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REVIEW ARTICLE

Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco – a review

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While carcinogenicity of smokeless tobacco (ST) to humans is well established the oral lesions that precede development of cancer are less well characterized. The clinical appearances of ST-associated lesions are variable. Epidemiological studies show a strong significant association of risk with chronic daily use but population differences are noted because of various commercial products in use. Morphological features observed are some what different to oral lesions caused by smoking and oral dysplasia in ST-associated lesions is less common. Effects of ST on oral keratinocytes observed *in vitro* include alterations in cell proliferation, apoptosis and activation of inflammatory markers. Genetic aberrations caused by ST include activation of *ras*, uncommon in smokers but mutational hot spots in *p53* encountered are similar to those in smokers.
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Introduction

Smokeless tobacco (ST) products contain a large array of carcinogens (1, 2) although the actual number found is fewer than in cigarette smoke (3). Benzo[a]pyrene and other polycyclic aromatic carcinogens (PAHs) are the most important carcinogenic agents in cigarette smoke but in unburnt tobacco, nitrosamines are the strongest carcinogens (4). The metabolites of nitrosamines, particularly nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3 pyridyl)-1-butane (NNK) are found locally in saliva of the oral cavity of ST users, as well as in their body fluids. These agents are known to cause toxic effects particularly cancer (4), and other cellular and DNA changes, either at the local placement sites or by indirect systemic effects. Epidemiological evidence for a significantly increased risk of oral cancer in ST users was recently reviewed (5, 6). Even ST products that are claimed to be low in nitrosamines are likely to raise the risk among users up to 30% of risk for oral cancer in smokers (7). Tobacco-specific N-nitrosamines (TSNA) levels in two US snuff brands were found to increase during 6 months storage at room temperature between 30% and 130% (8).

There are two main types of ST: chewing tobacco and snuff. Chewing tobacco in the form of loose leaf, cut or shredded, is universally available. Snuff for applying, dipping or sucking could be moist or is commercially available as portion bag packed products. Some regulations exist regarding sales and distribution. Finely powdered tobacco marketed as dry snuff is used nasally by few population groups. Worldwide several names are used to denote different ST products: plug, gutkha, khiwam, khaini, iq'milk, zarda, naswar, nass, chimo, toombak, shamma, gudhaku, gul, mishri, maras and moist snus.

This review examines the clinical, pathological, molecular and genotoxic effects of these products. Here the generic term ST will be used to denote the usage of any unburned tobacco while reviewing any toxic effect reported in the scientific literature associated with these numerous chewing, or dipping snuff products. Criteria for considering studies for review included (i) epidemiological studies on oral cancer on populations using ST, (ii) studies of cancer in experimental animals in one or more animal species (iii) studies describing *in vitro* tests using cultured cell systems, and (iv) genetic (molecular) studies on human oral tissues.

The main categories of snuff or chewing tobacco-induced oral mucosal soft tissue lesions reported are:

1 Oral squamous cell carcinoma
2 Verrucuos carcinoma
3 Leukoplakia
4 Erythroplakia

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5 Snuff Dippers' lesion/snus-induced lesion
6 Tobacco and lime users' lesion

Oral squamous cell carcinoma/verrucous carcinoma

Sundstrom et al. (9) described the clinical features of 23 oral cancers in snuff dipping Swedish males (age range 39–52 years). Their mean age was 76 years suggesting a long latent period for cancer development. Seventeen of these cancers were described as clinically exophytic and 11 had histologically bulbous invading fronts consistent with verrucous carcinoma. The authors however, did not attempt to classify these 23 oral cancers as squamous or verrucous. All cancers were in the anterior vestibulum where snuff was usually deposited and retained. Nine of these patients also had second primary tumors, oral or in other sites.

Hirsch et al. (10) reported eight oral cancer cases in Swedish snuff-dippers. Seven of this series were elderly male and had used snuff for longer than 20 years. Their cancers developed exactly at the location where the snuff was placed mostly on the upper vestibulum. All were pathologically confirmed as squamous cell carcinomas. Zatterstrom et al. (11) described a further case of well differentiated oral carcinoma in a 90-year-old Swedish man who had consumed snuff (snus).

Schildt et al. (12) recruited 410 oral cancer cases in a case–control study in Sweden. There were 106 active or ever users of oral snuff and 28 were ex-users. Among their cases 32.6% people had some lifetime exposure to oral snuff. Most common tumor site was the lip. An increased risk was found for lip cancer [odd ratio (OR) 1.8, CI 0.9–3.7] among ex-snuff users. Only one person of ex-users of snuff who had (oral) cancer had begun smoking after having quit using snuff confirming the long-term toxicity of snuff. In a second study by Lewin et al. (13) examining 83 Swedes who had ever used snuff, a high intensity of usage of Swedish snuff was associated with moderately, but not significantly, elevated risks [risk ratio (RR) = 1.7; 95% CI 0.8–3.9]. International Agency for Research on Cancer (IARC) Working Group re-examining these two Swedish publications concluded that the power of these studies was insufficient to demonstrate any possible cancer risk associated with snuff in the oral cavity (14). In a later study Rosenquist et al. (15) found no increased risk of oral cancer associated with the use of Swedish moist snuff (OR 1.1; 95% CI 0.5–2.5). With increased daily exposure (h/day) estimated risks were higher.

From the United States McGuirt and Wray (16) described the clinical profile of 116 patients with oral cavity cancer who were exclusive users of ST with no exposure to smoked tobacco or alcohol. The average age of the case-series was 78.4 years and average period of consumption was 55.5 years. Females were predominant (1:23 male to female ratio). A second primary tumor developed in the oral cavity of 18% (21 of 116) suggesting field cancerization. Forty-five of 91 who were followed up died of or with cancer.

McGuirt (17) earlier described a series of 76 patients with oral cancers who were all ST users. In this series 57 patients reported exclusive snuff use. Females were again predominant (1:3). Common lesion sites were alveolar ridge (32%) and buccal cavity (47%).

Winn et al. (18) in 30 snuff users (who were non-smokers) with cancer of the gum or buccal mucosa demonstrated a significant trend for the reported period of snuff use and their cancers. The risk increased with increasing length of exposure, with risk greatest for anatomic sites where the product was held in contact with oral mucosa (19).

In south Asia where oral cancer incidence is high (20). ST use is commonly reported. Tobacco is often mixed with areca nut, itself considered a carcinogen (21) or with lime (Calcium hydroxide). Several case series of oral cancer are described from countries in Asia, particularly India (22–25) with reference to tobacco chewing or oral snuff use. Recent case–control studies in India estimated population attributable risk at 66% to tobacco chewers and 49% to pan tobacco chewers for the development of oral cavity cancers (26, 27). Undoubtedly, ST is strongly associated with cancer reported from these countries (28). Buccal involvement is reported to be as high as 80% and cancers arise at the site of placement of the tobacco-containing quid, mostly the lower buccal sulcus or posterior buccal mucosa. The tumors appear as red granular areas, exophytic or tend to be ulcerative-infiltrative. Buccal carcinoma with extra oral fungation is not uncommon.

Toombak dipping – a form of snuff used in Sudan – is implicated as a toxic product causing oral cancer (29, 30). Idris et al. (30) documented 646 squamous cell carcinomas of the oral cavity from Sudan. In this series, 375 neoplasms were at the primary site of toombak application (lip, buccal, and floor of mouth). Toombak use was more common in people with cancers of lip, buccal or floor of mouth compared with other oral sites (58% vs. 19%). Five percent to 10% of the cases were under 30 years of age.

Shamma, sometimes known as Yemeni snuff, is a ST product that is usually held between the cheek and gum (gingiva). Ibrahim et al., (31) noted that all thirty eight Saudi patients in their case-series with oral cancer had used shamma. Cancer of the gum (gingiva) is the predominant site for oral cancer particularly in Jizan province where shamma is commonly used (32).

Nass use and associated oral and esophageal cancers are reported in descriptive studies from Uzbekistan or Uzbecks living in Central Asia and Pakistan (33).

In summary, in addition to chewing, tobacco snuff also carries an increased risk of developing oral cancer particularly when it contains a high level of TSNA.

Leukoplakia

Leukoplakias associated with oral use of snuff were described as homogeneous with a wrinkled surface (34). They were either non-elevated or only slightly elevated and were diffusely demarcated from the surrounding mucosa. Pindborg (35) reported some morphological

variations in ST-associated lesions in the form of discrete elevated keratinized striae particularly when involving non-keratinized mucosal sites. These striae, gave the appearance to the lesion described as 'pumice pattern'. A typically wrinkled appearance at the site of placement of the moist snuff and chewing tobacco was described by Axell et al. (36).

In studies in the United States, between 8% and 59% of ST users were found to have oral leukoplakia (37). The considerable range reported could be due to different ST products, length of use, and confusing terminology attributed to the term oral leukoplakia in some studies.

A 60-fold relative risk associated with ST and leukoplakia was reported by Grady et al. (38) for oral mucosal lesions including leukoplakia and snuff-induced lesions.

Among professional baseball players in the US (38) 196 of 423 (46.3%) current users had oral leukoplakia. Most of the lesions were in the mandibular labial/buccal gutter area with 42% in the anterior mandibular area. In 60% the lesion coincided with the site of placement of the quid. The risk was much higher among snuff users compared with ST users (OR 87 vs. 15). Users of Copenhagen and Skoal snuff were at highest risk. The severity of the leukoplakia lesion (graded 1–4 by the authors) increased with increasing amount of use, duration of use, suggesting a dose–response.

Sinusas et al. (39) investigated in detail 88 current users of ST among 220 professional baseball players. Oral leukoplakia was found in 25 of 88 current users (28.4%). Year round users had a significantly higher incidence rate and also higher grades of leukoplakia.

Among 565 US school children (age range 10–17 years) in whom 13.3% were ST users and nine leukoplakias were found, eight of which were in ST users (40).

Among 226 Navajo Indians aged 14–19 years 145 reported ST use. Thirty-seven of the ST users (25.5%) had oral leukoplakia. The duration and frequency of ST use were highly significant for leukoplakia (41). In a study of English coal miners who were tobacco chewers ($n = 280$) 10 (3.6%) were reported with leukoplakia (42).

Leukoplakia often preceded or is found at the time of diagnosis of oral cancer in ST/snuff users. In the series from North Carolina (16), 11 of 76 (14%) had prior leukoplakia excised before a cancer arose and 27 had co-existing leukoplakia suggesting the potential malignant nature of these lesions.

Betel-quid chewers in India who add tobacco to the quid chew approximately 7–12 g of tobacco per day. Mehta et al. (43) diagnosed leukoplakia in 117 of 3674 (1.8%) of betel-tobacco chewers in India. These were predominantly in men over the age of 30 years. Bilateral occurrence was observed in 12–23% of 880 leukoplakias reported (44). Gupta et al. (22) in a 10-year follow up study reported that 15 of 73 new leukoplakias in males occurred in betel-tobacco chewers and all 60 new leukoplakias among females occurred in chewers (non-smokers). Although leukoplakia occurs predominantly on the tongue in Western populations, in India buccal site is more common in tobacco chewers. Coexistence of oral leukoplakia with oral cancer is reported (45).

Multiple oral pre-malignant lesions associated with leukoplakia, notably erythroplakia, and submucous fibrosis were described in a cohort of tobacco chewers in Kerala, India. The presence of multiple oral pre-malignant lesions suggested an effect consistent with field cancerization because of prolonged chewing tobacco (46).

It is clear from these global studies undertaken in the USA, Scandinavia and parts of Asia and Africa that ST use is associated with the development of snuff-induced lesions and oral leukoplakia. There is clear site-specific involvement linked to the pattern of use, chewing and dipping and placement of ST in various sites. This to some extent is distinguishable from leukoplakia associated with smoking. In the US, ST-associated lesions are also found in younger age groups compared with smoking.

Erythroplakia

Only one study has looked at the association of chewing tobacco with oral erythroplakia (47). In this study in Kerala, India the adjusted OR for erythroplakia was 19.8 for individuals who had ever chewed tobacco. Erythroplakia was defined by WHO criteria but it is not clear how the authors excluded other red patches of oral mucosa (48) to diagnose erythroplakia.

Snuff dippers lesion

Axell et al. (49) and later Hirsch et al. (50) classified snuff induced oral mucosal lesions by four degrees. Greer and Poulsen (51) modified these criteria to fit three degrees (Table 1). Axell's criteria had a fourth category as the most severe being a white to yellowish brown, heavily wrinkled lesion with intervening deep, red furrows and/or heavy thickening (52).

Greer and Poulson (51) described oral mucosal alterations in 117 users of ST among high school children in Denver (US) when they had identified in a school survey among a total sample of 1119 students. Fifty had mucosal changes which appeared red or white in colour. The vast majority of lesions were white, corrugated and raised. Robertson et al. (53) in the US had noted that 46% of current users of ST (all

Table 1 Criteria used for classification of smokeless tobacco lesions (Axell et al. 1976, Greer & Poulson 1983)

Degree 1	Slight, superficial wrinkling of the mucosa. Color of the mucosa may range from normal to pale white or gray. Mucosa does not appear to be thickened
Degree 2	Distinct whitish grayish or occasionally reddish color change. Wrinkling is obvious, but there is no thickening of the mucosa
Degree 3	Mucosa is obviously thickened, with distinct whitish or grayish color change. Deep furrows are present within thickened areas

professional baseball players) had oral mucosal lesions located primarily at sites where ST quid was placed. Using Greer and Poulson (51) criteria for the detection of ST lesions among 245 male patients aged 15–77 years attending a dental practice in Oregan, Little et al. (54) recorded a high prevalence of mucosal lesions (78.6%), a quarter of which were in the most clinically advanced category (grade 3).

Kaugars et al. (55) investigated oral lesions that persisted for at least 7 days after discontinuation of ST use. Among white males in this group (mean age 29.3 years) 45 of 347 (13%) had mucosal alterations consistent with ST use.

In the US, Tomar et al. (56) found among 17 027 schoolchildren degree 3 lesions (see Table 1) to be more common among current snuff users (3%) compared with current tobacco-chewing subjects (2.6%). A quarter of all ST lesions found were on the mandibular anterior labial vestibule. A quarter of ST users examined in US also were reported with two or more lesions in the mouth (56). In a separate study 29% of current ST using Floridian students demonstrated oral lesions (not classified) (57).

Among 20 333 Swedes, the prevalence of snuff dippers lesion among males was 15.9%. Seventy-two (4.9%) were classified as grade 4 lesions (58).

Significant differences were noted in the prevalence of oral lesions caused by portion-bag snuff (2.9%) compared with the use of loose snuff (23.5%), suggesting that among users of portion-bag snuff oral lesions are less pronounced (59).

Twenty-one snuff-induced oral mucosal lesions were described by Jungell and Malmstrom (60) among 441 Finish military recruits. All lesions were found in the upper vestibular area where the snuff quid was placed. Clinically they appeared wrinkled, greyish white and slightly elevated. The only symptom reported was slight itching.

In summary, a snuff dippers lesion is a descriptive term preferred by US, Norwegian, Swedish and Sudanese authors describing oral keratoses predominantly among dippers. Oral lesions induced by this habit are site-specific and have pathognomic features that distinguish these white lesions from oral leukoplakia. However, the US authors' use of the two terms is arbitary and needs distinction and further clarification.

Tobacco-lime users lesion

An oral lesion in tobacco and lime users in Maharastra, India was described by Bhonsle et al. (61). This mucosal lesion coincided with the placement of the quid and could be scrapped off leaving a raw surface. Tobacco and lime mixture also called Khaini is usually retained in the anterior part of the mouth rather than chewed (62). Among Nepalese the habit is associated with white and red patches with rippled/fissured surface characteristics (25).

Nass made with local tobacco (partly cured), ash and lime used in Central Asian Republics of the former Soviet Republic and parts of Pakistan is significantly associated with the risk of oral leukoplakia. In 118 current nass users in Uzbekistan, the associated risk for oral leukoplakia (corrected for smoking and alcohol) was 3.9 (CI 2.6–5.7) (63).

Pathology of leukoplakia and snuff dipper's lesions

Histopathology of oral leukoplakia caused by ST and/or snuff-induced lesions were reported by Daniels et al. (64) and Greer et al. (65) from USA, Roed-Petersen & Pindborg, (34) Andersson et al. (66) and Jungell and Malmstrom (60) from Scandinavia and Idris et al. (67) from Sudan.

Common epithelial changes noted were hyperorthokeratosis, hyperparakeratosis, chevron pattern keratinization, pale surface staining , koilocytosis-like changes with vacuolated cells, and basal cell hyperplasia. Dysplasia was uncommon in the Sudanese biopsies reported (67) and Larsson et al. (68) noted that dysplasia may occasionally occur in snuff dipper's lesions. Although dysplasia was not found in the snuff dipper's lesions ($n = 29$) of moist snuff users in Sweden, an increased mitotic rate was found in a large majority (68). Kaugars et al. (69) found that women more likely had moderate to severe epithelial dysplasia than men ($P = 0.02$) but this may be because their lesions were detected a decade or so later or were in older women. Out of all pathological studies examining oral biopsies of ST users Kaugars et al. (69) recorded the highest prevalence of oral epithelial dysplasia (66.7% mild dysplasia; 5.4% severe dysplasia) but they noted that 91% of these biopsies were taken from the site of ST placement. However, majority of dysplasia changes were focal in nature. In a later study by the same group, 10 of 45 cases with ST lesions were diagnosed with dysplasia (four cases were focally mild; three mild; one severe). In the US, use of snuff was more frequently associated with development of oral mucosal lesions than was the use of chewing tobacco and snuff appeared to cause a greater variety of epithelial changes than did chewing tobacco (64). In Sweden, loose snuff users had more increased epithelial thickening compared with portion-bag snuff users who had less pronounced epithelial changes (66, 70, 71). Andersson et al. (70) in a study of biopsies from mucosal lesions in Sweden noted that the daily use of snuff caused a mixed tissue reaction of injury and repair. Koilocytic alterations noted in the epithelial keratinocytes in several studies [26 of 45 cases (65) and 22 of 141 cases (67)] suggest the presence of a cytopathic damage caused by a virus, possibly of human papillomavirus (HPV) in ST induced lesions (65, 67). However, a study using polymerase chain reaction performed on snuff-induced lesions from Scandinavia did not confirm any association of HPV or Epstein–Barr virus (EBV) (72).

Verrucous hyperplasia clinically indistinguishable from verrucous carcinoma has been described in ST users (73). The surface epithelium is highly keratinized, with corrugations and sharp or blunt processes. Some progress to verrucous carcinoma or may present as a co-existing lesion with carcinomas and are therefore

considered pre-cancerous. Commonly affected site is the alveolar mucosa.

Micronuclei are considered to be markers of abnormal mitoses. This morphological change in keratinocytes involves chromosomal breaks and missegregated chromatin, which result in the formation of separate smaller nucleus at the time of cell division. Micronucleus frequencies in exfoliated cells or cell scrapings have been validated as tissue-specific dosimeters of carcinogen exposure in humans. Several studies have shown an association of increased micronuclei and snuff use (74, 75). In 48 young adults, the frequency of micronucleated cells was significantly ($p < 0.01$) higher in the labial mucosa of exposed (2.22%) compared with unexposed individuals (0.27%) (76). Localized formation of micronuclei of the oral mucosa has also been described in Indian betel quid chewers with tobacco and Khaini chewers. Nair et al. (77) reported the frequency of micronuclei in exfoliated cells of human oral mucosa (betel quid chewers with tobacco; 4.83% ± 0.7%; tobacco and lime chewers, 5.2% ± 0.7%; controls 2.59% ± 0.4%). In several studies examining scraped or brushed mucosal cells of chewers from parts of India 2–6% had micronuclei (62). In another study in an Indian tribe chewing raw tobacco with lime 6.3% of cells were micronucleated (78). Ozkul et al. (79) reported doubling of micronuclei in Turkish ST (Maras powder) users compared with controls. The possibility of reversal of the formation of micronuclei using vitamin A or β-carotene supplements has been discussed (80).

Proliferation and differentiation markers were examined in 14 Finnish male snuff users, three of whom were also occasional smokers (81). Cell proliferation as determined by Ki67 staining was markedly reduced compared with controls. Altered CK 18 expression (but not CK19) was reported in the oral epithelium of some snuff users (five of 14).

Cellular atypia in buccal smears was more common in heavy toombak users (11+ quids a day) compared with cigarette smokers of similar frequency (11+ a day) but the authors remarked that the method is unreliable as cells are taken from the surface while abnormalities mostly occur at the base of the epithelium in the progenitor layers (82).

Ramaesh et al. (83) reported variations in cell and nuclear diameters in Sri Lankan tobacco chewers. While the nuclear diameter was increased the cell diameter was reduced compared with normal buccal cells, giving an increased nuclear to cytoplasmic ratio in chewers. In an electron microscopic examination widening of intercellular spaces was noted in the spinous layer (60) in Finnish snuff dippers.

A reduction in Langerhans cells in ST-associated oral mucosal lesions was reported by Daniels et al. (84) suggesting an impairment of immunologic protection. Higher levels of both interleukin (IL)-1α and β were observed in mucosal lesions at habitual ST placement sites (85) and this may be implicated in both the inflammatory response and epithelial proliferation.

Increased expression of keratins 13 and 14 in Sudanese snuff dippers was reported (86) indicating dysregulation of keratinocyte maturation and a third of the lesions also expressed K19 a basal keratin suggesting epithelial de-differentiation. Suprabasal expression of K19 was also reported by Luomanen et al. (87) in oral biopsies of 11 snuff users from Sweden. Increased tenascin expression was reported more conspicuous in biopsies of ST users than in smokers (88). This was distributed as a band under the epithelium. This suggested a marked connective tissue reaction to snuff suggesting an epithelial-mesenchymal interaction either inflammatory or pre-neoplastic in nature.

An amorphous deposit in the lamina propria of the oral mucosa where the snuff is habitually placed was noted from Denmark 40 years ago (89). Several investigators subsequently commented on the presence of a similar histological appearance initially regarded as amyloid (90) but later thought to be non-amyloid (50, 91) and speculated as collagen by Axell et al. (49). Idris et al. (92) by electronmicroscopy studies later characterized this amorphous, eosinophilic, acellular deposit of varying in size with a fibrillar texture at the margins, in 25 oral snuff induced lesions from the Sudan as collagen.

Human volunteer studies

Several groups have experimented on humans by short-term application of ST on oral tissues (sites) (93, 94). The study groups (19 males; mean age 25 ± 1.4 years) were regular snuff users but placed moist snuff on a new mucosal site during the experiment. The authors reported erythema, ulceration and white striae at the place of application in as few as 2–7 days. By 7 days, 56% of subjects displayed white striated lesions (93). Rapid development of ST lesions in human volunteers is somewhat contrasting to reported lesions in chronic users (24). Significantly increased mucosal concentrations of IL-1α and prostaglandin E_2 (PGE_2) were also reported at new sites of snuff placement, both molecules with immune and inflammatory functions. These data are similar to what was earlier reported on 18 male ST users exhibiting an increased gingival inflammation at new placement sites of ST (95).

Healthy volunteers ($n = 20$) switching to a snuff brand with a lower pH and nicotine content of snuff demonstrated significantly less pronounced clinical and histological changes at experimental sites (96). This indicated that nicotine in snuff caused the biological effects noted on oral mucosa.

Exposure of human buccal mucosa to 1.5–2.5 g of ST (in Ringer's solution) caused dilatation of intercellular spaces of the epithelium and altered barrier function suggesting that ST may facilitate buccal transport of substances at application sites (97).

Animal carcinogenecity studies

Several laboratory studies have examined the carcinogenicity of ST in experimental animals by oral, subcutaneous administration, intravaginal instillation or skin or oral application (98). Many animal models (species) have been used particularly mouse, rat, hamster and

baboon. Studies conducted previous to 1985 were reviewed by the IARC expert working group (28). The working group concluded that at that point of time there was limited evidence in experimental animals for carcinogenicity of ST. Several new studies using animal models have been reported in the literature in the past two decades. Two most convincing studies were performed on Sprague–Dawley rats by surgically creating a test canal in the lower lip (99, 100). In both studies, a significantly increased yield of both malignant and benign oral tumors was reported, compared with controls.

In a further experimental study on Syrian golden hamsters conducted for a shorter duration although no cancers were reported in treated cheek pouches, other benign tumors and mucosal changes of metaplastic (101) or dysplastic nature were found. Sub-epithelial hyalinization noted in humans (see above) was also found in rat mucosa in one study (102).

A further interesting finding reported by Chen, (102) was a change of ploidy status; 25% of buccal epithelial cells of tobacco-treated rats were tetraploid and 5% octaploid suggesting that the mitotic process could be altered following ST application. These data are confirmatory of ploidy alterations noted in hamster cheek pouch in carcinogenesis models (103). Altered ploidy status is now considered a significant putative marker for dysplasia with potential for malignant transformation (104), but due to inaccurate reporting of data by Sudbo et al. (104) these results await revalidation by other groups.

In more recent experimental *in vivo* studies 10 male golden Syrian hamsters were treated with a ST extract (105). An upregulation of Cox-2 was demonstrated by immunohistochemistry in tobacco treated hamster tissues. Cox-2 is an important mediator in carcinogenesis, and Cox-2 is upregulated in oral mucosa of smokers (106). Recently it has been suggested that inhibiting Cox-2 would be a promising strategy for preventing oral and head cancer (107).

In vitro studies

Effects of snuff extract on epithelial growth and differentiation were studied in HaCaT cells grown *in vitro* (108). Snuff exposed cultures did not show any increase in cell proliferation measured by Ki 67 staining but did show a disturbance in the differentiation process by a decrease of CK 10 and flaggrin expression. Murrah et al. (109) however, demonstrated that ST extracts are capable of increasing cell proliferation and growth effects of human oral epithelial cells in culture similar to the proliferation effects shown in human oral mucosa in tobacco users (110). Wang et al. (111) also reported increased cell proliferation of human keratinocytes by low doses of ST and by both low and high doses of ST on fibroblasts in organotypic culture.

Fox et al. (112) demonstrated that cell death following long-term snuff exposure *in vitro* was not a result of apoptosis but was related to epithelial–mesenchymal interactions resulting in the loss of cell adhesion. Contradictory results were reported by Mangipudy and Vishwanatha (113) in a hamster cheek model treated with ST. ST caused a dose-dependent induction of apoptosis and this was shown to be mediated by nitric oxide. A fourfold rise in apoptotic cells was shown in keratinocytes treated with ST extracts at varying concentrations from 100 μg/ml to 300 μg/ml (114). These authors also demonstrated 51–85% reduction in apoptotic cells when simultaneously treated with antioxidants.

Increased PGE_2 secretion was seen when peripheral blood mononuclear cells were cultured with 1% ST (nicotine concentration 117.5 μg/ml) extracts (115) relative to control cultures, although gingival mononuclear cells were not further activated. When subjected to 5% or 10% ST (nicotine concentration 560–1118 μg/ml) extracts, oral keratinocytes grown from gingival sites were found to produce increased amounts of PGE_2 as well as IL-1β (116). The levels of IL-1β were not as dramatic as compared with PGE_2. PGE_2 is a regulator of keratinocyte proliferation and these experimental findings may also indicate host mechanisms to cell injury.

Seyedroudbari and Khan (117) studied the effects of an aqueous smokeless tobacco extract (STE) on tumor necrosis factor α (TNF-α) and IL-1β production, besides proliferation of lymphocytes. A macrophage cell line J 774-A1 was used in the assay to assess the effect of STE on secretion of pro-inflammatory cytokines TNF-α and IL-1β (both secreted by macrophages). The effect of STE on lymphocyte proliferation was studied in female BALB/C mice which were sacrificed and the spleen cells extracted and grown in media containing various concentrations of STE. Tritiated thymidine uptake assay was used to determine the rate of cell proliferation. Enzyme-linked immunosorbent assay (ELISA) assay for detecting levels of TNF-α and IL-1β secreted by macrophage cells in response to STE revealed a substantial (six- to eightfold) increase of both molecules at medium doses of STE (1.25–5 mg/ml). At higher concentration this effect was not seen. STE also induced lymphocytic proliferation.

Activation of complement was demonstrated *in vitro* using aqueous extracts of loose leaf chewing tobacco, dry snuff and moist snuff (118). This may contribute to inflammation at sites where snuff is placed locally resulting in focal inflammation of mucosal tissue.

Genetic aberrations

DNA adducts

DNA adduct formation is central to the carcinogenic process (119). Studies on examining DNA adducts in human oral tissues with a known exposure to ST are limited.

Sister chromatid exchanges

Several studies have shown the presence of chromosomal aberrations (CAs) and sister chromatid exchanges (SCEs) in snuff dippers' lesions (with hyperkeratosis)

(68, 74–76, 120). In most studies, compared with controls the frequencies of CA and SCEs were higher (Table 2). This observation is also reflected in oral squamous cell carcinomas (OSCCs) of ST users in comparison with non-user controls (Table 2) (77, 79, 121–130).

Verma (130) also examined CAs (chromosomal breaks and gaps) in tobacco chewers and compared values with non-tobacco chewers, and with other chewing habits such as areca nut and betel quid. Chromosomal breaks were 0 for controls, 2.8, 4.6 and 6.0 for betel quid, areca nuts and tobacco users respectively. There was a statistically significant correlation between years of tobacco exposure and occurrence of breaks and gaps. SCE frequency among tobacco chewers was the highest (11.53/cell) as compared with controls (2.11/cells), betel quid users (5.81/cell) and areca nut users (8.39/cell).

Micronuclei formation, chromosomal breaks and SCE described above *in vivo* also have been reproduced *in vitro* exclusively using CHO cell systems (Table 3) (131–135).

These reported laboratory data confirm that preneoplastic changes initiated by tobacco use are representative in the chromosomal complement of keratinocytes harvested from ST users. Thus ST (in addition to age) has been cited as a major stimulator of SCE.

Genomic instability

Genomic instability could be reflected by microsatellite alterations in specific target regions of chromosomes. Primary oral squamous cell carcinomas of 77 Indian tobacco chewers were analyzed by Mahale & Saranath (136). Using a panel of 11 microsatellite markers on chromosome 9, an overall 48 of 77 (62%) of the patients demonstrated microsatellite alterations including 27% microsatellite instability and 52% loss of heterozygosity (LOH). A majority of the alterations occurred on the p arm at 9p21-23 that may be indicative of involvement of *p16* (*CDKN2*) tumor suppressor gene (see below) located on this chromosome.

Table 2 Frequencies of chromosomal aberrations, sister chromatid exchange and micronucleated cells in smokeless tobacco users and non-users

Population	*Habit group*	*n*	*Chromosomal aberrations (CA/per cell)*	*Sister chromatid exchange (SCE; mean frequency/cell)*	*Micronucleated cells (MNC; frequency %)*	*Reference*
Indian	Non-chewers	10			0.47	Stich et al. 1982 (121)
	Pan + Tobacco	10			2.2–3.0	
	Khaini	27			6.78	
Indian	Non-chewers	10			0.3–0.9	Stich et al. 1982 (122)
	Pan + Tobacco	10			3.3–13.1	
Indian	Non-chewers	20		5.32 ± 1.21		Ghosh & Gosh 1984 (123)
	BQ no Tobacco	18		9.84 ± 2.37		
	BQ + Tobacco	15		12.13 ± 3.58		
Indian	No habit	15	0.05 ± 0.003	6.17 ± 0.01	0.18	Dave et al. 1991 (124)
	Pan masala	10	0.12 ± 0.009	7.59 ± 0.11	0.7	
	Pan masala + T	5	0.12 ± 0.014	7.56 ± 0.11	0.82	
Indian	Non-chewers	15	0.05 ± 0.004			Adhvaryu et al. 1991 (125)
	AN + L + T	45	0.09 ±			
Indian	Non-chewers	27			2.59 ± 0.4	Nair et al.1991 (79)
	BQ + T	35			4.83 ± 0.7	
	Lime + T	35			5.20 ± 0.7	
Indian	Non-user	102			0.35	Das & Das 1992 (127)
	Tobacco paste	120			2.06	
Indian	Non-users	15			1.9 ± 0.19	Kayal et al. 1993 (128)
	Mawa	20			6.9 ± 0.54	
	Lime + Tobacco	14			5.9 ± 0.49	
	Dry snuff	12			5.66 ± 0.39	
Indian	Non-users	13			1.0 ± 0.32	Kayal et al. 1993
	Masheri	16			3.19 ± 0.63	
Indian	Non-users	40		20.29 ± 0.52*		Trivedi et al. 1995 (129)
	Tobacco chewers	40		21.59 ± 0.59*		
Indian	Non-users	10		2.11		Verma 1998 (130)
	Betel quid	23		5.81		
	Areca nut			8.39		
	Tobacco			11.53		
US	Non-users	24		6.78 ± 0.15	9.0 ± 0.27	Livingston et al. 1990 (76)
	Snuff	24		6.70 ± 0.18	20 ± 2.22	
US	Non-users	15			1.58 ± 0.58	Tolbert et al. 1991 (64)
	Snuff	38			3.79 ± 0.56	
US	Non-users	19			4.6	Roberts et al. 1997 (65)
	Snuff	22			21.6	
Turkish	Non-users	15			0.84 ± 0.22	Ozkul et al. 1997 (79)
	Maras ST	25			1.86 ± 0.26	

T, tobacco; BQ, betel quid; AN, areca nut; L, lime.

Table 3 Frequency of chromosomal aberrations, sister chromatid exchanges and micronucleated cells in experimental systems *in vitro* following treatment with ST

Experimental system	*Agent*	*Dose/condition*	*Chromosomal aberrations (CA/per cell)*	*Sister Chromatid Exchange (SCE; mean frequency/cell)*	*Micronucleated cells (MNC; frequency %)*	*References*
CHO cells	Controls	20 ul/ml	0.09 ± 0.03	9.72 ± 0.43	0.5	Jaju et al. 1992 (131)
	Controls + S9		0.08 ± 0.03	9.48 ± 0.58	0.4	
	Pan masala		0.77 ± 0.10	16.76 ± 0.92	2.7	
	Pan masala + S9		0.16 ± 0.04	16.48 ± 0.95	2.9	
	Pan masala + tobacco		0.51 ± 0.09	16.08 ± 1.01	2.9	
	Pan masala + tobacco + S9		0.23 ± 0.06	13.36 ± 1.07	2.6	
CHO cells	Controls	DMSO extract	0.09 ± 0.03	9.72 ± 0.43	0.5	Patel et al. 1994 (132)
	Controls + S9		0.08 ± 0.04	9.48 ± 0.58	0.4	
	Pan masala		0.46 ± 0.08	17.36 ± 0.92	3.7	
	Pan masala + S9		o.13 ± 0.03	18.44 ± 0.83	2.5	
	Pan masala + T		0.53 ± 0.09	16.24 ± 0.82	3.4	
	Pan masala + T + S9		0.19 ± 0.05	18.12 ± 0.84	2.9	
CHO cells	Controls		7			Patel et al. 1994 (133)
	Pan masala	+0.2% Ethanol	32			
	Pan masala + Tobacco	+0.2% Ethanol	26			
CHO cells	Controls	15 ul/ml	0.07	6.76	0.18	Trivedi et al. 1994 (134)
	Betel leaf extract		0.14	7.76		
	Pan masala		0.09	8.84	0.7	
	Pan masala + T		0.1	9.2	0.82	
CHO cells	Controls		0.11 ± 0.002	9.69 ± 0.25		Trivedi et al. 1995 (135)
	AN + T		0.21 ± 0.007	11.77 ± 0.32		

T, tobacco.

Mutations in oncogenes and tumor suppressor genes

Occurrence of multiple mutated genes in cancers in tobacco users is consistent with the chronic barrage of DNA damage by metabolically activated tobacco carcinogens (3). One of the early molecular studies undertaken in Indian ST chewers was an analysis of the *ras* gene and subsequently several studies on many other individual oncogenes, tumor suppressor and DNA repair genes have been undertaken. These studies are summarized below:

H-RAS, Ki-RAS, N-RAS

High incidences of *H-ras* mutations (codons 12, 13 or 61) were reported in oral carcinomas in the Indian tobacco chewing population (137) in comparison with smokers from industrialized Western populations (138, 139). All three studies were undertaken in the same laboratory providing internal controls. In a subsequent study from south India, mutational frequencies of *H*, *Ki* and *N-ras* genes were also reported from an analysis of 46 ST associated OSCCs (140). Nine of 46 (19.5%) tumors analyzed had point mutations in *H-ras* codon 12 (in six cases), and in codon 13 (one case). A rare *H-ras* mutation at codon 59 (one case) and a point mutation in *N-ras* codon 12 were also reported. Five G → T transversions and four G → A transitions were observed. In the latter study, all *ras* mutation positive tumor samples were negative for *p53* mutations. Although tobacco chewing of the study subjects was known, the confounding effects of areca nut and betel quid were not reported.

p53

The gene most widely studied and frequently found to be mutated in oral squamous cell carcinomas in ST users is *p53*. *p53* mutations are also reported in potentially malignant lesions of the oral cavity (leukoplakia and snuff dippers lesions), indicating that these events may be linked temporally to DNA damage caused by ST.

Studies on *p53* mutations associated with ST report frequent mutations in oral cancers from population groups examined in Sudan, Norway, Sweden, USA and India (141–147). The *p53* mutations reported in these studies were analyzed using the IARC Tp53 mutation database. The analysis (Fig. 1) revealed 51 mutations, which included 41 missense (80.3%), one nonsense (2%), seven silent (13.7 %), two frameshift (3.9%.) mutations and two deletions. The mutational hot spots were at codons 141, 175, 179, 205, 237, 248, 268, 272, 273, 282 and 290 (Fig. 2). The mutational hot spots at codons 175, 248 and 273 are common to all cancers. The mutational hot spot at codon 205 has been reported in Head and Neck cancer including the oral cavity among smokers.

The *p53* mutation spectrum comprised of 47% GC → AT transitions (29.4%at CpG sites and 17.6% non-CpG sites), 19.26% GC → TA transversions; 3.92% GC → CG transversions; 1.96% AT → TA transversions, 17.6% A → G transitions, 5.88% A → C transitions, and 3.92% deletions (Fig. 3).

The high level of G to A transitions (47%) in oral cancers from ST users includes the mutation events scored as G → A and as C → T. As the latter correspond to G to A changes occur on the non-coding, transcribed strand of genomic DNA (by convention the base changes induced by a mutation are read on the coding, non-transcribed strand). Smokers, non-snuff dippers and tobacco with betel quid users have been excluded from the analysis and therefore the data represent the DNA changes associated with ST use.

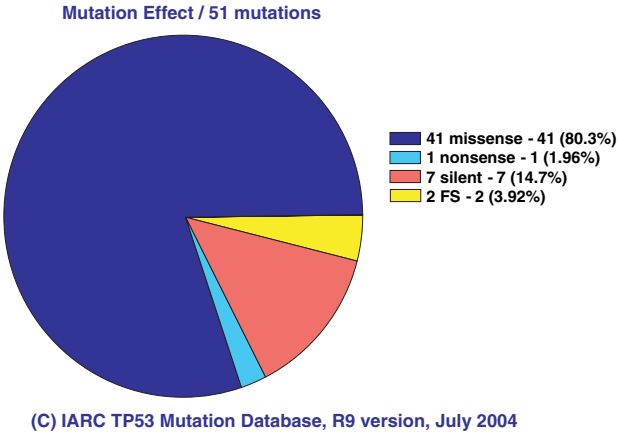

Figure 1 Mutation effects of p53 mutations in OSCCs from users of smokeless tobacco. [All the Figures were obtained by on-line analysis using November 2004 update of IARC TP53 mutation database (http://www.iarc.fr/p53/Index.html)].

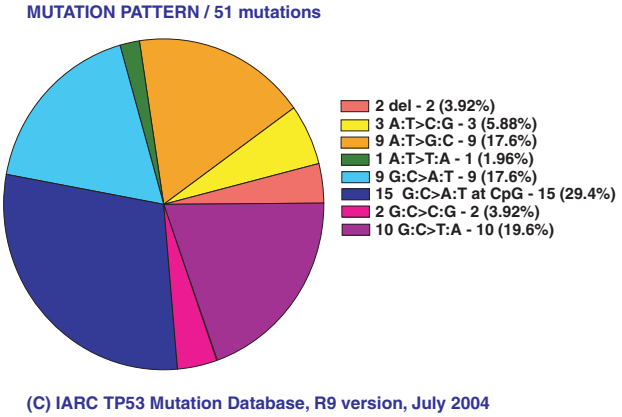

Figure 3 Mutation pattern of p53 mutations in OSCCs from users of smokeless.

The types of base changes seen along the entire *p53* coding sequence occur in a random manner, although the hot spots reported in human cancers are also found in these oral cancers from the ST users. All these hot spots are within the DNA binding domain of the p53 spanning codons 173–312.

The *p53* mutational hot spots at codons 157, 158 and 245 reported in lung cancers from tobacco smokers are not found in ST users. However, the mutational hot spots at codons 248 and 273 are common in tumors in both ST users and smokers. These two codons are the most commonly mutated ones in the entire *p53* mutation database and have been encountered in most human cancers (148). These two residues contact between p53 protein and its DNA target, hence mutation at these residues has been proposed to affect p53 function (149). Mutations at these residues have been shown to abrogate the transcriptional activation capacity of p53 for its downstream target genes such as $p21^{waf1}$ and bax. *p53* mutations are generally more common in smokers than non-smokers (150, 151).

G → T transversions have been described as a molecular signature of tobacco smoke mutagens in smoking associated lung cancers (150, 152, 153). Pfeifer et al. (148) by a systemic study of the published literature (148, 154) confirmed and extended the existence of a specific mutation pattern in lung cancers of smokers. The major carcinogens in ST are TSNAs, while PAHs are the predominant carcinogens in tobacco smoke. Hence it was of interest to analyze the currently available literature on *p53* mutations in oral squamous cell carcinomas from ST consumers (snuff dippers from USA, Sudan, Norway, Sweden and tobacco chewers from South Asia including India). Mutations G:C → T:A, C:G → T:A, G:C → T:A, which are known to be associated with TSNAs were found to be most common in oral squamous cell carcinomas in ST users (155).

Expression of p53 protein has been examined in snuff induced oral lesions by immunohistochemical analysis. Ibrahim et al. (155) showed discordant results and

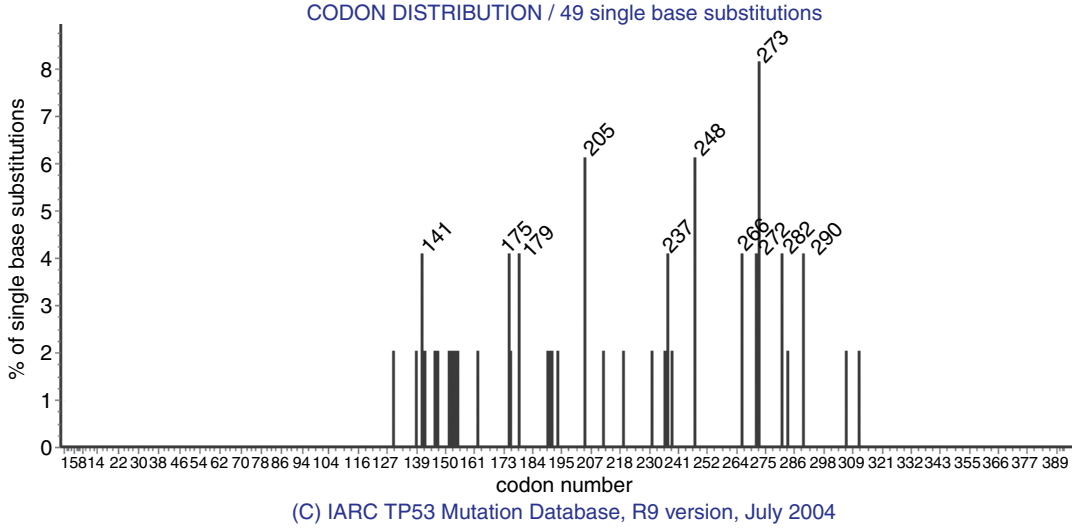

Figure 2 Codon distribution of p53 mutations in OSCCs from users of smokeless tobacco.

Merne et al. (81) showed low p53 immunopositivity while Wood et al. (156), Wedenberg et al. (157), and Schildt et al. (158) reported significantly elevated expression of p53 in snuff-induced oral lesions. It is difficult to determine whether positive p53 staining is due to mutation of p53 or elevated wild type p53 expression, where in p53 protein is bound to another protein and retained in the tissue, but in an inactive state.

Baral et al. (159) analyzed co-expression of c-myc and p53 in 48 patients with OSCC from the eastern part of India who were habituated to ST such as pan with tobacco (betel quid) or tobacco paste (gudakhu). Twenty-two of 48 (45.8%) OSCCs were p53 positive and 27 of 48 (56.3%) were c-myc positive. The epithelium adjacent to p53-positive carcinoma was also p53-positive in 50% cases, but the epithelium surrounding p53 negative tumors was p53 negative.

In a cohort of Indian subjects, comparison of p53 protein expression in 22 baseline biopsies of oral precancerous lesions that transformed to cancer 4–25 years later, against 68 pre-cancers that did not transform over the same period, did not show significant relationship between p53 protein expression and malignant transformation (160). However, the gain of p53 protein expression in nine of 10 biopsies that did not show p53 expression at baseline did so in serial biopsies taken just before these lesions underwent progression to squamous cell carcinoma, indicating that p53 alteration could be a late phenomenon in ST induced oral cancer.

DAPK, p16, MGMT and GSTP1

Kulkarni and Saranath (161) studied concurrent hypermethylation of *DAPK*, *p16*, *MGMT* and *GSTP1* in 60 primary oral tumors from habitual tobacco chewers and corresponding adjacent clinically and histopathologically normal mucosa as well as buccal epithelial scrappings from 20 normal healthy non-users of tobacco.

Promoter hypermethylation was observed in 40 of 60 (66.7%); 41 of 60 (68.3%) and 31 of 60 (51.7%) of OSCCs in *p16*, *DAPK* and *MGMT* respectively. Amongst the adjacent mucosa analyzed, 30 of 60 (50%), 36 of 60 (60%) and 16 of 60 (26.7%) tissues demonstrated promoter hypermethylation of *p16*, *DAPK* and *MGMT* respectively. No correlation could be established between hypermethylation of these genes and clinico-pathological parameters in patients.

$p21^{WAF1}$

Mutations in exon 2 of $p21^{WAF1}$, the cyclin-dependent kinase inhibitor gene were found in OSCCs in six of 14 (43%) toombak users, compared with two of 14 (14%) in Sudanese non-users, five of 35 (14%) in American/British and six of 27 (22%) in Scandinavian smokers (162). OSCCs from toombak dippers showed 13 different mutations distributed as 10 (77%) transitions and three (23%) transversions, while non-snuff-dippers revealed 33 different mutations distributed as 14 (42%) transitions and 190 (58%) transversions. No significant difference was observed in mutation frequencies for $p21^{WAF1}$ in Sudanese toombak dippers and non-snuff-dippers from Sudan/Scandinavia or the USA/UK. Mutation frequencies of $p21^{WAF1}$ in Sudanese Blacks (eight of 48; 17% toombak users/non-users) did not differ significantly from those in white people (11 of 62; 18%). All OSCCs from Sudanese toombak dippers showed correlation between *p21* exon 2 mutations and tumor suppressor gene *p53* mutations in exons 5–9.

APC and MCC

Sikdar et al. (163) reported LOH at *APC* and *MCC* genes in 40 OSCCs and 57 leukoplakia patients from Eastern India. Amongst the patients with OSCC 58% were tobacco chewers, while among the leukoplakia patients only 10% were tobacco chewers. Four of 16 (25%) OSCCs, three of which were tobacco chewers and one of 29 (3%) of leukoplakias (also a tobacco chewer) showed LOH at APC. No LOH was observed at *MCC* gene in any of the OSCCs or leukoplakias.

EGFR

Saranath et al. (164) reported *EGFR* gene amplification in 19 of 66 (29%) OSCCs in the tobacco chewing Indian population.

FHIT

Kannan et al. (165) showed lack of exonic mutation in *FHIT* gene (exons 5–9) in 55 OSCCs from tobacco chewers from South India, suggesting that *FHIT* gene may not be linked to chewing tobacco related oral carcinogenesis.

INGI

Mutational analysis of *INGI* (a candidate tumor suppressor gene) was carried out in 71 Indian OSCCs, (15 of which harbored *p53* mutations). All the patients were habitual tobacco chewers for more than 10 years. None of the OSCCs revealed any mutations. Hence *INGI* mutation is likely to be rare in Indian oral carcinomas (166).

Other cellular proteins

Nagpal et al. (167) showed high accumulation of signal transducers and activators of transcription 3 (Stat 3) in 53 of 90 (58.9%) and medium Stat 3 staining in 21 of 90 (23.3%) HNSCC patients with tobacco chewing habits for more than 10 years. Apoptotic index did not show correlation to Stat 3 expression.

High apoptosis rate and a high bax expression were reported in OSCCs from Norway compared with those from Sudan irrespective to toombak use (168). However, no significant differences were observed in apoptosis, bax, bc1-2 and Ki-67 in OSCCs from Sudan in relation to toombak use or *p53* gene status. Teni et al. (169) reported aberrant expression of bcl-2 and bax in oral lesions comprising leukoplakia and oral sub-mucous fibrosis as well as in oral cancers in tobacco chewers in India.

Conclusions

The pathology of oral soft tissue lesions associated with the use of ST indicates features of pre-malignancy and

carcinomas at the site of application or dipping the product. The studies following short-term application of ST in humans describe epithelial morphological changes similar to long standing snuff-induced lesions. In experimental *in vitro* systems, ST has shown effects on cell proliferation, apoptosis and activation of inflammatory mediators. Convincing animal carcinogenesis studies are reported both using chewing tobacco and snuff products commercially available for human consumption in Europe and in North America. The mutational spectrum reported high incidence of *H-ras* mutations in tobacco chewing oral carcinomas in Indian population in comparison with smokers from Western populations. Analysis of *p53* mutations showed that G → A, C → T and G → T mutations that are associated with TSNAs have been found to be most common in ST associated oral cancers.

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