Original Article

Analysis of Epstein–Barr Virus Infection in Oral Potentially Malignant Disorders and Oral Cancer: A Cross-Sectional Study

Jintana Pankam[1], Puangwan Lapthanasupkul[2], Nakarin Kitkumthorn[3], Dulyapong Rungraungrayabkul[4], Boworn Klongnoi[1,5], Siribang-on Piboonniyom Khovidhunkit[1,6]

[1]Development of Disease Management Model for Oral Cancer with an Integration Network of Screening, Surveillance, and Treatment from Primary Care Unit to Tertiary Care in Nakhon Ratchasima Province Project, Faculty of Dentistry, Mahidol University, Departments of [2]Oral and Maxillofacial Pathology, [3]Oral Biology, [4]Oral Medicine and Periodontology, [5]Oral and Maxillofacial Surgery, [6]Advanced General Dentistry, Faculty of Dentistry, Mahidol University, Bangkok, Thailand

Received : 15-12-22
Revised : 16-03-23
Accepted : 26-03-23
Published : 29-06-23

ABSTRACT

Aims and Objectives: The primary objective of this study is to determine the prevalence of Epstein–Barr virus (EBV) in oral potentially malignant disorders (OPMDs) and oral cancer (OC) in a group of Thais using polymerase chain reaction (PCR) and Epstein–Barr encoding regions (*EBERs*) *in situ* hybridization (ISH). The secondary objective is to investigate the risk factors of OC and the association between the presence of EBV and risk factors of OC/site of oral lesions. **Materials and Methods:** Sixty-one participants attending the screening project for OC and OPMDs at the Northeastern district hospitals of Thailand were recruited. Information related to risk factors and biopsy tissues for histopathological diagnosis was collected. Sixty-seven paraffin tissue blocks, including 52 OPMDs and 15 OC specimens, were investigated for EBV infection, using PCR analysis with latent membrane protein-1 (*LMP-1*) primer and *EBERs* ISH. Pearson's Chi-square or Fisher's exact test was used to analyze the differences in variables between participants with OPMDs and OC, as appropriate. The association between EBV infection and related risk factors was analyzed using logistic regression with a significant level at 0.05. **Results:** Using PCR analysis, 8 of 67 specimens (11.94%) were positive for *LMP-1*. Three cases of OPMDs were positive for both *LMP-1* PCR and *EBERs* ISH. Regarding risk factors of OC, the two most common risk factors were betel nut chewing (52.46%) and working in sunlight (42.62%). The habit of taking alcohol was significantly different between the OC and the OPMDs groups ($p = 0.009$). The association between *LMP-1* and the lesion at the tongue was statistically significant, with odds ratio = 4.900 (95% confidence interval = 1.046–22.943; $p = 0.044$). **Conclusions:** The prevalence of EBV infection in this group of participants was low. However, OPMDs at the tongue exhibited a significant association with EBV infection.

Keywords: *Epstein–Barr virus, LMP-1, EBERs, oral cancer, oral potentially malignant disorders, risk factor*

Introduction

According to a recent report from the National Cancer Institute of Thailand 2020, the incidence of oral cancer (OC) has increased over the past decade. In Thai males, OC is the sixth most common cancer, whereas it is the tenth most common cancer in Thai females.[1] Oral potentially malignant disorders (OPMDs) are mucosal lesions that carry a significant

Access this article online

Quick Response Code:

Website: https://journals.lww.com/jpcd

DOI: 10.4103/jispcd.JISPCD_235_22

Address for correspondence: Dr. Siribang-on Piboonniyom Khovidhunkit,
Department of Advanced General Dentistry,
Faculty of Dentistry, Mahidol University,
6 Yothi Rd. Rajthewee, Bangkok 10400, Thailand.
E-mail: siribangon.pib@mahidol.edu

How to cite this article: Pankam J, Lapthanasupkul P, Kitkumthorn N, Rungraungrayabkul D, Klongnoi B, Khovidhunkit SP. Analysis of Epstein–Barr virus infection in oral potentially malignant disorders and oral cancer: A cross-sectional study. J Int Soc Prevent Communit Dent 2023;13:221-8.

potential for subsequent progression to OC.[2] OPMDs include leukoplakia, erythroplakia, erythroleukoplakia, oral submucous fibrosis, palatal lesions in reverse smokers, oral lichen planus, oral lichenoid reactions, graft versus host disease, oral lupus erythematosus, actinic cheilitis, and some hereditary conditions such as dyskeratosis congenita and epidermolysis bullosa.[2] The overall malignant transformation (MT) rate of OPMDs is 7.9%, indicating the seriousness of the problem.[3] The major risk factors contributing to OPMDs and OC are well established, including tobacco use, betel quid chewing, and alcoholism.[4]

Besides these lifestyle habits, viral infection has also been documented as a risk factor for OC.[5] Epstein–Barr virus (EBV), a double-stranded DNA virus of about 170 kb, is one of the most common viruses that cause several human diseases. EBV infection is ubiquitous, and people are usually infected in their childhood or early adolescence.[6] EBV-determined nuclear antigen-1 (*EBNA-1*) is required for self-replication of the virus, whereas *EBNA-2* and latent membrane protein-1 (*LMP-1*) are related to the immortalization of infected cells *in vitro*.[7] The oral cavity and pharyngolarynx are the main portals through which environmental pathogens enter the human body. Although EBV has been known to infect B cells of the pharyngeal tonsils via saliva, it also infects epithelial cells, leading to several epithelial malignancies such as nasopharyngeal carcinoma (NPC), gastric carcinoma, and breast carcinoma.[8] A recent study demonstrated EBV infection in OPMDs and normal mucosal samples, suggesting that the oral cavity is an environment favoring latent EBV infection.[9] In addition, several reports of EBV-related oral squamous cell carcinoma (OSCC) have been published worldwide.[10-12] Still, very few studies on the association between OC/OPMDs and EBV infection have been conducted in Thailand.

The primary objective of the study was to determine the prevalence of EBV infection in OPMDs and OC in a group of Northeastern Thais. The secondary objective was to identify the risk factors of OPMDs and OC and the association between the presence of EBV and the risk factors of OC. In addition, the comparison between *LMP-1* and Epstein–Barr encoding regions (*EBERs*) expression in OC and OPMDs was also performed.

Materials and Methods

The study protocol was approved by the committee on the human right to human experimentation (COA.NO.MU-DT/PY-IRB 2019/050.3107, COA. NO.MU-DT/PY-IRB 2020/058.2909, and COA. NO.MU-DT/PY-IRB 2019/041.0307). The ethical guidelines of the Declaration of Helsinki were followed in this study. All details about the volunteers and their identities were anonymous. Each subject was given verbal and written information about the nature of the research, and written informed consent was signed. Inclusion criteria were participants 40 years old and older, having OPMDs or OC. Moreover, information related to documented risk factors of OC, such as tobacco smoking, use of smokeless tobacco, being secondhand smokers, alcohol consumption, betel nut chewing habit, working in sunlight for an extended time, and a history of head and neck cancer was also collected. The participants who had other lesions or missing risk factor data were excluded from the study.

The sample size calculation was conducted using an infinite population proportion formula with the prevalence from Reddy *et al*.[13] Sixty-seven formalin-fixed paraffin-embedded (FFPE) tissue blocks from 61 participants were retrieved from the screening project for OPMDs and OC at district hospitals in the Northeastern area of Thailand. Board-certified oral and maxillofacial pathologists have done the histopathological diagnosis of these lesions. These FFPE tissue blocks included 9 hyperkeratosis, 16 mild epithelial dysplasia, 9 moderate epithelial dysplasia, 8 severe epithelial dysplasia, 12 OSCC, 10 oral lichen planus/oral lichenoid reaction, and 3 verrucous carcinoma specimens.

In situ hybridization

Biopsy specimens were fixed in formalin, then dehydrated, embedded in paraffin, and sectioned by a routine histopathological method. To examine the presence of the EBV genome in the specimens, *EBER-in situ* hybridization (ISH) was performed on an automated stainer (Benchmark XT; Ventana Medical Systems, Inc., Tucson, Arizona) using the INFORM *EBER* probe (Ventana Medical Systems, Inc.). Visualization was performed using the ISH iView Blue Detection Kit with ISH Protease 2, and Nuclear Fast Red (Ventana Medical Systems, Inc.) was used as contrast. An NPC specimen was used as a positive control.

DNA extraction

First, the FFPE blocks of desired samples were sectioned to obtain tissue sections of about 3–4 ribbons with 10-µm thickness using a microtome (LEICA RM 2255) and collected in a 1.5-mL sterile tube. DNA extraction was subsequently done by QIAamp DNA FFPE Tissue (QIAgen, Hilden, Germany) according to the manufacturer's protocol. The DNA concentration was measured using a NanoDrop 2000 spectrophotometer (ND-1000 Spectrophotometer,

NanoDrop Technologies, Wilmington, Delaware), and the optical density ratio $260_{OD}/280_{OD}$ greater than 1.8 was used as the accepted ratio for DNA purity and polymerase chain reaction (PCR). The processed DNA was kept at −20 °C until further use.

Polymerase chain reaction

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified for the quality control of DNA. The PCR mixture for *GAPDH* primers contained 0.1 µL of each 20-µM primer, 1 µL of 10× buffer, 0.8 µL of 25 mM $MgCl_2$, 1 µL of 50 ng/µL of the investigated DNA, 0.2 µL of 10-mM deoxynucleotide triphosphates, 0.5 unit of *Taq* DNA polymerase (Thermo Scientific™, Vilnius, Lithuania), and sterile distilled water to the total volume of 10 µL. All samples positive for *GAPDH* were then subjected to the PCR for EBV investigation. The PCR mixture for *LMP-1* primers to detect EBV contained 25-µM primers; other components were similar to those used for the *GAPDH* amplification. The PCR condition of each primer set was carried out according to the study by Wanvimonsuk *et al*.[14] All PCR products were subjected to 1% agarose gel electrophoresis. The gel was stained with Intron redsafe™ and analyzed under ultraviolet transillumination (Geldoc, Bio-Rad®, Hercules, California). PCR analysis was conducted in triplicate in EBV-positive samples to confirm the results. DNA extracted from NPC and deionized sterile water were the positive and the negative controls, respectively.

Statistical analyses

Statistical analysis was performed using IBM SPSS for Windows, version 27.0 (IBM Corp., Armonk, New York). The association between EBV infection and related risk factors was analyzed using Pearson's Chi-square, Fisher's exact test and logistic regression with a significant level at 0.05. The relationship between ISH and PCR was analyzed by diagnostic test.

Results

The demographic and risk behavior data are shown in Table 1. All participants were older than 40 years old, with a mean age of 69.03 ± 10.68 years at diagnosis. Most participants were in the 6th–7th decade of life and mainly were female (73.77%). All participants presented with at least one risk factor. Betel nut chewing (52.46%) was the most common risk factor among the participants, followed by working in sunlight (42.62%) and being a second-hand smoker (29.51%). Alcohol consumption (58.33%) was the risk factor most frequently seen in the OC group, whereas in the OPMDs group, betel nut chewing (55.10%) was the risk factor most commonly observed [Table 1]. Statistical analysis revealed that alcohol drinking habit in the OPMDs group was significantly different from that in the OC group ($p = 0.009$) [Table 1].

Amplification for latent membrane protein-1 by polymerase chain reaction

GAPDH was detected in all of the DNA samples (100%) for the DNA quality test. Out of 67 DNA samples, eight cases (11.94%) were positive for EBV. Figure 1 shows PCR amplification of *GAPDH* (150-bp DNA) and *LMP-1* (129 bp). Regarding the risk factors for OC, participants with smoking habits exhibited the highest percentage (21.43%) of EBV expression [Table 2]. Half of the positive EBV samples were excised from the tongue. Six of the positive samples were mild epithelial dysplasia, and the remaining two were oral lichen planus/oral lichenoid reaction and hyperkeratosis [Table 2].

EBERs expression by *in situ* hybridization

Of the 67 OPMDs and OC samples subjected to ISH, three cases (4.48%) were positive for *EBERs* [Table 2]. The highest prevalence of *EBERs* expression was observed in smokers (14.29%). Interestingly, all EBV-positive samples were obtained from the tongue. The histopathological diagnosis of the three positive samples constituted two cases of mild epithelial dysplasia and a case of hyperkeratosis [Table 2]. In both mild epithelial dysplasia and hyperkeratosis, nuclear positivity for *EBERs* was observed in the superficial layer of the epithelium. Representative pictures of positive samples for *EBERs* are shown in Figure 2.

Comparison between latent membrane protein-1 and Epstein–Barr encoding regions expression

Among eight positive PCR cases, three cases were also positive for *EBERs*. Only the samples from the tongue were positive for both *LMP-1* and *EBERs*. Most of the EBV-positive specimens, either by PCR (six of eight cases) or ISH (two of three cases), were diagnosed as mild epithelial dysplasia [Table 2]. Furthermore, the diagnostic test demonstrated that the PCR technique exhibited 100% sensitivity and 92.31% specificity with a positive predictive value of 37.50% and a negative predictive value of 100%.

Associations of latent membrane protein-1 and risk factors/site of lesions

The associations of *LMP-1* and risk factors/site of lesions were analyzed by logistic regression [Table 3]. No statistically significant association between risk factors and the presence of *LMP-1* was revealed [Table 3]. However, a statistically significant association between the lesions from the tongue and the presence of *LMP-1* was observed (odds ratio [OR] = 4.900; 95% confidence interval [CI] = 1.046–22.943; $p = 0.044$).

Table 1: Demographic and risk behaviors of participants with OPMDs and OC

Characteristics	Total cases (n = 61) n (%)	OPMDs (n = 49) n (%)	OC (n = 12) n (%)	p value[a]
Male	16 (26.23)	10 (20.41)	6 (50.00)	0.063
Female	45 (73.77)	39 (79.59)	6 (50.00)	
Age (year)				0.467
40–59	9 (14.75)	7 (14.29)	2 (16.67)	
60–79	43 (70.49)	36 (73.47)	7 (58.33)	
≥80	9 (14.75)	6 (12.24)	3 (25.00)	
Mean ± SD	69.03 ± 10.68	68.08 ± 10.13	72.92 ± 12.42	
Range	43–94	43–91	54–94	
Tobacco smoking				0.124
Nonsmoker	47 (77.05)	40 (81.63)	7 (58.33)	
Smoker	14 (22.95)	9 (18.37)	5 (41.67)	
Use of smokeless tobacco				>0.999
Nonsmoker	42 (68.85)	35 (71.43)	7 (58.33)	
Smoker	16 (26.23)	14 (28.57)	2 (16.67)	
N/A	3 (4.92)	0 (0.00)	3 (25.00)	
Being secondhand smoker				>0.999
No	37 (60.65)	31 (63.27)	6 (50.00)	
Yes	18 (29.51)	15 (30.61)	3 (25.00)	
N/A	6 (9.84)	3 (6.12)	3 (25.00)	
Alcohol drinking				0.009
Nondrinker	45 (73.77)	40 (81.63)	5 (41.67)	
Drinker	16 (26.23)	9 (18.37)	7 (58.33)	
Betel nut chewing				0.404
Nonchewer	29 (47.54)	22 (44.90)	7 (58.33)	
Chewer	32 (52.46)	27 (55.10)	5 (41.67)	
Working in sunlight				0.468
No	35 (57.38)	27 (55.10)	8 (66.67)	
Yes	26 (42.62)	22 (44.90)	4 (33.33)	
History of head and neck cancer				0.554
No	57 (93.44)	46 (93.88)	11 (91.67)	
Yes	3 (4.92)	2 (4.08)	1 (8.33)	
N/A	1 (1.64)	1 (2.04)	0 (0.00)	

OC = oral cancer, OPMDs = oral potentially malignant disorders, SD = standard deviation

[a]p value from Pearson's Chi-square or Fisher's exact test

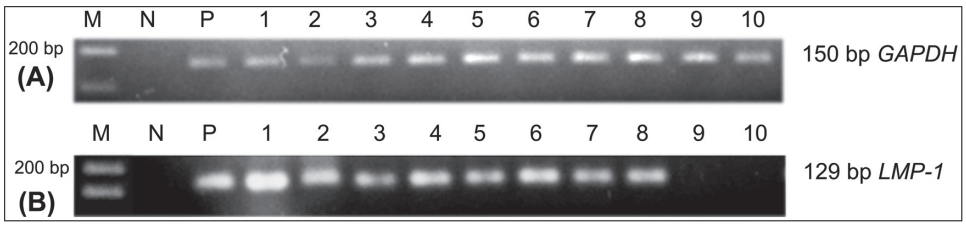

Figure 1: PCR amplification of *GAPDH* (A) and *LMP-1* (B) genes: Lane M: 100 bp DNA ladder, N: Negative control (dH_2O), P: Positive control (NPC), Lanes 1–6: Positive samples diagnosed with mild epithelial dysplasia, Lane 7: Positive sample diagnosed with oral lichen planus/oral lichenoid reaction, Lane 8: Positive sample diagnosed with hyperkeratosis, and Lanes 9–10: Negative EBV samples diagnosed with severe epithelial dysplasia and OSCC, respectively. EBV = Epstein–Barr virus, *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase, *LMP-1* = latent membrane protein-1, NPC = nasopharyngeal carcinoma, OSCC = oral squamous cell carcinoma, PCR = polymerase chain reaction

Discussion

According to PCR analysis, the prevalence of EBV DNA in OPMDs and OC was 11.94% (8/67), with the highest prevalence in the mild epithelial dysplasia samples (75%). However, only two cases of mild epithelial dysplasia and one case of hyperkeratosis showed positivity for *EBERs* [Table 2]. Most of the previous studies in Thailand focused on the presence of EBV in OSCC rather than OPMDs. For example, a study in

Northern Thailand by Iamaroon *et al.*[15] demonstrated a lack of *EBERs* expression in OSCC. Consistently, we could not detect EBV DNA in any of the OSCC cases by PCR analysis. In contrast, Acharya *et al.*[16] detected EBV DNA in 45.05% of OSCC cases, and 18.08% of controls from oral exfoliated cells retrieved from OSCC lesions or normal oral mucosa, respectively. In contrast, a study by Rahman *et al.*[9] in Central Thailand using

Table 2: Risk factors/clinicopathologic characteristics and EBV infection

Characteristic	*n* (%)	Positive PCR *n* (%)	Positive ISH *n* (%)
Oral cancer risk factor (*n* = 61 participants)			
Tobacco smoking	14 (22.95)	3 (21.43)	2 (14.29)
Being secondhand smoker	18 (29.51)	3 (16.67)	1 (5.59)
Use of smokeless tobacco	16 (26.23)	3 (18.78)	1 (6.25)
Alcohol drinking	16 (26.23)	1 (6.25)	1 (6.25)
Betel nut chewing	32 (52.46)	3 (9.38)	1 (3.13)
Working in sunlight	26 (42.62)	5 (19.23)	2 (7.69)
History of head and neck cancer	3 (4.92)	0	0
Site of lesions (*n* = 67 specimens)			
Buccal mucosa	28 (41.79)	2 (7.14)	0
Tongue	14 (20.90)	4 (28.57)	3 (21.43)
Lip	11 (16.42)	1 (9.09)	0
Other sites	14 (20.90)	1 (7.14)	0
Histopathological diagnosis (*n* = 67 specimens)			
Hyperkeratosis	9 (13.43)	1 (11.11)	1 (11.11)
Mild epithelial dysplasia	16 (23.88)	6 (37.50)	2 (12.50)
Moderate epithelial dysplasia	9 (13.43)	0	0
Severe epithelial dysplasia	8 (11.94)	0	0
Squamous cell carcinoma	12 (17.91)	0	0
Oral lichen planus/oral lichenoid reaction	10 (14.93)	1 (10.00)	0
Verrucous carcinoma	3 (4.48)	0	0

EBV = Epstein–Barr virus, ISH = *in situ* hybridization, PCR = polymerase chain reaction

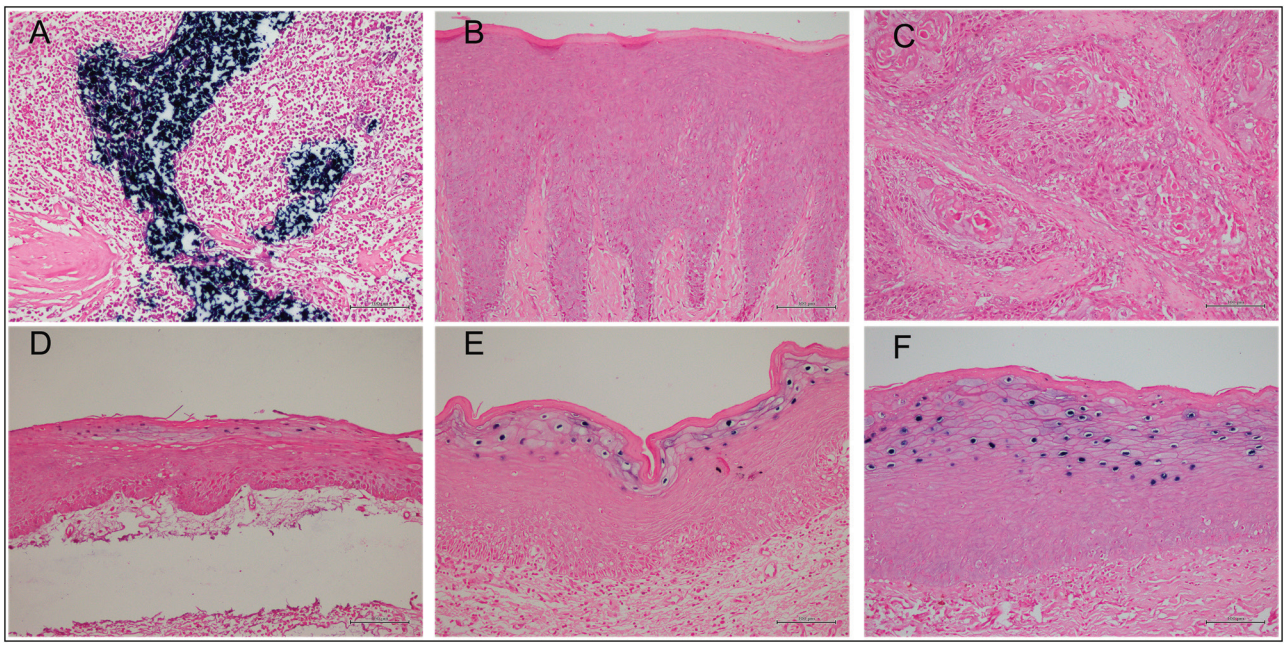

Figure 2: *EBERs* expression (original magnification, 200X): (A) Positive control: NPC, (B) Negative control from normal oral mucosa, (C) Representative of negative *EBERs* from OC: OSCC, (D–F) Representative pictures of positive samples diagnosed with mild epithelial dysplasia (D and E) and hyperkeratosis (E). *EBERs* = Epstein–Barr encoding regions, NPC = nasopharyngeal carcinoma, OC = oral cancer, OSCC = oral squamous cell carcinoma

Table 3: Association of risk factors/site of lesions and EBV PCR positivity

Risk factors (n = 61)	OR adj. (95% CI)	p value[a]
Tobacco smoking (yes vs. no)	17.341 (0.795–378.054)	0.070
Secondhand smoker (yes vs. no)	0.45 (0.040–5.046)	0.517
Alcohol drinking (yes vs. no)	0.099 (0.005–2.028)	0.133
Betel nut chewing (yes vs. no)	1.760 (0.129–23.965)	0.671
Working in sunlight (yes vs. no)	1.917 (0.174–21.125)	0.595
Site of lesions (n = 67)	OR (95% CI)	p value[b]
Tongue vs. nontongue	4.900 (1.046–22.943)	0.044

CI = confidence interval, EBV = Epstein–Barr virus, OR = odds ratio, PCR = polymerase chain reaction

[a]p value from multivariable logistic regression

[b]p value from univariable logistic regression

immunohistochemistry revealed that all (100%) of the biopsy specimens, including 10 normal oral mucosa, 69 oral leukoplakia with or without epithelial dysplasia, and 36 OSCC expressed *LMP-1* protein. Moreover, an increasing trend in percentages of *LMP-1* protein expression was observed among normal mucosa (26.36%), oral leukoplakia without dysplasia (28.03%), oral leukoplakia with dysplasia (34.15%), and OSCC (59.67%).[9] The diversity in EBV prevalence may result from various factors, such as geographical differences, sample collection techniques, sample preparation, and EBV detection methods.

Of the eight EBV DNA-positive OPMDs cases, only 37.5% (3/8) expressed *EBERs* within the nuclei in the epithelium, indicating the subcellular localization of latent EBV infection.[17] A comparison of the EBV detection method between PCR and ISH revealed that PCR showed a high sensitivity of 100% and specificity of 92.31% for EBV detection. PCR analysis and ISH possess both advantages and disadvantages. The PCR technique is a highly sensitive method for EBV DNA detection.[18] A study conducted in China compared three methods for detecting EBV in FFPE tissues of Hodgkin's lymphoma.[18] The PCR technique exhibited the highest rate of detection (74.6%), followed by ISH for *EBER-1* RNA (67.8%) and immunohistochemistry for *LMP-1* protein (66.1%). In addition, the PCR technique is less expensive and more approachable than the ISH technique. We used primers to detect the *LMP-1* gene of EBV in FFPE tissues. However, this method cannot determine the subcellular localization of EBV within the lesion or distinguish between EBV-driven diseases and bystander EBV infection. *EBERs* RNA is expressed in all latency stages of EBV-infected cells.[19] Hence, *EBERs* expression, considered a standard technique for EBV detection in human specimens, might solve this drawback.[20]

Among EBV-positive OPMDs cases, mild epithelial dysplasia (six of eight) was the most common lesion being positive for EBV, followed by hyperkeratosis (one of eight) and oral lichen planus/oral lichenoid reaction (one of eight). Our findings were consistent with a previous study, in which *EBER-1* RNA was frequently detected in oral epithelial dysplasia: 62.5% in mild dysplasia and 38.5% in severe dysplasia.[21] Kikuchi *et al*.[11] also demonstrated higher expression of *EBERs* in severe epithelial dysplasia (94.4%) compared with OSCC (34.7%). These findings suggested that the degree of epithelial dysplasia might not be the factor influencing EBV infection.[11]

The second objective of this study was to determine the risk factors of OPMDs and OC in these participants. Betel nut chewing habit (52.46%) and working in sunlight (42.62%) were the two most common risk factors among them [Table 1]. Betel nut chewing is frequently associated with the occurrence of OPMDs,[22] whereas sunlight exposure is strongly related to lip cancer.[23] In our study, most participants were farmers; therefore, they had a long duration and intense sunlight exposure, which could induce OPMDs or OC of the lip. Although only about one-third of the participants (26.23%) had the habit of taking alcohol, a significant difference in alcohol-taking habit was observed between the OC and OPMDs. This could be because the number of participants with OC was low compared with that with OPMDs, and more than half of participants with OC consumed alcohol. A previous study in Thailand demonstrated that alcohol consumption and betel quid chewing were significantly associated with OSCC (adjusted OR = 6.15 and 11.34, respectively).[16] Another study investigating the associated risk factors of OPMDs in Northeastern Thailand indicated that betel nut chewing, smoking, and alcohol consumption were strongly associated with an increased risk for OPMDs (adjusted OR = 8.81; OR = 7.53; and OR = 4.57, respectively).[24] These findings and ours suggested that betel nut chewing habit and alcohol consumption are still prominent in Northeastern Thais, and a program to persuade these people to quit these risk factors should be taken into account.

The association between the presence of EBV and risk factors of OC/site of oral lesions was further evaluated. No association between the presence of EBV and risk factors of OC was found. However, the presence of EBV was associated with tongue lesions. A previous study revealed that the predominant oral site for EBV-positive OSCC was the lateral border of

the tongue.[9,25] A study by Shahrabi-Farahani *et al.*[25] demonstrated a relationship between EBV and OSCC in Iranian patients and showed the presence of EBV in 72.3% of the tongue OSCCs. In our study, 50% and 100% of EBV-positive OPMDs detected by PCR and ISH, respectively, were located on the tongue. Additionally, logistic regression analysis suggests that OPMDs at the tongue possessed a high risk of EBV infection compared with other sites (OR = 4.90; 95% CI = 1.046–22.943; p = 0.04). This result raises the notion that latent infection of EBV may be often found at the tongue as observed in oral hairy leukoplakia,[26] an EBV-associated condition usually located at the lateral borders of the tongue.

In this study, various methods were conducted to compare and confirm EBV expression in OPMDs and OC. In addition, the association between OC risk factors and EBV expression also were observed, though many risk factors showed no significant difference with EBV expression by statistical analysis. The limitations of this study include a small number of categories of biopsy specimens. Most of the specimens were mild epithelial dysplasia, whereas there were very few cases of moderate and severe epithelial dysplasias. Moreover, the tissues included in this study were FFPE tissues, in which the qualities of DNA and RNA may be inferior to the fresh tissues. Therefore, more samples with a considerable number of each category of dysplasia would further be recruited. Moreover, our specimens came from only one center; hence, for generalizability, a multicenter study should, therefore, be considered.

Conclusion

In conclusion, our study indicated that the prevalence of EBV infection in this group of participants was low. Most of the positive EBV samples were mild epithelial dysplasia, whereas the presence of EBV was significantly associated with OPMDs lesions of the tongue. No statistically significant association between risk factors and the presence of *LMP-1* was found by logistic regression analysis.

Acknowledgements

The authors would like to thank Mr. Dusit Bumalee, Assistant Professor Dr. Vanvisa Sresumatchai and Associate Professor Dr. Natchalee Srimaneekarn for their advice and support.

Financial support and sponsorship

This study was supported by Mahidol University, Grant/Award number: (052/2562), to Siribang-on Piboonniyom Khovidhunkit, Boworn Klongnoi, and Vanvisa Sresumatchai under the Development of Disease Management Model for Oral Cancer with an Integration Network of Screening, Surveillance, and Treatment from Primary Care Unit to Tertiary Care in Nakhon Ratchasima Province Project.

Conflicts of interest

The authors declare no potential conflicts of interest.

Authors contributions

J.P.: performed experiments, data curation, and wrote original manuscript. P.L.: prepared specimens, performed some parts of the experiments, gave some comments, and reviewed the manuscript writing. N.K.: supplied some materials and gave some comments for the manuscript writing. D.R.: performed some parts of the experiments, data analysis, wrote and reviewed manuscript. B.K.: supplied a part of the research fund and prepared some OSCC specimens and clinical data. S.P.K.: responsible for overall concept, supervised the project, established, reviewed and edited the manuscript, and supplied research fund.

Ethical policy and institution review board statement

The study protocol was approved by the committee on the human right to human experimentation of the Faculty of Dentistry/Faculty of Pharmacy, Mahidol University (COA.NO.MU-DT/PY-IRB 2019/050.3107, COA.NO.MU-DT/PY-IRB 2020/058.2909 and COA.NO.MU-DT/PY-IRB 2019/041.0307).

Patient declaration of consent

Informed consent was obtained from all subjects involved in this study.

Data availability statement

The data presented in this study are available on request from the corresponding author. The data are not publicly available because of ethical restrictions.

List of Abbreviations

DNA: deoxyribonucleic acid
EBERs: Epstein–Barr encoding regions
EBNA-1: EBV-determined nuclear antigen-1
EBV: Epstein–Barr virus
FFPE: formalin-fixed, paraffin-embedded
GAPDH: glyceraldehyde-3-phosphate dehydrogenase
ISH: *in situ* hybridization
LMP-1: latent membrane protein-1
NPC: nasopharyngeal carcinoma
OC: oral cancer
OPMDs: potentially malignant disorders
OSCC: oral squamous cell carcinoma
OSF: oral submucous fibrosis
PCR: polymerase chain reaction

References

1. Insamran W, Sangrajrang S. National cancer control program of Thailand. Asian Pac J Cancer Prev 2020;21:577-82.
2. Warnakulasuriya S. Clinical features and presentation of oral potentially malignant disorders. Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:582-90.
3. Iocca O, Sollecito TP, Alawi F, Weinstein GS, Newman JG, De Virgilio A, *et al*. Potentially malignant disorders of the oral cavity and oral dysplasia: A systematic review and meta-analysis of malignant transformation rate by subtype. Head Neck 2020;42:539-55.
4. Siewchaisakul P, Wang ST, Peng SM, Sarakarn P, Chen LS, Chen TH, *et al*. Effect of metabolic syndrome on incidence of oral potentially malignant disorder: A prospective cohort study in Taiwan. BMJ Open 2020;10:e041971.
5. Foy JP, Bertolus C, Boutolleau D, Agut H, Gessain A, Herceg Z, *et al*. Arguments to support a viral origin of oral squamous cell carcinoma in non-smoker and non-drinker patients. Front Oncol 2020;10:822.
6. Cui X, Snapper CM. Epstein Barr virus: Development of vaccines and immune cell therapy for EBV-associated diseases. Front Immunol 2021;12:734471.
7. El-Sharkawy A, Al Zaidan L, Malki A. Epstein-Barr virus-associated malignancies: Roles of viral oncoproteins in carcinogenesis. Front Oncol 2018;8:265.
8. Farrell PJ, White RE. Do Epstein-Barr virus mutations and natural genome sequence variations contribute to disease? Biomolecules 2021;12:17.
9. Rahman R, Poomsawat S, Juengsomjit R, Buajeeb W. Overexpression of Epstein-Barr virus-encoded latent membrane protein-1 (LMP-1) in oral squamous cell carcinoma. BMC Oral Health 2019;19:142.
10. Vanshika S, Preeti A, Sumaira Q, Vijay K, Shikha T, Shivanjali R, *et al*. Incidence of HPV and EBV in oral cancer and their clinico-pathological correlation—A pilot study of 108 cases. J Oral Biol Craniofac Res 2021;11:180-4.
11. Kikuchi K, Noguchi Y, de Rivera MW, Hoshino M, Sakashita H, Yamada T, *et al*. Detection of Epstein-Barr virus genome and latent infection gene expression in normal epithelia, epithelial dysplasia, and squamous cell carcinoma of the oral cavity. Tumour Biol 2016;37:3389-404.
12. Naqvi SU, Khan S, Ahmed A, Lail A, Gul S, Ahmed S. Prevalence of EBV, CMV, and HPV in oral squamous cell carcinoma patients in the Pakistani population. J Med Virol 2020;92:3880-3.
13. Reddy SS, Sharma S, Mysorekar V. Expression of Epstein-Barr virus among oral potentially malignant disorders and oral squamous cell carcinomas in the south Indian tobacco-chewing population. J Oral Pathol Med 2017;46:454-9.
14. Wanvimonsuk S, Thitiwanichpiwong P, Keelawat S, Mutirangura A, Kitkumthorn N. Distribution of the Epstein-Barr virus in the normal stomach and gastric lesions in Thai population. J Med Virol 2019;91:444-9.
15. Iamaroon A, Pattanaporn K, Pongsiriwet S, Wanachantararak S, Prapayasatok S, Jittidecharaks S, *et al*. Analysis of 587 cases of oral squamous cell carcinoma in northern Thailand with a focus on young people. Int J Oral Maxillofac Surg 2004;33:84-8.
16. Acharya S, Ekalaksananan T, Vatanasapt P, Loyha K, Phusingha P, Promthet S, *et al*. Association of Epstein-Barr virus infection with oral squamous cell carcinoma in a case-control study. J Oral Pathol Med 2015;44:252-7.
17. AbuSalah MAH, Gan SH, Al-Hatamleh MAI, Irekeola AA, Shueb RH, Yean Yean C. Recent advances in diagnostic approaches for Epstein-Barr virus. Pathogens 2020;9:226.
18. Qi ZL, Han XQ, Hu J, Wang GH, Gao JW, Wang X, *et al*. Comparison of three methods for the detection of Epstein-Barr virus in Hodgkin's lymphoma in paraffin-embedded tissues. Mol Med Rep 2013;7:89-92.
19. Herbert KM, Pimienta G. Consideration of Epstein-Barr virus-encoded noncoding RNAs EBER1 and EBER2 as a functional backup of viral oncoprotein latent membrane protein 1. mBio 2016;7:e01926-15.
20. Tonoyan L, Chevalier M, Vincent-Bugnas S, Marsault R, Doglio A. Detection of Epstein-Barr virus in periodontitis: A review of methodological approaches. Microorganisms 2020;9:72.
21. Jiang R, Gu X, Moore-Medlin TN, Nathan CA, Hutt-Fletcher LM. Oral dysplasia and squamous cell carcinoma: Correlation between increased expression of CD21, Epstein-Barr virus and CK19. Oral Oncol 2012;48:836-41.
22. Klongnoi B, Sresumatchai V, Clypuing H, Wisutthajaree A, Pankam J, Srimaneekarn N, *et al*. Histopathological and risk factor analyses of oral potentially malignant disorders and oral cancer in a proactive screening in northeastern Thailand. BMC Oral Health 2022;22:613.
23. Ugelvig Petersen K, Volk J, Kaerlev L, Lyngbeck Hansen H, Hansen J. Cancer incidence among merchant seafarers: An extended follow-up of a Danish cohort. Occup Environ Med 2018;75:582-5.
24. Juntanong N, Siewchaisakul P, Bradshaw P, Vatanasapt P, Chen SL, Yen AM, *et al*. Prevalence and factors associated with oral pre-malignant lesions in northeast Thailand. Asian Pac J Cancer Prev 2016;17:4175-9.
25. Shahrabi-Farahani M, Karimi E, Mostaan LV, Saba S, Yazdani N, Amoli MM. Association between Epstein-Barr virus and tongue squamous cell carcinoma in Iranian patients. Pathol Res Pract 2018;214:130-3.
26. Almazyad A, Alabdulaaly L, Noonan V, Woo SB. Oral hairy leukoplakia: A series of 45 cases in immunocompetent patients. Oral Surg Oral Med Oral Pathol Oral Radiol 2021;132:210-6.