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Betel Quid Chewing and Alcohol Consumption Co- Variate with Detection of Epstein - Barr Virus (EBV) DNA in Oral Squamous Cell Carcinoma Tissues: Preliminary Evidence from a Subset of an Unmatched Case-Control Study

Manosha Lakmali Perera[1*], Irosha Rukmali Perera[2], P. Kirupakaran[3] and Suresh Shanmuganathan[4]

[1]*School of Dentistry and Oral Health, Griffith University, Queensland, Australia (Alumni)*

[2]*Preventive Oral Health Unit, The National Dental Hospital (Teaching), Colombo, Sri Lanka*

[*]Corresponding Author
Manosha Lakmali Perera, School of Dentistry and Oral Health, Griffith University, Queensland, Australia.

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Abstract

Introduction: *There is a dearth of information on the detection of oncogenic EBV in oral squamous cell carcinoma tissues in the Sri Lankan context. This subset of an unmatched case-control study aims to detect EBV-DNA in oral squamous cell carcinoma tissues of a group of Sri Lankan male patients.*

Method: *Subset was selected representing the vast majority of OSCC patients in Sri Lanka, from an unmatched case-control study. Incisional biopsies of cases and excisional biopsies of controls were collected and stored at -800 C. DNA was extracted from frozen specimens, and Real-Time PCR was performed to detect target DNA of this ubiquitous virus, in histologically confirmed 27 Oral Squamous Cell Carcinoma (OSCC) cases and clinically diagnosed 26 Fibroepithelial-Polyp (FEP) controls with better quality DNA. Socio-demographic and substance abuse data were collected from 29 cases and 25 controls by a pretested interviewer-administered questionnaire, and the data were entered and analyzed using the SPSS-21 Statistical Package.*

Results: *Descriptive and inferential statistics were used to determine the significance of qualitative and quantitative data statistically. The overall EBV prevalence was 34/53 (64.2%). In OSCC cases the EBV positivity was higher 21(77.8%) than the FEP controls 13 (50.0%), and this difference was statistically significant (p = 0.035). The cases were higher betel quid chewers (p = 0.003) and heavy alcohol consumers (p =0.001) than the control group.*

Conclusion: *Betel quid chewing and alcohol consumption co-variate with the detection of Epstein-Barr virus (EBV) DNA in oral squamous cell carcinoma tissues which warrants further investigations.*

Keywords: Epstein Barr Virus, Co-Factor, Sri Lanka, Association

1. Introduction

The viral aetiology of global malignancies reported is approximately 13% -15% and the mode of transmission of these viruses is considered as population and geographic-specific, depending on lifestyle-related risk habits and anthropometry measures of individuals [1-4].

Universal Epstein Barr Virus (EBV), also known as HHV-4, is one of the oncogenic viruses belonging to the subfamily Gammaherpesvirinae within the family Herpesviridae. This virus is the main cause of nasopharyngeal cancer, which has a distinct biological entity. A causal association with EBV remains hitherto unresolved to this date, despite oral cancers being a constituent component of head and neck cancers (HNSCC) [3,4]. However, a meta-analysis revealed that the pooled OR with 95% CI between EBV infection and OSCC risk was 5.03 (1.80–14.01) with significant heterogeneity observed (I2 = 87%). The subgroup analysis indicated that the year of publication, study location, economic level, sample size, tissue type, detection method and marker, control type, and language might explain potential sources of heterogeneity [5]. As reported by the authors, publication bias was not observed, and sensitivity analysis showed stable results. Hence, the evidence suggests a possible association of EBV infection with an increased risk of OSCC. However, it is not known whether this association is mediated by oral risk habits [5].

Furthermore, EBV is the causative agent of gastric carcinoma and lymph proliferative disorders such as Burkitt's and Hodgkin's lymphomas and an influential factor in the epithelial hyper proliferation and abnormal keratinization of oral hairy leukoplakia, a benign lesion common in immunosuppressed

patients as well as B cell lymphomas. Nevertheless, any co-variation of EBV with oral risk habits is yet to be established globally as well as in Sri Lanka. In light of these findings, the present study aimed to explore the detection of Epstein-Barr virus (EBV) DNA in oral squamous cell carcinoma tissues of a group of Sri Lankan male patients [6].

2. Methodology

A subset from an unmatched case-control study representing the vast majority of OSCC patients was used for this study as described previously [4]. Each participant provided written informed consent. Ethical approval for this study was obtained from the University of Peradeniya, Sri Lanka (FRC/ FDS/ UOP/E/2014/32), and the Griffith University Human Research Ethics Committee, Australia (DOH/18/14/HREC). Deep tissue samples (~100 mg each) were dissected from fresh incisional biopsies for OSCC cases and excisional biopsies for FEP stored at -800C, avoiding contamination from the tumour surface. DNA was extracted with the Gentra Puregene Tissue kit (Qiagen) strictly adhering to the manufacturer's protocol for solid tissue. [Cat no. 158689] as described previously [4]. The quality of extracted DNA was assessed by β-globin PCR with the primers PCO3 and PCO4. The Real–Time PCR assay was set up to amplify 106 bp length of EBV- DNA from primer sequences as described previously 4. A 10- µl volume of each test sample was placed into the wells of a white 384-well plate in an Eppendorfep Motion 5075. Real-time PCR performed using a Quant 6- real-time machine as described previously [4]. The absence of PCR inhibitors in extracted DNA was confirmed by β-globin PCR assay with the primers PCO3 and PCO4 4. Data entering and analysis made by SPSS-21 Statistical Package. Statistical significance of qualitative and quantitative data was obtained by; Descriptive statistics for percentage, frequency, mean, and standard deviation [4]. Chi-Square test for assessing the relationship between categorical variables, T-test for examining the relationship between quantitative variables and Fisher's exact for comparing groups (where cell counts were <5)

Assay	Primers	Annealing Temperature	Sequence (5'-3')	Reference sequence	Nucleotide Position	Amplicon Length
EBV EBNA-1	EBNA-1 01.1 EBNA-1 01.2	55^{0}C	CCGGTGTGTTCGTATAGGAC GGGAGACGACTCAATGGTGTA	NC_009334	98,005 98,025	106 bp

Table 01: Primers used for RT- PCR EBV EBNA-1 assay [8]:

Table 01 describes the primer specifications of Manosha EBV EBER-1 FastTaq 2016-04-18_analysis to detect EBNA-1 target DNA.

3. Results

Variable	Cases n=29	Controls n=25	p value	
Age mean ± SD in years Gender	61.62±9.21 Male	49.96 ±13.38	**0.0001***	(p<0.05)
	N % 29 (100.0)	N % 25 (100)		

Table 1: Distribution of Cases and Controls by Age and Sex

* *t- test to compare means of to examine the relationship between quantitative variables, such as age.*

As per the Table 01, the mean ± SD age of cases was 61.62±9.21 years where as the mean ± SD age of the controls was 49.96 ±13.38 years. This difference in age groups was statistically significant (p= 0.0001). This may be due to typical older age group of males affected by OSCC compared to relatively younger age group of males who presented with FEPs.

Variable	Cases n=29 N %	Controls n=25 N %	p-value	
Betel Quid Chewing Habit				
Never	0 (0.0)	4 (16.0)	**0.003****	(p<0.05)
Past	5 (20.0)	2 (8.0)		
Sometimes	1 (4.0)	7 (28.0)		
Daily	23 (76.0)	12 (48.0)		
Total	**29 (100.0)**	**25 (100.0)**		

Smoking Habit				
Never	5 (17.2)	8 (32.0)	**0.418****	(p>0.05)
Past	8 (27.6)	6 (24.0)		
Sometimes	4 (13.8)	5 (20.0)		
Daily	12(41.4)	6 (24.0)		
Total	**29 (100.0)**	**25 (100.0)**		
Alcohol Consumption				
Never	3 (10.3)	3 (12.0)	**0.001****	(p<0.05)
Past	6 (20.7)	2 (8.0)		
Sometimes	4 (13.8)	16 (64.0)		
Weekly	16 (55.2)	4 (16.0)		
Total	**29 (100.0)**	**25 (100.0)**		

Table 2: Distribution of the Cases and Controls by Established Risk Habit Profile

*** Fisher's exact test to compare groups (cell counts <5)*

Table 2 demonstrates the risk habit profile: betel chewing, smoking and alcohol consumption among cases and controls. Accordingly, the vast majority (76.0%) of OSCC cases were daily betel chewers. Exactly, 20% reported past betel chewing habits. In contrast, 48.0% of FEP controls were daily betel chewers as well. Of them, 28.0% chewed betel sometimes. Despite every betel chewing habit among cases and controls, the majority of cases chewed betel daily and these differences were statistically significant (p = 0.003). In the smoking habit, 41.4% of cases and 24.0% of controls were smoking daily. Compared to controls, cases were ever smokers but these differences were not statistically significant (p=0.418). With regards to alcohol consumption, the majority (55.2%) of cases were weekly drinkers of alcohol whereas the main (64.0%) of controls was sometime drinkers of alcohol. Overall, despite both groups being alcohol consumers, cases reported a significantly higher weekly drinking of alcohol (p=0.001), (Table, 2).

Target EBV- DNA	Cases n=27	Controls n=26	Total	p-value
	N (%)	N (%)	N (%)	
Positive	21 (77.8)	13 (50.0)	34(64.2)	0.035***(p<0.05)
Negative	06 (22.2)	13 (50.0)	19(35.8)	
Total	27 (100.0)	26 (100.0)	53(100.0)	

Table 3: Distribution of Cases and Controls by EBV Status

* Chi-square Test of Statistical Significance

Table 06 presents the distribution of Cases and Controls by EBV-DNA status. In Table 06, the overall detection of EBV among 53 Tissue samples was 64.2%. Among OSCC cases (n=27) occurrence of target EBV –DNA was as high as 77.8% while this occurrence among FEP controls (n=26) was lower (64.2%) than that for the cases and these differences were statistically significant (p<0.05).

4. Discussion

According to evidence on EBV serology, the global population has shown up to 90% of seropositivity [2-4]. EBV establishes latency in a minor proportion of B lymphocytes located in the lymphoid tissues, including those of the head and neck, notably Waldeyer's ring, and in the lining epithelia of the naso and oropharynx, and in salivary glands where they replicate under the immunosuppressive conditions and shed in secretions of the upper aerodigestive tract, as asymptomatic in most individuals [7]. Against this backdrop, the presence of EBV-DNA in oral cancer tissues in a representative subset of OSCC tissues of male oral cancer patients needs ca utious interpretation. Relatively higher EBV and IgG antibody titre (1:640–≥1:1280) were detected in Sri Lankan oral squamous cell carcinoma (OSC) patients in a study conducted more than two decades ago. Detection of target EBV-DNA in OSCC cases was much higher (77.8%) than (35.0%) in the past study conducted by Jalouli and colleagues [8]. A statistically significant difference (p= 0.035) in the detection of EBV-DNA in OSCC cases compared with FEP controls was found in our study. Corroborating our findings, increased EBV occurrence in OSCC cases was found in Taiwan (82.5%), Japan (76.6%), Yamen (73.3%), and Hungary (73.8%) [4].

In the oral risk habit comparison, all cases (100%) but controls (84%) were present or past betel quid chewers. The difference between the two groups was statistically significant (p= 0.003). Moreover, the majority (55.2%) of cases were regular drinkers whereas the majorities (64.0%) of controls were occasional drinkers. Despite both groups being alcohol consumers, cases reported significantly higher weekly drinking of alcohol and those differences were statistically significant (p=0.001).

Corroborating our findings, 81.7% of OSCC cases in the Yemen study reported Shamma use (a type of smokeless tobacco product used in that country) and EBV was detected among 73.3% of cases compared to 60.8% of controls [4]. In contrast, in our study, there was no significant difference in the occurrence of EBV among OSCC cases and controls in the Yemen study [4]. Another study reported smoking and alcohol consumption were not associated with EBV occurrence among OSCC and oral lichen planus patients [13]. Thus, there is promising evidence to speculate increased occurrence of EBV among OSCC and OPMD patients compared to healthy controls. This could be due to either conducive immunosuppressive conditions or cofactor assistance of ingredients in betel quid and alcohol to activate and replicate latent EBV in the head and neck region. Nevertheless, more studies are warranted with methodological rigorousness to establish the association between lifestyle-related risk habits and EBV occurrence in oral cancer patients. The small sample size and inability of surface sterilization using 70% alcohol to prevent surface contamination by salivary EBV were limitations of the present study.

Thus, there is promising evidence to speculate increased occurrence of EBV among OSCC and OPMD patients compared to healthy controls. This could be due to either conducive immunosuppressive conditions or cofactor assistance of ingredients in betel quid and alcohol to activate and replicate latent EBV in the head and neck region. Nevertheless, more studies are warranted with methodological rigorousness to establish the association between lifestyle-related risk habits and EBV occurrence in oral cancer patients. The small sample size and inability of surface sterilization using 70% alcohol to prevent surface contamination by salivary EBV were limitations of the present study.

5. Conclusions and Recommendations

Our preliminary findings indicate a possible co variation of betel quid chewing with or without tobacco and alcohol consumption with a higher occurrence of EBV- DNA in OSCC tissues among oral cancer patients. However, our findings should be validated and confirmed by rigorous case-control studies with larger sample sizes, controlled for potential confounders. This will provide more conclusive evidence for the putative association of lifestyle-related-oral risk habits (as co-factors) with active replication of EBV in OSCC cases compared with benign mucosal lesions. It is better to plan in-vitro experiments to expand the knowledge of chemical stimuli in aromatic and aliphatic compounds in smokeless tobacco, areca nut and alcohol that can activate the latency-to-lytic switch of EBV in cultured cell lines. If the causal inference is proven, vaccination of high-risk population can prevent the causative role of EBV in carcinogenesis.

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Conflicts of Interest: Authors declare no conflict of interest.

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Ethics statement: Ethics clearance were obtained from the University of Peradeniya, Sri Lanka (FRC/ FDS/ UOP/E/2014/32), and the Griffith University Human Research Ethics Committee, Australia (DOH/18/14/HREC).

Authors' Contributions

Perera ML: Prepared the proposal, conducted data and sample collection, performed all the laboratory experiments, analyzed data and prepared the manuscript.

Perera IR: Sri Lankan Supervisor in this study analysed data collect by the questioner and edited this manuscript.

Kirupakaran P: Supervised clinical diagnosis and provided intellectual input in manuscript writing.

Shanmuganathan S: Provided intellectual input in sample collection protocol and manuscript writing.

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