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Epstein–Barr Virus and Human Papillomavirus in Snuff-induced Lesions of the Oral Mucosa

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Epstein–Barr virus (EBV) and human papillomavirus (HPV) have been associated with benign as well as malignant oral lesions. We examined 43 patients with snuff-induced lesions and 22 control patients with clinically healthy oral mucosa for the presence of HPV and EBV. Polymerase chain reaction was performed on fresh frozen oral biopsies with degenerate consensus primers for HPV and nested primers for EBV. None of the 43 snuff-induced lesions or the 22 control biopsies were HPV-positive. Seven of the 43 (16.3%) snuff-induced lesions and one of the 22 (4.5%) controls were positive for EBV. The snuff-induced lesions were classified according to clinical severity, grade 1 lesions being the least severe and grade 4 the most severe. Eleven percent of grade 2 lesions, 15.8% of grade 3 lesions and 20% of grade 4 lesions were EBV-positive. Neither EBV nor HPV seem to be associated with snuff-induced lesions. *Key words: oral snuff, polymerase chain reaction.*

INTRODUCTION

The association between human papillomavirus (HPV) and the development of benign and malignant genital tumours is well established. A large number of studies have also examined the relationship between HPV and cancer in the head and neck region (1, 2). The associations between smokeless tobacco keratoses and reactive, putative premalignant lesions and HPV have also been studied (1, 2).

The Epstein–Barr virus (EBV) is widespread in the normal population and 70–90% of adults have demonstrable EBV antibodies. EBV is strongly associated with oral hairy leukoplakia which is a non-precancerous lesion of the tongue often seen in HIV-infected patients (3). EBV is also associated with infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma and polymerase chain reaction (PCR) has been used successfully to detect EBV in the saliva of patients with these diseases (4). In contrast to Burkitt's lymphoma, EBV DNA is detected in epithelial cells of nasopharyngeal carcinoma (1). The association between EBV and malignant and pre-malignant lesions of the oral cavity has also been studied in a few investigations (5, 6). Suggestions have been made that HPV and EBV are cofactors, as are tobacco and alcohol consumption, in the development of oral squamous cell carcinoma in the immunocompromised patient (7).

The aim of the present study was to examine the possible relationship between presence of HPV and EBV and Swedish smokeless tobacco exposure in snuff-induced oral lesions using a PCR-based method.

MATERIALS AND METHODS

Patients

Biopsies were obtained from macroscopic snuff lesions in 43 patients [41 men and 2 women, mean age 43.4 ± 10.6 (± SD) years] who volunteered for a tobacco cessation programme and from healthy oral mucosa in 22 control patients [21 men and 1 women, mean age 41.6 ± 7.4 (± SD) years] at the Department of Oral & Maxillofacial Surgery, Göteborg University, Sweden. The biopsies were taken from the vestibular frontal region in the maxilla. Control patients had no ongoing smokeless tobacco habits. Informed consent was obtained from all patients. The patients had no ongoing or previous history of EBV- or HPV-associated disease. The study was approved by the Ethics Committee of Göteborg University.

Clinical methods

The clinical appearance of the snuff-induced lesions was graded according to the criteria given by Axell et al. (8):

Grade 1 "A superficial lesion with a colour similar to the surrounding mucosa with slight wrinkling and no obvious thickening"
Grade 2 "A superficial whitish or yellowish lesion with wrinkling and no obvious thickening"
Grade 3 "A whitish–yellowish to brown wrinkled lesion with intervening furrows of normal mucosal colour and obvious thickening"
Grade 4 "A marked whitish–yellow to brown and heavily wrinkled lesion with intervening deep and reddened furrows and/or heavy thickening"

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The snuff habits of the patients were elicited as follows:

1. Number of years with snuff habit (a)
2. Daily exposure to snuff in hours (b)
3. Daily consumption of snuff in grammes (c)
4. Total exposure (TE) = a × b × c

Tissue processing and morphological examination

Biopsies were obtained under local anaesthesia (20 mg/ml lidocaine + 12.5 µg adrenaline; Astra, Södertälje, Sweden). The specimens were immediately placed in 99% alcohol, kept at room temperature for 24 h and then stored at −20°C until analysed. The biopsies were sectioned (5 µm) and the epithelium identified.

DNA extraction from fresh biopsies

Fresh frozen biopsies were weighed and homogenized in a brass mortar (cooled to −70°C in liquid nitrogen) and disolved in 450 µl sterile TE buffer (50 mM EDTA; 10 mM autoclaved Tris–HCl pH 7.5) to which 50 µl 10 mg/ml proteinase K was added. The sample was incubated in a water bath at 37°C overnight. The samples then underwent phenol–chloroform DNA extraction according to standard procedures. During the DNA extraction process, negative controls were included to exclude contamination during the process.

PCR HPV

The PCR reaction was carried out according to standard procedure described previously (9). As a positive control in every PCR assay, SiHa cell lines (ATCC No. HTB 35) and HeLa cell lines (ATCC No. CCL 2) were used. As a negative control in every PCR assay, a reaction mixture with water was used. Every assay was also controlled with a parallel reaction of β-actin (9).

PCR EBV

The primers used in the nested PCR assay and the PCR conditions have been described elsewhere (10). DNA from the EBV-positive Raji cell line (ATCC No. CCL 86) was used as a positive control sample in the PCR assay. As a negative control, a reaction mixture with water was used. Every EBV reaction was controlled with a parallel reaction of β-actin (9).

Gel electrophoresis

The PCR products were analysed by gel electrophoresis in a 3% agarose gel. The amplified product was visualized by staining the gel with ethidium bromide (1 µg/ml in Tris borate buffer) and inspected under ultraviolet illumination.

Southern blot hybridization

Southern blotting was used to confirm the negative HPV results with the consensus primers. The products of PCR with consensus primers were hybridized to consensus probes GP1/G2. The procedure was performed according to the standard method described earlier (9).

Statistics

Differences in EBV prevalence between snuff users and controls were analysed with Fisher's exact test. For comparison of EBV prevalence between the different groups of snuff users, the Mann–Whitney U-test was used. ANOVA was used to compare continuous snuff habit data between the different groups of snuff users. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

Clinical evaluations

The severity of the snuff-induced lesions in relation to age and snuff habits is shown in Table I. The average duration of the snuff dipping habit was 21.5 ± 8.4 ($\pm$ SD) years and the average daily snuff consumption was 4.9 ± 2.6 ($\pm$ SD) grammies. There was no significant difference with regard to duration of snuff-dipping habit, daily snuff consumption or daily snuff exposure in hours between the clinical groups. However, when the TE was compared between the clinical groups, a statistically significant difference was seen between patients with grade 4 and grade 2 lesions and between patients with grade 3 and grade 4 lesions ($p = 0.0359$).

HPV status

In all 65 specimens a visible PCR product was seen at the expected size of β-actin in the agarose gel and considered positive for β-actin. The 43 snuff-induced lesions and the 22 controls had no visible PCR product on the agarose gel at the expected size of the HPV consensus primer product. They were also negative with the Southern blot hybridization analysis and therefore considered negative for HPV.

EBV status

EBV positivity was seen in 7 of the 43 (16.3%) snuff-induced lesions and in 1 of the 22 (4.5%) control specimens. One of 9 (11%) grade 2 lesions, 3 of 19 (15.8%) grade 3 lesions and 3 of 15 (20%) grade 4 lesions were EBV-positive (Table II). Neither the difference in EBV positivity between snuff-induced lesions and control biopsies ($p = 0.2484$) or that between patients with different grades of lesions ($p = 0.1369$) was statistically significant. EBV status in

Table I. *Degree of clinical severity in relation to age and snuff habits of the patients*

Clinical grade	No.	Age in years (mean ± SD)	Duration of habit in years (mean ± SD)	Daily exposure in hours (mean ± SD)	Daily consumption in grammes (mean ± SD)	Total exposure (mean ± SD)
2	9	43.4 ± 4.6	20.2 ± 7.0	14.6 ± 1.0	3.8 ± 2.0	1065 ± 608
3	19	44.1 ± 8.6	20.9 ± 8.2	15.2 ± 2.4	4.5 ± 2.1	1370 ± 893
4	15	42.7 ± 10.9	23.1 ± 9.7	15.4 ± 2.2	6.2 ± 3.1	2419 ± 2015
Total	43	43.4 ± 10.6	21.5 ± 8.4	15.1 ± 2.1	4.9 ± 2.6	1672 ± 1444
Controls	22	41.6 ± 7.4	–	–	–	–

Table II. *Prevalence of EBV and HPV in snuff-induced lesion*

Snuff lesion grade	*n*	HPV (*n*)	EBV (*n*)	EBV (%)
2	9	0	1	11.1
3	19	0	3	15.8
4	15	0	3	20.0
Controls	22	0	1	4.5

relation to age and snuff habits is shown in Table III. There was no statistically significant difference between the EBV-positive and EBV-negative patients with regard to snuff habits or TE.

DISCUSSION

The histological appearance of snuff-induced lesions closely resembles that of oral leukoplakias, which are considered to be premalignant lesions. While the aetiology of oral leukoplakias in most cases is unknown, snuff-induced lesions have a known aetiology which makes it easy to measure the smokeless tobacco exposure and then to make comparisons with other variable, i.e. viral presence. However, clinical investigations have failed to show any carcinogenic effect of oral snuff in a Swedish population (11). No HPV positivity was found in snuff-induced lesions in this investigation, which was surprising because previous analyses of other oral lesions have detected HPV in lichenoid lesions (27.3%), leukoplakias (29.6%) and oral cancers (12.5%) (12). The evidence for an interaction between HPV and smokeless tobacco in these snuff-induced lesions therefore seems to be weak.

Previous Scandinavian studies on snuff-induced lesions were also negative with regard to HPV (13, 14) and the same was reported by Shroyer and Greer (15) who found no HPV-positive smokeless tobacco keratoses in 10 investigated lesions. Shroyer and Greer compared PCR with *in situ* hybridization and found PCR to be the more effective and reliable method. In our study, we used the more sensitive PCR-based method, while Ibrahim et al. (13) and Praetorius et al. (14) used *in situ* hybridization. The absence of HPV in the control group in our study corresponds well to the finding of Eike et al. (16), who also found 0% HPV positivity in normal oral mucosa.

Praetorius et al. (14), studied the presence of EBV in 24 snuff-induced lesions in a Danish population with the less sensitive *in situ* hybridization technique, and reported no EBV-positive specimens. Horiuchi et al. (6) found EBV in 5.3% of the oral leukoplakias and increasing EBV positivity in the more dysplastic lesions. They gave three possible explanations: (1) EBV infection may be involved in the carcinogenesis of oral squamous cell epithelium; (2) EBV easily infects squamous cell carcinoma cells; and (3) EBV exists in cancer cells as a passenger with no biological role. Cruz et al. (5) found 77.8% EBV positivity in premalignant lesions and increased EBV positivity in oral squamous cell carcinomas when compared with clinically normal oral mucosa. The fact that they did

Table III. *EBV status in relation to age and snuff habits*

EBV status	No.	Age in years (mean ± SD)	Duration of habit in years (mean ± SD)	Daily exposure in hours (mean ± SD)	Daily consumption in grammes (mean ± SD)	Total exposure (mean ± SD)
Positive	7	41.0 ± 9.3	24.7 ± 9.3	15.1 ± 1.7	5.1 ± 2.1	2126 ± 1468
Negative	36	43.9 ± 10.8	21.0 ± 8.2	15.1 ± 2.2	4.9 ± 2.7	1584 ± 1443

not find any EBV in a cell line derived from an EBV-positive tumour suggested to them that EBV is not present in the tumour cells or that EBV presence is not important for *in vitro* propagation of tumour cells. In our study, EBV most probably exists as a passenger, as no clinical evidence of malignant transformation was documented in the snuff-induced lesions. No statistically significant difference was found between snuff lesions and clinically normal mucosa, thus supporting this assumption.

Subjects with snuff-induced lesions have been shown to have a deficiency in their local immune defence. The number of Langerhans cells is decreased in comparison with healthy oral mucosa and small salivary glands are found to be irreversibly destroyed by the snuff habit (17, 18). Also, oral exposure to snuff significantly depresses peripheral blood natural killer cell activity in the rate (19). In congruence, this might result in increased shedding of EBV from natural reservoirs. However, the results of our study do not support this theory as no difference was seen between subjects with snuff lesions and controls with regard to EBV positivity. Even though 16% of the snuff lesions were EBV-positive, the decreased local immune defence does not seem to affect EBV prevalence. The patients had no known history of EBV-associated diseases even thought 70–90% of adults in the general population have demonstrable antibodies against EBV. A majority of snuff users inserted the snuff using their bare fingers, possibly leading to the transfer of viral infectants, and this therefore could contribute to the differences found in virus prevalences. However, this does not seem to be the case in this study.

The EBV prevalence in clinically healthy oral mucosa found in this study is also low compared with that found in other studies (5, 20). Mao and Smith (20) discuss the possibility that EBV DNA was present in saliva that contaminated the smears. The PCR technique does not allow localization of the virus within the tissue and the possibility that EBV might be disseminated in the saliva or be present in infiltrating lymphocytes cannot be excluded, even though maximum effort was made to separate epithelium from connective tissue. However, Horiuchi et al. (6) used DNA *in situ* hybridization procedure to confirm that EBV genomes were localized in the nucleus of cancer cells in $\approx 60\%$ of squamous cell carcinomas and 25% of cancer *in situ* cases showing positive PCR results.

In this investigation, we have confirmed a lack of correlation between mucosal lesions of the oral cavity in snuff users and the detection of EBV DNA and HPV DNA by PCR of samples from these lesions. Both HPV and EBV have been suggested to be involved in oral cancer development. The use of snuff does not seem to enhance infections with either EBV or HPV.

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REFERENCES

1. Syrjänen S. Viral infections in oral mucosa. Scand J Dent Res 1992; 100: 17–31.
2. Praetorius F. HPV-associated diseases of oral mucosa. Clin Dermatol 1997; 15: 399–413.
3. Sandvej K, Krenacs L, Hamilton-Dutoit S, Rindum J, Pindborg J, Pallesen G. Epstein-Barr virus latent and replicative gene expression in oral hairy leukoplakia. Histopathology 1992; 20: 387–95.
4. Saito I, Nishimura S, Kudo I, Fox R, Moro I. Detection of Epstein-Barr virus and human herpes virus type 6 in saliva from patients with lymphoproliferative diseases by the polymerase chain reaction. Arch Oral Biol 1991; 36: 779–84.
5. Cruz I, Brule AVd, Steenbergen R, et al. Prevalence of Epstein-Barr virus in oral squamous cell carcinomas, premalignant lesions and normal mucosa—a study using the polymerase chain reaction. Oral Oncol 1997; 33: 182–8.
6. Horiuchi K, Mishima K, Ichijima KK, Sugimura M, Ishida T, Kirita T. Epstein-Barr virus in the proliferative diseases of squamous epithelium in the oral cavity. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995; 79: 57–63.
7. Flaitz C, Nichols M, Adler-Storthz K, Hicks M. Intraoral squamous cell carcinoma in human immunodeficiency virus infection. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1995; 80: 55–62.
8. Axell T, Mörnstad H, Sundström B. The relation of the clinical picture to the histopathology of snuff dippers' lesions in a Swedish population. J Oral Pathol 1976; 5: 229–36.
9. Ting Y, Manos M. Detection and typing of genital human papillomaviruses. In: Innis M, Gelfand D, Sninsky J, White T, eds. PCR protocols. A guide to methods and applications. San Diego, CA: Academic Press, 1990: 356–67.
10. Voog E. Genital viral infections. Studies on human papilloma virus and Epstein-Barr virus. University of Göteborg, 1996.
11. Schildt EB, Eriksson M, Hardell L, Magnusson A. Oral snuff, smoking habits and alcohol consumption in relation to oral cancer in a Swedish case-control study. Int J Cancer 1998; 77: 341–6.
12. Sand L, Jalouli J, Larsson PA, Hirsch JM. Human papilloma viruses in oral lesions, Anticancer Res 2000; 20: 1183–8.
13. Ibrahim S, Warnakulasuriya K, Idris A, Hirsch JM, Johnson N, Johannessen A. Expression of keratin 13,

14 and 19 in oral hyperplastic and dysplastic lesions from Sudanese and Swedish snuff-dippers: Association with human papillomavirus infection. Anticancer Res 1998; 18: 635–46.
14. Praetorius F, Syrjänen S, Padayachee A, Pindborg J. Human papillomavirus and snuff-induced oral leukoplakias. In: Hausen HZ, Gissman L, eds. The Ninth International Papillomavirus Workshop. Heidelberg, 1990: 255.
15. Shroyer K, Greer RO Jr. Detection of human papillomavirus DNA by in situ DNA hybridization and polymerase chain reaction in premalignant and malignant oral lesions. Oral Surg Oral Med Oral Pathol 1991; 71: 708–13.
16. Eika A, Buchwald C, Rolighed J, Lindeberg H. Human papillomavirus (HPV) is rarely present in normal oral and nasal mucosa. Clin Otolaryngol 1995; 20: 171–3.
17. Daniels T, Hansen L, Greenspan J, et al. Smokeless tobacco-associated epithelial and Langerhans cell changes. Smokeless tobacco or health. Monograph 2. NIH, Bethesda, Maryland, USA 1992: 66–73.
18. Hasséus B, Wallström M, Österdahl BG, Hirsch JM. Immunotoxic effects of smokeless tobacco on the accessory cell function of rat oral epithelium. Eur J Oral Sci 1997: 105: 45–51.
19. Johansson S, Hirsch JM, Johnson D. Effect of repeated oral administration of tobacco snuff on natural killer-cell activity in the rat. Arch Oral Biol 1991; 36: 473–6.
20. Mao E, Smith C. Detection of Epstein-Barr virus (EBV) DNA by the polymerase chain reaction (PCR) in oral smears from healthy individuals and patients with squamous cell carcinoma. J Oral Pathol Med 1993; 22: 12–7.

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