IMMUNOHISTOCHEMICAL DETECTION OF p53 IN NON-MALIGNANT AND MALIGNANT ORAL LESIONS ASSOCIATED WITH SNUFF DIPPING IN THE SUDAN AND SWEDEN

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Immunohistochemistry was used to examine the expression of p53 in pre-malignant oral lesions and oral squamous-cell carcinomas (SCCs) from Swedish and Sudanese snuff-dippers, as well as in pre-malignant oral lesions and oral SCCs from non-snuff-dippers from the Sudan, Sweden and Norway. Of the 14 SCCs from Sudanese snuff-dippers, 21% (3/14) expressed p53. Of the 64, 60 and 41 SCCs from non-snuff-dippers from the Sudan, Sweden and Norway, 64% (9/14), 65% (39/60) and 68% (28/41) expressed p53, respectively. A statistically significant difference in expression of p53 was found in SCCs from Sudanese snuff-dippers compared to those from non-snuff-dippers from all or any of the 3 countries. None of the suspected pre-malignant oral lesions from Sudanese snuff-dippers or non-snuff-dippers expressed p53. Only 2 out of the 15 oral fibro-epithelial hyperplastic lesions from Swedish snuff-dippers expressed p53. Some of the oral epithelial dysplastic lesions, as well as the carcinoma in situ lesions from Norwegian non-snuff-dippers, expressed p53, while the oral fibro-epithelial hyperplastic lesions did not. The low relative frequency of p53 expression found in oral SCCs from snuff-dippers compared to those from non-snuff-dippers might suggest differences in mechanisms of oncogenic action induced by snuff. Alternatively, the pathogenesis of malignant oral lesions from snuff-dippers may follow a p53-independent pathway. In view of the unusually high levels of the tobacco-specific nitrosonamines (TSNA) found in the type of snuff used in the Sudan, investigations of p53 mutations or oncoproteins are needed.

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Squamous-cell carcinoma (SCC) of the head and neck ranks as the sixth most common malignant neoplasm world-wide (Parkin et al., 1993). In the developing countries, oral cavity and pharynx combined represent the third most common site of cancer (Johnson, 1991). The number of cases of this type of cancer is particularly high in Asia (Parkin et al., 1993) as well as in Africa (Idris et al., 1995), and is now also increasing in the developed countries (Johnson, 1991). The etiology of oral SCCs has been associated with the use of various forms of tobacco and/or alcohol consumption (Binnie et al., 1983; Johnson and Warnakulasuriya, 1993). In Norway, the use of snuff is relatively uncommon (Kraft and Svendsen, 1996) and oral-cancer incidence is also low (Hakulinen et al., 1986). The prevalence of oral cancer is particularly high in the Sudan (16.38%; Idris et al., 1995) compared to Sweden (2.1%; Ostman et al., 1995) and Norway (1.9% for males; 2.5% for females; Hakulinen et al., 1986). Descriptive and case-control studies from the Sudan (Elbeshir et al., 1989; Idris et al., 1994, 1995) have documented an association between oral-cancer incidence and use of snuff (a mixture of tobacco and sodium bicarbonate). In Sweden, a possible link between oral-cancer incidence and snuff dipping has also been suggested (Hirsch and Johansson, 1983; Hirsch et al., 1984).

The tumor-suppressor gene p53 encodes a nuclear phosphoprotein that plays an important role in cell proliferation and differentiation (Harlow et al., 1985; Finlay et al., 1989). A deregulation of cell growth and differentiation occurs when this gene is deactivated by mutation or deletion, or by a virus (Harris, 1991). Several studies have reported the relationship between cigarette smoking, alcohol consumption (Field et al., 1991; Brennan et al., 1995; Lazarus et al., 1996), betel and tobacco chewing (Kaur et al., 1994) and expression of p53 in oral SCCs. However, some studies have shown a lower prevalence of p53 expression in oral SCCs in tobacco chewers (Ranasinghe et al., 1993a, b; Thomas et al., 1994).

The objective of the present study was to determine the relative frequency of p53 expression in suspected oral pre-malignant lesions and in oral SCCs in relation to use of snuff, as a marker for p53 mutation.

MATERIAL AND METHODS

During the period November 1993 to August 1995, 39 patients (29 males and 10 females, mean age 56.9 ± 2.44 SE, SD 15.28, range 18–94 years) with suspected premalignant oral lesions and oral squamous-cell carcinomas (SCCs) were identified. Some of these patients were interviewed (Idris et al., 1994) on use of snuff, years of usage, frequency of daily consumption, position of quid placement in the mouth and use of other tobacco products. All these patients were found to use snuff with a mean dipping period of 21 years (Table 1). The remaining (n = 17) patients did not use snuff or any other form of tobacco. From each patient a surgical tissue sample was taken, fixed in 10% buffered formalin and dispatched to the Department of Oral Pathology and Forensic Odontology, University of Bergen, where it was embedded in paraffin.

From the period March 1992 to December 1993, 15 patients (all males, mean age 39.5 ± 1.03 SE, SD 12.5, range 23–74 years) suspected of having snuff-induced oral lesions, presented at the Department of Oral and Maxillofacial Surgery, Faculty of Odontology, Göteborg. The patients were interviewed on snuff use, years of usage, frequency of daily consumption, position of quid placement in the mouth and use of other tobacco products. All these patients were found to use snuff with a mean dipping period of 11 years (Table 1). After detailed history and clinical examination of the lesions, a surgical tissue sample was taken, fixed in 10% buffered formalin, and further embedded in paraffin. From the period March 1988 to December 1993, hospital records of 60 patients (27 males and 33 females, mean age 60.17 ± 1.11 SE, SD

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Received: May 20, 1996 and in revised form September 5, 1996.
were randomly selected from the files of the Department of Oral Pathology, Faculty of Odontology, Göteborg. From the hospital records, only 8 patients (6 males and 2 females) reported cigarette smoking, with no further information on the amount smoked daily or weekly. Howevcr, no data on alcohol consumption was available. Similarly, no data on alcohol consumption was available.

14.72, range 44–95 years) previously diagnosed with pre-malignant or malignant oral lesions at the Department of Oral and Maxillofacial Surgery, Faculty of Odontology, Göteborg, were randomly selected from the files of the Department of Oral Pathology, Faculty of Odontology, Göteborg. From the hospital records, only 8 patients (6 males and 2 females) reported cigarette smoking, with no further information on the amount smoked daily or weekly. However, no data on alcohol consumption was available. From the period January 1990 to November 1994, hospital records of 60 patients (30 males and 30 females, mean age 64.04 ± 1.72 SD, SD 14.42, range 31–88 years), previously diagnosed with pre-malignant or malignant oral lesions, were randomly selected from the files of the Department of Pathology, Haukeland University Hospital, Bergen. There were 12 fibro-epithelial hyperplasias, 2 epithelial dysplasias, 5 carcinomas in situ and 41 oral SCCs. From the hospital records, only 11 patients (37%, all males) reported cigarette smoking, with no further information on the amount smoked daily or weekly. Similarly, no data on alcohol consumption was available.

The site distributions of the biopsy specimens (n = 174) selected from the Sudan, Sweden and Norway are shown in Table II. Data (except names and other identification numbers) including age, sex, site of the lesion and previous histopathological diagnosis, were made available to the investigators. As positive controls, sections of formalin-fixed, paraffin-embedded tissue specimens from 5 carcinomas of the cervix that were previously p53-positive were included from the Department of Pathology, Haukeland University Hospital, Bergen. As negative controls, formalin-fixed, paraffin-embedded tissue specimens of oral mucosa from 5 normal patients with no history of snuff dipping, cigarette smoking, use of any other form of tobacco and/or alcohol consumption were obtained from the same department.

**Tissue preparation**

From each biopsy specimen (n = 174), 5 sections (5 μm) were prepared. One section was stained with hematoxylin and eosin (H. and E.) to evaluate the histopathological diagnosis. The remaining sections were used for immunohistochemistry.

**Evaluation of the H. and E.-stained sections**

The H. and E.-stained sections were examined with a light microscope. The diagnoses were then confirmed and the lesions were graded as fibro-epithelial hyperplasia, dysplasia (mild, moderate or severe), carcinoma in situ, or SCC. The SCCs were classified as well, moderately or poorly differentiated according to Cawson and Eveson (1987).

**Immunohistochemistry**

Monoclonal antibodies (MAbs). As described (Ibrahim et al., 1992), 2 MAbs (DO-7 and DO-1) were used for detection of p53. DO-7 (DAKO-p53, DAKO, Copenhagen, Denmark) recognizes epitopes residing between amino acids 35–45 of human wild-type and mutant p53 (Vojtesek et al., 1992). It was diluted at 1:100 in PBS, pH 7.2, containing 5% BSA. DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) reacts with an amino-acid terminal epitope (residues 37–45) of human wild-type and mutant p53 (Vojtesek et al., 1992). It was diluted 1:1,000 in PBS/5% BSA.

For immunohistochemistry, a streptavidin-biotin complex protocol was employed. Briefly, tissue sections were deparaffinized in xylene and rehydrated through graded ethanol. Before incubation with the primary MAbs, sections were treated with 0.1% (mg/ml) Protease (bacteroid type xxiv, Sigma, St. Louis, MO) at 37°C for 10 min, and then heated in a microwave oven at a high power setting (700W) and at a lower power setting (425W) for 5 min, respectively. Endogenous peroxidase activity was blocked by using 1% hydrogen peroxide in methanol for 30 min, and the sections were thereafter washed in PBS. The sections were incubated for 30 min with normal rabbit serum (X902, DAKO) diluted 1:10 in PBS/5% BSA, then incubated overnight (18–20 hr) with the primary antibody at room temperature (20–22°C). After washing in PBS, the sections were incubated with biotinylated, rabbit anti-mouse IgG (E354, DAKO) used at a dilution of 1:200 in PBS/5% BSA, for 60 min. The sections were washed in PBS, then incubated for 30 min with an avidin biotin complex (ABC, Vector, Burlingame, CA) for 30 min, washed and slightly counterstained with hematoxylin (20 sec) dissolved in water and mounted with a water-soluble mounting medium (Immuno-mount, Shandon, Pittsburgh, PA). Control sections were incubated in duplicate with PBS or normal rabbit serum instead of the primary antibody.

**Evaluation of the immunohistochemistry**

Whole-tissue sections (including the epithelium subjacent to the non-malignant, pre-malignant and malignant areas when present in the specimens) were examined with a light microscope for p53-positive nuclear staining. The staining was recorded as positive (+; > 10% of all the tumor cells positive) for nuclear p53 staining and as negative (−; < 10% positive cells) for negative staining.

**Statistical analysis**

Using Chi-square statistical analyses at p < 0.05 significance level, the difference in the level of expression of p53 in the suspected pre-malignant and malignant oral SCCs from the Sudan, Sweden and Norway was investigated. p53 expression was also correlated with snuff dipping and/or cigarette smoking.

**RESULTS**

**Light microscopic evaluation**

Histopathological evaluation of the oral tissue specimens is shown in Tables III and IV. There were no differences in the histological pictures of the oral SCCs from snuff-dippers and non-snuff-dippers from the Sudan.

**p53 expression**

Descriptions of p53 expression (DO-7, DO-1), snuff dipping and sites of the oral lesions are shown in Tables III and IV. Of the 14 oral SCCs from Sudanese snuff dippers, 21% (3/14)
The negative control sections from normal oral mucosal snuff-dippers, 13% (2/15) expressed p53 (Table IV), compared to those from non-snuff-dippers from the Sudan, Norway and Sweden respectively. The difference in expression of p53 was statistically significant in the oral SCCs from Sudanese snuff-dippers compared to those of non-snuff-dippers from all 3 countries (Table III). The difference in p53 expression in the oral SCCs from non-snuff-dippers from the 3 countries collectively was not statistically significant (Chi-square test).

Of the 14 oral SCCs from Sudanese non-snuff-dippers, 65% (9/14) expressed p53, while none of the 14 oral SCCs from Swedish non-snuff-dippers, 65% (9/14) expressed p53. p53 expressed (in the atypical surface epithelium as well as in the infiltrating malignant epithelial tissue) in the oral SCCs of Sudanese snuff dippers (Fig. 1a, b) and in those of non-snuff-dippers from the Sudan (Fig. 2a), Sweden (Fig. 2b) and Norway (Fig. 2c) was localized to the nuclei, and no cytoplasmic staining was seen.

Statistical analysis of the difference in expression of p53 in the oral SCCs from Sudanese non-snuff-dippers compared to those from non-snuff-dippers from all 3 countries was significant (Table III). The difference in expression of p53 was statistically significant in the oral SCCs from Sudanese snuff-dippers compared to those from non-snuff-dippers from the Sudan, Norway and Sweden (Table III). The difference in p53 expression in the oral SCCs from non-snuff-dippers from the 3 countries was not statistically significant (Chi square = 0.663). In addition, there was a statistically significant difference between the anatomical site of the oral lesions and p53 expression in the oral SCCs from snuff-dippers and those from non-snuff-dippers in the 3 countries (Table III). The difference in p53 expression in the oral SCCs from Norwegian non-snuff-dippers, 80% (4/5) and 50% (1/2) expressed p53, respectively, (Table IV) and the staining was localized to nuclei of the epithelial basal-cell layer. The ages of the 2 patients were 36 and 50 years, and both had dipped snuff for more than 15 years. Of the 5 carcinomas in situ and the 2 epithelial dysplasias from Norwegian non-snuff-dippers, 80% (4/5) and 50% (1/2) expressed p53, respectively, (Table IV) and the staining was localized to nuclei of the epithelial basal-cell layer. None of the 12 fibro-epithelial hyperplasias from Norwegian non-snuff-dippers expressed p53.

**DISCUSSION**

In the present study, a significantly lower relative frequency (p = 0.013) of p53 expression was found in oral SCCs of...
frequency of p53 expression in the same lesions. Several mechanisms have been suggested to explain the findings of lower relative frequency or absence of p53 expression. Among these mechanisms are the occurrence of allelic deletions of the p53 locus (Blount et al., 1991), nonsense mutations (Hollstein et al., 1991), certain intronic mutations (Takahashi et al., 1990), or p53 degradation by the human papilloma virus (HPV) E6 protein (Scheffner et al., 1990). In addition, formation of p53 complexes with other cytoplasmic proteins has been suggested (Harlow et al., 1985; Pinhasi-Kimhi et al., 1986). The lower relative frequency of p53 expression found in oral SCCs from Sudanese snuff-dippers, in the presence of high levels of tobacco-specific nitrosamine (TSNA) found in snuff, supports the possible occurrence of one of these mechanisms. In non-tobacco-related neoplasms, however, over-expression of p53 has not been shown to be a marker for mutations (Thompson et al., 1992; Barnes et al., 1992).

The high relative frequency of p53 expression found in oral SCCs from non-snuff-dippers was also similar to that observed in other studies (Gusterson et al., 1991; Schipper et al., 1991). In addition, expression of p53 in the oral SCCs of cigarette smokers from Sweden and Norway was consistent with that reported in other studies (Brennan et al., 1995; Lazarus et al., 1996). These data suggest that a history of cigarette smoking is associated with high incidence of p53 expression, as reported by Lazarus et al. (1996). Nevertheless, no data on history of alcohol consumption were available from patients from the Sudan (a predominantly Muslim population), Sweden and Norway.

Epidemiological studies from the Sudan (Idris et al., 1994, 1995, 1996) have suggested a causal relationship between snuff dipping and oral SCCs. Sudanese snuff has been reported to contain unusually high levels of TSNA, namely N'-nitrosonornicotine (NNN) and 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butane (NNK), exceeding the levels reported in any kind of smokeless tobacco including Swedish snuff (Hoffmann and Adams, 1981; Blide et al., 1987; Brunnemann et al., 1987; Idris et al., 1991). However, a study from the USA (Lazarus et al., 1995), using polymerase chain reaction and single-stranded conformation polymorphism analysis, has reported p53 mutations in a patient with severe oral epithelial dysplasia who had dipped snuff for more than 60 years. The lower relative frequency of p53 expression found in oral SCCs from Sudanese snuff-dippers questions the role of TSNA in induction of oral SCCs through p53 inactivation.

In conclusion, the present study has shown a lower relative frequency of p53 expression in oral SCCs as well as in oral fibro-epithelial hyperplasias from Sudanese and Swedish snuff-dippers, respectively, compared to oral lesions from non-snuff-dippers. The validity of the immunohistochemical techniques for detection of p53 and/or other genetic lesions should be examined carefully to understand the pathogenesis of snuff-induced oral SCCs. Studies of p53 mutations within the coding sequence regions of the p53 protein are therefore needed. In addition, the role of other oncogenes and co-carcinogens has to be investigated in snuff-related hyperplastic and premalignant lesions, as well as in oral SCCs, to reveal the possible mechanisms of initiation and progression of snuff-induced oral lesions.

ACKNOWLEDGEMENTS

This study was supported by Colgate Forskningsfond, Colgate-Palmolive Norway A/S. We are grateful to the Toomsk Research Center and Oral Cancer Campaign for providing the Sudanese material. We are also grateful to Ms. G. Albrechtsen for advice on statistical analysis and Ms. G. Øjordsbakken for skilled technical assistance.
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