ORIGINAL ARTICLE

Presence of Human Papillomavirus and Epstein–Barr Virus in Squamous Lesions of the Tongue

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ABSTRACT

Background: *Several studies have suggested a possible role of human papillomavirus (HPV) and Epstein–Barr virus in the pathogenesis of oral premalignant lesions. This study aimed to investigate the correlation between squamous dysplasia of the tongue and expression of p16 and Ki67 immunohistochemically as well as HPV genotypes with real-time polymerase chain reaction (PCR).*

Methods: *Twenty-three tongue biopsies were stained immunohistochemically for p16, Epstein–Barr virus and Ki67 and real-time PCR and chromogenic* in-situ *hybridization for HPV.*

Results: *Dysplasia was diagnosed in 16 of 23 cases without invasive carcinoma and suspicious for dysplasia (n=17) and HPV infection (n=6). These were subjected to chromogenic in-situ hybridization for HPV DNA (HPV-III family 16). There was no immunoreactivity for Epstein–Barr virus. p16 was positive in 4/16 (25%) of dysplastic lesions. One lesion was positive for HPV by chromogenic* in-situ *hybridization, and one case was positive by real-time PCR for HPV.*

Conclusion: *This evidence suggested that HPV infection but not Epstein–Barr virus infection plays a role in pathogenesis of squamous dysplasia localized tongue.*

Keywords: Epstein–Barr virus, human papillomavirus, p16, PCR, squamous dysplasia, tongue.

INTRODUCTION

Several studies have suggested a possible role of human papillomavirus (HPV) and Epstein–Barr virus (EBV) in the pathogenesis of oral carcinoma and presence of HPV and EBV in oral premalignant lesions (OPLs) (1–3) and oral squamous benign lesions (3, 4–7). Any single method of detection has limitations (8). Human papillomavirus-induced carcinogenesis is associated with low pRb protein levels which leads to subsequent p16 upregulation (1). p16 protein normally acts to block cell cycle progression at G1 to S transition; therefore, inactivation of the p16 gene enables unregulated cell growth (6). Oral premalignant lesion is the most reliable predictor of malignant development (5). Histopathologic evaluation of dysplasia is subjective, and there are differences inter- and intra-observer (9–11). This study aimed to investigate the correlation between localized squamous dysplasia of the tongue (TSD) and expression of p16 and Ki67 immunohistochemically and HPV genotypes with real-time polymerase chain reaction (PCR).

MATERIALS AND METHODS

Twenty-three tongue biopsy specimens diagnosed as pre-neoplastic (n = 17) and suspicious lesions for dysplasia and HPV infection (n = 6) with coilocytic changes microscopically, which have no invasive carcinoma. Cases were selected from the pathology archives of Kartal Dr. Lütfi Kırdar City Hospital during the period of 2010–2013. All the procedures were approved by the Ethical Committee of Kartal Dr. Lütfi Kırdar City Hospital. All biopsy specimens were re-evaluated for dysplasia, ulceration, inflammation, intraepithelial inflammatory infiltration, achantosis, rete condition, apoptosis, suprabasal mitosis, parakeratosis, foreign

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body reaction, and vascularization. Histological diagnoses of pre-neoplastic oral lesions were determined according to the WHO 2005 classification of oral epithelial dysplasia. All cases were stained immunohistochemically for p16, anti-EBV and Ki67, chromogenic *in-situ* hybridization (CISH) for HPV DNA (HPV III family 16) and real-time PCR for HPV.

Immunohistochemical studies were performed using Bond™ Polymer Refine Detection method (Leica Biosystems Newcastle Ltd, Newcastle upon Tyne, UK) with diaminobenzidine as the chromogen and haematoxylin as the nuclear counterstain. All immunohistochemical assays were performed using the Leica BOND-MAX™ automated system. Included antibodies were anti-p16 (clone R19-D; DB Biotech, Kosice, Slovak Republic; 1:100 dilution), anti-Ki67 (clone SP6; Biocare medical, CA, USA; 1:100 dilution) and anti-EBV (clones CS1, CS2, CS3 and CS4, Leica Microsystems, UK; 1:100 dilution). All antibodies were diluted with Lab Vision Antibody Diluent (TA-125-AD). Epstein–Barr virus and p16 staining were scored as strong, weak or negative on the basis of nuclear and/or cytoplasmic staining. Weak cytoplasmic staining or reactivity in cells less than 5% was interpreted as negative. Diffuse (more than 80% or focal 5%–80%) strong staining was scored as positive (13). Ki67 staining was evaluated and scored according to the limited localization of basal layer (score:0) or extending to the upper layer (score:1).

All cases were investigated for the presence of HPV DNA by the CISH method and analysed the presence of HPV DNA, HPV III family 16 for HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. Real-time PCR for HPV performed by using the 'HPV sign® Q24 complete' kit allows HPV virus detection and genotyping using 'Rotor-Gene' and 'PyroMark Q24' instrument system (Qiagen, Germany) for paraffin-embedded tissues. The 'HPV sign® Q24 complete' kit allows to identify the HPV genotypes present in the sample using Identify PyroMark SW 1.0 or the equivalent IdentiFire™ software (Biotage AB, Sweden), which analyse and align the determined sequence with the library supplied in the kit. After amplification of DNA extracted from the biopsy on Rotor-Gene, detection and genotyping are respectively performed through melt curve analysis and Pyrosequencing. 'PyroMark™ Q96 ID system' (Qiagen), genotype-specific and 30-base pair log sequences were obtained with sequencing primers HPV 1 seq primer, HPV 2 seq primer, HPV 3 seq primer and HPV 4 seq primer. Alignment of sample sequences against the HPV library's genotype-specific sequences was performed with the 'PyroMark™ IdentiFire software 1.0' (Qiagen). The 'HPV sign® Q24 complete' kit allows detection and identification of the HPV HR type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59; HPV IR type 26, 30, 53, 66, 67, 68a, 70, 73, 82, and 85; and HPV LR type 6, 11, 40, 42, 43, 44, 54, 55, 61, and 69.

Statistical assessments were performed by using SPSS 17 for Windows (SPSS Inc, Chicago, IL, USA). Pearson Chi-square test was used for evaluation of the presence of dysplasia, suprabasal mitosis in squamous epithelium, p16 positivity and Ki67 positivity in the basal layer of squamous epithelium. The statistical significance level was established at $p < 0.05$ and confidence interval was 95%.

The Spearman correlation test was used to evaluate the relationship between low-grade dysplasia, high-grade dysplasia, non-dysplastic lesions and EBV, Ki67, p16 expression, presence of ulceration, inflammation, intraepithelial inflammatory infiltration, achantosis, parakeratosis, apoptosis, and increase of vascularity.

RESULTS

Cases comprised 9 men and 14 women with age ranging from 20 to 81 years (mean age = 54 years). Sixteen cases of dysplastic epithelial lesions comprised 4 cases of high-degree (25%) and 12 cases of low-degree (75%) dysplasia. Age range of 16 cases of TSD was 20–67 years with mean age of 49.2 years. Cases of dysplasia were 68.7% in age groups of 60–69 years. Seven lesions have no dysplastic alterations, have a squamous proliferation with irregular rete ridges (n = 7, 100%), parakeratosis (n = 7, 100%), inflammation (n = 6, 85.7%), apoptosis (n = 2, 28.5%), ulceration (n = 1, 14.2%), and increase in capillary vessels (n = 6, 85.7%).

p16 was positive in 50% of high-grade dysplasia (n = 2/4) and 16.7% of low-grade dysplasia (n = 2/16), which were not found as statistically significant. However, there was a weak positive correlation (Spearman correlation test; $r = 0.245$, $p > 0.05$). Gender wise distribution of cases of TSG showed a male predominance in p16 positivity with one out of four males. The p16 reaction was also identified at the some normal elements such as fibroblasts, glandular acinus, muscle fibres, ductal epithelium, and endothelium.

Epstein–Barr virus was positive in two of four cases with severe dysplasia and none of low grade dysplasia and non-dysplastic lesions. There was a moderate positive correlation between EBV and high-grade dysplasia (Spearman correlation test; $r = 0.486$, $p = 0.018$).

Immunoexpression analysis of Ki67 did not reveal statistically significant differences between the expression of markers and histopathological parameters, except Ki67 whose increased expression was associated with the decrease in high-degree dysplasia (Spearman correlation test; $r = 0.700$, $p = 0.000$).

One case with high-grade dysplasia was positive (Figure) for HPV by CISH and another case with low-grade dyspalasia was positive for HPV by real-time PCR. HPV type was not determined in both positive cases, probably due to insufficient amount. The case with high-grade dysplasia positive for HPV by CISH was also positive for p16. However, the case with low-grade dysplasia and HPV positive by PCR was negative for p16.

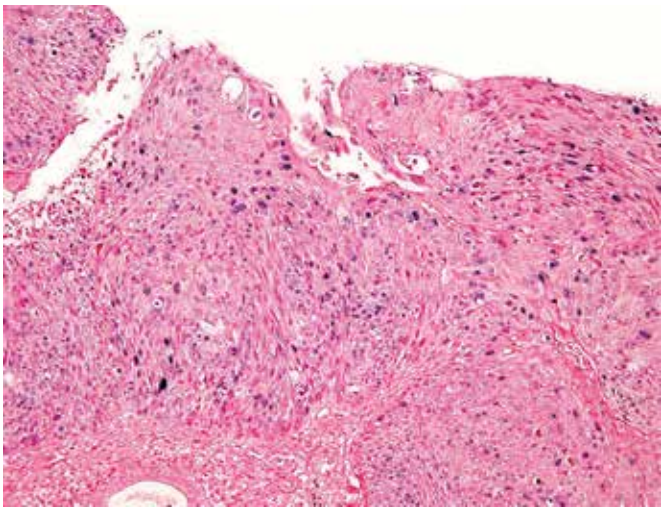

Figure: Chromogen *in-situ* hybridization for HPV III family 16 for high-grade dysplasia on tongue (CISH, ×200).

There was no correlation between the nature of the lesion and presence of ulceration, inflammation, intraepithelial inflammatory infiltration, acanthosis, parakeratosis, and increase of vascularity. There was a positive moderate correlation between the nature of the lesion and the presence of apoptosis which was not statistically significant, while p value was near to significant value (Spearman correlation test; $r = 0.407$, $p = 0.054$).

DISCUSSION

Most common risk factors for development of oropharyngeal cancers are tobacco and alcohol. The role of HPV in oral and oropharyngeal carcinoma was first proposed by Syrjanen *et al* in 1983 (12). It is found both in an episomal form and in an integral form. It has also been suggested that HPV may be latent for a long time in the episomal format in the oral mucosa, hence being responsible for initiation and development of a tumoural growth. This tumoural growth may occur as a result of a multicarcinogenic interaction together with some other carcinogens and co-carcinogens (13,14). Hoffmann *et al* found HPV 16 antigen expression in 66.7% of cases of leukoplakia with the majority of cases with mild dysplasia (15). Although Fregonesi *et al* found HPV 16/18 positivity of 40% in OPLs with various grades of dysplasia (16), Abdelsayed found no significant association between epithelial dysplasia and HPV status (17).

Dragomir found that p16 was present at nuclear and cytoplasmic level in 22 of the 34 analysed cases (64.7%). The intensity of p16 reaction was found in tumour and at the level of adjacent dysplastic epithelium, labelled cells being located mainly basal and parabasal and sometimes located in the entire epithelium and p16 proved to be a specific marker for dysplasic epithelium (2). Jiang *et al* researched the presence of EBV in 23 samples of normal oral squamous epithelium, including 5 from tonsil, 12 from base of tongue and 6 from normal margins of dysplasia, 29 cases of squamous dysplasia and 26 cases of oral squamous cell carcinoma (OSCC). Expression of EBER1 was not detected in the normal margin epithelium, but were found in 5/8 mild, 6/8 moderate and 5/13 severe grade dysplasia tissues. The frequency and levels of expression in OSCC epithelial tissues did not differ from those in severe grade dysplastic epithelium (3).

Our study showed 2 of 16 (12.5%) HPV positivity with CISH in TSD, which is low but in accordance with the literature (1, 4, 6, 15, 18). In the case of high dysplasia with HPV, positive immunohistochemical expression of p16 protein was also found. One another HPV-positive case with PCR could not be typed with genotyping. Thus, the investigation of HPV genotyping is not useful in identifying dysplasic lesions using aged archived paraffin blocks or due to insufficient quantity (18). There was a correlation between high-grade dysplasia and Ki67, p16, EBV expression and apoptosis .

CONCLUSION

This study did not reveal a statistically significant relationship between HPV and EBV and neoplastic changes. It appears that HPV and EBV do not play major role in TSD, although HPV involvement cannot completely be ruled out in lesions of the tongue.

AUTHORS' NOTE

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTION

KB: participated in the design of the study, conceived the study, analysis and interpretation of data, ŞK: acquisition of data, HİK: helped to draft the manuscript, NK: acquisition of data. All authors read and approved the final manuscript.

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