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Smokeless tobacco increases aneuploidy in oral HPV16 E6/E7-transformed keratinocytes *in vitro*

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BACKGROUND: The scope of this work was to study synergism between human papillomavirus (HPV) infection and tobacco *in vitro*, both known to be independent risk factors for oral cancer.
METHODS: HPV-positive and HPV-negative oral keratinocytes and oral HPV-negative fibroblasts were exposed to smokeless tobacco extract (STE) prepared from the Scandinavian (STE1) and US-type (STE2) snuff. Cell cycle profiles were determined with flow cytometry, and HPV E6/E7 mRNA expression in HPV-positive cells was assayed using RT-qPCR.
RESULTS: The exposure of HPV-positive keratinocytes with STE2 increased the number of aneuploid cells from 27% to 80% of which 44% were in S-phase, while none of the diploid cells were in S-phase. The changes after STE1 exposure were less than seen after STE2: from 27% to 31% of which 34% were in S-phase. STE had no effect on HPV16 E6/E7 expression in HPV-positive keratinocytes. In oral spontaneously transformed, HPV-negative keratinocytes, the number of aneuploid cells at G2-M stage increased after STE1 and STE2 exposure from 3% to 9% and 7%, respectively. In HPV-negative oral fibroblasts, the number of cells at G2-M phase increased from 11% to 21% after STE1 and 29% after STE2 exposure.
CONCLUSIONS: The effect of STE varied in the cell lines studied. STE2 increased significantly the proportion of aneuploid cells in HPV-positive oral keratinocytes, but not HPV16 E6/E7 expression. This indicates that tobacco products may enhance the effects of HPV 16 and the risk of DNA aneuploidy increasing risk to malignant transformation.

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Introduction

Smoking and alcohol consumption are the main risk factors for oral cancer, explaining nearly 80% of these cancers (1). In 2009, IARC concluded that there is sufficiently evidence that HPV16 can cause oral cancer. Currently, approximately 10–30% of oral cancers are associated with HPV while the figures are much higher for oropharyngeal cancer varying from 50% up to 80% (2–4). According to tentative evidence, patients with head and neck carcinoma (HNSCC) were classified as HPV-positive and HPV-negative, with better and worse prognosis, respectively, and the smoking history was not found to have association with etiology or patient survival (5, 6). However, more recent data have shown that smoking affects the outcome of HNSCC both with and without HPV infection. Patients with HPV infection alone have the best prognosis, and heavy smokers with HPV infection have an intermediate prognosis, whereas HPV-negative heavy smoking do present with the worst outcome (7, 8).

In 1993, the first evidence was provided that current smoking increases the risk of HPV infection in the uterine cervix (OR = 2.7; 95% CI 1.7–4.3) (9). The risk of cervical squamous cell carcinoma is increased among current smokers, in a dose-dependent manner (10). Smoking also seems to increase the risk of HPV infection and virus-associated diseases at other anatomic sites such anal and male genital tract as well as head and neck region (7, 11–13).

Important *in vitro* models for HPV-induced oral carcinogenesis have been developed. Li and coworkers showed already in 1992 that HPV16-immortalized cell lines could be transformed by exposure to either benzo[a]pyrene or methanesulfonic acid ethyl ester (14). Similar multistep events in HPV carcinogenesis have been described in genital keratinocytes, *in vitro* (15–17). A functional interaction has been described between tobacco smoke and HPV16 E6/E7 in malignant transformation of lung cancer cells *in vitro* (18). Moreover, tobacco carcinogens do not affect only keratinocytes but also oral fibroblasts (19).

In this study, we used (i) spontaneously transformed oral and skin keratinocytes, (ii) HPV-transformed oral keratinocytes, and (iii) normal oral fibroblasts as a model for testing the effects of two varieties of snuff (Scandinavian-type snuff and US-type snuff) on the cell cycle profile and transcription

of HPV16 E6 and E7 oncogenes analyzed by flow cytometry and real-time PCR, respectively. The hypothesis was that snuff exposure alters the cell cycling of HPV16-transformed cells more than that of the other cell lines.

Materials and methods

Cell lines

Three human keratinocyte cell lines, HMK, IHGK, and HaCaT, and oral fibroblasts were used. The HMK oral keratinocyte cell line was established originally from a biopsy specimen of a healthy gingiva, but the cells were found to be spontaneously transformed (20). HMK cells were grown in KGM [keratinocyte-SFM with calcium (Gibco, Invitrogen Co. Paisley, UK)]. IHGK cells are normal gingival keratinocytes that have been immortalized with HPV16 (21). IHGK cells proliferate only in enriched keratinocyte growth medium [defined keratinocyte serum-free media (DK-SFM); Gibco] with low calcium and without serum. IHGK cells below 100 passages were used in this study. HaCaT cells, a spontaneously immortalized skin keratinocyte cell line (22), were maintained in Dulbecco's modified Eagle's medium (D-MEM) (Gibco BRL, Paisley, UK), supplemented with 1% non-essential amino acids (Gibco BRL), 2 mM L-glutamine (Gibco BRL), 10% fetal bovine serum (Gibco), 100 μg/ml streptomycin, and 100 IU/ml penicillin (Gibco). Fibroblasts established from the gingival biopsy were cultured in Dulbecco's minimal essential medium (D-MEM), supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 50 μg/ml streptomycin, 100 U/ml penicillin, and 10% fetal calf serum. All experiments used the same cell batch of each cell line and were run parallel to ensure consistent results. All cells were grown in 75-mm^3 bottles until monolayers for the experiment. The cell line authentication was indirect in this work by constant levels of HPV E6 and E7 in the IHGK cell line and the other cells lines staying negative.

Smokeless tobacco extracts (STE)

Smokeless tobacco extracts were prepared from the commercial loose snuff using the method described by Hirsch and coworkers (23). STE1 was prepared from Ettan (Gothia Snus, Sweden) and STE2 from 1S3 reference snuff (University of Kentucky, Kentucky, USA). The chemical profiles of the snuffs are provided as Table 1. Briefly, 1 g of moist snuff was mixed with 10 ml of cell culture medium. The mixture was incubated for 2 h at 37°C. Then, it was centrifuged twice for 10 min to remove snuff powder. The solution was sterilized by filtration through a 0.45- and 0.2-μm filter. The 10% solution obtained was then diluted to a 0.25% final concentration for the experiments. The snuff extracts were administered to the monolayer cultures in the cell culture medium for 2 days. Control cells were treated similarly but without any snuff in medium. Three parallel cultures were run for each study group. At harvest, 500 000 cells from each experiment were collected for flow cytometry, and the rest of the cells were collected in Trizol and kept at −70°C for RNA extraction.

Flow cytometry

Cell cycle profiles were determined from all cell types investigated using the commercial CycleTEST PLUS DNA reagent kit, the FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA, and BD Biosciences Pharmingen, San Diego, CA, USA), and MODFITLT cell cycle analysis software (Verity Software House, Topsham, ME, USA). Samples were prepared, and determinations were conducted according to the manufacturer's instructions.

Table 1 Analysis of STE1 and STE2 on nicotine and methyl tert-butyl ether dissolving compounds carried out with gas chromatography, mass selective detector followed by a recognition with mass spectrometry database

Snuff	*Compound*	*mg/ml*
STE1 (Ettan snuff)	Nicotine	0.60
	Nicotyrine*	0.03
	3-Oxo-α-ionol	0.01
STE2 (1S3 reference snuff)	Nicotine	0.70
	Nicotyrine*	0.02
	2.3′-Dipuridyl	0.01
	Tetrahydro-4,4-7a-trimethyl-2-benzofuranone	0.01

*3-(1-methylpyrrol-2-yl)pyridine.

RNA extraction

Total RNA was isolated from the IHGK cells by CsCl gradient centrifugation. Cells were first lysed with GIT buffer (4 M guanidine isothiocyanate, 1 M Na-citrate, beta-mercaptoethanol 0.7%, Na-lauryl sarcosine 0.5% pH 7.0), and the cell suspension was mixed with 5.7M cesium chloride (with 2.5% 1M Na-citrate) and ultracentrifuged (30 000 rpm, 21 h, +20°C). RNA was collected from the bottom of the ultracentrifuge tube, impurities were extracted with phenol/chloroform (1:1), and RNA was precipitated with ethanol.

Expression of HPV16 E6 and E7 mRNA in IHGK cell lines

Human papillomavirus 16 E6 and E7 mRNA expression was assayed using the TaqMan real-time quantitative PCR method as described previously (24). The primers and probes were designed using the Primer Express program V2.0 (PE Applied Biosystems, Foster City, CA, USA) (Table 2). The E6 and E7 probes were labeled with 6-carboxyfluorescein at the 5′ end and Dark Quencher (Scandinavian Gene Synthesis AB, Koping, Sweden) at the 3′ end. The amplification conditions were 2 min at 50°C, 10 min at 95°C, and a two-step cycle of 95°C for 15 s and 60°C for 60 s for a total of 40 cycles. Real-time quantifications in triplicates were performed using the ABI PRISM 7700 Sequence Detector. The results were normalized against GAPDH mRNA levels.

Statistical analysis

T-test with MEDCALC, version 12.5.0.0. (MedCalc Software Acacialaan, Ostend, Belgium), was used to analyze the HPV16 E6 and E7 mRNA expression levels after STE exposure.

Results

Table 3 shows the results of flow cytometric analysis of cell lines with and without exposure to STE1 and STE2.

Table 2 HPV16 probes and primers used in this study

HPV16	*Name*	*Sequence*	*Length*	*Mol Wt*
E6				
Probe	16E6PRO	(6-FAM) CAG GAG CGA CCC AGA AAG TTA CCA CAG TT	29	10050
Primer 1	16E6-FP	GAG AAC TGC AAT GTT TCA GGA CC	23	7073
Primer 2	16E6-RP	TGT ATA GTT GTT TGC AGC TCT GTG C	25	7685
E7				
Probe	16E7PRO	(6-FAM) CCA GCT GGA CAA GCA GAA CCG GAC	24	8511
Primer 1	16E7-FP	CAG CTC AGA GGA GGA GGA TGA A	22	6883
Primer 2	16E7-RP	CAC ACT TGC AAC AAA AGG TTA CAA TAT T	28	8549

Table 3 Flow cytometric analysis of the cell lines exposed to STE1 and STE2

	DNA diploid					*DNA aneuploid*				
	Total	*G0-G1*	*G2-M*	*S*	*G2/G1*	*Total*	*G0-G1*	*G2-M*	*S*	*DI/G2/G1*
HMK										
Co	0.00	0.00	0.00	0.00	–	100.0	50.96	3.40	45.64	2.01/1.92
Tetraploid										
STE1	0.00	0.00	0.00	0.00	–	100.0	47.79	8.53	43.68	1.97/1.92
STE2	0.00	0.00	0.00	0.00	–	100.0	55.29	7.16	37.55	1.99/1.90
IHGK										
Co	72.64	33.86	15.05	51.09	2.00	27.36	58.78	41.22	0.00	1.23/1.84
Diploid/aneuploid										
STE1	69.21	47.92	14.43	38.65	2.00	30.79	54.14	11.23	34.63	1.23/1.94
STE2	19.47	86.95	13.05	0.00	2.00	80.53	51.87	3.92	44.21	1.23/1.98
HaCaT										
Co	0.00	0.00	0.00	0.00	–	100.0	68.20	5.43	26.37	1.92/1.93
Tetraploid										
STE1	0.00	0.00	0.00	0.00	–	100.0	70.61	5.38	24.01	1.99/1.92
STE2	0.00	0.00	0.00	0.00	–	100.0	72.76	5.52	23.58	2.00/1.92
Fibroblasts										
Co	100.0	83.04	5.91	11.05	2.00	0.00	0.00	0.00	0.00	–
Diploid										
STE1	100.0	67.29	11.67	21.04	2.00	0.00	0.00	0.00	0.00	–
STE2	100.0	51.01	19.81	29.18	1.99	0.00	0.00	0.00	0.00	–

Co, Control.
Cell lines were highlighted in bold.

Spontaneously transformed oral keratinocytes (HMK) were totally DNA tetraploid with a DNA index of 2.01. A slight decrease in DNA synthesis was found after exposure to STE2, while both STE1 and STE2 exposure increased the number of the HMK cells at G2-M phase from 3.4% to 8.5% and 7.2%, respectively. Of HPV-transformed keratinocytes (IHGK), more than two-thirds were DNA diploid and the rest were clearly DNA aneuploid with a DNA index of 1.23. The proportion of aneuploid IHGK cells increased significantly after exposure to STE2 from 27.4% to 80.5%. At the same time, the percentage of DNA diploid cells in the S-phase dropped sharply, and the percentage of DNA aneuploid cells in the S-phase rose steeply from 0% to 44.2%. The same pattern, however, in a much weaker form, also occurred after exposure to STE1.

Exposure to STE1 or STE2 also increased the proportion of DNA diploid IHGK cells in the G0-G1-phase but not DNA aneuploid cells. Furthermore, it decreased the percentage of DNA aneuploid cells in the G2-M phase from 41.2% to 11.2% (STE1) and 3.9% (STE2), but not the percentage of DNA diploid cells. Spontaneously immortalized skin keratinocytes (HaCaT) were found to be hypotetraploid with a DNA index of 1.92. Neither of the STEs had any considerable effect on this cell line when compared to controls. Gingival fibroblast cells were totally DNA diploid. A twofold rise in DNA synthesis and G2-M-phase cells was found after exposure to STE1 and a threefold rise in S-phase and G2-M-phase cells after exposure to STE2 when compared to controls.

The effects of STE1 and STE2 on GAPD normalized HPV16 E6 and E7 oncogene expression in IHGK cells are shown in Fig. 1. No statistically significant differences were found in HPV16 E6 or HPV16 E7 oncogene expression after STE1 (E6, $P = 0.253$; E7 $P = 0.856$) or STE2 (E6 $P = 0.769$; E7 $P = 0.814$) exposures as compared to the non-exposed control cultures.

Discussion

A multistep model for HPV-induced oral carcinogenesis has been shown, where cells do not undergo senescence but enter an extended life span with shortening of telomere DNA (25). In some of the immortal clones surviving beyond crisis, elevation of telomerase activity and stabilization of telomere length were observed. Furthermore, the E6 and E7 oncoproteins of 'high-risk' HPV disrupt the cell cycle

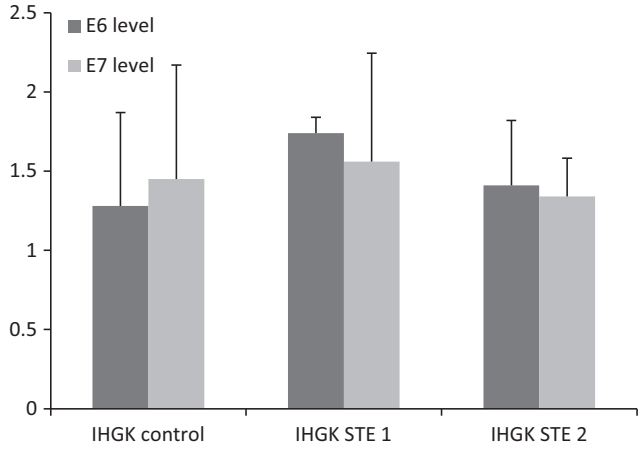

Figure 1 HPV16 E6 mRNA and E7 mRNA expression in IHGK cells after 2-day exposure with STE1 or STE2.

control and DNA repair in HPV-immortalized keratinocytes and enhance mutation frequency resulting from genomic instability. However, HPV infection alone failed to give rise to a tumorigenic cell population requiring additional exposure to chemical carcinogens. Our results with HPV-transformed oral keratinocyte cell line fit to this model. Both STEs increased the proportion of aneuploidy in IHGK cell line, but the US-type snuff, STE2, was more potent in increasing the aneuploidy of IHGK cells than the Scandinavian-type snuff, STE1. This most likely is due to the different chemical profiles of the snuffs (Table 1). Interestingly, neither STE1 nor STE2 exposure had effect on the HPV16 E6 and E7 oncogene expression.

The transforming properties of high-risk HPVs reside primarily in the E6 and E7 oncogenes, which induce genomic instability already in pre-neoplastic lesions, when the viral genome still persists in an episomal state (26). Cell cycle progression is regulated at several checkpoints including the G1-, the G2-, the spindle assembly-, and the post-mitotic checkpoints, whose defects contribute to genomic instability. In the present study, we found that in unexposed IHGK cell cultures, 73% of the cells were diploid and they were mostly in S-phase, while aneuploidy cells were mostly at G0-G1 phase. After STE1 and STE2 exposure, the amount of aneuploid cells increased from 27% to 31% and 81%, respectively. After STE2 exposure, 44% of the aneuploid cells were in the S-phase, while none of the diploid IHGK cells were in the S-phase. This suggests that STE2 provides a growth advantage for HPV16 E6/E7-transformed cells with aneuploidy over the diploid cells. Another explanation could be that diploid cells become aneuploid after STE exposure. However, this is not supported by the fact that none of the diploid cells were in the S-phase, while 44% of the aneuploid cells are in the S-phase after STE2 exposure.

HPV16 E7 oncoprotein disrupts the G1/S-cell cycle checkpoint at multiple levels to promote unscheduled entry into S-phase and viral genome replication by the host cell DNA replication machinery (27, 28). Genomic stability is maintained, in part, by the strict control of centriole duplication, which begins in late mitosis/early G1-phase of the cell division cycle following centriole separation (29). HPV16 E7 has been found to rapidly induce centriole over-duplication, in part, through the simultaneous formation of more than one daughter centriole at single maternal centrioles (28, 30). However, the role of E7 in cell cycling would not entirely explain the growth advantage of aneuploid HPV16 E6/E7 cells over diploid cells found in our experiments. The role of HPV16 E6 in cell cycling in G2 checkpoint control might be critical in this respect. It has been shown that E6 does not affect the mitotic spindle checkpoint (31). Importantly, however, reduced G2 checkpoint function in E6-expressing cells might produce stress on the mitotic spindle and thereby stimulate endo-reduplication. Inactivation of G2 checkpoint function in E6-expressing cells is postulated to be the result of a selective process whereby damaged chromosomes resulting from telomere crisis cause cells with intact G2 checkpoint function to slow growth (32). Cells with reduced G2 checkpoint function have a growth advantage, and they are permitted to enter mitosis with damaged chromosomes (33). These cells with reduced G2 checkpoint function also will tend to enter mitosis before chromatids have been sufficiently de-catenated, which could explained the increased number of IHGK cells with aneuploidy especially after STE2 exposure.

Smokeless tobacco efficiently delivers nicotine and tobacco-specific carcinogens (34, 35). The major accepted pathway of mechanisms of tobacco carcinogenesis involves carcinogen–DNA adduct formation and consequent mutations in critical genes (36). Of the two snuffs, STE2 induced more marked changes than STE1. STE1 and STE2 are based on different mechanisms, which may partly be explained by differences in their chemical composition. The nicotine content of the STE2 is much higher (2.52%) than that of the Scandinavian type used (0.8%) (37). However, we could not confirm this as both snuff extracts used here had nearly similar nicotine contents (analysis by the Laboratory of Toxicology, Regional Institute of Occupation Health, Turku, Finland) (Table 1). Our *in vitro* results are in line with the epidemiological reports showing greater risk of oropharyngeal cancer with STE2, the North American snuff, than STE1, the Scandinavian type of snuff (38).

Although we found the significant increase of aneuploidy in HPV16 E6/E7-transformed IHGK cells after STE exposure, there was no upregulation in HPV16 E6 and E7mRNA expression. Firstly, this may reflect the cell culture conditions where epithelial cells are grown as monolayers without any differentiation. Furthermore, IHGK cell line contains only E6 and E7 genes and not LCR or E2 which are of importance in regulation of E6 and E7 transcription. The other explanation may be that the increase in the amount of aneuploidy is not directly associated with the increase in E6 or E7 expression. It has been suggested that the progression of epithelial cells to cancer is usually a result of both HR-HPV persistent infection as well as cofactors, of which tobacco may be the most important one (39, 40). Indeed, even tobacco use alone increases the number of aneuploid nuclei in clinically healthy oral epithelium (41). Oral dysplasia among smokers has also been significantly linked to DNA aneuploidy (42). Our results support the early findings of 1990s on functional interaction between tobacco

and HPV16 E6/E7 (43–45). The same has been shown with cervical cell lines where HPV16-immortalized cervical cells were more susceptible to DNA damage than their HPV16-negative counterparts (46).

We also tested other cells not related to HPV. Our results showed that STE exposure in these cells were not similar as found in IHGK cell line. In spontaneously transformed oral HMK cell line, STE1 and STE2 suppressed DNA synthesis while it was increased in fibroblasts. It was recently shown that in lung cells, nicotine caused an override of DNA-damage-induced G1/S restriction (47). A twofold rise in DNA synthesis and G2-M-phase cells was also found in fibroblasts after exposure to STE1, and a threefold rise in S-phase and G2-M-phase cells after exposure to STE2, as compared to controls.

It is currently acknowledged that carcinogenesis is not determined only by malignant keratinocytes, but also by the tumor stroma (48). So-called cancer-associated fibroblasts promote tumor growth and progression (49). Our results suggest that STEs may promote normal fibroblasts to a more active direction. Active fibroblasts are associated with increased number of fibroblasts, capillary density as well as type-I-collagen and fibrin deposition *in vivo* (50). Nicotine itself has been found to induce apoptosis of human gingival fibroblasts and generate reactive oxygen species, which again may lead to oxidative stress (19). Clinically, tobacco and snuff use is known to increase oral mucosal fibrosis as well as hyperkeratosis. Because similar effect of snuff was not found in HMK keratinocytes, which all were aneuploidy, these results may reflect the effect of STE in aneuploid cells without HPV infection, resulting only in increase of cells at G2-M phase. As expected, spontaneously transformed skin cell line, HaCat, was resistant to STE exposure without any changes in cell cycling implicating biologic differences between mucosal and skin keratinocytes.

To conclude, STEs, and especially the North American type of STE, increased the aneuploidy of HPV16 E6/E7-transformed oral epithelial cells. Our results suggest a possible synergy between tobacco components and viral oncogenes, providing a growth advantage for aneuploid cells. Growth advantage of aneuploid epithelial cells and activation of oral fibroblasts further support the malignant transition when tobacco exposure is not disconnected.

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Conflict of interest

No conflict of interest.