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

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**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 (39%) 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.

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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 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 participants 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 *p53* gene, restriction enzyme BstUI (New England Biolabs, Beverly, MA, USA) was used to identify the *p53* genotypes. For the in vitro amplification of *p53* gene fragments from each sample, the primers for exon 4 were chosen using the computer software package programme Vector NTI 5.1, Align x1.0 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 MgCl₂, and 10 pmol of each of the primers. A hot start at 95°C for 10 min 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 Agarar; Marine Bio Products 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
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 numbers 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-nitrosonornicotine (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**
