_T (threshold cycle) values obtained from serially diluted (10^{-5} to 10^{-10}) positive control and the EBV copy number for clinical samples was calculated by plotting the results (C_T values) on the standard curve plot. The viral load in each sample was expressed as the number of viral DNA copies per 50 ng of purified DNA.

Statistical analysis

Independent sample *t*-test was performed to calculate the significance of EBV copy number among OSCC and OPMD. The odds and risk ratio was calculated for the samples containing ≥ four log count of EBV copy number by Chi-square test with 95% confidence interval. A p-value of <0.05 was considered significant.

Result

Majority of the patients belonging to OSCC and OPMD had the habit of either smoking or chewing tobacco, with or without the habit of alcohol consumption. OPMD group comprised 23 patients *viz.,* OSMF ($n = 7$), Erythroplakia ($n = 6$), Leukoplakia ($n = 7$), erythroleukoplakia ($n = 3$). Table −1, 2 &3 shows the prevalence of EBV DNA copy number in all the three groups (OPMD, OSCC and Health). All the samples in three groups showed the presence of EBV DNA. Majority of the samples in the study groups (Group A& Group B) showed significantly high DNA copy numbers. Among Group A ($n = 19$) only three samples had low DNA copy number ($1.5 – 5.0 \times 10^2$), while the remaining 16 samples possessed significantly high DNA Copy numbers ($\geq 1.0 \times 10^4$). Among Group B excluding one sample (1.2×10^2) all others had high DNA copy numbers ($1.0 \times 10^4 – 1.3 \times 10^{5)}$. Conversely, the EBV DNA copy numbers were very low in the control group (group C). (Fig −1) Among the seven OSCC patients without habits, six patients' samples harboured a very high EBV copy number. Significant difference between grading/habits and EBV DNA copy number were not observed both in Group A (OSCC) and Group B (OPMD) subjects. (Tables 1& 2) The risk ratio/odds ratio for Group A and Group B were2.822/ 5.328 and 4.66/ 85.25 respectively. A very high statistical significant difference ($p = 0.000$) was observed between the study groups (group A, $p = 0.000$ and group B, $p = 0.000$) and healthy group (group C) concerning the DNA copy numbers of EBV (Table 3).

Discussion

Subjects with absence of established risk factors may also develop OSCC. This suggests the presence of other additional factor which may include infections with oncogenic viruses. EBV, an oncogenic human herpes virus recognized as the first human tumor virus has been associated with various types of epithelial and B-lymphocyte cell malignancies. EBV infection is observed as a latent infection lifelong among asymptomatic individuals [14–17].

Accumulating evidence suggests the association of Epstein-Barr virus to increased risk of OSCC [18]. In the present study, Real- Time PCR was targeted to amplify the highly conserved EBNA1 gene of 73-bp size. EBNA1 gene which codes for EBV nuclear antigen 1is essential for the conservation of the EBV genome in the infected cell. EBNA 1, a highly conserved gene in all EBV strains was chosen because they encode the product, Epstein-Barr nuclear antigen 1, which is important in viral pathogenesis [5,7].

To the best of our knowledge none of the earlier studies have reported 100% positivity of EBV in diseased and healthy subjects. Moreover, all the earlier studies were qualitative. Hence, we designed the present study to quantify the EBV DNA copy in the exfoliated cells of OSCC, OPMD and healthy subjects. The results of the present study show a very high mean EBV DNA copy number among OPMD and OSCC patients compared to healthy subjects. Significant odds and risk ratio was observed for EBV among group A and group B. Earlier eight studies

Table 1.
Prevalence of EBV – EB nuclear antigen 1 DNA load, pathology parameters & habits among Group A (OSCC).

S.no	Sample Id	EBV copy no	EBV copies	Grade	localization	Habits
1	K6	13,292	1.3×10^4	WD	Tongue	Tobacco Chewing+smoking+alcohol
2	K7	16,005	1.6×10^4	WD	Buccal mucosa	Tobacco Chewing+smoking+alcohol
3	K8	13,818	1.3×10^4	WD	Buccal mucosa	No habits
4	K10	23,733	2.3×10^4	WD	Buccal mucosa	Tobacco Chewing+ alcohol
5	K11	13,174	$1.3 \times 10^{4*}$	WD	Tongue	No habits
6	K14	10,663	1.0×10^4	MD	Floor of the mouth	Tobacco Chewing+smoking+alcohol
7	K16	13,600	1.3×10^4	MD	Buccal vestibule	Tobacco Chewing+smoking+alcohol
8	K19	11,491	1.1×10^4	PD	Palate	Smoking+alcohol
9	K20	34,259	$3.4 \times 10^{4*}$	MD	Buccal vestibule	No habits
10	K21	12,196	$1.2 \times 10^{4*}$	MD	Buccal vestibule	No habits
11	K23	24,148	2.4×10^4	MD	Buccal mucosa	Tobacco Chewing
12	K27	18,505	$1.8 \times 10^{4*}$	WD	Buccal mucosa	No habits
13	K28	18,409	1.8×10^4	MD	Buccal vestibule	Tobacco Chewing
14	K36	13,708	$1.3 \times 10^{4*}$	WD	Tongue	No habits
15	K37	43,691	4.3×10^4	WD	Retromolar area	Tobacco Chewing + alcohol
16	K40	14,256	1.4×10^4	WD	Buccal mucosa	Tobacco Chewing + alcohol
17	K51	502	5.0×10^2	WD	Buccal mucosa	Tobacco Chewing + alcohol
18	K58	153	$1.5 \times 10^{2*}$	WD	Floor of the mouth	No habits
19	K64	289	2.8×10^2	WD	Buccal mucosa	Tobacco Chewing+smoking+alcohol

*EBV load in patients with no habits, WD= Well differentiated squamous cell carcinoma MD= Moderately differentiated squamous cell carcinoma, PD= Poorly differentiated squamous cell carcinoma.

Table 2.
Prevalence of EBV – EB nuclear antigen 1 DNA load, pathology parameters & habits among Group B (OPMD).

S.no	Sample Id	EBV copy no	EBV copies	OPMD	localization	Habits
1	K1	12,077	1.2×10^4	Leukoplakia	Buccal mucosa	Tobacco Chewing
2	K2	18,173	1.8×10^4	Leukoplakia	Buccal mucosa	Smoking+Alcohol
3	K3	22,208	2.2×10^4	OSMF	Buccal mucosa	Tobacco Chewing+Alcohol
4	K4	24,160	2.4×10^4	Erythroplakia	Buccal mucosa	Tobacco Chewing +Alcohol
5	K5	11,869	1.1×10^4	Leukoplakia	Buccal mucosa	Smoking
6	K9	14,472	1.4×10^4	Leukoplakia	Buccal mucosa	Smoking+ Tobacco Chewing+Alcohol
7	K12	10,432	1.0×10^4	OSMF	Buccal mucosa	Tobacco Chewing+Alcohol
8	K13	11,748	1.1×10^4	Erythroplakia	Buccal mucosa	Smoking+Tobacco Chewing +Alcohol
9	K15	38,409	3.8×10^4	Erythroplakia	Buccal mucosa	Smoking+Tobacco Chewing +Alcohol
10	K17	11,098	1.1×10^4	Erythroleukoplakia	Buccal mucosa	Smoking+Tobacco Chewing +Alcohol
11	K18	20,504	2.0×10^4	Leukoplakia	Buccal mucosa	Smoking
12	K22	26,498	2.6×10^4	OSMF	Buccal mucosa	Tobacco Chewing
13	K 24	26,816	2.6×10^4	Erythroleukoplakia	Buccal mucosa	Smoking+Tobacco Chewing +Alcohol
14	K25	18,647	1.8×10^4	Leukoplakia	Tongue	Tobacco Chewing +Alcohol
15	K26	20,082	2.0×10^4	Erythroplakia	Buccal mucosa	Smoking+Tobacco Chewing +Alcohol
16	K29	12,862	1.2×10^4	Erythroleukoplakia	Buccal mucosa	Smoking+ +Alcohol
17	K30	17,602	1.7×10^4	Leukoplakia	Buccal mucosa	Tobacco Chewing
18	K31	14,655	1.4×10^4	Leukoplakia	Buccal mucosa	Tobacco Chewing
19	K32	138,233	1.3×10^5	OSMF	Buccal mucosa	Tobacco Chewing
20	K33	14,856	1.4×10^4	Leukoplakia	Buccal mucosa	Tobacco Chewing
21	K34	11,979	1.1×10^4	OSMF	Buccal mucosa	Smoking+Tobacco Chewing +Alcohol
22	K35	12,503	1.2×10^4	OSMF	Buccal mucosa	Tobacco Chewing
23	K44	126	1.2×10^2	Erythroleukoplakia	Buccal mucosa	Smoking+Tobacco Chewing +Alcohol

OSMF= Oral submucous fibrosis.

Table 3.
Comparison of EBV DNA Load in OSCC/ OPMD and healthy subjects.

OSCC and health Group	mean	Std deviation	t	P value
A (OSCC) *n* = 19	15,573.3	10,725.3	4.554	0.000*
C (Health) *n* = 39	3014.23	9419.06	4.352	0.000*
OPMD and health				
B (OPMD) *n* = 23	22,174.3	26,415.5	4.126	0.000*
C (Health) *n* = 39	3014.23	9419.06	3.355	0.003

T- Test, * = highly significant p value.
OSCC= Oral squamous cell carcinoma.
OPMD = Oral potentially malignant disorders.

have reported the prevalence of EBV in the paraffin-embedded tissues of OSCC patients by conventional PCR [19–26]Among the eight studies, four have used the primers that target EBV-DNA (BAM-HW) and have reported a very low prevalence of EBV in the tissues of OSCC patients and normal tissues [19–21,25]. The 100% presence of EBV among OSCC patients is well in line with Cruz et al. Conversely, the prevalence of EBV in the healthy cohorts does not agree with Cruz et al. study [20]. Three other studies have used primers targeting EBV-DNA [22–24]. One study has used primers targeting EBV-DNA (EBNA2) [26]. The results of the present study do not agree with Sand et al. and Bagan et al. who have reported 37.9% / 40% and 7.4% / 0% of EBV DNA in the tissues of OSCC patients/normal tissue respectively by nested PCR [27,28]. This study has reported 100% prevalence compared to a Japanese study, who has shown 83.33% of EBV DNA (BAMHW) by *in situ* hybridization [29].

The present study has detected a very high prevalence of EBV in the oral exfoliated cells of OSCC patients and healthy controls compared to a study among Thailand population by Sulav Acharya et al. 2015who has detected EBV (OSCC=45.05%, healthy subjects = 18.08%) by nested PCR using primers specific for EBV DNA polymerase [30]. The results

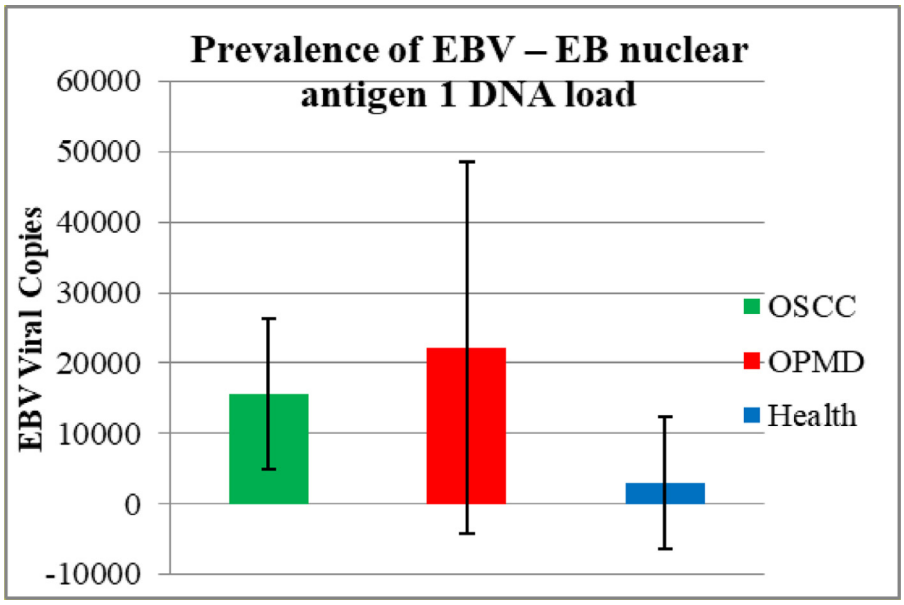

Fig. 1. Bar chart with error bars showing the EBV DNA levels in the exfoliated epithelial cells of Group A (OSCC), Group B (OPMD) and Group C (Healthy subjects).

of our study reveals a very high prevalence of EBV compared to Jiang R et al. 2012 who has reported 52.40% and 30.4% EBV positivity in OSCC and in healthy subjects respectively by RT qPCR [31]. Correspondingly, the present study shows a very high prevalence of EBV DNA compared to Jamshid Jalouli et al. 2012 who has reported 55% EBV positivity in OSCC by nested PCR [32]. Our study revealed a very high prevalence of EBV compared to a north Indian study by Jamshid Jalouli et al. 2010by PCR/DNA sequencing (25% in OSMF and 29% in OSCC) and Sand LP et al. 2002by nested PCR (37.9% in OSCC and 7.3% in healthy persons) [27,33].

Real - time PCR was chosen as it is an excellent method for Quantification of DNA. In comparison to earlier studies, our study stands unique as we have reported EBV DNA in all the samples of all the three groups. This finding may be attributed to the primers that were designed to amplify all the EBV strains as these primers possess highly conserved genes. EBV DNA presence in the oral epithelial cells of all the samples reveals that the oral cavity may be a reservoir for EBV. Even though all samples (study and control) showed the presence of EBV DNA, a highly significant EBV-DNA copy number was observed among Group A and Group B. (Fig 1) This finding of the present study and the presence of a highly significant EBV copy number in patients without any habits suggests a strong association of EBV risk to OPMD and OSCC. The significant odds and risk ratio among the OPMD and OSCC group may associate EBV as a risk factor for these conditions. Quantification of this oncogenic virus in the oral exfoliated cells of OSCC and OPMD patients may be suggested to associate them to these conditions. A coordinated effort by basic medical scientists and clinical investigators will improve our rational approach for applying and interpreting EBV tests in oral pathology clinical settings.

Conclusion

Given the inconsistencies observed in the previous studies, we aimed to quantify the EBV DNA by targeting the EBNA1 gene by real time PCR in the oral exfoliated cells of oral squamous cell carcinoma, oral potentially malignant disorders and healthy cohorts. All the subjects in the three cohorts harboured EBV DNA. In spite of 100% prevalence in all three groups, a highly significant EBV copy was observed in the oral exfoliated cells of patients with OSCC and OPMD compared to healthy subjects. The large variation in the levels of EBV DNA shown by the error bars (Fig –1) strongly recommend that the quantification of EBV DNA in the oral epithelial cells of OPMD and OSCC patients may add a diagnostic value in assessing the role of EBV in these conditions. The high odds and risk ratio obtained for EBV DNA copy further supports a strong link of this virus to OSCC and OPMD. Similar studies with a large sample size will throw more light in the association and diagnostic value of EBV to OSCC and OPMD.

Ethics approval and consent to participate

Ethical approval was given by The Institutional Ethics Committee (Ethics committee DCGI Registration No. ECR/761/Inst/TN/2015), Sree Balaji Dental College and Hospital. IEC Approval No: SBDCH/IEC/03/2016/1. Written consent was obtained from the participants.

Authors' contributions

GK, SLS & KM wrote the proposal, GK and SLS participated in data collection and analysis, VN & KM participated in the analysis, KM, SLS, VN & KMKM participated in drafting, writing, reviewing of the manuscript for publication. All the authors read and approved the manuscript.

Declaration of Competing Interest

None.

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References

[1] CF Lopes, BB de Angelis, HM Prudente, BV de Souza, SV Cardoso, RI. de AzambujaRibeiro, Concomitant consumption of marijuana, alcohol and tobacco in oral squamous cell carcinoma development and progression: recent advances and challenges, Arch Oral Biol 57 (8) (2012) 1026–1033 Aug.

[2] JA Nemes, L Deli, Z Nemes, IJ. Márton, Expression of p16(INK4A), p53, and Rb proteins are independent from the presence of human papillomavirus genes in oral squamous cell carcinoma, Oral Surg Oral Med Oral Pathol Oral Radiol Endod 102 (2006) 344–352.

[3] D Martin, JS. Gutkind, Human tumour associated viruses and new insights into the molecular mechanisms of cancer, Oncogene 27 (2009) S31–S42.
[4] R Metgud, M Astekar, M Verma, A. Sharma, Role of viruses in oral squamous cell carcinoma, Oncology Rev 6 (2) (2012) e21.
[5] P Matthew, Kurzrock R Thompson, Epstein - Barr virus and cancer, Clin cancer Res 10 (2004) 803–821.
[6] R. Kuppers, B cells under influence: transformation of B cells by Epstein - Barr virus, Nat Rev Immunol 10 (2003) 201–212.
[7] M Gonzalez-Moles, J Gutiérrez, I Ruiz, JA Fernandez, M Rodriguez, J. Aneiros, Epstein-Barr virus and oral squamous cell carcinoma in patients without HIV infection: viral detection by polymerase chain reaction, Microbios 96 (1998) 23–31.
[8] B Gruhne, R Sompallae, MG. Masucci, Three Epstein-Barr virus latency proteins independently promote genomic instability by inducing DNA damage, inhibiting DNA repair and inactivating cell cycle checkpoints, Oncogene 28 (2009) 3997–4008.
[9] SY Huang, CY Fang, CH Tsai, et al., N-methyl-N-nitro-N-nitroso guanidine induces and cooperates with 12-O-tetradecanoylphorbol-1, 3-acetate/sodium butyrate to enhance Epstein-Barr virus reactivation and genome instability in nasopharyngeal carcinoma cells, Chem Biol Interact 188 (2010) 623–634.
[10] S Johansson, JM Hirsch, D Johnson, et al., Effect of repeated oral administration of tobacco snuff on natural killer- cell activity in the rat, Arch Oral Biol 36 (1991) 473–476.
[11] LM Sopori, W. Kozak, Immunomodulatory effects of cigarette smoke, J Neuroimmunol 83 (1998) 148–156.
[12] L Sand, M Wallstroem, J Jalouli, P-A Larsson, J-M. Hirsch, Epstein- Barr virus and human papillomavirus in snuff-induced lesions of the oral mucosa, Acta Otolaryngol 120 (2000) 880–884.
[13] MD Fellner, KA Durand, MA Rodríguez, L Irazu, LV Alonio, MA. Picconi, Duplex real-time PCR method for Epstein–Barr virus and human DNA quantification: its application for post-transplant lymphoproliferative disorders detection, Braz J Infect Dis 18 (2014) 271–280.
[14] MP Thompson, R. Kurzrock, Epstein-Barr virus and cancer, Clin Cancer Res 10 (2004) 803–821.
[15] RT Javier, JS. Butel, The history of tumor virology, Cancer Res 68 (2008) 7693.
[16] SR Prabhu, DF. Wilson, Evidence of Epstein-Barr Virus Association with Head and Neck Cancers: A Review, J Can Dent Assoc 82 (2016).
[17] E Grywalska, J. Rolinski, Epstein-Barr virus-associated lymphomas, Semin Oncol 42 (2015) 291–303.
[18] Y She, X Nong, M Zhang, M Wang, Epstein-Barr virus infection and oral squamous cell carcinoma risk: a meta-analysis, PLoS ONE 12 (10) (2017) e0186860 2017.
[19] EJ Van Rensburg, S Engelbrecht, HW Van, E Raubenheimer, BD. Schoub, Detection of EBV DNA inoral squamous cell carcinomas in a black African population sample, In Vivo (Brooklyn) 9 (1995) 199–202.
[20] WE VanHeerden, EJ van Rensburg, S Engelbrecht, EJ. Raubenheimer, Prevalence of EBV in oral squamouscell carcinomas in young patients, Anticancer Res 15 (1995) 2335–2339.
[21] I Cruz, VDBA Rd, PJ Snijders, CJ Meijer, JM Walboomers, GB Snow, et al., Prevalence of Epstein-Barrvirus in oral squamous cell carcinomas, premalignant lesions and normal mucosa– a study using the polymerase chain reaction, Oral Oncol 33 (1997) 182–188.
[22] XQ Ding, ZY. Zhu, Relationship between Epstein-Barr virus infection and oral squamous cell carcinoma, Acad J SUMS 18 (1997) 140–141 153.
[23] WL Chen, LW Tong, QL Deng, Human papillomavirus type 16 and Epstein-Barr virus relative to oralsquamous cell carcinoma, J Oral MaxillofacSurg 8 (1998) 23–25 (in Chinese).
[24] J D'Costa, D Saranath, V Sanghvi, AR. Mehta, Epstein-Barr virus in tobacco-induced oral cancers andoral lesions in patients from India, J Oral Pathol Med 27 (1998) 78–82.
[25] A Kis, E Feher, T Gall, I Tar, R Boda, ED Toth, et al., Epstein-Barr virus prevalence in oral squamouscell cancer and in potentially malignant oral disorders in an eastern Hungarian population, Eur J Oral Sci 117 (2009) 536–540.
[26] K Kikuchi, Y Noguchi, M Hoshino, H Sakashita, T Yamada, H Inoue, et al., Detection of Epstein-Barrvirus genome and latent infection gene expression in normal epithelia, epithelial dysplasia, and squamouscell carcinoma of the oral cavity, Tumour Biol 37 (2016) 3389–3404.
[27] LP Sand, J Jalouli, PA Larsson, JM. Hirsch, Prevalence of Epstein-Barr virus in oral squamous cell carcinoma,oral lichen planus, and normal oral mucosa. Oral Surg. Oral Med. Oral Pathol, Oral Radiol Endod 93 (2002) 586–592.
[28] JV Bagan, Y Jiménez, J Murillo, R Poveda, JM Díaz, C Gavaldá, et al., Epstein-Barr virus in oral proliferative verrucous leukoplakia and squamous cell carcinoma: a preliminary study, Med. Oral .Patol .Oral .Cir. Bucal. 13 (2008) E110–E113.
[29] M Shimakage, K Horii, A Tempaku, K Kakudo, T Shirasaka, T. Sasagawa, Association of Epstein-Barr virus with oral cancers, Hum Pathol. 33 (2002) 608–614.
[30] S Acharya, T Ekalaksananan, P Vatanasapt, K Loyha, P Phusingha, S Promthet, et al., Association of Epstein-Barr virus infection with oral squamous cell carcinoma in a case–control study, J. Oral Pathol Med 44 (2015) 252–257.
[31] R Jiang, X Gu, TN Moore-Medlin, CA Nathan, LM. Hutt-Fletcher, Oral dysplasia and squamous cell carcinoma: correlation between increased expression of CD21, Epstein-Barr virus and CK19, Oral Oncol 48 (9) (2012) 836–841.
[32] Jalouli Jamshid, Miranda M. Jalouli, Sapkota Dipak, Salah O. Ibrahim, Per-Anders Larsson, Lars Sand, Human Papilloma Virus, Herpes Simplex Virus and Epstein Barr Virus in Oral Squamous Cell Carcinoma from Eight Different Countries, Anticancer Research 32 (2012) 571–580.
[33] Jamshid Jalouli, Salah O, Ravi Mehrotra Ibrahim, Miranda M. Jalouli, Dipak Sapkota, Per-Anders Larsson, Jan-M. Hirsch, Prevalence of Viral (HPV, EBV, HSV) Infections in Oral Submucous Fibrosis and Oral Cancer from India, Acta Otolaryngol 130 (2010) 1306–1311.