



# Prevalence of Co-infection by Human Papillomavirus, Epstein- Barr Virus and Merkel Cell Polyomavirus in Iranian Oral Cavity Cancer and Pre-malignant Lesions

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## Article type: ABSTRACT

### Original Article

Human papillomavirus (HPV) is recognized as the most important risk factor in oral cavity cancer and pre-malignant lesions; however, the etiological association of concomitant infection with other oncogenic viruses as a co-factor has not been definitively proven. The present study aimed to determine the prevalence of co-infection with HPV, Epstein–Barr virus (EBV) and Merkel Cell PolyomaVirus (MCPyV) in oral cavity lesions in Iranian patients. One hundred and fourteen oral cavity samples, including 33 oral squamous cell carcinoma, 28 oral lichen planus, 16 oral epithelial dysplasia and 37 oral irritation fibromas were analyzed for the HPV, EBV and MCPyV infection by quantitative real-time PCR. According to histological features 32.5% and 28.9% of cases were oral irritation fibroma and oral squamous cell carcinoma, respectively. Infection with at least two viruses was detected in 21.1% of patients. In this group, co-infection with HPV/EBV was identified in 37.5% of cases, HPV/MCPyV in 29.2%, EBV/MCPyV in 12.5%, and HPV/EBV/MCPyV in 20.8%. There was no statistically significant difference between multiple infections and anatomical locations of cancer. The prevalence of triple viral infection (HPV/EBV/MCPyV) in well differentiated tumors was higher than EBV or MCPyV single infection. This study revealed that co-infection of HPV, EBV and MCPyV can be detected in both malignant and non-malignant oral cavity tissues, and co-infection with all three viruses in well differentiated tumors can be shown as a synergistic hypothesis of the pathogenic role of these viruses in oral malignant transformation.

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## Introduction

Oral cavity cancer is the 16<sup>th</sup> most common cancer worldwide, with an expected number of 377,000 new cases diagnosed in 2020 (1). The oral cavity includes different sites, including buccal mucosa, gingiva, oral tongue, floor of mouth, and palate. These sites may have different opportunities of exposure to viral infections due to their different locations. Therefore, it will be important to investigate the impact of viral infections on definite sites of the oral cavity (2). The global incidence of lip and oral cancers is 4 per 100,000 people annually. In Iran, the high annual incidence (1400 cases per 100 thousand) of oral cancers is a serious concern (3).

A viral infection of oral tissues is often encountered in dental practice. Many viral infections are related to tumor formation and, therefore, quick reporting and recommendation to oral disease management can be necessary in dental practice (4).

Human papillomavirus (HPV) was recognized by the international agency for research on cancer (IARC) to be an important human oncogenic producing tumor in the head and neck region (5, 6). The role of HPV virus, especially HPV16 in head and neck squamous cell carcinoma is well established (7–11).

Epstein-Barr virus (EBV) belongs to the *Herpesviridae* family, and is an important pathogenic virus that infects B cells in the oropharyngeal epithelium. The transmission of EBV infection occurs following the contact with oral secretions, saliva on fingers, toys or other substances. EBV replicates in epithelial cells of the oropharynx, and viruses are commonly shed in the saliva (12).

The role of EBV has been shown in several epithelial malignancies, including nasopharyngeal, gastric, and lymphoepithelial carcinomas (13-15). Recently, some studies have suggested a relationship between EBV and oral cavity lesions (16-21). Other reported associated conditions are oral hairy leukoplakia, liver cancers, salivary lymphoepithelial carcinomas and oral squamous cell carcinoma (OSCC). However, the association between EBV and OSCC remains controversial.

In a case-control study (22), HPV oral infection was powerfully related to a sub-group of oropharyngeal squamous cell carcinoma, in which high-risk sexual behaviors (i.e., oral, vaginal) were reported, regardless of alcohol and tobacco use. Despite the relationship between oral HPV infection and sexual behavior, a Finnish HPV Family Study (23) has shown that persistent high-risk HPV infection in a mother is a major risk factor for oral and genital infections by this virus in her children; this exposure appears to be modulated by the immune system.

Many studies have reported the existence of both HPV and EBV in some types of head and neck cancers. In Blanco's review study, they discussed the potential role and contribution of HPV/EBV co-infection in head and neck carcinogenesis, as well as the mechanisms that are potentially involved. Blanco et al. reported that HPV infection in OSCCs ranges between 6% to 58% worldwide, meanwhile, EBV infection has been detected in 25.9%–82.5% of OSCCs and HPV/EBV co-infection was evidenced in 6.5–37.5%. Also, an absence of EBV/HPV co-infection has been reported in oral cancers, conversely (24).

Merkel cell polyomavirus (MCPyV) has a circular dsDNA, and belongs to Polyomaviridae family. MCPyV large T antigen (LT-Ag) has a potential to be oncogene and interact with pRb, p53 and Hsc70 that disrupt the regulation of cell cycle proliferation (25).

It seems that MCPyV is widespread among healthy people. It has been shown that the prevalence of MCPyV seropositivity was 0% to 43% in infants and children of 2-5 years old, respectively and more than 80% in persons older than 50 years (26). However, MCPyV has been established as the etiological factor in the majority of Merkel cell carcinoma, a rare and very aggressive skin cancer (27), and MCPyV genome fragments have been detected in the upper aerodigestive tract including esophagus and oral cavity (15, 27-29). This virus has not yet been accurately associated with any other human disease probably because MCPyV DNA was identified in cutaneous swabs from clinically healthy persons with a prevalence of 40–100% (26, 30). The specific way of transmission remains to be clarified, and could involve mucosal, cutaneous, fecal–oral, or respiratory ways (30).

If the upper aerodigestive tract is exposed persistently to this oncogenic virus, malignancy may develop in this region. Additionally, detection of MCPyV DNA has also been recently reported in patients with different forms of oral cancers (28, 31, 32). The oral tongue and the floor of the mouth are the most common sites of squamous cell carcinoma within the oral cavity, accounting for more than 50% of cases (33, 34). Many studies presented the role of some viruses in the progress of head and neck squamous cell carcinoma (HNSCC). But they generally paid attention to one type of virus, whereas few recent reports have evaluated the potential association among the infection of two or three oncogenic viruses and tumorigenesis. Therefore, the present study is the first investigation that associates HPV, EBV, MCPyV co-infection in oral lesions in the Iranian population, and evaluates the positive samples in terms of viral copy number per cell.

## Material and Methods

### Patients and tissue samples

In this cross-sectional study, a total of 114 extracted DNA with OSCC, oral lichen planus (OLP), epithelial dysplasia and oral irritation fibroma (IF) from a previously reported data bank (35) were included. Briefly, from each paraffin embedded tissue block, 10 sections of 5- $\mu$ m-thick tissue slices were collected in a microcentrifuge tube. To dissolve the paraffin, the tissue sections were incubated 3 times in 500  $\mu$ L xylene for 10 min at 60 °C and washed with absolute ethanol. DNA was isolated using the DNA Extraction Mini Kit from Tissue (Yekta TajhizAzma, Tehran, Iran) according to the manufacturer's instructions. In brief, for tissue digestion and protein removal, 200  $\mu$ L tissue lysis buffer and 20  $\mu$ g proteinase K (10 mg/mL) were added to each tube. Samples were subsequently incubated at 56 °C overnight.

The quality and quantity of purified DNA was determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). In addition, DNA integrity in each sample was assessed by human RNase P gene (22) amplification as described previously (36). Preparation of plasmids containing cloned target sequences of MCPyV LTag, EBV EBER and human RNase P gene (real-time PCR standards) was described previously (20, 36).

The clinical and demographic characteristics of patients, including gender, age, anatomical location of the tumor, and tumor grade were collected from the Department of Dental Pathology records. The study protocol was approved by the Ethics Committee of Babol University of Medical Sciences (number: IR.MUBABOL.HRI.REC.1400.158).

### HPV DNA detection and genotyping

HPV-16 and HPV-18 genotypes' specific real-time PCR was conducted using a Rotor-Gene® Q (Qiagen GmbH, Hilden, Germany) real-time PCR system by the primer sets and TaqMan probe designed for the E6 and E7 gene (Table 1). Real time PCR cycling conditions for HPV-16 were as follows: initial denaturation (95 °C for 5 min), following 40 cycles including denaturation (95 °C for 15 s) and annealing/extension (60 °C for 30 s). Real-time PCR thermal cycles for HPV-18 were performed as follows: initial denaturation (95 °C for 5 min), following 40 cycles including denaturation (95 °C for 10 s) annealing (54.5 °C for 45 s) and extension (72 °C for 45 s). In addition, SYBR Green Real Time PCR with L1 General Primers MY09 and MY11 were performed as previously described (37). DNA extracted from CaSki and HeLa cell lines were used as positive controls for HPV-16 and HPV-18, respectively.

#### EBV DNA detection and quantitation

Detection and quantitation of EBV DNA sequences were performed by the primer sets and TaqMan probe specific (Table 1) for the EBER gene of EBV as described elsewhere (20). Each reaction consisted of 100 ng extracted DNA, 12.5 µL YTA qPCR Probe Master Mix (Yekta TajhizAzma, Tehran, Iran), 0.3 µM each primer, and 0.2 µM dual-labeled probe in a 25 µL total reaction. Each real-time PCR run included reaction mixtures without DNA template as a non-template control (NTC) and DNA extracted from supernatant of EBV-producing B-cell line (B95-8) was used as a positive control (38).

#### MCPyV DNA detection and quantitation

Detection and quantitation of the MCPyV DNA was done by quantitative real-time PCR assay, according to a previously described procedure (35). Real-time PCR was performed using a Rotor-Gene Q real-time PCR system (Qiagen, Hilden, Germany) using primer sets and TaqMan probes specific for the MCPyV LT-Ag gene and the human RNase P gene (Table 1).

**Table 1.** Sequences of primers and specific probes.

| Target Gene                 | Primer and Probe          | Sequences (5'-3')                       |
|-----------------------------|---------------------------|-----------------------------------------|
| Human RNase P (22)          | RNP F-Primer              | 5'-AGATTTGGACCTGCGAGCG-3'               |
|                             | RNP R-Primer              | 5'-GAGCGGCTGTCTCCACAAGT-3'              |
|                             | RNP Probe                 | FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1        |
| MCPyV LTA <sub>g</sub> (23) | LTA <sub>g</sub> F-Primer | 5'-CCAAACCAAAGAATAAAGCACTGA-3'          |
|                             | LTA <sub>g</sub> R-Primer | 5'-TCGCCAGCATTGTAGTCTAAAAAC-3'          |
|                             | LTA <sub>g</sub> Probe    | HEX-AGCAAAAACACTCTCCCCACGTCAGACAG-BHQ1  |
| EBV EBER (24)               | EBER-F-Primer             | 5'- TGACGTAGTCTGTCTTGAGGAGATG -3'       |
|                             | EBER-R-Primer             | 5'- CGTCTCCTCCCTAGCAAAACC -3'           |
|                             | EBER-Probe                | FAM-TGCAAAACCTCAGGACCTACGCTGC-TAMRA     |
| HPV16-E6 (38)               | E6 F-Primer               | 5'- GACCCAGAAAGTTACCACAGTTA -3'         |
|                             | E6 R-Primer               | 5'- ATTAGAATGTGTGTACTGCAAGC -3'         |
|                             | E6-Probe                  | 5'-FAM- GCACAGAGCTGCAAACAAC -BHQ1-3'    |
| HPV18-E7 (38)               | E7 F-Primer               | 5'-GGAAGAAAACGATGAAATAGATGGA-3'         |
|                             | E7 R-Primer               | 5'-CACACTTACAACACATACACAACA-3'          |
|                             | E7-Probe                  | 5'- Hex-ACCAGCCCCGACGAGCCGAACCA-BHQ1-3' |

## Statistical analysis

Statistical analysis was done by SPSS version 22 software. The chi-square test or exact test (in case of sparse data) was used to compare the study groups' differences for categorical. A p- value of less than 0.05(<0.05) was considered statistically significant. The two figures were depicted via GraphPad Prism version 6.

## Results

### Demographic and histopathological characteristics of samples

The current cross-sectional research included 114 oral lesions. According to the histopathologic diagnosis, study participants were divided into group of: 33 subjects with OSCC confirmed diagnosis and 81 subjects without neoplasia. Among the subjects in non-neoplastic group, 28 (24.6%), 16 (14%), and 37 (32.5%) had OLP, epithelial dysplasia, and IF lesions, respectively. There was no statistically significant difference between age ( $P = 0.49$ ) and gender ( $P = 0.19$ ) in neoplastic and non-neoplastic groups (Tables 2 and 3). The samples with OSCC were classified according to tumor differentiation grade (34) as follows: out of the 33 samples, 20 (60.6%) were classified as well-differentiated, 10 (30.3%) moderately differentiated, and 3 (9.1%) poorly differentiated. The non-neoplastic oral samples were categorized based on histopathologic criteria: 16 (19.8%) were diagnosed with oral dysplasia, 12 (14.8%) with erosive, 16 (19.8%) with reticular in OLP groups and 37 (45.7%) had irritation fibroma. In terms of location of oral lesions, in neoplastic group, the most common location was the gingiva with 12 (36.4%) cases, followed by tongue, buccal mucosa, lip and floor of the mouth with 9 (27.3%), 7 (21.2%), 3 (9.1%) and 2 (6.1%) cases, respectively. Out of the 81 non-neoplastic oral specimens, 44 (54.3%) cases were located at the buccal mucosa, followed by 16 (19.8%), 10 (12.3%), 8 (9.9%), and 3 (3.7%) cases in the tongue, gingiva, lip, and floor of the mouth, respectively.

**Table 2.** Prevalence of HPV, EBV and MCPyV according to demographic data in patients with oral lesions.

|               | Total<br>N (%) <sup>a</sup> | Single infection [n (%)] |             |             |                    | P-<br>value | Multiple infections [n (%)] |               |               |                   |                    | P-<br>value |
|---------------|-----------------------------|--------------------------|-------------|-------------|--------------------|-------------|-----------------------------|---------------|---------------|-------------------|--------------------|-------------|
|               |                             | HPV                      | EBV         | MCPyV       | Total <sup>b</sup> |             | HPV/<br>EBV                 | HPV/<br>MCPyV | EBV/<br>MCPyV | HPV/EBV/<br>MCPyV | Total <sup>c</sup> |             |
| Sex           |                             |                          |             |             |                    |             |                             |               |               |                   |                    |             |
| Male          | 45<br>(39.5)                | 6<br>(75)                | 3<br>(37.5) | 4<br>(30.8) | 13<br>(44.8)       | 0.19        | 2<br>(22.2)                 | 3<br>(42.9)   | 1<br>(33.3)   | 1<br>(20)         | 7<br>(29.2)        | 0.86        |
| Female        | 69<br>(61.5)                | 2<br>(25)                | 5<br>(62.5) | 9<br>(69.2) | 16<br>(55.2)       |             | 7<br>(77.8)                 | 4<br>(57.1)   | 2<br>(66.7)   | 4<br>(80)         | 17<br>(70.8)       |             |
| Total         | 114<br>(100)                | 8<br>(100)               | 8<br>(100)  | 13<br>(100) | 29<br>(100)        |             | 9<br>(100)                  | 7<br>(100)    | 3<br>(100)    | 5<br>(100)        | 24<br>(100)        |             |
| Age<br>(Year) |                             |                          |             |             |                    |             |                             |               |               |                   |                    |             |
| =< 50         | 45<br>(39.5)                | 3<br>(37.5)              | 1<br>(12.5) | 6<br>(46.2) | 10<br>(34.5)       |             | 4<br>(44.4)                 | 5<br>(71.4)   | 1<br>(33.3)   | 3<br>(60)         | 11<br>(45.8)       |             |

|                           |              |             |             |             |              |      |             |             |             |            |              |      |
|---------------------------|--------------|-------------|-------------|-------------|--------------|------|-------------|-------------|-------------|------------|--------------|------|
| 51-69                     | 50<br>(43.8) | 3<br>(37.5) | 5<br>(62.5) | 3<br>(23.1) | 11<br>(37.9) | 0.49 | 5<br>(55.6) | 2<br>(28.6) | 1<br>(33.3) | 2<br>(40)  | 12<br>(50)   | 0.21 |
| >=70                      | 19<br>(16.7) | 2<br>(25)   | 2<br>(25)   | 4<br>(30.8) | 8<br>(27.6)  |      | 0<br>(0.0)  | 0<br>(0.0)  | 1<br>(33.3) | 0<br>(0.0) | 1<br>(4.2)   |      |
| <b>Location of lesion</b> |              |             |             |             |              |      |             |             |             |            |              |      |
| Buccal mucosa             | 51<br>(44.7) | 3<br>(37.5) | 2<br>(25)   | 4<br>(30.8) | 9<br>(31)    | 0.28 | 6<br>(66.7) | 1<br>(14.3) | 1<br>(33.3) | 2<br>(40)  | 10<br>(41.7) | 0.18 |
| Floor of mouth            | 5 (4.4)      | 1<br>(12.5) | 0<br>(0.0)  | 1<br>(7.7)  | 2<br>(6.9)   |      | 0<br>(0.0)  | 0<br>(0.0)  | 2<br>(66.7) | 0<br>(0.0) | 2<br>(8.3)   |      |
| Gingiva & Alveolar mucosa | 22<br>(19.3) | 2<br>(25)   | 3<br>(37.5) | 0<br>(0.0)  | 5<br>(17.2)  |      | 2<br>(22.2) | 2<br>(28.6) | 0<br>(0.0)  | 1<br>(20)  | 5<br>(20.8)  |      |
| Labial mucosa             | 11<br>(9.6)  | 0<br>(0.0)  | 2<br>(25)   | 3<br>(23.1) | 5<br>(17.2)  |      | 0<br>(0.0)  | 2<br>(28.6) | 0<br>(0.0)  | 1<br>(20)  | 3<br>(12.5)  |      |
| Tongue                    | 25<br>(21.9) | 2<br>(25)   | 1<br>(12.5) | 5<br>(38.5) | 8<br>(27.6)  |      | 1<br>(11.1) | 2<br>(28.6) | 0<br>(0.0)  | 1<br>(20)  | 4<br>(16.7)  |      |

a: total of all samples; b: total among single infected samples; c: total among multiple infected samples.

### HPV detection and high risk viral genotypes in oral lesions

HPV DNA was detected in 29 (25.4%) out of 114 tested samples, of which 8 (27.6 %) were infected with a single HPV and 16 (55.2%) with two or more viral infections (multiple infection). HPV genotypes 16 and 18 were not recognized in all positive samples. Specifically, HPV DNA was detected in 9 out of the 33 neoplastic oral lesions (27.3%). Based on tumor differentiation, 25% of well differentiated, 20% of moderately differentiated and 66.7% of poorly differentiated tumors were HPV DNA positive. In addition, HPV DNA was detected in 20 out of the 81 samples from non-neoplastic group (24.7%). According to histopathologic diagnosis, 12.5% of oral dysplasia, 46.2% of erosive, 18.8% of reticular and 24.3% of samples with IF were HPV DNA positive. There was no statistically significant difference in HPV DNA positivity between neoplastic and non-neoplastic groups ( $P = 0.066$ ).

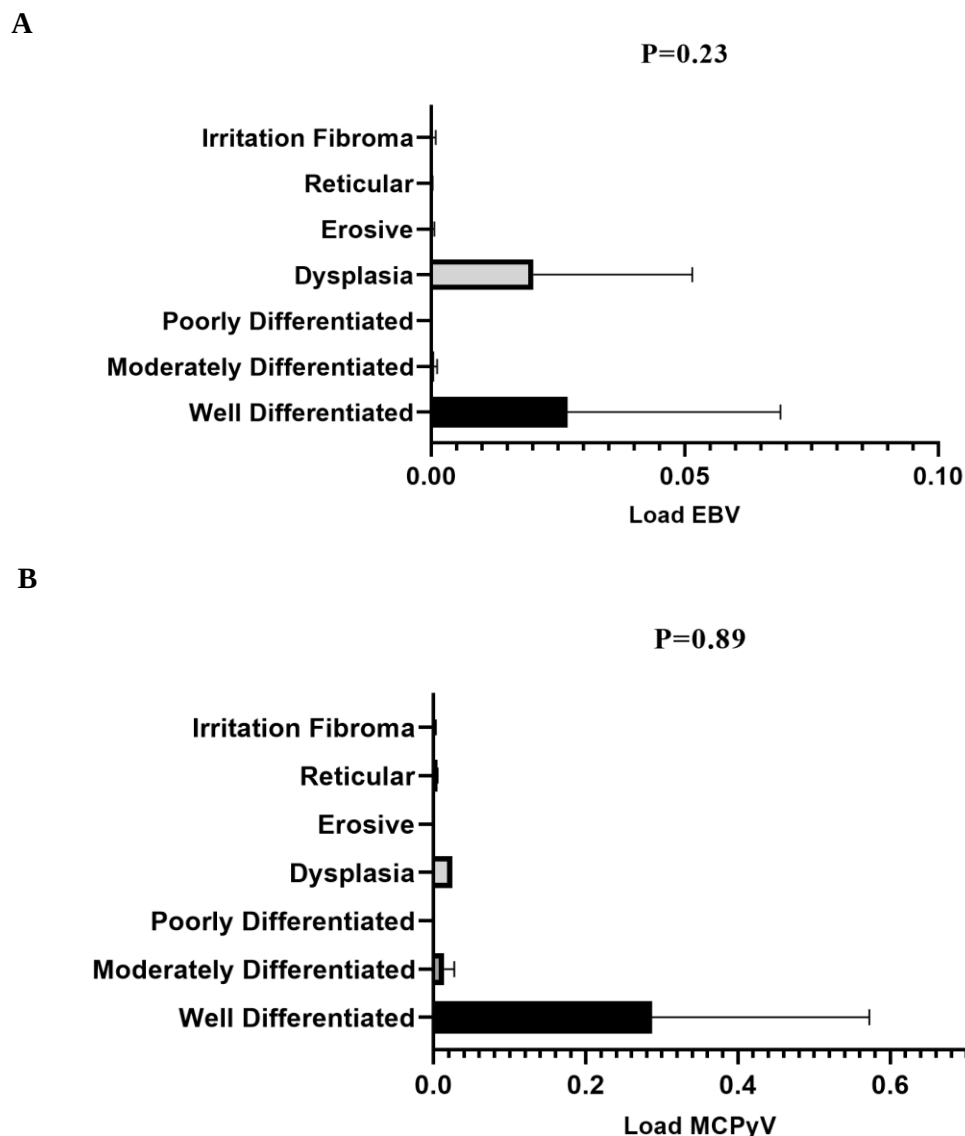
### Detection and quantitation of EBV in oral lesions

Of the 114 tested specimens, EBV DNA was detected in 25 (21.9%) subjects. EBV DNA was observed in oral samples of 10 out of the 33 (30.3%) neoplastic cases and 15 out of the 81 (18.5%) non-neoplastic cases. There was no statistically significant difference between neoplastic and non-neoplastic groups regarding EBV DNA positivity ( $P = 0.143$ ). Also, EBV DNA was detected in 35% (7/20) of well differentiated, 20% (2/10) of moderately differentiated and 33.3% (1/3) of poorly differentiated tumors. In non-neoplastic samples, IF with 24.3% (9/37) had the most EBV DNA prevalence followed by erosive, reticular, and dysplasia samples with 23.1% (3/13), 12.5% (2/16), and 6.3% (1/16) EBV DNA positivity respectively. Based on the location of oral lesions in neoplastic samples, 14.3% (1/7) of buccal mucosa, 50% (1/2) of floor of oral cavity, 41.7% (5/12) of gingiva, 33.3% (1/3) of lip, and 22.2% (2/9) of tongue samples were EBV DNA positive. In non-neoplastic oral lesions, 22.7% (10/44) of buccal mucosa, 25%



(2/8) of lip, 33.3% (1/3) of floor of oral cavity, 10% (1/10) of gingiva, and 6.3% (1/16) of tongue samples were EBV DNA positive.

The mean EBV DNA load was 0.10506429 and 0.003427631 per cell in neoplastic cases and non-neoplastic samples, respectively. There was no statistically significant difference between neoplastic cases and non-neoplastic samples regarding mean EBV DNA load ( $P = 0.23$ ). Additionally, the mean EBV copy number was higher in well differentiated (0.2870966), moderately differentiated (0.02785336) tumors and dysplasia (0.02497262) compared to other histopathologic groups; however, this difference was not statistically significant ( $P = 0.23$ ) (Figure 1A).



**Fig. 1. Viral DNA load according to histopathological characteristics.** A: Mean EBV DNA load; B: Mean MCPyV DNA load. Data were assessed according to viral DNA copies per cell in different histopathological groups. The P-value was determined by the KruskalWallis test.

### Detection and quantitation of MCPyV in oral lesions

In the current study, neoplastic and non-neoplastic oral samples were tested for the existence of MCPyV DNA by quantitative real-time PCR. Out of 114 specimens, the MCPyV DNA was detected in 28 (24.6%). MCPyV DNA was detected in oral samples of 8 out of 33 neoplastic cases (24.2%) and 20 out of 81 specimens in non-neoplastic group (24.7%). There was no statistically significant difference in MCPyV DNA positivity between neoplastic and non-neoplastic groups ( $P = 0.471$ ). Regarding the histopathological diagnosis in neoplastic cases, MCPyV infection was detected in 30% (6/20) of well differentiated, 20% (2/10) of moderately differentiated, but in none of the poorly differentiated tumors. In non-neoplastic group, 18.7% (3/16) of oral dysplasia, 30.8% (4/13) of erosive, 12.5% (2/16) of reticular and 29.8% (11/37) of IF samples were positive for MCPyV DNA. According to the location of oral lesions in neoplastic cases, 14.3% (1/7) of buccal mucosa, 50% (1/2) of floor, 8.3% (1/12) of gingiva, 66.7% (2/3) of lip, and 33.3% (3/9) of tongue samples were MCPyV DNA positive. In non-neoplastic oral lesions, 15.9% (7/44) of buccal mucosa, 50% (4/8) of lip, 66.7% (2/3) of floor, 20% (2/10) of gingival, and 31.3% (5/16) of tongue samples were MCPyV DNA positive.

The mean MCPyV DNA load was 0.013963416 and 0.003112143 per cell in neoplastic cases and non-neoplastic samples, respectively. There was no statistically significant difference between neoplastic cases and non-neoplastic samples regarding mean MCPyV DNA load ( $P = 0.89$ ). Additionally, the mean MCPyV copy number was higher in well differentiated (0.026910492) tumors and dysplasia (0.020213939) compared to other histopathologic groups however, this difference was not statistically significant ( $P = 0.89$ ) (Figure 1B).

### Viral co-infection in oral lesions

In total, 46.5% (53/114) of the oral lesion samples had at least one viral infection. Co-infection of double and triple infection with HPV, EBV and MCPyV was seen in 24 (21.1%) oral lesions samples. Of 24 co-infected samples, 7 (29.2%) were found in neoplastic cases and 17 (70.8%) in non-neoplastic oral samples. There was no statistically significant difference between neoplastic and non-neoplastic samples regarding concomitant double and triple viral infection (Tables 3 and 4).

**Table 3.** Prevalence of HPV, EBV and MCPyV according to clinical characteristics in patients with oral lesions.

|                               | Total N (%) <sup>a</sup> | Single infection [n (%)] |          |          |                    | P-value | Multiple infections [n (%)] |            |            |                |                    | P-value |
|-------------------------------|--------------------------|--------------------------|----------|----------|--------------------|---------|-----------------------------|------------|------------|----------------|--------------------|---------|
|                               |                          | HPV                      | EBV      | MCPyV    | Total <sup>b</sup> |         | HPV+ EBV                    | HPV+ MCPyV | EBV+ MCPyV | HPV+EBV+ MCPyV | Total <sup>c</sup> |         |
| <b>Type of lesion</b>         |                          |                          |          |          |                    |         |                             |            |            |                |                    |         |
| OSCC                          | 33 (28.9)                | 3 (37.5)                 | 3 (37.5) | 4 (30.8) | 10 (34.5)          | 0.56    | 3 (33.3)                    | 0 (0.0)    | 1 (33.3)   | 3 (60)         | 7 (29.2)           | 0.66    |
| OLP                           | 28 (24.6)                | 3 (37.5)                 | 0 (0.0)  | 2 (15.4) | 5 (17.2)           |         | 3 (33.3)                    | 2 (28.6)   | 1 (33.3)   | 1 (20)         | 7 (29.2)           |         |
| Dysplasia                     | 16 (14)                  | 1 (12.5)                 | 1 (12.5) | 2 (15.4) | 4 (13.8)           |         | 0 (0.0)                     | 1 (14.3)   | 0 (0.0)    | 0 (0.0)        | 1 (4.2)            |         |
| IF                            | 37 (32.5)                | 1 (12.5)                 | 4 (50)   | 5 (38.5) | 10 (34.5)          |         | 3 (33.3)                    | 4 (57.1)   | 1 (33.3)   | 1 (20)         | 9 (37.5)           |         |
| <b>Histological diagnosis</b> |                          |                          |          |          |                    |         |                             |            |            |                |                    |         |



|                                |                      |                    |                    |                     |                     |      |                    |                    |                    |                    |                     |      |
|--------------------------------|----------------------|--------------------|--------------------|---------------------|---------------------|------|--------------------|--------------------|--------------------|--------------------|---------------------|------|
| OSCC well differentiated       | 20<br>(17.5)         | 1<br>(12.5)        | 2<br>(25)          | 3<br>(37.5)         | 6<br>(20.7)         |      | 2<br>(22.2)        | 0<br>(0.0)         | 1<br>(33.3)        | 2<br>(40)          | 5<br>(20.8)         |      |
| OSCC moderately differentiated | 10<br>(8.8)          | 1<br>(12.5)        | 1<br>(12.5)        | 1<br>(7.7)          | 3<br>(10.3)         |      | 0<br>(0.0)         | 0<br>(0.0)         | 0<br>(0.0)         | 1<br>(20)          | 1<br>(4.2)          |      |
| OSCC poorly differentiated     | 3<br>(2.6)           | 1<br>(12.5)        | 0<br>(0.0)         | 0<br>(0.0)          | 1<br>(3.4)          | 0.85 | 1<br>(11.1)        | 0<br>(0.0)         | 0<br>(0.0)         | 0<br>(0.0)         | 1<br>(4.2)          | 0.73 |
| Dysplasia                      | 16<br>(14)           | 1<br>(12.5)        | 1<br>(12.5)        | 2<br>(15.4)         | 4<br>(13.8)         |      | 0<br>(0.0)         | 1<br>(14.3)        | 0<br>(0.0)         | 0<br>(0.0)         | 1<br>(4.2)          |      |
| Erosive lichen planus          | 12<br>(10.5)         | 1<br>(12.5)        | 0<br>(0.0)         | 1<br>(7.7)          | 2<br>(6.9)          |      | 2<br>(22.2)        | 2<br>(28.6)        | 0<br>(0.0)         | 1<br>(20)          | 5<br>(20.8)         |      |
| Reticular lichen planus        | 16<br>(14)           | 2<br>(25)          | 0<br>(0.0)         | 1<br>(7.7)          | 3<br>(10.3)         |      | 1<br>(11.1)        | 0<br>(0.0)         | 1<br>(33.3)        | 0<br>(0.0)         | 2<br>(8.3)          |      |
| Irritation fibroma             | 37<br>(32.5)         | 1<br>(12.5)        | 4<br>(50)          | 5<br>(38.5)         | 10<br>(34.5)        |      | 3<br>(33.3)        | 4<br>(57.1)        | 1<br>(33.3)        | 1<br>(20)          | 9<br>(37.5)         |      |
| <b>Type of lesion</b>          |                      |                    |                    |                     |                     |      |                    |                    |                    |                    |                     |      |
| Cancerous                      | 32<br>(28.1)         | 3<br>(37.5)        | 3<br>(37.5)        | 4<br>(30.8)         | 10<br>(34.5)        |      | 3<br>(33.3)        | 0<br>(0.0)         | 1<br>(33.3)        | 3<br>(60)          | 7<br>(29.2)         |      |
| Pre-cancerous                  | 16<br>(14)           | 1<br>(12.5)        | 1<br>(12.5)        | 2<br>(15.4)         | 4<br>(13.8)         | 0.56 | 0<br>(0.0)         | 1<br>(14.3)        | 0<br>(0.0)         | 0<br>(0.0)         | 1<br>(4.2)          | 0.66 |
| Non-Cancerous                  | 66<br>(57.9)         | 4<br>(50)          | 4<br>(50)          | 7<br>(53.8)         | 15<br>(51.7)        |      | 6<br>(66.7)        | 6<br>(85.7)        | 2<br>(66.7)        | 2<br>(40)          | 16<br>(66.7)        |      |
| <b>Total</b>                   | <b>114<br/>(100)</b> | <b>8<br/>(100)</b> | <b>8<br/>(100)</b> | <b>13<br/>(100)</b> | <b>29<br/>(100)</b> |      | <b>9<br/>(100)</b> | <b>7<br/>(100)</b> | <b>3<br/>(100)</b> | <b>5<br/>(100)</b> | <b>24<br/>(100)</b> |      |

a: total of all samples; b: total among single infected samples; c: total among multiple infected samples.

**Table 4.** Prevalence of single and multiple infections with HPV, EBV and MCPyV in infected patients.

#### All infections

|            |            |            |            |
|------------|------------|------------|------------|
| HPV        | EBV        | MCPyV      |            |
| 29 (54.7%) | 25 (47.2%) | 28 (52.8%) | 53 (46.5%) |

#### Single infection

|           |           |            |            |
|-----------|-----------|------------|------------|
| HPV       | EBV       | MCPyV      |            |
| 8 (27.6%) | 8 (27.6%) | 13 (44.8%) | 29 (25.4%) |

#### Co-infections

|           |           |           |               |            |
|-----------|-----------|-----------|---------------|------------|
| HPV+EBV   | HPV+MCPyV | EBV+MCPyV | HPV+EBV+MCPyV |            |
| 9 (37.5%) | 7 (29.2%) | 3 (12.5%) | 5 (20.8%)     | 24 (21.1%) |

## Discussion

Viral diseases can be related to the progress and development of periodontal diseases (4). Infectious particles are essential but not enough for cancer initiation or progression (5, 38, 39). In patients infected with single virus type, secondary co-infection with another virus can help as an important co-factor that may affect beginning and/or progression of tumors.

HPV, EBV and MCPyV are well known tumorigenic viruses related to the progress of different cancers. There are many researches in the literature concerning the role of viruses in the progression of head and neck squamous cell carcinoma (HNSCC). But they are mostly dedicated to single or two types of virus (predominantly HPV and EBV). However, only some of recent studies have evaluated the probable relationship between the infection of more than two tumorigenic viruses and oncogenesis (21). Vanshika *et*

*al.* in 108 oral cancer patients in India found that the incidence of EBV was 27.8%, HPV 16 13%, and reported a complete absence of HPV 18 by real time PCR. Also, they noticed a co-infection by EBV and HPV in 5.6% of cases (40).

According to Vazquez-Guillen *et al.* in a retrospective study of 195 laryngeal specimens of squamous cell carcinoma, HPV DNA was detected in 47.7% of samples. EBV DNA was detected in 27.7% tumor tissue samples of which 46.3% were in co-infection with HPV. MCPV DNA was detected in 5.6% cases of which 45.4% were in co-infection with an HPV. According to these results, HPV-52 was the most prevalent high risk-HPV, which may propose that this and other HPV types as well as HPV-16 and 18 could be considered for prophylaxis (41).

The present study is the first new comment that associates HPV, EBV and MCPyV co-infection in oral lesions in the Iranian patients. To study whether these 3 tumorigenic viruses could have a role in the etiology of oral cancer in Northern Iran, a cross-sectional study was designed and a total of 114 malignant, pre-malignant and non-malignant oral samples were tested for HPV, EBV and MCPyV infections.

In the current study, HPV infection in malignant lesions (27.3%) was greater than non-malignant cases (24.7%). HPV infections in oral lesion was mainly caused by genotypes other than HPV-16 and 18, which might be explained by a wide-ranging of different HPV genotypes that can affect oral lesion pathogenesis. These documents are consistent with a number of reports, which recognized different and putative new HPV genotypes in malignant and non-malignant oral samples (8, 42, 43).

In the current study, EBV DNA was found in 30.3% of malignant and 18.5% of non-malignant oral samples. Even though EBV infection was correlated to risk of oral cancer, but the result was not statistically significant. Jaloluli *et al.* (44) detected the presence of EBV in 55% of samples from eight different countries.

In the present study, MCPyV DNA was detected in malignant, pre-malignant and non-malignant oral samples. But, MCPyV infection in non-malignant lesions (24.2%) was similar to malignant cases (24.7%). According to Muñoz *et al.*, of the total of 120 HNSCCs, 15 were positive for MCPyV (12.5%). In subjects without cancer, only one (1.8%) case was positive for MCPyV (45).

The clinical and molecular consequences of co-infection with HPV, EBV and MCPyV in oral lesions from Iranian patients are unknown. Previously, we reported the presence of single HPV infection in esophageal squamous cell carcinoma samples (46), as well as EBV (20) and MCPyV (35) in oral lesions from Iranian patients. Thus, a possible collaboration between HPV, EBV and MCPyV in oral cancer is an interesting point that needs to be studied in the future, as previously reported in esophageal cancer.

Some authors discover an association between EBV infection (particularly co-infection with HPV) and OSCC and oropharyngeal cancer (47-49). Furthermore, some investigators showed that co-infection by multiple tumorigenic viruses can be an important risk factor in the progress of OSCC (21, 48-50). In our study, similar to Polz-Gruszka *et al.* (50), HPV-EBV co-infection was detected in 7.9 % of samples (Table. 4). This low percentage cannot support the hypothesis that co-infection plays a role in OSCC. However, it cannot be completely excluded.

Co-infections occur commonly in regions with high prevalence of infectious agents, particularly in developing countries (51). Drop *et al.* (21) in a study on 53 oral cavity samples in Poland indicated

HPV/EBV co-infection in 26.4% (14/53) of patients with oral cancer and Deng *et al.* (52), in research performed in Japan, showed HPV/EBV co-infection in 1% of patients with head and neck cancer and in 10% of patients with nasopharyngeal carcinoma.

Jiang *et al.* (48) suggest that co-infected cells can have a greater oncogenic potential than normal cells, and that co-infection by HPV and EBV may have a more profound effect on invasion than propagation.

In a previous study, we found co-infections in 33/168 (19.6%) of esophagus samples and 16 (16.0%) and 17 (25.0%) concomitant double and triple infection with HPV, EBV and MCPyV in neoplastic and non-neoplastic esophageal samples, respectively (15). The prevalence of HPV/EBV, HPV/MCPyV, EBV/MCPyV and HPV/EBV/MCPyV co-infections was 2.4%, 13.1%, 3%, and 1.2% of esophageal samples, respectively. But these double and triple infections were detected in 24/114 (21.1%) of oral lesions with 7.9%, 6.1%, 2.6% and 4.3% of HPV/EBV, HPV/MCPyV, EBV/MCPyV, and HPV/EBV/MCPyV co-infections, respectively.

Many infected pathogens frequently lead to irritation of tissues or organs, which can cause the initiation of oncogenesis. Al Moustafa *et al.* suggested that HPV and EBV co-infections have an important role in starting neoplastic transformation of human oral epithelial cells (53). However, it is not clear which virus, HPV or EBV, contributes to the first infection in co-infected patients (54). But, the reports by Makielski *et al.* (55) showed that infection with HPV in the oral cavity may increase the ability of epithelial cells to provision the EBV life cycle, increasing therefore EBV-related pathogenesis in the oral cavity. Also, Guidry and Scott (56) proposed that co-infection of HPV/EBV increases EBV persistence both via latency or improved viral replication and by over expression of HPV oncogenes.

In comparison with prior reports in our region, the present study was performed with a larger variety of malignant and non-malignant oral samples, and to our knowledge, this is the first report in the EMRO region (World Health Organization / Regional Office for the Eastern Mediterranean) regarding the effect of these three tumorigenic viruses (HPV, EBV, and MCPyV) in oral lesions by very sensitive real-time PCR technique.

According to our previous report, a low copy number of MCPyV DNA was detected in malignant and non-malignant esophageal samples (15). The difference between MCPyV DNA loads (as a copy per cell) in malignant and non-malignant patients was not statistically significant. Low copy numbers of MCPyV DNA in both malignant and non-malignant esophageal tissue samples might be described by simple persistent viral replication in esophagus as a viral shedding from another organ (e.g., respiratory tract) to esophagus or as a passenger virus without any pathological outcome.

In summary, in this research we assessed the prevalence of HPV, EBV and MCPyV in oral lesions from Iranian patients for the first time. MCPyV increased when compared with EBV and HPV, suggesting an association of MCPyV infection with these lesions. However, no relation between MCPyV infection and age, gender, tumor localization or differentiation status was observed.

Regarding HPV infection, in the present study, genotype specific PCR was limited to HPV16 and HPV18 and specific PCR for low-risk HPV6, HPV11 and non-HPV16, non-HPV18 high risk genotypes were not performed. This is a limitation of the present study and absence of other high risk HPV genotypes (non-HPV16, non-HPV18 types) and more prevalent low risk subtypes (HPV6 and HPV11) should be interpreted

with caution. A possible role for high HPV types other than 16 and 18 in the pathogenesis of oral lesions and its association with the geographical area (Northern Iran) may be hypothesized, but further epidemiological investigations should be done to prove this hypothesis. In addition, the results for EBV positivity in oral lesions should be interpreted cautiously, since PCR positivity may be due to EBV in oral epithelial cells (including carcinoma cells) as well as B lymphocytes. To differentiate the cell lineage infected with this virus, in situ hybridization for EBERs and/or immunohistochemistry for EBV-encoded proteins should be done in future studies. Moreover, genetic alterations in host cell machinery may support the establishment of latent EBV infection, which is believed to be an initiation phenomenon for EBV epithelial carcinogenesis (57). Further future investigations over host genetic alterations may shed more light on development of epithelial malignancies by EBV.

In conclusion, in Iranian patients with oral lesions, co-infection with at least one virus was detected in 21.1% of cases. In this group, co-infection with HPV/EBV was recognized in 37.5% of cases, HPV/MCPyV in 29.2%, HPV/MCPyV in 12.0%, and HPV/EBV/MCPyV in 20.8%. No difference of multiple infections in different locations of lesion was observed.

The prevalence of multiple infections in IF was more frequent than HPV, EBV or MCPyV single infection, and this situation was also observed in well differentiated tumors. Regarding lesion localization, buccal mucosa and gingiva were more frequent in multiple viral infections than in single infection.

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## Conflict of Interest

The authors declare no conflict of interest.

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