p53 Codon 72 Polymorphism in Oral Exfoliated Cells in a Sudanese Population

LARS SAND[1], MIRANDA M. JALOULI[1], JAMSHID JALOULI[1], DIPAK SAPKOTA[2,3] and SALAH O. IBRAHIM[2,3]

[1]Department of Surgical Sciences, Oral and Maxillofacial Surgery,
Medical Faculty, Uppsala University, Uppsala, Sweden;
Departments of [2]Biomedicine and [3]Clinical Dentistry Periodontology,
University of Bergen, Bergen, Norway

Abstract. *Earlier studies have investigated the tumor suppressor gene p53 as a co-factor in the development of oral squamous cell carcinoma (OSCC). Our previous studies have indicated that chronic use of Sudanese snuff (toombak) and the presence of human papilloma virus (HPV) may be involved in the high prevalence of OSCC in Sudan. This study investigated the prevalence of p53 codon 72 polymorphism in brush biopsies obtained from a Sudanese population. A total of 174 individuals were included in the study; chronic toombak users (n=152) and non-users (n=22). DNA was extracted from all the samples and genotyped for the codon 72 polymorphism by polymerase chain reaction/ restriction fragment length polymorphism. The Arg/Pro genotype was found in 53% of the 174 study participants, compared to 21% found with Arg/Arg and 26% found with Pro/Pro. Stratifying by toombak use, 28 (18%), 45 (29%) and 79 (52%) of the 152 samples from toombak users had Arg/Arg, Pro/Pro and Arg/Pro respectively, compared to 9 (41%), 0 (0%) and 13 (59%) found in the 22 samples from non users. The differences between the samples from toombak users and non users in Arg/Arg and Pro/Pro codon 72 polymorphism and HPV infection were statistically significant (p<0.05). Our study indicated that a high prevalence of the genotype Arg/Pro at the p53 codon 72 may contribute to susceptibility to OSCC, especially in combination with the use of carcinogenic tobacco-specific nitrosamine (TSNA)-rich toombak. Our observations warrant an in-depth study for understanding the role of p53 polymorphism in human oral cancers.*

Correspondence to: Lars Sand, Department of Surgical Sciences, Oral and Maxillofacial Surgery, Medical Faculty, Uppsala University, SE-751 85 Uppsala, Sweden. Tel: +46 186116450 , Fax: +46 18559129, e-mail: lars.sand@hotmail.com

The *p53* tumor suppressor gene is considered the guardian of the genome (1). The basic function of *p53* is to respond to cellular stress with apoptosis, cell cycle arrest and senescence. During neoplastic progression, *p53* is often mutated and fails to efficiently perform these functions (2). Mutant *p53* not only functions as a tumor suppressor but can also exert tumor-promoting effects (3). Expression and mutations of *p53* protein has been examined in snuff- induced oral lesions (4, 5), with a high incidence of mutations found in oral squamous cell carcinoma (OSCC) from Sudan snuff, *toombak*, users (6). Polymorphism of the *p53* gene is a known risk factor for cancer development (7). A single nucleotide polymorphism at the *p53* codon 72 exon 4, which encodes either arginine (Arg) (8) or proline (*Pro*), in the p53 protein has been reported as a risk factor in cancer development (9). It is known that each individual inherits a *p53* genotype that can be heterozygous (*Arg/Pro*) or homozygous for either arginine (*Arg/Arg*) or proline (*Pro/Pro*) at the *p53* codon 72 locus (7). Common genetic variants of *p53*, such as polymorphism at codon 72, may contribute to cancer development (9). Although this polymorphism may represent a risk factor, none of the *p53* codon 72 genotypes has been conclusively associated with increased risk for malignancy (9, 10). It has been demonstrated in epidemiological studies investigating hereditary factors (11) that the *Pro/Pro* genotype is strongly associated with ethnicity and is considered to be an important confounding factor, while other studies have shown a wide range of variation (12, 13). OSCC is a major health problem, and the incidence rates are highest in developing countries, with males being more affected than females (14). The primary risk factors for development of OSCC have not been fully elucidated. However, tobacco use and alcohol consumption are considered to be the main etiological factors (15, 16). In the Sudanese population, both cigarette smoking and alcohol consumption are less common than the use of non-smoked tobacco, locally known as *toombak*, which is a widespread habit (17). The use of *toombak* has been shown to produce a variety of pathological changes, such as hyperkeratosis and dysplasia (18). The high incidence of

OSCC reported from Sudan has been strongly attributed to the use of *toombak* (18). An unusually high level of carcinogenic tobacco-specific nitrosamines (TSNAs) has been detected in *toombak* which is considered to be the main cause of tumors of the oral cavity (19). Human papilloma virus (HPV) and also other virus species may be important risk factors in OSCC development (20-23). The objective of the present study was to examine the prevalence of *p53* codon 72 polymorphism in brush biopsies obtained from a Sudanese population and relate the findings to the frequent use of *toombak* and high prevalence of OSCC in Sudan.

Patients and Methods

Patients and oral tissue specimens. The study subjects selected were random *toombak* consumers or non *toombak-* using volunteers living in the city of Khartoum, Sudan. Regardless of gender, the study participates were 18 years of age or older and non-alcohol users. Brush biopsies were collected from the site of placement of the *toombak* quid (labial or buccal sulcus) (n=152, 5 females, 147 males; age range 18-70 years, mean 38.5±standard deviation (SD) 12.3 years) and from either the buccal mucosa or the labial sulcus of non-users (n=22, all females; age range 28-70, mean 38.8±12.1 years). For collection of the brush biopsies, superficial scrapes of the labial sulcus or the buccal mucosa were carried out with soft toothbrushes, by performing five to ten gentle strokes (from a high to low position) in the selected areas. The cells from the brushes were suspended in tubes containing 10% phosphate-buffered saline (PBS). The participants subsequently gargled with saline, and the resulting suspension was added to the same tube. Thereafter, the tubes were centrifuged twice at 1000 rpm for 5 minutes and the cells were collected and were frozen at –70˚C until further use for DNA extraction.

DNA extraction. DNA was extracted from all the samples used in this study, using a standard protocol as described in the DNeasy Tissue Kit Handbook (Cat No. 69506; Qiagen GmBH, Hilden Germany). Purified DNA was quantified by spectrophotometry (DU® 530 Life Science UV/Visible Spectrophotometer; Beckman Coulter, Fullerton, CA, USA).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. For the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of codon 72 of the *p53* gene, restriction enzyme BstUI (New England Biolabs, Beverly, MA, USA) was used to identify the *p53* genotypes. For the in vitro amplification of codon 72 of the *p53* gene fragments from each sample, the primers for exon 4 were chosen using the computer software package programme Vector NTI 5.1, Align ×1.0 Version 5.0 for Windows™ (InforMax BioSuite ™ Inc. North Bethesda, MD, USA) and were purchased from (MWG-Biotech AG, Ebersberg, Germany). The two primers were: forward, 5'-CTG CTC TTT TCA CCC ATC TAC AGT CC-3'; reverse, 5'-CTC AGG GCA ACT GAC CGT GCA AG-3'. The PCR was carried out in a Gene Amp® PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA). The 50 µl PCR mixture consisted of 1-3 µl of the genomic DNA solution, 200 µM of each of the four deoxynucleotide triphosphates (dNTPs), 0.20 U of AmpliTaq®Gold DNA polymerase (5 U/µl; PE Applied Biosystems), 2 mM MgCl$_2$, and 10 pmol of each of the primers. A hot start at 95˚C for 10 min was followed by 40 cycles of

amplification, where each cycle consisted of denaturation at 95˚C for 40 s, annealing at 58˚C for 60 s, and extension at 72˚C for 60 s. The last PCR cycle was followed by a final extension at 72˚C for 7 min. As negative controls for the amplification reactions, PCRs without DNA were used as a template. Human placental DNA was used as wild-type control. After confirmation of the amplified fragment of expected size (317 bp) on an agarose gel, the PCR products were digested with ten units of restriction enzyme BstUI at 60˚C for 16 hours. The DNA fragments were subjected to electrophoresis through a 3% agarose gel and stained with 0.5 µg/ml ethidium bromide. The results obtained were confirmed by digesting the PCR products with two units of the restriction enzyme BtgI at 37˚C for 16 hours.

Gel electrophoresis. Aliquots of 15 µl of the PCR product were analyzed on 2% agarose gel (DNA Agar; Marine Bio *Pro*ducts Inc, Quincy, MA, USA) containing 0.5 g/mol of ethidium bromide (Merck KGaA, Darmstadt, Germany), and visualized under ultraviolet light. The size of the amplified product was determined by comparison with a base-pair (bp) ladder size marker (Gene Ruler, 100bp, 50bp DNA Ladder Plus; Fermentas, St, Leon-Rot, Germany).

Data analysis. Chi-square test and logistic regression analysis were used to evaluate the distribution of the frequency of codon 72 *p53* genotypes among the samples studied. For all statistical analyses, SPSS statistical programme, version 16.0 for windows (Statistical Package for the Social Sciences, IBM, Chicago Illinois, USA) was used. The values were considered significantly different at *p*<0.05.

Results

A comparison of the distribution of the *p53* codon 72 polymorphism was performed between the brush biopsies from the *toombak* users and non users. Odds ratio (OR) for *Arg/Arg*, *Arg/Pro* and *Pro/Pro* genotypes were OR=1.95 (95% CI=0.753-5.066, *p*=0.164) and OR=2.607 (95% CI=1.949-3.487, *p*<0.0001), respectively. The genotyping results from the both biopsies are presented in Table I.

Pro/Pro homozygosity was found only in the brush biopsies from chronic *toombak* users. The differences in the proportion of the *Arg/Arg* and the *Pro/Pro* in the chronic *toombak* users was statistically significant when compared to those in the non users (*p*=0.016 and *p*<0.0001, respectively); however, for the *Arg/Pro* (*p*=0.535) genotype, there was no statistically significant difference. An example of the PCR-RFLP for *p53* codon 72 polymorphism found in the brush biopsy from chronic toombak users is shown in Figure 1.

Discussion

In this work, the distribution of the *p53* codon 72 genotypes was examined in brush biopsies obtained from a Sudanese population. The subjects studied were both chronic *toombak* users and non users. We found a dominant occurrence of the *Arg/Pro* heterozygote in the studied population (53%). Earlier studies have shown sharp ethnic differences in codon 72 allelic frequencies. A study by Beckman *et al*. focused on the
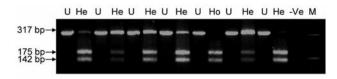
Figure 1. An example of the PCR-RFLP for p53 codon 72 polymorphism found in the brush biopsy from chronic toombak users examined (U, uncut; He, heterozygous; Ho, homozygous pro/pro; -Ve, negative control; M, PCR marker).

Table I. *Prevalence of p53 codon 72 polymorphism.*

	p53 Polymorphism, n (%)		
Group (n)	*Arg/Arg*	*Arg/Pro*	*Pro/Pro*
Total brush biopsies 174	37 (21)	92 (53)	45 (26)
Toombak users 152	28 (18)	79 (52)	45 (29)
Toombak non users 22	9 (41)	13 (59)	0 (0)
p-value	0.016	0.535	<0.0001

northern hemisphere, and found that the frequency of *Pro72* allele shows a North-South gradient, from 0.17 in Swedish Saamis to 0.63 in Nigerians (13). In Western Europe, USA, Central, South America and Japan, the most common allele is *Arg72* (frequency 0.60-0.83). Other studies however, have shown frequencies of *Pro72* greater than 0.40 in Afro-Americans (24, 25) and in Chinese (26, 27). Shi *et al*. suggested that these latitude-dependent variations are related to winter temperature and not to UV radiation, and that this was associated with high frequency of *Arg72* in Eastern Asia individuals from 67 different populations mostly from China (28). Studies regarding the influence of the different *p53* genotypes on cancer development have come to various conclusions, but putative associations have been also challenged by other studies (29, 30). Several studies have reported occurrence of *Pro/Pro* genotype in cases of human cancer examined from several populations, suggesting that individuals with the *Pro/Pro* genotype are more susceptible to cancer development (11, 31-37). In a specific study, Storey *et al*. suggested that the HPV E6 oncoprotein, binds to and induces the degradation of *p53*, resulting in the presence of either Pro or Arg at position 72 (38). Comparison of patients with HPV-associated tumors and the normal population, revealed a seven fold higher susceptibility of individuals homozygous for arginine alleles of codon72 *p53* (*Arg/Arg*) to HPV-associated tumorigenesis than of heterozygotes (*Arg/Pro*). They concluded that the arginine-encoding allele represents a significant risk factor in the development of HPV-associated cancer (38). In earlier work on a Sudanese population by Jalouli *et al*., HPV was found in higher amounts on healthy individuals compared to patients with dysplasia and OSCC (20). OSCCs constitute a major health problem in Sudan and the disease is linked to the use of *toombak* (39). *Toombak* has been found to contain high levels of the potent carcinogenic components TSNA, in particular *N*-nitrosonor-nicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (19). The level of NNN and NNK in *toombak* has been found to be up to 560-fold, which is higher than that reported for North American or European *snuff* (19). It has been reported that cancer occurs at the site where the *toombak* quid is regularly placed (39). Previously we examined *p53* gene mutations in cases of OSCC from

Sudanese users of *toombak* and found a high level of mutations (exons 5-9) in these tumors compared with OSCCs from non-*toombak* users from Sudan and Scandinavia (6). An interesting study by Fan *et al*. showed that the *Arg/Pro* contributes to heritable susceptibility for smoke-induced lung carcinoma (40). In our study, we found a dominant prevalence of the *Arg/Pro* heterozygote (53%). Multiple factors seem to be important when the high prevalence of OSCC in Sudan (39) is investigated. The high concentration of TSNA in *toombak* (19), the higher proportion of HPV infection in healthy individuals (20), and a possible genetic predisposition associated with higher prevalence of *Arg/Pro* genotype in the Sudanese population, may all independently or synergistically contribute to OSCC in Sudan.

Conclusion

To our knowledge, this is the first report showing a high prevalence of the *Arg/Pro* genotype in a Sudanese population. Our study might indicate that presence of the *Arg/Pro* genotype at *p53* codon 72 may contribute to susceptibility to the development of OSCC, especially in combination with the TSNA-rich *toombak*. These observations warrant further studies involving *toombak* associated OSCC in order to determine the possible role of the *Arg/Pro* heterozygote genotype of *p53* at codon 72 as a risk factor for the development of OSCC.

References

1   Lane DP: Cancer. *p53*, guardian of the genome. Nature *358*: 15-16, 1992.

2   Athar M, Elmets CA and Kopelovich L: Pharmacological activation of *p53* in cancer cells. Curr Pharm Des *17*: 631-639, 2011.

3   Suzuki K, Matsubara H. Recent advances in *p53* research and cancer treatment. J Biomed Biotechnol *2011*: 978312, 2011.

4   Wedenberg C, Jonsson A and Hirsch JM: Assessment of *p53* and Ki-67 expression in snuff-induced lesions. Br J Oral Maxillofac Surg *34*: 409-413, 1996.

5   Warnakulasuriya KA and Ralhan R: Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco – a review. J Oral Pathol Med *36*: 63-77, 2007.

6   Ibrahim SO, Vasstrand EN, Johannessen AC, Idris AM, Magnusson B and Nilsen R *et al*: Mutations of the *p53* gene in oral squamous-cell carcinomas from Sudanese dippers of nitrosamine-rich *toombak* and non-snuff-dippers from the Sudan and Scandinavia. Int J Cancer *81*: 527-534, 1999.

7   Hollstein M, Sidransky D, Vogelstein B and Harris CC: *p53* mutations in human cancers. Science *253*: 49-53, 1991.

8   van Benthem BH, Spaargaren J, van Den Hoek JA, Merks J, Coutinho RA and Prins M: Prevalence and risk factors of HSV-1 and HSV-2 antibodies in European HIV infected women. Sex Transm Infect *77*: 120-124, 2001.

9   Shen H, Solari A, Wang X, Zhang Z, Xu Y, Wang L *et al*: P53 codon 72 polymorphism and risk of gastric cancer in a Chinese population. Oncol Rep *11*: 1115-1120, 2004.

10 Wang YC, Lee HS, Chen SK, Chang YY and Chen CY: *Prognostic* significance of *p53* codon 72 polymorphism in lung carcinomas. Eur J Cancer *35*: 226-230, 1999.

11 Twu CW, Jiang RS, Shu CH and Lin JC: Association of *p53* codon 72 polymorphism with risk of hypopharyngeal squamous cell carcinoma in Taiwan. J Formos Med Assoc *105*: 99-104, 2006.

12 Sjalander A, Birgander R, Kivela A and Beckman G: *p53* polymorphisms and haplotypes in different ethnic groups. Hum Hered *45*: 144-149, 1995.

13 Beckman G, Birgander R, Sjalander A, Saha N, Holmberg PA, Kivela A *et al*: Is *p53* polymorphism maintained by natural selection? Hum Hered *44*: 266-270, 1994.

14 Franceschi S, Bidoli E, Herrero R and Munoz N: Comparison of cancers of the oral cavity and pharynx worldwide: etiological clues. Oral Oncol *36*: 106-115, 2000.

15 Franceschi S, Levi F, Dal Maso L, Talamini R, Conti E, Negri E *et al*: Cessation of alcohol drinking and risk of cancer of the oral cavity and pharynx. Int J Cancer *85*: 787-790, 2000.

16 Bosetti C, Negri E, Franceschi S, Conti E, Levi F, Tomei F *et al*: Risk factors for oral and pharyngeal cancer in women: a study from Italy and Switzerland. Br J Cancer *82*: 204-207, 2000.

17 Idris AM, Ibrahim YE, Warnakulasuriya KA, Cooper DJ, Johnson NW and Nilsen R: Toombak use and cigarette smoking in the Sudan: estimates of prevalence in the Nile state. Prev Med *27*: 597-603, 1998.

18 Idris AM, Ibrahim SO, Vasstrand EN, Johannessen AC, Lillehaug JR, Magnusson B *et al*: The Swedish snus and the Sudanese *toombak*: are they different? Oral Oncol *34*: 558-566, 1998.

19 Idris AM, Nair J, Friesen M, Ohshima H, Brouet I, Faustman EM *et al*: Carcinogenic tobacco-specific nitrosamines are present at unusually high levels in the saliva of oral snuff users in Sudan. Carcinogenesis *13*: 1001-1005, 1992.

20 Jalouli J, Ibrahim SO, Sapkota D, Jalouli MM, Vasstrand EN, Hirsch JM *et al*: Presence of human papilloma virus, herpes simplex virus and Epstein-Barr virus DNA in oral biopsies from Sudanese patients with regard to *toombak* use. J Oral Pathol Med *39*: 599-604, 2010.

21 Jalouli J, Ibrahim SO, Mehrotra R, Jalouli MM, Sapkota D, Larsson PA *et al*: Prevalence of viral (HPV, EBV, HSV) infections in oral submucous fibrosis and oral cancer from India. Acta oto-laryngologica *130*: 1306-1311, 2010.

22 Sand L, Jalouli J, Larsson PA and Hirsch JM: Human papilloma viruses in oral lesions. Anticancer Res *20*: 1183-1188, 2000.

23 Sand L, Wallstrom M, Jalouli J, Larsson PA and Hirsch JM: Epstein-Barr virus and human papillomavirus in snuff-induced lesions of the oral mucosa. Acta Otolaryngol *120*: 880-884, 2000.

24 Jin X, Wu X, Roth JA, Amos CI, King TM, Branch C *et al*: Higher lung cancer risk for younger African-Americans with the *Pro/Pro p53* genotype. Carcinogenesis *16*: 2205-2208, 1995.

25 Weston A, Perrin LS, Forrester K, Hoover RN, Trump BF, Harris CC *et al*: Allelic frequency of a *p53* polymorphism in human lung cancer. Cancer Epidemiol Biomarkers Prev *1*: 481-483, 1992.

26 Ngan HY, Liu VW and Liu SS: Risk of cervical cancer is not increased in Chinese carrying homozygous arginine at codon 72 of *p53*. Br J Cancer *80*: 1828-1829, 1999.

27 Peixoto Guimaraes D, Hsin Lu S, Snijders P, Wilmotte R, Herrero R, Lenoir G *et al*: Absence of association between HPV DNA, TP53 codon 72 polymorphism, and risk of oesophageal cancer in a high-risk area of China. Cancer Lett *162*: 231-235, 2001.

28 Shi H, Tan SJ, Zhong H, Hu W, Levine A, Xiao CJ *et al*: Winter temperature and UV are tightly linked to genetic changes in the *p53* tumor suppressor pathway in Eastern Asia. Am J Hum Genet *84*: 534-541, 2009.

29 Matakidou A, Eisen T and Houlston RS: TP53 polymorphisms and lung cancer risk: a systematic review and meta-analysis. Mutagenesis *18*: 377-385, 2003.

30 Li Y, Qiu LX, Shen XK, Lv XJ, Qian XP and Song Y: A meta-analysis of TP53 codon 72 polymorphism and lung cancer risk: evidence from 15,857 subjects. Lung Cancer *66*: 15-21, 2009.

31 Granja F, Morari J, Morari EC, Correa LA, Assumpcao LV and Ward LS: *Pro*line homozygosity in codon 72 of *p53* is a factor of susceptibility for thyroid cancer. Cancer Lett *210*: 151-157, 2004.

32 Yu MW, Yang SY, Chiu YH, Chiang YC, Liaw YF and Chen CJ: A *p53* genetic polymorphism as a modulator of hepatocellular carcinoma risk in relation to chronic liver disease, familial tendency, and cigarette smoking in hepatitis B carriers. Hepatology *29*: 697-702, 1999.

33 Wang YC, Chen CY, Chen SK, Chang YY and Lin P: *p53* codon 72 polymorphism in Taiwanese lung cancer patients: association with lung cancer susceptibility and prognosis. Clin Cancer Res *5*: 129-134, 1999.

34 Tsai MH, Lin CD, Hsieh YY, Chang FC, Tsai FJ, Chen WC *et al*: *Pro*gnostic significance of the proline form of *p53* codon 72 polymorphism in nasopharyngeal carcinoma. Laryngoscope. *112*: 116-119, 2002.

35 Buyru N, Tigli H and Dalay N: P53 codon 72 polymorphism in breast cancer. Oncol Rep *10*: 711-714, 2003.

36 Kawajiri K, Nakachi K, Imai K, Watanabe J and Hayashi S: Germ line polymorphisms of *p53* and CYP1A1 genes involved in human lung cancer. Carcinogenesis *14*: 1085-1089, 1993.

37 Wu X, Zhao H, Amos CI, Shete S, Makan N, Hong WK *et al*: *p53* Genotypes and Haplotypes Associated With Lung Cancer Susceptibility and Ethnicity. J Natl Cancer Inst *94*: 681-690, 2002.

38 Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F *et al*: Role of a *p53* polymorphism in the development of human papillomavirus-associated cancer. Nature. *393*: 229-234, 1998.

39 Idris AM, *Pro*kopczyk B and Hoffmann D: Toombak: a major risk factor for cancer of the oral cavity in Sudan. Prev Med *23*: 832-839, 1994.

40 Fan R, Wu MT, Miller D, Wain JC, Kelsey KT, Wiencke JK *et al*: The *p53* codon 72 polymorphism and lung cancer risk. Cancer Epidemiol Biomarkers Prev *9*: 1037-1042, 2000.