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Assessment of p53 and Ki-67 expression in snuff-induced lesions

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SUMMARY. **An immunohistochemical study of snuff-induced lesions with a monoclonal antibody (DO-7) specific to p53 mutant and wildtype antioncogene product demonstrated nuclear overexpression of the mutant protein in 45.9 nuclear profiles/mm² epithelium (SEM 10.8; $n=15$) compared with only 0.18 positively stained nuclear profiles/mm² in the control group (SEM 0.18; $n=4$). Furthermore, the biopsy material was also stained with the antibody Ki-67, which has been shown to be excellent for the estimation of the growth fraction in both normal and malignant human tissues. Ki-67 stained positive in 566.1 nuclear profiles/mm² epithelium (SEM 85.0; $n=15$) in the snuffgroup compared with 20.2 nuclear profiles/mm² (SEM 4.9; $n=4$) in the control group. To the best of our knowledge, this is the first study showing overexpression of p53 protein and Ki-67 in snuff-induced lesions. The results may indicate that the p53 gene is involved in the initial events leading to subsequent malignant transformation of oral mucosa exposed to snuff. Furthermore, mutations of the p53 gene have been associated with increased cellular proliferation with greater risk of perpetuation of mutations and malignant transformation.**

INTRODUCTION

The prevalence of snuff-dippers in Sweden is the highest in the world. The snuff used is non-fermented highly alkaline (pH 8–9) moist snuff. Different brands contain varying amounts of chemicals and the quantities of carcinogenic tobacco-specific-N-nitrosamines may range from 5.5 to 106 p.p.m.[1] The snuff habit very often starts in a young age group, thus exposing growing individuals for large amounts of carcinogenic substances. There exist different opinions in the literature as to whether snuff-dipping is a dangerous form of tobacco consumption or not. Kirkland[2] and Russel *et al.*[3] consider snuff-dipping to be more harmless than smoking. However, there are numerous studies reporting on the possible link between the use of snuff and the development of oral squamous cell carcinoma.[1,4–9] Oral cancer is the sixth most common cancer in the world,[10–11] accounting for approximately 4% of all cancers and 2% of all cancer deaths.[12] Its etiology is complex and both endogenous as well as exogenous factors are of importance. Clearly tobacco is one of the most predominant exogene agents which, together with alcohol, is responsible for over 0.75 of the 20 000 reported oral cancer deaths in Europe per year.[13]

There are two types of genes which are strongly implicated in the development of the malignant phenotype.[14] The first group is the oncogenes which through mutations and/or amplifications give rise to proteins leading to abnormal cell growth. The second group is the tumour suppressor genes, which encodes proteins with the ability to suppress cell division. During the development of a tumour, the tumour suppressor genes very often become mutated or deleted and thus lose their normal function to negatively regulate cell growth. The protein p53 is the product of one such well-characterized tumour suppressor gene and thought to be involved in the regulation of DNA replication. In its wild-type form, the p53 protein suppress cell proliferation and transformation.[15–16] However, the p53 gene is a frequent target for mutations and when mutated loses its capacity for growth suppression, allowing unrestricted proliferation.

The p53 protein is present in extremely low concentrations in normal cells, owing to a very short half life[17] and is virtually undetectable by immunohistochemical techniques.[18,19] Hence, the ability to detect the protein in tumours is probably synonymous with the presence of a mutation, since mutation has been shown to stabilize the protein and extending its half-life.[15] In a few recently published studies, a strong correlation between betel and/or heavy tobacco use and overexpression of p53 protein in oral dysplasia and squamous cell carcinomas has been noted.[20–23] However, as far as we know, there have been no reports evaluating the immunohistochemical expression of p53 protein in snuff-dippers lesions. To investigate this possibility, we immunostained 15 lesions from snuff-dippers for presence of the p53 protein. Furthermore, the tissues were also stained with the antibody Ki-67, which reacts with two nuclear proteins present in all phases of the cellcycle except G0. Ki-67 has been shown to be excellent for the estimation of the growth fraction in both normal and malignant human tissues[24] and the antibody is now the usual standard for the assessment of cell proliferation.[25]

MATERIALS AND METHODS

Case selection

Upper lip biopsies from fifteen outpatients (Department of Oral Surgery, Göteborg University, Sweden) were used in the present study. All patients (14 men and one woman, mean age 39.5 ± 12.5 years ($\bar{x} \pm SD$)) were regular snuff-dippers and non-smokers who exhibited snuff-induced lesions, such as a whitish-yellowish to brown wrinkled lesion with intervening normal or reddened furrows. The tissue specimens were obtained through incisional biopsies, randomly enclosing the upper vestibular mucosa, submucosa and minor salivary glands down to the underlying muscular layer. Only clinically changed parts of the mucosa were included.

Samples of normal oral mucosa were obtained from four patients (three women and one man, mean age 37.3 ± 14.4($\bar{x} \pm SD$)) through upper lip biopsies. The patients did not have any past or present history of snuff-dipping or tobacco consumption. The alcohol consumption for both groups was not recorded.

Throughout the study, the ethical rules for research described in the Declaration of Helsinki (1975) were followed.

Immunohistochemical method

The immunohistochemical studies were performed using routinely processed sections, fixed in 4% formaldehyde in phosphate buffer, pH 7.4, for 2–4 days and embedded in paraffin. The specimens were cut in 4 μm-thick sections, and the sections rehydrated and processed with the avidin-biotin-peroxidase complex (ABC) method, following digestion with citric acid used in conjunction with a 10 min microwave treatment for antigen retreival. In brief, the sections were incubated with the primary antisera followed by the secondary biotinylated antibody and the ABComplex (No.K 355, Dako A/S Denmark). The primary antibodies were monoclonal mouse anti-human p53 antibody, DO7 (No.M 7001, Dako A/S, Denmark) and polyclonal rabbit anti-human Ki-67 antigen (No.A 047, Dako A/S, Denmark) diluted 1:100 in 4% normal serum in 0.5 M Tris-saline buffer (TRIS), pH 7.6. The p53 antibody recognizes epitopes residing between amino acids 19–26 of human wild-type and mutant p53. The incubations were performed at room temperature for 30 min. The secondary antibodies were biotinylated rabbit anti-mouse and goat anti-rabbit. The antibodies were diluted 1:200 and 1:300 in Tris, respectively. The immunoreactions were visualized by a chromogen substrate solution consisting of 0.6 mg/ml 3,3'-diaminebenzidine (No. SK-4100, Vector laboratories, USA) and 0.01% hydrogen peroxidase (No. M7209, E Merck, Germany) in Tris for 5 min. Non-specific endogenous peroxidase staining was reduced by section immersion in hydrogen peroxidase before the antibody incubations. Staining specificity was assessed by omission of the primary or secondary antisera or by incubation of the primary antiserum previously absorbed with the antigen. The sections were thoroughly rinsed in Tris before and after incubation, counterstained with Harris hematoxylin and after dehydration were mounted in Eukitt. Positive controls consisted of sections obtained from two strongly p53-positive invasive squamous cell carcinomas.

Positive labelling of p53 and Ki-67 was quantified by determining the number of positive cells expressing nuclear staining in seven randomly selected high-power fields (× 40 objective) from three section planes of the tissue block. The section planes were approximately 200 μm apart. All positively stained nuclear profiles per square millimetre of the epithelium were counted.

The arithmetic mean value for the number of positively stained nuclear profiles/mm^2 was calculated for each of the test groups (snuffgroup $n = 15$, controlgroup $n = 4$). To analyse the divergence from the mean value the standard error of the mean (SEM) was used.

RESULTS

Clinical evaluations

The age and snuff habit of the patients are summarized in Table 1. The mean age of the patients was 39.5 ± 12.5 ($\bar{x} \pm SD$) years and the mean number of years with the snuff-dipping habit was 15.6 ± 6.3($\bar{x} \pm SD$). On average, the patients kept the quid for 13.1 ± 3.8 h($\bar{x} \pm SD$), and the mean number of grams of snuff used daily was 36.1 ± 17.6($\bar{x} \pm SD$).

Histomorphology

Overall, the snuff-induced lesions were histomorphologically characterized by evenly distributed, slight to moderate hyperparakeratinization. Increased epithelial thickness was noted in a majority of the specimens. Atrophic lesions were rare and seen in one specimen only. Vacuolated epithelial cells were frequently noted in the superficial cell layers as well as varying degrees of stromal chronic inflammation (Fig. 1). Lymphocytes and plasma cells were the predominating inflammatory cells and were also seen scattered within the epithelium of three specimens. Increased numbers of mitotic figures were not apparent. No specimen showed any sign of epithelial dysplasia or evidence of invasion of squamous carcinoma. Candidal infection was absent in all biopsies. In a majority of the specimens, salivary gland tissue were present and in general showed slight to moderate fibrosis and chronic, mild sialadenitis.

Table 1 – Age and exposure data ($n = 15$) ($\bar{x} \pm SD$)

Age (years)	Hours of daily snuff use	Grams of snuff used daily	Years with regular snuff habit
39.5 ± 12.5	13.1 ± 3.8	36.1 ± 17.6	15.6 ± 6.3

Fig. 1 – Snuff-induced lesion showing hyperparakeratosis, marked acanthosis and presence of vacuolated cells in the superficial layers of the epithelium (H&E × 100).

Immunohistochemistry

The results of the p53 and Ki-67 staining are summarized in Table 2. Positive staining for p53 protein was noted in 45.9 nuclear profiles/mm^2 epithelium (SEM 10.8) in lesions from snuff-dippers compared with 0.18 nuclear profiles/mm^2 (SEM 0.18) in the controlgroup. The positively stained cells were found more or less scattered among negative cells (Fig. 2), and the intensity of the staining varied between lesions and individual cells. Staining was always confined to the nuclei, and the positive cells were in general found close to the basal cell layers. There was no clear relationship between the number of p53-positive epithelial cells and the histomorphological changes found in the lesion. Nuclei of the positive control cells were positive, and negative controls were non-reactive.

Ki-67 reactivity was recognised as diffuse or dot-like nuclear staining in epithelial cells located in basal and suprabasal cell layers of both normal mucosa and mucosa from snuff-dippers (Fig. 3). However, the number of positively stained nuclear profiles/mm^2 differed markedly between the two groups. The group with snuff-induced lesions showed 566.1 positive nuclear profiles/mm^2 (SEM 85.0) compared with only 20.2 positive nuclear profiles/mm^2 (SEM 4.9) in the control group (see Table 2).

Table 2 – Number of positively stained nuclear profiles of p53 protein and Ki-67 per mm^2 of the epithelium

		Arithmetic mean value		Range	
		$\bar{x}$	SEM	Max	Min
p53	snuffgroup	45.9	10.8	172.9	11.5
	controlgroup	0.18	0.18	0.73	0
Ki-67	snuffgroup	566.1	85.0	1152	106
	controlgroup	20.2	4.9	32.0	14.6

DISCUSSION

The principal finding of the present study is that aberrant expression of the p53 protein was found in all snuff-induced lesions, but was virtually absent in tissue specimens from the control group. The p53 positive epithelial cells showed staining intensity varying between weak to strongly positive. Furthermore, the positive cells were not seen lying in clusters but rather appearing singly among negative cells. Mutations of the p53 gene are said to be the single most common genetic aberration in all human neoplasms,[26–27] and results in expression of a more stable gene product, thus allowing visualization of this oncoprotein by simple immunohistochemical methods. In studies from the UK, a moderate to high prevalence of p53 overexpression in upper aerodigestive tract squamous cell carcinomas has been found. A range of values were reported for oral cancer: 35%,[28] 50%,[29] and 80%[23] and for head and neck carcinomas: 35%[30] and 67%.[23] Thus, mutation of the p53 gene may be said to be a prevalent finding in malignancies in the oral mucosa and head and neck region. However, a few studies have shown that immunopositivity is not always synonymous with mutation. For example, enhanced detection of p53 protein without mutations has been documented in breast cancer[27,31] and in non-neoplastic tissues such

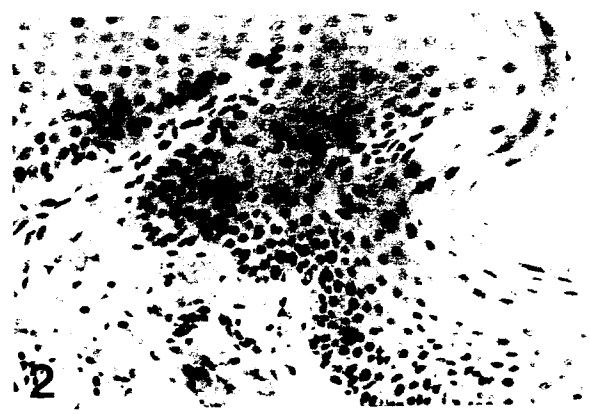

Fig. 2 – Positive p53 nuclear staining in scattered epithelial cells in the basal and suprabasal cell layers in snuff-induced lesion. (Mab DO-7, with light hematoxylin counterstain, ×250.)

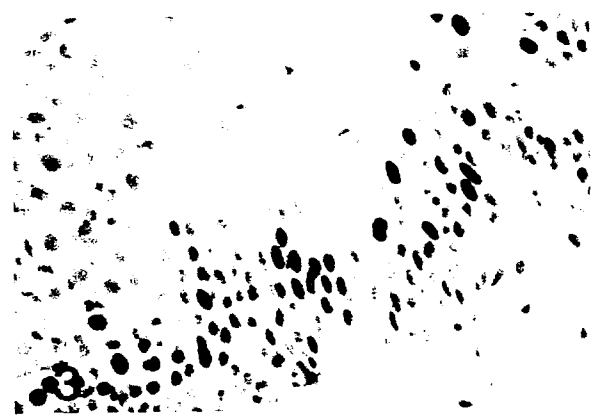

Fig. 3 – Positive Ki-67 nuclear staining in epithelial cells located in the basal and suprabasal cell layers in snuff-induced lesion. (Pab Ki-67, with light hematoxylin counterstain, ×400.)

as psoriatic skin.[32] Furthermore, fixation, temperature, and detection methods, such as antigen retrieval, may also modify the stability of the protein.[33] However, in the present study, there were virtually no p53 positive cells in samples obtained from normal oral mucosa. Also, Ogden et al.[29] assessed the expression of p53 protein in normal, benign and malignant oral mucosa and found not one case of p53 positivity in normal or benign oral lesions. Furthermore, a strong association between tobacco use in the form of smoking or chewing, and p53-positive tumours has been described for lung cancer[34] and head and neck cancer and oral dysplasias.[20–22] Only 1 of 10 cancers from non-smokers was found to be positive for the p53 protein.[20]

The present results may indicate that overexpression of the p53 gene contributes to subsequent malignant cell transformation related to snuff-dipping. Accordingly, alterations of the p53 gene may be involved in initial events leading to snuff-induced oral cancer. Interestingly, not one of the snuff-induced lesions showed any clinical or histopathological signs of epithelial dysplasia or squamous cell carcinoma, thus confirming earlier findings that overexpression of p53 protein is an early event in tumorgenesis.[30,35–37]

Finally, the quantification of Ki-67 positive cells yielded significantly higher numbers in snuff-induced lesions compared with normal oral mucosa. This difference most probably reflects the fact that squamous epithelium exposed to tobacco in the form of snuff very frequently responds with an increased epithelial thickness corresponding to a higher cellular turnover rate. Increased cellular proliferation has been shown to result from the expression of defective p53 protein, with subsequent greater risk of perpetuation of mutations and malignant transformation.[38–41]

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