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The Association between Epstein-Barr virus (EBV) Past Infection with the Risk of Oral Squamous Cell Carcinoma (OSCC)

(Hubung Kait antara Jangkitan Lepas Virus Epstein Barr (EBV) dengan Risiko Karsinoma Sel Skuama Mulut (OSCC))

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ABSTRACT

*The association of Epstein-Barr virus (EBV) with oral cancer has been widely reported in the past. However, previous studies mainly focused on the current infection of EBV without acknowledging the possibility of past infection in patients which may lead to oral cancer development. The present study aims to investigate the correlation between past EBV infections with Oral Squamous Cell Carcinoma (OSCC). Both Immunoglobulin M (IgM) and Immunoglobulin G (IgG) antibodies against EBV were screened to detect the presence of EBV in sera of OSCC patients using Enzyme-Linked Immunosorbent Assay (ELISA). The use of IgM antibody against EBV confirms current infection in patients, whereas IgG antibody would predict past infection throughout patients' lifetime. Through the present study, we would be able to confirm whether patients with past EBV infection have a significant risk in developing oral cancer. ELISA tests were carried out to detect the presence of EBV IgG and IgM in 206 OSCC and control serum samples. Statistical analysis was performed using SPSS 12.0.1. Our results had shown that 96.6% (*n *= 199) of OSCC samples and 97.2% (*n *= 130) control were positive with EBV VCA IgG, however, none of the OSCC and control samples was positive for EBV VCA IgM. The presence of EBV VCA IgG in both OSCC and control suggest that past EBV infection does not play a significant role as a risk indicator for OSCC. Therefore, the association between EBV and OSCC was not well demonstrated in this study.*

Keywords: Enzyme-linked immunosorbent assay; Epstein-Barr virus; immunoglobulin G (IgG); immunoglobulin M (IgM); oral squamous cell carcinoma

ABSTRAK

*Hubung kait virus Epstein-Barr (EBV) dengan kanser mulut telah banyak dilaporkan sebelum ini. Namun demikian, kajian terdahulu lebih tertumpu kepada jangkitan semasa EBV tanpa mengambil kira kebarangkalian jangkitan masa lalu pada pesakit yang boleh menyebabkan perkembangan kanser mulut. Tujuan kajian ini dijalankan adalah untuk mengkaji korelasi antara jangkitan EBV yang lalu dengan Karsinoma Sel Skuama Mulut (OSCC). Kedua-dua antibodi Imunoglobulin M (IgM) dan Imunoglobulin M (IgG) terhadap EBV telah dikesan menggunakan Asai Imunojerapan Berpaut Enzim (ELISA) untuk mengenal pasti kehadiran EBV dalam sera pesakit OSCC. Antibodi IgM terhadap EBV digunakan untuk mengesahkan jangkitan semasa pada pesakit, manakala antibodi IgG dapat menjangkakan kebarangkalian jangkitan masa lalu sepanjang hayat pesakit. Melalui kajian ini, kami boleh mengesahkan sama ada pesakit yang mengalami jangkitan EBV yang lalu mempunyai tahap risiko tinggi dalam permulaan OSCC. Ujian ELISA dijalankan untuk mengesan kehadiran EBV IgG dan IgM dalam 206 sampel serum OSCC dan kawalan. Analisis statistik dijalankan menggunakan SPSS 12.0.1. Berdasarkan hasil kajian ini, didapati 96.6% (*n *= 199) sampel OSCC dan 97.2% (*n *= 130) sampel kawalan, positif dengan EBV VCA IgG. Akan tetapi, tiada sampel OSCC dan kawalan positif terhadap EBV VCA IgM. Kehadiran IgG VCA EBV dalam kedua-dua sampel OSCC dan kawalan mencadangkan bahawa jangkitan EBV yang lalu tidak memainkan peranan penting sebagai penanda risiko terhadap OSCC. Oleh yang demikian, hubung kait antara EBV dan OSCC tidak dapat dibentangkan dengan baik dalam kajian ini.*

Kata kunci: Asai imunojerapan berpaut enzim; imunoglobulin G (IgG); imunoglobulin M (IgM); karsinoma sel skuama mulut; virus Epstein-Barr

INTRODUCTION

Oral cancer is classified under the Head and Neck cancer. It was reported as the sixth most prevalent cancer worldwide (Tang et al. 2015). Oral cancer has been ranked as the 17th most common cancer worldwide and 16th most common cancer with a high mortality rate (Ferlay et al. 2018). Consequently, the mortality rate of oral cancer remains high, which is at approximately 50% of the overall mortality rate (Le Campion et al. 2017). Oral squamous cell carcinoma (OSCC) which originates from

the mucosal lining represents 90% of oral cancer and remains as a major health problem in many parts of the world (Sand & Jalouli 2014; Syrjänen et al. 2011). The development of OSCC has been widely associated with several known risk factors such as tobacco and betel quid chewing, alcohol consumption and smoking (Acharya et al. 2015; Rosnah et al. 1999). However, there are several other risk factors that have been identified in association with the development of OSCC such as ionising radiation, genetic predisposition, Human Papilloma Virus (HPV) and Epstein Barr Virus (EBV) infection (Kumar et al. 2016; Scully & Bagan 2009).

One of the prominent viruses associated with oral cancer is EBV. EBV is a member of the herpes virus family which infects approximately 90% of the world's adult population (Danielsson et al. 2018; Sand et al. 2002). EBV is also well known to be associated with infectious mononucleosis, Burkitt's lymphoma, B-cell lymphoma, Hodgkin's disease, peripheral T-cell lymphoma, nasopharyngeal carcinoma, oral hairy leukoplakia, gastric carcinoma and OSCC (Macsween & Johannessen 2014; Patel 2014; Prabhu & Wilson 2016; Yen et al. 2009). EBV primary infection occurs within the early years of life with no symptoms, and the virus persists throughout the life in the B-lymphocytes and leads to the shedding of the virus particles from the oral epithelium when re-activated (Jayasooriya et al. 2015; Purushothaman & Verma 2014; Shimakage et al. 2002). In primary infection, EBV infects and replicates in the oropharynx epithelial cells and expresses proteins causing cell proliferation (Cohen 2015; Thompson & Kurzrock 2004). In this phase, antibodies IgG, IgM and IgA are produced in response to EBV Viral Capsid Antigen (VCA) (Hanlon et al. 2014; Thompson & Kurzrock 2004). EBV infected individuals carry EBV throughout their whole life without presenting any illness and these individual usually show an elevated EBV VCA-IgG (Abbott et al. 2017; Riordan et al. 1996).

EBV has been strongly associated with nasopharyngeal carcinoma. In recent years, EBV was found in numerous tumours such as breast (Arbach et al. 2006; Mezher et al. 2017), cervix (de Lima et al. 2018), gastric (Rugge et al. 2015), or gastrointestinal tract (Mirzaei et al. 2018), kidney (Bamoulid et al. 2017; Creager et al. 1998), lung (Wang et al. 2016), skin (Litvinov et al. 2016), and thyroid (Stamatiou et al. 2016). However, the exact role of the virus in the development of cancer remains uncertain. Although several reports have suggested the association of EBV with OSCC, the exact contribution of EBV to the development of oral carcinogenesis is still unclear (Kumar et al. 2016; Sand & Jalouli 2014).

The present study aims to investigate the correlation between past EBV infections with OSCC. Indirect Enzyme-Linked Immunosorbent Assay (ELISA) was applied to detect the presence of IgG and IgM antibodies against EBV VCA in OSCC sera.

Materials and Methods

Sample Collection

A total of 206 OSCC sera and 134 sera from non-OSCC individuals (control group) were obtained from the Malaysian Oral Cancer Database & Tissue Bank System (MOCDTBS) (Rosnah et al. 2013) coordinated by the Oral Cancer Research & Coordinating Centre (OCRCC), Faculty of Dentistry, University of Malaya. Approval for this study was obtained from the Medical Ethics Committee of Faculty of Dentistry, University of Malaya (ref no: DF DR1307/0077(U)).

Detection of Antibodies Against EBV VCA

VCA of EBV IgG and IgM antibodies in patient sera were detected using EBV VCA IgG and IgM ELISA kits based on the manufacturer's protocols (Diagnostic Automation Inc, CA, USA). These ELISA micro-well strips were pre-sensitized with EBV antigen by passive absorption. All sera samples were diluted to obtain a uniform concentration using the provided sample diluents. The sample diluents for EBV VCA IgM ELISA contains antihuman IgG. This anti-human IgG precipitated and removed IgG and rheumatoid factor leaving only IgM in the samples to react with the immobilized antigen. The diluted sera samples were then added to the strips and incubated for 25 min at 25°C. After thorough washing, Peroxidase Conjugates goat anti-human IgG (γ chain specific)/ IgM (μ chain specific) was added in and the strips were further incubated for 25 min at 25°C. The wells were subsequently washed and 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was pipetted into each well and incubated for 15 min at 25°C. The peroxidase substrate solution was added to terminate the enzyme-substrate reaction. These changes were then measured using 450 nm using a microplate reader (Tecan Infinite m200 Pro, Tecan Group Ltd., Mannedorf) spectrophotometrically. EBV antibody's (IgG or IgM) valence in the samples was detected through optical density (OD) according to the manufacturer's protocol and was further correlated to the Calibrator. The cut-off level for seropositivity was determined according to the manufacturer's guidelines. The positive and negative controls were provided with the kit.

Statistical Analysis

All statistical analyses in this study were conducted using the Statistical Package for the Social Sciences (SPSS) software version 12.0.1. In addition, to determine whether variables such as EBV IgG/IgM, gender, race and age were predictors of OSCC, logistic regression analysis was conducted. The assumption on independent errors, normality, multicollinearity, nomoscedasticity, and outliers were examined (Kerishnan et al. 2016).

Results and Discussion

EBV belongs to the herpes virus family which infects approximately 90% of the world's adult population (Danielsson et al. 2018; Sand et al. 2002). EBV infection is associated with various cancers such as infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma, oral hairy leukoplakia and OSCC (Macsween & Johannessen 2014; Patel 2014).

EBV infection was reported to be correspondent to the increased risk of OSCC (She et al. 2017). Studies have shown that EBV infected individuals usually carry the virus throughout their whole life without any symptoms and present elevated EBV VCA-IgG (Hanlon et al. 2014; Jenson 2011). Therefore, it had been suggested that the past or the long-term exposure to viruses may contribute to the onset of OSCC (Gupta & Metgud 2013). Similarly, in our previous association study on Human Papillomavirus (HPV) 16 in OSCC, HPV16 IgG antibodies were identified as a significant indicator of OSCC risk factor and further supporting that past exposure to HPV could increase the risk of OSCC (Kerishnan et al. 2016).

To identify the presence of EBV in patients, many of the previous studies applied methods such as *in situ* hybridization and polymerase chain reaction (PCR) to detect viral deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) transcripts (Syrjänen et al. 2011; Yen et al. 2009). However, these methods are expensive, time-consuming and unable to determine past exposure of EBV (Petter et al. 2000; Salehi et al. 2016). Therefore, in the present study, the presence of both IgG and IgM antibodies against EBV were determined using ELISA. Through IgG and IgM screening, the patients' past and current exposure status against EBV were confirmed. The presence of IgM antibodies against the virus directly shows acute or current exposure, whereas, IgG antibodies against virus indicate past exposure to the virus.

Demographic Profile

A total of 206 OSCC patients (cases) and 134 non-OSCC patients (control) representing a mean age of 58.8±14.2 and 33.5±8.6, respectively, were included in this investigation. Based on the socio-demographic profiles of OSCC patient recruited in this study, female (67.0%) Indians (49.5%) were found to be the highest number of patients diagnosed with OSCC (Table 1).

Although the high incidence of oral cancer is generally associated with tobacco smoking, betel quid chewing and alcohol consumption, viruses are also known to play a role in OSCC development (Kumar et al. 2016; Polz-Gruszka et al. 2014). The general causative virus for OSCC includes EBV (Jalouli et al. 2010; Sand & Jalouli 2014).

EBV VCA Serological Analysis

To evaluate the seropositivity of EBV VCA in OSCC (n=206) and control (n=134) patients, EBV VCA ELISA assays were used to detect both EBV VCA IgG and EBV VCA IgM. Based on the analysis, 96.6% of OSCC patients and 97.2% of control were seen positive for EBV VCA IgG, whereas EBV VCA IgM was not detected in both OSCC patients and control (Table 2).

EBV was reported as the most common and widespread human virus with lifelong latent infection (Evans 2013). Most of the normal adults carry EBV throughout their life with no ill effects and these patients usually showed an elevated EBV VCA IgG (Odumad et al. 2011; Riordan et al. 1996). Studies have shown that EBV is able to form a latent infection, of which it remains dormant with low viral expression and minimal cytopathic effects for infection (Sand & Jalouli 2014). Furthermore, since it takes years for cancer to develop, long persistence of EBV infection may likely to contribute to the occurrence of cancer (Farrell 2019). Therefore, our results further support previous studies on the association between EBV and OSCC.

TABLE 1. Socio-demographic profile of OSCC patients and control

	OSCC samples (n=206)		Control samples (n=134)	
	No. of patients	%	No. of patients	%
Gender				
Male	68	33.0	83	61.9
Female	138	67.0	51	38.1
Ethnicity				
Malay	47	22.8	91	67.9
Chinese	35	17.0	30	22.4
Indian	102	49.5	12	9.0
Others	22	10.7	1	0.7
Age (mean ± SD)	206	58.8± 14.2	134	33.5± 8.6

TABLE 2. Percentage of distribution for EBV VCA IgG/IgM antibodies among the OSCC patients and control

	OSCC samples (*n*=206)		Control samples (*n*=134)	
	No. of patients	%	No. of patients	%
EBV VCA IgG				
Positive	199	96.6	130	97.2
Negative	7	3.4	4	2.8
EBV VCA IgM				
Positive	0	0	0	0
Negative	206	100	134	100

TABLE 3. Logistic regression analyses in predicting the risk factors in OSCC

	B	SE	Odd Ratio	P-value	95% C.I for Lower	95% C.I for Upper
EBV VCA IgG	-0.14	1.11	0.87	0.90	0.10	7.67
Gender (Female)	1.39	0.47	4.01**	0.00	1.59	10.07
Race (Indian)						
(Indian vs Malay)	-2.26	072	0.10**	0.00	0.03	0.43
(Indian vs Chinese)	-1.71	0.81	0.18*	0.03	0.04	0.88
(Indian vs Others)	1.11	1.71	3.04	0.52	0.11	86.71
Age	0.14	0.02	1.15**	0.00	1.10	1.19
Constant	-6.55	1.69	0.00	0.00		

Note: $R^2 = 0.561$ (Cox and Snell), 0.774 (Nagalkerke). Model χ^2 (7) = 254.3, $p<0.001$. *$p<0.05$, **$p<0.01$.

* several data's were obtained from Kerishnan et al. (2016)

On the contrary, none of the OSCC and control was found to be positive for EBV VCA IgM. These results demonstrate that both OSCC patient and control exhibit past EBV infection in early life and therefore seropositivity was only observed in EBV VCA IgG antibodies and not in EBV VCA IgM. The association between viral IgM and OSCC was not well-defined due to the fact that the onset of carcinogenesis by viral infection is influenced by the past infection or the latency of a virus (de Oliveira et al. 2016; Sand & Jalouli 2014).

RISK INDICATOR OF OSCC

To assess whether a certain variable could significantly predict the risk of OSCC, a logistic regression analysis was used. Based on these analyses, independent variables such as gender (female), and race (Indian) and age were identified as a significant contributor in predicting the risk of OCSS (Table 3). However, EBV was not found significant compared with the other variables further suggesting that past EBV infection may not play a significant role as a risk indicator for OSCC.

CONCLUSION

The association of EBV with the development of OSCC has been extensively studied in the past. Even though this association has been widely reported previously (Sand et al. 2014), no safe conclusion was drawn from this (Sand & Jalouli 2014). Similarly, based on our current EBV IgG and IgM results, seropositivity was found in both OSCC and control sample. Therefore, the association between EBV and OSCC was not well demonstrated in this study, further suggesting that past EBV infection does not play a significant role as a risk indicator for OSCC.

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