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 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 human papillomavirus genotypes with Real-time PCR.

Methods: Twenty three tongue biopsies were detected with immunohistochemically for p16, Epstein-Barr virus and Ki67 and Real-time PCR and chromogenic in-situ hybridization for human papillomavirus.

Results: Twenty three tongue biopsy specimens diagnosed as pre-neoplastic and suspicious lesions for dysplasia (n:17) and human papillomavirus infection (n:6) microscopically, have no invasive carcinoma. Chromogenic in-situ hybridization for human papillomavirus DNA (HPV-III family16), There was no immureactivity for Epstein-Barr virus. p16 positivity was found in 25% of dysplasia. One lesion was positive for human papillomavirus by Chromogenic in-situ hybridization and one case was positive by Real-time PCR for human papillomavirus.

Conclusions: These evidences suggested that human papillomavirus infection plays a role in pathogenesis of squamous dysplasia localized tongue, but not Epstein-Barr virus infections.

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

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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 (OPL) (1-3) and oral squamous benign lesions(3,4-7). Single way for detection have limitations(8). HPV 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 the G1 to S transition; therefore, inactivation of the p16 gene enables unregulated cell growth(6). OPLs is most reliable predictors of malignant development(5). Histopathologic evaluation of dysplasia is subjective and there is differences inter- and intra-observer(9-11). This study aimed to investigate the correlation between squamous dysplasia localized tongue (TSD) and expression of p16 and Ki67 immunohistochemically and HPV genotypes with Real-time 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, have no invasive carcinoma. Cases were selected from the pathology archives of Dr.Lütfi Kirdar Kartal Education and Research Hospital between the years of 2010-2013. All the procedures were approved by the Ethical Committee of Dr.Lütfi Kirdar Kartal Education and Research Hospital. All biopsy specimens were re-evaluated for dysplasia, ulceration, inflammation, intraepithelial inflammatory infiltration, achantosis, rete condition, apoptosis, suprabasal mitosis, parakeratosis, foreign 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 with immunohistochemically for p16, anti-
Epstein-Barr virus 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, UK) with diaminobenzidine as the chromogen and hematoxylin as the nuclear counterstain. All immunohistochemical process was 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). EBV 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 CISH method and analyzed 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.
“PyroMarkTM Q96 ID system” (Qiagen, Germany), 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 “PyroMarkTM 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, 59, HPV IR, type 26, 30, 53, 66, 67, 68a, 70, 73, 82, 85 and HPV LR type 6, 11, 40, 42, 43, 44, 54, 55, 61, 69.

Statistical assessments were performed by using SPSS 17 for Windows. Pearson chi-square test was used for evaluation of the presence of dysplasia, suprabasal mitosis in squamous epithelium, p16 positivity, 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 ranging in age 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 years to 67 years with mean age of 49,2 years. Cases of the dysplasia were 68,7% in age groups of 60 to 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 positivity was found 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 1 out of 4 males. The p16 reaction was also identified at the some normal elements such as fibroblasts, glandular acinus, muscle fibers, ductal epithelium and endothelium.

EBV was positive in 2 of 4 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; $r = 0.486$, $p=0.018$).

Immunoeexpression 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 1) for HPV by CISH and another case with low grade dysplasia was positive for HPV by Real-time PCR. Both two positive cases were not successfully typed, probably due to insufficient quantity. 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 with PCR was negative for p16.

There was no correlation between the nature of the lesion and presence of ulceration, inflammation, intraepithelial inflammatory infiltration, achantosis, parakeratosis, an 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 factor for development of oropharyngeal cancers are tobacco and alcohol. HPV in oral and oropharyngeal carcinoma was first proposed by Syrjanen et al. in 1983 (12). HPV has been found to be 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 tumoral growth. This tumoral growth may occur as a result of a multicarcinogenic interaction together with some other carcinogens and co-carcinogens (13,14). Hoffmanna 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 analyzed cases (64.7%). The reaction was found in tumor and at the level of adjacent dysplastic epithelium, labeled cells being located mainly basal and parabasal and sometimes located in the entire epithelium and p16 proved to be a specific marker for dysplastic epithelium (2). Jiang et al. researched 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, that is, 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.

CONCLUSIONS

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 of 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.
REFERENCES


Figure: Chromogen *in-situ* hybridisation for HPV III family 16 for high grade dysplasia on tongue (CISH, x200).