Prevalence of Epstein-Barr virus (EBV) in Oral Exfoliated Cells: A Pilot Prospective Study of Oral Squamous Cell Carcinoma

Sulav Acharya[*1], Pensiri Phusingha[1], Patravoot Vatanasapt[2], Supannee Promthet[3], Tipaya Eklaksananan[1], Chamsai Pientong[1]

[1]Department of Microbiology, Faculty of Medicine, Khon Kaen University, Thailand

[2]*Department of Otorhinolaryngology, Faculty of Medicine, Khon Kaen University*, Thailand

[3]*Department* of Epidemiology, *Faculty of Public* Health, Khon Kaen University, Thailand

Background and Objectives: Oral squamous cell carcinoma (OSCC) is a subtype of head and neck squamous cell carcinoma (HNSCC) that accounts for more than 90% of HNSCC. Smoking, chewing betel quid and alcohol consumption has been reported as major risk factors for OSCC. Epstein-Barr virus (EBV), an oncogenic DNA virus is responsible for causing different types of cancer such as Burkitt's lymphoma, gastric carcinoma and nasopharyngeal carcinoma. To explore the association of EBV with OSCC, this study investigated the prevalence of EBV infection in oral exfoliated cells of case-control study of OSCC.

Methods: Oral exfoliated cells collected from OSCC cases and controls with normal healthy mucosa were extracted for DNA and qualified by polymerase chain reaction (PCR) using housekeeping gene primers. The qualified DNA was detected for EBV-DNA using PCR. The PCR product from EBV positive cases were confirmed by dot blot hybridization using EBV specific probe and nested PCR using specific primers.

Results: Tongue was the most common site of these OSCC cases. The prevalence of EBV in OSCC cases (44%) was statistically significant different from control (19%). According to anatomical site of lesion among OSCC cases, prevalence of EBV was highest in gum cancer cases (85.7%).

Conclusions: This result demonstrates the association of EBV with OSCC and suggests that EBV may be an etiological risk factor for OSCC. The mechanism behind the etiologic role of EBV in OSCC must be further studied.

Key words: Epstein-Barr virus, Oral squamous cell carcinoma

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Introduction

The malignancy of oral cavity originated from squamous cell is named as oral squamous cell carcinoma (OSCC), accounts for more than 90% of head and neck squamous cell carcinoma (HNSCC). OSCC is the sixth most common cancer with the high morbidity and mortality annually worldwide. The overall and disease-free survival rates are 56% and 58%, respectively[1].

Smoking, betel quid chewing and alcohol consumption have been reported as major risk factors for OSCC. Besides these well known risk factors, biological oncogenic viruses might play important role in OSCC. An oncogenic Epstein-Barr virus (EBV) is a double stranded DNA virus of the human herpesvirus family which is able to persist lifelong in the human body. EBV

infections are common and worldwide in distribution, with over 90% of adult being serology positive. It is found to be the causative agent of infectious mononucleosis and is closely associated with Burkitt's lymphoma[2], nasopharyngeal carcinoma[3], and EBV-induced disorders in immune deficient patients[4]. However, only a handful of other epithelial-cell malignancies of the head and neck have been linked to EBV infection[5-7].

Objectives

In this study, we aimed to investigate the prevalence of EBV in oral exfoliated cells from case-control study of OSCC to establish the association of EBV with OSCC.

Methods

Specimens were collected from 200 patients (cases and controls) at Srinagarind hospital and Khon Kaen hospital in Khon Kaen, Thailand. All clinical history was documented that includes age, histological grading, disease stage and risk factors.

Cases: Samples were collected from 100 patients, who were diagnosed of oral cancer in site of lip, tongue and oral cavity (C00-C10) excluding C07 and C08 (International Classification of Diseases for Oncology, third edition, 2000) and diagnosed with histological confirmed squamous cell carcinoma.

Controls: Samples were collected from 100 patients, who were diagnosed with other diseases but not cancer or chronic infection in the head and neck and individually matched with case subject in sex, age (± 3 years) who was admitted in the same hospital.

Sample Collection

Exfoliated oral squamous cells were collected using conical cytobrush by scratching 10 rounds at site of buccal in oral cavity of patient.

DNA extraction

DNA was extracted from exfoliated oral cells by Gentra Puregene DNA extraction kit (Qiagen, Hilden, Germany) according to manufacturer's protocol for exfoliated cells.

EBV detection by polymerase chain reaction

The polymerase chain reaction (PCR) was performed to qualify the extracted DNA and amplify region of DNA polymerase BNLF1 (EBV) for the detection of EBV. -globin was used as an internal control with forward primer -globin/PC04 (5'-CAACTTCATCCACGTTCACC-3') and reverse primer -globin/GH20 (5'-GAAGAGCCAAGGACAGGTAC-3') which amplify 268bp product. The primers EBV-F (5'-CGA GTC ATC TAC GGG GAC ACG GA-3') and EBV-R (5'-AGC ACC CCC ACA TAT CTC TTC TT-3') were used to amplify a 194 bp product. PCR was performed in final volume of 25 ตl containing 0.2 mM of each dNTP, 0.2 ตM of each primer, 0.025 U of Taq DNA polymerase and 4 mM of $MgCl_2$ in 1X PCR buffer (Invitrogen). Thermal profile was 95^0C for 2 min; 40 cycles of 95^0C°C for 30 s, 53^0C for 1 min and 72^0C for 30 s; and a final extension at 72^0C for 5 min. DNA from the EBV-positive B95-8 cell line was used as a positive control.

EBV confirmation by dot blot hybridization:

The denatured 15ตl EBV positive PCR product was blotted in the nylon membrane (Hybond N+) by the mini–blotter. Pre-hybridization was done with the hybridization solution (5XSSC, 1%SDS, 10% dextran sulfate). Then, hybridization was done with the hybridization solution along with denatured EBV probe sequence (5'-ACC CGG AGC CTG TTT GTA GC-3'biotin) and incubated overnight. After that membrane was washed with 2XSSC/0.1%SDS and 1XSSC/0.1%SDS, subsequently. Finally, the membrane was washed with 1XSSC. Then the membrane was blocked with 1%skim milk/1XPBS. After that the membrane was incubated with Streptavidin-conjugated horse radish peroxidase. Next the membrane was washed with 1XPBS/0.15% tween20. Finally the

signal was detected by adding substrate (SuperSignal West Pico Chemiluminescent Substrate).

Confirmation by nested PCR

The samples which were PCR positive but dot-blot negative was further confirmed by nested PCR. The EBV F (5'-ACC CGG AGC CTG TTT GTA GC-3') and EBV R (5'-GGA GAA GGT CTT CTC GGC CTC-3') primers were used to amplify a 54 bp product from the PCR positive product from the first round PCR. PCR was performed in a final volume of 25 ตl containing 0.2 mM of each dNTP, 0.2 ตM of each primer, 0.025 U of Taq DNA polymerase and 4 mM of $MgCl_2$ in 1X PCR buffer (Invitrogen). Thermal profile was 95^0C for 2 min; 40 cycles of 95^0C for 30 s, 47^0C for 1 min and 72^0C for 30 s; and a final extension at 72^0C for 10 min.

Results

Out of total 200 samples, 100 samples were control and 100 samples were cases that consisted of different lesion site as shown in Figure 1. The lesion at tongue was the most common. Of total, 63 samples were positive for EBV-DNA by PCR at 194bp as shown in Figure 2(A). Confirmation was done by dot blot hybridization in which 46 samples out of 63 samples were positive by dot blot hybridization as shown in Figure 2(B). The remaining 17 samples which were positive by PCR but negative by dot blot hybridization was further confirmed by nested PCR as shown in Figure 2(C). Overall prevalence of EBV in cases and controls were 44% and 19% as shown in the Figure 3. The prevalence of EBV in cases when compared with controls was statistically significant ($p<0.001$) using chi-square test. After individual analysis of data according to their anatomical site of origin in the oral cavity the prevalence of EBV among cases was found to be highest in gum (alveolar ridge) with the prevalence of 85.7% and overall distribution is as shown in Figure 4.

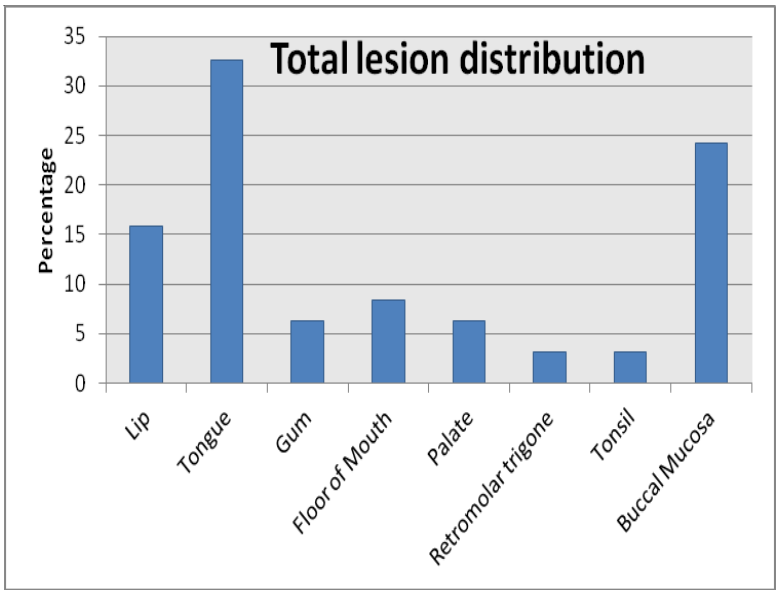

Figure 1 Demonstrate the distribution of total lesion in cases

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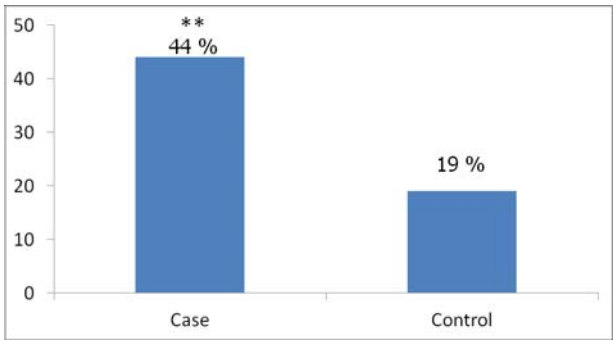

Figure 3 Overall prevalence of EBV in case and control samples from OSCC. ** ($p<0.001$)

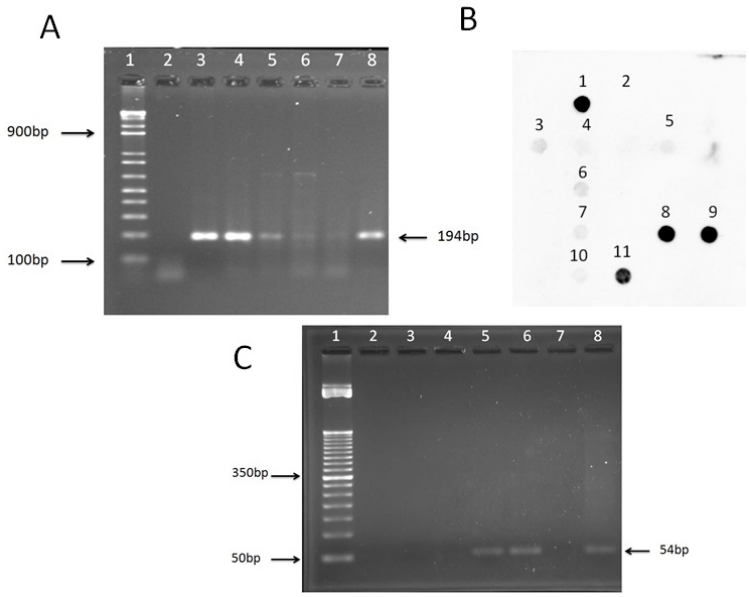

Figure 2 Detection of EBV-DNA by PCR and confirmation by dot blot hybridization and nested PCR. (A) Demonstrate PCR product with Lane 1 Ladder, Lane 2 negative control, Lane 3-6 EBV- DNA positive band, Lane 7 EBV-DNA negative and Lane 8 positive control band (B) Dot blot hybridization result showing Dot 1 positive control, 2 as a negative control, 3-11 EBV positive Dot (C) The nested PCR product with lane 1 ladder, lane 2 negative control, Lane 3, 4 and 7 EBV-DNA negative, Lane 5-6 EBV-DNA positive band and Lane 8 positive control band.

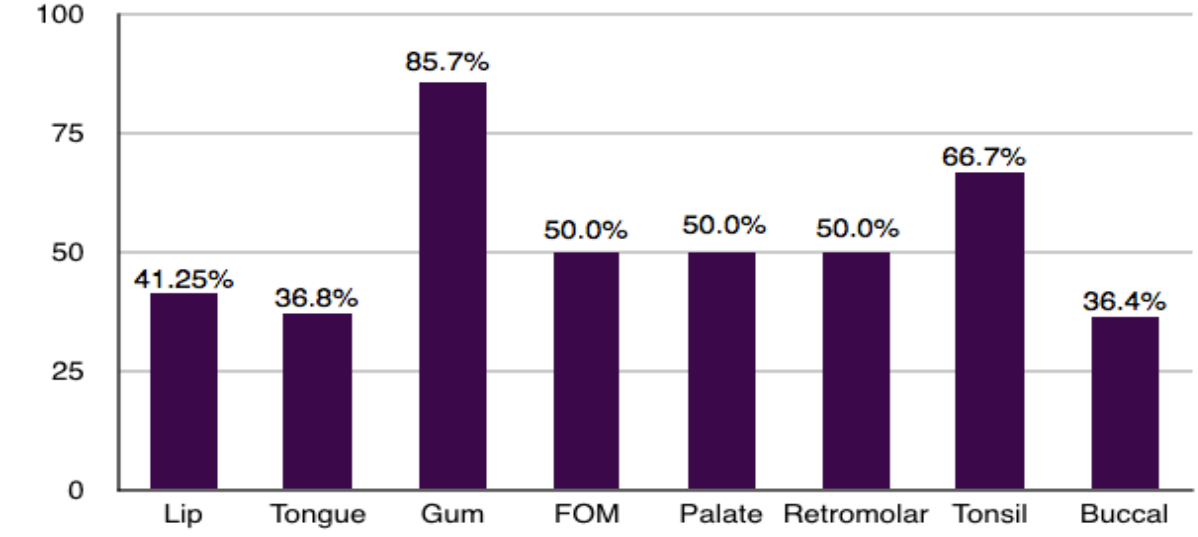

Figure 4 Prevalence of EBV in the cases according to different anatomical lesion sites.

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This pilot prospective study of oral squamous cell carcinoma (OSCC) investigated EBV infection in oral exfoliated cells by PCR and dot blot hybridization technique to evaluate an association of EBV with OSCC. The result shows that prevalence of EBV infection in OSCC case was significantly higher than control. There are various studies carried out in the different parts of the world regarding the prevalence of EBV in OSCC but few evidence of case-control study of EBV in OSCC. Several studies showed the prevalence of EBV in OSCC ranging from 15 % to 82.5%. In 1999, Kobayashi I *et al* studied in OSCC cases and reported that the prevalence of EBV in the OSCC with 15.2%[8]. .Another study performed in Sweden, in 2002 by Sand LP et al. demonstrated that the prevalence of EBV in OSCC was 37.9%[9]. In our case-control study of OSCC, similar results showed that the prevalence of EBV in case was 44% whereas 19% in control. The significant difference was shown between cases and control. It suggested the association of EBV and OSCC. The OSCC cases with lesion site at gum were frequently found with EBV detection. Interestingly, the prevalence of EBV may be depended on the used technique. By microarray analysis, Yen C-Y *et al* studied OSCC in Taiwan and detected high prevalence of EBV in OSCC (82.5%)[10]. This result supported the association of EBV with OSCC. However, the mechanism of EBV infection associated with OSCC development remains obscure and still needs further investigation. In addition, the correlation of EBV with other risk factors needs to be carried out to know the EBV as independent or dependent risk factors for OSCC.

Conclusion

The significantly high prevalence of EBV in the oral exfoliated cells from cancer samples when compared with the samples without cancer suggests EBV as an important etiological risk factor of OSCC. This result leads us to further explore the mechanism of association of EBV with OSCC in the lesion cells and FFPE samples.

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