Int. J. Cancer: **81,** 527–534 (1999)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

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

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Using PCR-SSCP/DNA sequencing methods, we analyzed 14 oral squamous-cell carcinomas (OSCCs) and 8 pre-malignant oral lesions from different Sudanese patients for prevalence of mutations in exons 5 to 9 of the *p53* gene in relation to toombak-dipping status. OSCCs (14 from Sudan, 28 from Scandinavia), and 3 pre-malignant oral lesions from Sudanese non-dippers were used as controls. A statistically significant increased incidence in mutations of the *p53* gene was found in OSCCs from toombak dippers (93%; 13/14), as compared with those from non-dippers in Sudan (57%; 8/14) and in Scandinavia (61%; 17/28) respectively. In OSCCs from dippers, mutations were found in exons 5 to 9, while in those from non-dippers they were found in exons 5, 7, 8, 9, and no mutations were found in exon 8 in any of the OSCCs from Sudan. Certain types of mutations, however, were similar with respect to exposure to toombak. OSCCs from dippers showed 15 transversions, 9 transitions, 3 insertions and one deletion, compared with 7 transversions, 2 transitions and one deletion found in OSCCs from Sudanese non-dippers, and 9 transversions, 17 transitions and 2 insertions found in those from non-dippers in Scandinavia. No mutations were found in any of the non-malignant oral lesions in relation to dipping or non-dipping status. These findings suggest that (*i*) the use of toombak plays a significant role in induction of increased *p53* gene mutations, (*ii*) mutations observed were similar to those induced by tobacco-specific *N*-nitrosamines (TSNAs) in experimental animal models and those already reported in toombak dippers, (*iii*) types of mutations associated with TSNAs were similar in the exposed and the control groups, (*iv*) a novel mutation in exon 6 was found in the OSCCs from toombak dippers, (*v*) the *p53* exons 5 (codon 130), 6 (codons 190, 216) and 7 (codons 229, 249, 252) mutations are probable hot spots for toombak-related OSCCs. Further studies are necessary to validate the increased incidence and exon locations of the *p53*-gene mutations as a biomarker of malignant transformation in populations in which the oral use of tobacco is habitual. *Int. J. Cancer* 81:527–534, 1999.
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Globally, the incidence of oral squamous-cell carcinomas (OSCCs) is on the increase, especially in developing countries, where oral-health-care resources are meagre (Johnson, 1991). The most frequently mutated tumor-associated gene yet identified is the *p53* gene found in mutated form in more than 50% of all human tumors (Hollstein *et al.,* 1991). In OSCCs, 98% of *p53*-gene mutations have been described in exons 5 to 8, in codons 238–248 (exon 7) and 278–281 (exon 8); and these DNA regions are likely hot spot areas (reviewed in Raybaud-Diogène *et al.,* 1996). These mutations have been found to show variations in different countries in relation to geographical location as well as ethnicity (Greenblatt *et al.,* 1994; Raybaud-Diogène *et al.,* 1996). For example, in the USA most mutations were detected in exon 7, while in Sweden and Japan they were detected in exon 8 (Greenblatt *et al.,* 1994; Raybaud-Diogène *et al.,* 1996). Several reports have investigated the association between p53 expression and gene mutation in OSCCs from patients with habits of oral use of tobacco and/or alcohol drinking. p53-protein over-expression has been shown to positively correlate with patient history of cigarette smoking and alcohol drinking (Field *et al.,* 1991, 1994; Brennan *et al.,* 1995; Lazarus *et al.,* 1996*b*), betel-nut and tobacco chewing (Kaur *et al.,* 1994) and toombak dipping in Sudan (Lazarus *et al.,* 1996*a*). Toombak is a form of oral snuff extensively used in Sudan (Idris *et al.,* 1995). In OSCCs, it has been illustrated that there is a wide spectrum of *p53*-gene mutations in cigarette smokers who also consume large quantities of alcohol, predominately at the cytidine-phosphate-guanosine (CpG) sites. These sites have been suggested as endogenous mutational ''hot spots,'' for those who do not smoke cigarettes or drink alcohol (Brennan *et al.,* 1995). *p53*-gene mutations have been reported from 2 OSCCs from toombak dippers, one at codon 285 ($CGG^{arg} \rightarrow TGG^{trp}$) and the other in intron 6 (AT → GC), while no mutations were found in an OSCC from a non-dipper (Lazarus *et al.,* 1996*a*). The tobacco-specific nitrosamines (TSNAs), present at inordinately high levels in Sudanese toombak, are believed to play an integral role in the development of tobacco-related OSCCs in Sudan (Idris *et al.,* 1995; Lazarus *et al.,* 1996*a*).

We have reported a significantly lower relative frequency of p53-protein expression in OSCCs from toombak-dippers compared with those from non-dippers in Sudan and Scandinavia (Ibrahim *et al.,* 1996). Although the early protein 6 (E6) of the high-risk human-papilloma-virus (HPV) types has been shown to induce degradation of the p53 protein (Scheffner *et al.,* 1990), we could not detect the HPV genome in OSCCs from toombak-dippers or non-dippers (Ibrahim *et al.,* 1998). In contrast, a significantly higher level of expression of keratin 13, 14 and 19 was found in the same OSCCs from Sudanese toombak dippers than in those from non-dippers in Sudan and in Scandinavia (Ibrahim *et al.,* 1998).

Epidemiological studies from Sudan have suggested a causal relationship between oral use of toombak and the development of OSCCs (Idris *et al.,* 1995). In Scandinavia, however, the relation between the oral use of snuff and the development of OSCCs is subject to controversy (Socialstyrelsen, 1996). Since the *p53* tumor-suppressor gene is a likely candidate for toombak-induced genetic alterations, the objective of the present study was to determine the prevalence of *p53* point mutations in the OSCCs and non-malignant oral lesions from Sudan already studied (Ibrahim *et al.,* 1996), together with control OSCCs and non-malignant oral

Grant sponsors: Richard With-Johnsen og Hustru Fanny's Fond; Gades Legat; Norwegian Cancer Society; The L. Meltzer Høyskolefond.

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Received 4 August 1998; revised 18 November 1998

lesions from non-dippers in Sudan and Scandinavia used in our earlier studies (Ibrahim *et al.*, 1996, 1998). Polymerase-chain-reaction-single-strand-conformation-polymorphism (PCR-SSCP) and DNA-sequencing techniques were used for molecular analysis, and the findings were correlated with the habitual oral use of snuff.

MATERIAL AND METHODS

Oral tissue samples

Formalin-fixed, paraffin-embedded tissue specimens of OSCCs (n = 14) and of non-malignant oral lesions (n = 8) from individual Sudanese toombak dippers, and OSCCs (n = 14) and non-malignant oral lesions (n = 3) from individual Sudanese non-dippers described earlier (Ibrahim *et al.*, 1996), were included in the present work. No data on current or previous history of cigarette smoking were available for any of the patients selected from Sudan. As controls, 28 formalin-fixed, paraffin-embedded tissue specimens of OSCCs from non-dippers in Scandinavia (17 in Norway 11 in Sweden) were included, as have been described earlier (Ibrahim *et al.*, 1996). Of the Scandinavian controls, 11 Norwegian patients (all males) and 8 Swedish patients (6 males, 2 females) reported cigarette smoking, with no information on the number of cigarettes smoked daily or weekly. No data on alcohol consumption were available.

DNA extraction

From the paraffin blocks, 2 or 3 10-µm-thick sections were cut, and total cellular DNA was extracted following the procedures described by Wright and Manos (1990) and Wang *et al.* (1994) with slight modifications. After repeated steps of de-waxing in xylene, centrifuging and re-suspending in absolute ethanol, the formed pellet was dried at 37°C overnight, re-suspended in 100 µl of digestion buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA and 0.5% Tween) containing 200 µg/ml of proteinase K (Sigma, St. Louis, MO) and incubated at 37°C for 5 days. Fresh enzyme was added each day, the final volume being 210 µl. Thereafter, an equal volume of 20% Chelex-100 (BioRad, Hercules, CA) solution containing 0.1% SDS and 1% Tween 20 was added. For recovery of DNA, the mixture was incubated at 37°C for 30 min, boiled at 95°C for 8 min, centrifuged at 12,000 g for 1 min; thereafter the supernatant was carefully collected into a fresh tube. DNA was quantified using a spectrophotometer (Beckman, DU 530, Life Sciences, Fullerton, CA).

Polymerase chain reaction and single-strand conformation polymorphism (PCR-SSCP)

A two-round nested PCR protocol was used. For the first PCR-round, exons 5 to 9 of the *p53* gene were amplified *in vitro* from each sample using primers described by Ryberg *et al.* (1994), as shown in Table I. Primers were obtained from (MedProbe, Oslo, Norway). The PCR was carried out in the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). In this round of PCR amplification, the 50-µl PCR reaction mixture consisted of 1 µl of the genomic DNA solution, 200 µM of each of the 4 deoxynucleotide triphosphates (dNTPs), 0.25 U AmpliTaq Gold DNA polymerase (5 U/µl, Applied Biosystems), 1.25 (for primer exon 5), 1 (for primer exons 6 and 7) and 2.5 (for primer exons 8/9) mM $MgCl_2$ respectively, and 10 pmol of each of the primers. A ''hot start'' at 94°C for 10 min was followed by 40 cycles of amplification, where each cycle consisted of 40 sec de-naturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C. The last PCR cycle was followed by a final extension at 72°C for 7 min. PCR reactions without DNA as a template were used as negative controls. Human placental DNA was used as normal control. Determination of the PCR amplification product was done by electrophoresis and staining with 0.5 µg/ml ethidium bromide in a 3% agarose gel; the amplified samples were recorded. Therafter, from the PCR products, 0.1 µl for the amplified samples and 1 µl for the non-amplified ones were re-amplified with the second round primers (obtained from MedProbe) described by Gaidano *et al.* (1991), as shown in Table I. All the conditions were the same as for the first round PCR, except that 2 mM $MgCl_2$ was used with all the primers, applying 0.25 U of the enzyme AmpliTaq DNA polymerase (5 U/µl, Applied Biosystems). Determination of this second-round PCR amplification product was done as for the first-round PCR, and the amplified samples were recorded. Before the SSCP and DNA-sequencing analysis of the amplified samples, second-round PCR products were purified, either by the QIAquick Gel Extraction Kit or by the QIAquick PCR Purification Kit, with use of a microcentrifuge as described in QIAquick Spin Handbook (QIAquick, QIAvac; Qiagen, Chatsworth, CA).

For the SSCP analysis, 3 µl of the purified PCR products were mixed with an equal volume of double-distilled water, and the mixture was heated for 5 min at 95°C to de-nature DNA, rapidly cooled on ice for a few min, then 1.4 µl of the mixture was directly pipetted onto the PhastGel Sample Applicator 8/1 µl (Pharmacia, Uppsala, Sweden), immediately followed by loading onto a gel. Electrophoresis was carried out using the semi-automated electrophoresis system (PhastSystem) on polyacrylamide-gel electrophoresis (PAGE), where 20% homogenous native gels were employed at 4°C for exons 5, 6 and 9 with samples separated for 400, 500 and 600 Vh, respectively, and at 15°C for exon 7 and at 20°C for exon 8 with samples separated for 450 and 270 Vh respectively. DNA bands were visualized with silver staining (PhastGel DNA silver-

TABLE I – FIRST- AND SECOND-ROUND PCR PRIMERS

Exon	Product length (nts)	Sequence
First-round PCR primers		
5	299	5′TTC AAC TCT GTC TCC TTC CT-3′
		5′GCA ATC AGT GAG GAA TCA GA-3′
6	224	5′TGG TTG CCC AGG GTC CCC AG-3′
		5′TGG AGG GCC ACT GAC AAC CA-3′
7	718	5′AGG CGC ACT GGC CTC ATC TT-3′
		5′AGG GGT CAG CGG CAA GCA GA-3′
8 and 9	445	5′TTG GGA GTA GAT GGA GCC T-3′
		5′AGT GTT AGA CTG GAA ACT TT-3′
Second-round PCR primers		
5	245	5′TTC CTC TTC CTG CAG TAC TC-3′
		5′ACC CTG GGC AAC CAG CCC TGT-3′
6	189	5′ACA GGG CTG GTT GCC CAG GGT-3′
		5′AGT TGC AAA CCA GAC CTC AG-3′
7	188	5′GTG TTG TCT CCT AGG TTG GC-3′
		5′GTC AGA GGC AAG CAG AGG CT-3′
8	213	5′TAT CCT GAG TAG TGG TAA TC-3′
		5′AAG TGA ATC TGA GGC ATA AC-3′
9	131	5′GCA GTT ATG CCT CAG ATT CAC-3′
		5′AAG ACT TAG TAC CTG AAG GGT-3

TABLE II – MUTATIONS OF THE *p53* GENE (EXONS 5–9) IN ORAL SQUAMOUS-CELL CARCINOMAS FROM SUDANESE TOOMBAK-DIPPERS AND FROM NON-DIPPERS IN SUDAN AND SCANDINAVIA

	Exon 5		Exon 6		Exon 7		Exon 8		Exon 9	
	Tumor number	Codon/mutation	Tumor number	Codon/mutation	Tumor number	Codon/mutation	Tumor number	Codon/mutation	Tumor number	Codon/mutation
Sudanese Toombak-dippers (n = 13/14)	1/2	130/CTC → CTT (S)	3	190/CCT → CCG* (S)	2	229/TGT → TGG*	8/12	299/CTG → CTTG (1) 305/AAG → TAA* (stop)	12	310/AAC → TTG*
	1	166/TCA → GCA*	5	216/GTG → GGG*	6	229/TGT → TA (−)			3/11	310/AAC → ACC*
	3	132/AAG → ATG*			7	237/ATG → ATA			13	312/ACC → AGC*
		133/ATG → GATG (1) 148/GAT → TGA* (stop)			8	239/AAC → TAA* (stop)				
	4	139/AAG → GAG 142/CCT → CTT			3 9/3 10 11/2	242/TGC → TAC 249/AGG → AAG 245/GGC → GAA 252/CTC → CAC*				
Non-snuff-dippers Sudanese (n = 8/14)	14/15	132/AAG → ATG*			16	240/AGT → CCC* 249/AGG → AAG			20	322/CCA → CGA 323/CTG → CG (−)
					17/18	244/GGC → TGA* (stop)			21	323/CTG → GGG*
					19	254/ATC → GTC				
Scandinavian (n = 17/28)	22/23	130/CTC → CTT (S)			26	238/TGT → CGT	26	279/GGG → GAG	36	308/CTG → CTA 315/TCT → TGT*
	24	136/CAA → CCA*			26/27/28	246/ATG → ATA	33	281/GAC → GCC*		
	25	146/TGG → TAG (stop)			26/27	248/CGG → CGA	32/34	299/CTG → CTTG (1) 305/AAG → TAA* (stop)	27	308/CTG → CTC* (S)
					28	248/CGG → CAA	35	306/CGA → TGA (stop)	33	309/CCC → TCC 323/CTG → CGG*
					29	244/GGC → GAA				
					30	239/AAC → ACC*			30	322/CCA → CGA*
					31	246/ATG → ATA				
					32	249/AGG → AAG				

(S), silent mutation; *Transversion; (I), insertion leading to frameshift and stop codon; (−), deletion. All other mutations were transitions. Only tumors 5, 14–16, 19–21, 23–25, and 30–35 were previously found positive for p53 protein expression by immunohistochemistry.

staining kit). Under all the SSCP runnings, purified PCR products of human placental DNA were included and processed under the same conditions.

DNA sequencing

After the initial PCR/SSCP analysis of all the samples was completed, a non-radioactive cyclic sequencing reaction was performed for the samples to be sequenced using the manufacturer's protocol for the ABI PRISM BigDye Cycle Sequencing Ready Reaction Kit (Applied Biosystems). In the 10-µl amplification reaction, 2 µl of the purified PCR products of the fragments which showed mobility shifts in the SSCP analysis, 4 µl from the BigDye reaction mixture, and 1.5 pmol from the PCR primers of the second round PCR were amplified. Sequencing was done on the automated ABI PRISM 377 DNA Sequencer (Applied Biosystems), and was carried out in both directions to confirm the findings. The software packages Vector NTI 5.1 and Align X 1.0 (InforMax BioSuite), Version 5.0 for Windows (InforMax, North Bethesda, MD) were used to analyze results of sequencing.

Statistical analysis

The 2-tailed Fischer's exact test (at $p < 0.05$ significance level) was used to compare analysis of *p53* mutations in the OSCCs from the Sudan and from Scandinavia. *p53* mutations were correlated with snuff dipping and/or cigarette smoking.

RESULTS

PCR and SSCP analysis for p53 *mutations*

For the 2-round nested PCR protocol, a prominent increase in the total number of samples amplified was seen after the second-round PCR. All samples that amplified with this round were subjected to further analysis. For the PCR-SSCP analysis, sample DNA fragments showing mobility shifts in comparison with those of normal cellular DNA were sequenced. Amplified DNA showing only mutant bands was used directly for sequencing.

Determination of the mutated sequences

A summary of the mutations (transitions, transversions, insertions and/or deletions) of the *p53* gene detected from the OSCCs from toombak dippers and in those from non-dippers in Sudan and Scandinavia is shown in Table II. There were no mutations detected in samples from the non-malignant oral lesions from toombak dippers or non-dippers in Sudan. Figure 1*a* (exon 7; from a carcinoma from a Sudanese toombak dipper) and Figure 1*b* (exon 8; from a carcinoma from a non-dipper), present examples of the band shifts found in the PCR-SSCP analysis for a tumor DNA with no band shifts in the normal placental DNA (*i*), and the sequencing results for both normal placental DNA (*ii*) and tumor DNA (*iii*). Of the 14 OSCCs investigated from toombak dippers, mutations in exons 5 to 9 were found in 93% (13/14), compared with 57% (8/14) and 61% (17/28) in those from non-dippers in Sudan and in Scandinavia respectively. In Figure 2, the association of toombak dipping and/or non-dipping with the relative frequencies of mutations of the *p53* gene found in the OSCCs from toombak dippers in Sudan compared with those from non-dippers in Sudan and/or Scandinavia is shown. For the OSCCs from toombak dippers, the mutations were found distributed in exons 5 (4 tumors; 29%), 6 (2 tumors; 14%), 7 (8 tumors; 57%), 8 (2 tumors; 14%) and 9 (4 tumors; 29%), while for those from non-dippers in Sudan and Scandinavia, mutations were found distributed in exons 5 (6 tumors; 14%), 7 (11 tumors; 26%), 8 (5 tumors; 12%; only in the Scandinavian OSCCs) and 9 (6 tumors; 14%) (Table II).

The difference in mutations of the *p53* gene was statistically significant in the OSCCs from Sudanese toombak dippers when compared either with those from non-dippers in Sudan and in Scandinavia ($p = 0.04$) or only with those in Scandinavia ($p = 0.02$). The difference in *p53*-gene mutations in the OSCCs from non-dippers in Sudan and in Scandinavia was not statistically significant ($p = 0.91$). In addition, no statistically significant difference was found in *p53* mutations for the OSCCs from toombak dippers/non-dippers from Sudan ($p = 0.07$).

Relationship between p53 *mutations and toombak dipping*

The *p53*-gene-mutated OSCCs from toombak dippers (Table II) showed 15 transversions (5 A:T to T:A in exons 5, 7–9; 4 T:A to G:C in exons 5, 6 and 7; 2 T:A to A:T in exon 7; 2 A:T to C:G and one C:G to G:C in exon 9; and one G:C to T:A in exon 5) and 9 transitions (5 G:C to A:T in exon 7; 3 C:G to T:A and one A:T to G:C in exon 5). There were 3 insertions of one base pair in exons 5 and 8 (resulting in frameshifts and stop codon), and deletion of one base pair in exon 7 (Table II). In exon 5, 2 transition mutations were silent, and one transversion resulted in a stop codon (Table II). In exon 6, one transversion was a silent mutation, while 3 transversions (one in exon 7, and 2 in exon 8) resulted in stop codons (Table II).

For the OSCCs from Sudanese and Scandinavian non-dippers (Table II), there were 7 transversions (2 A:T to T:A in exon 5; 2 C:G to G:C in exon 9; 2 G:C to T:A in exon 7; one A:T to C:G in exon 7), 2 transitions (G:C to A:T and A:T to G:C found in exon 7) and deletion of one base pair in exon 9 for the Sudanese carcinomas, as compared with 9 transversions (3 A:T to C:G in exon 5, 7 and 8; 2 C:G to G:C in exon 9; 2 A:T to T:A in exon 8; one G:C to C:G in exon 9; one T:A to G:C in exon 9), 17 transitions (12 G:C to A:T in exons 5, 7–9; 4 C:G to T:A in exons 5, 8 and 9; one T:A to C:G in exon 7) and 2 insertions of one base pair (resulting in frameshifts and stop codon) in exon 8 for the Scandinavian OSCCs (Table II). For the Sudanese OSCCs, one transversion in exon 7 resulted in a stop codon, while for the Scandinavian ones, 2 transitions were silent and one resulted in a stop codon in exon 5, one transition and one transversion in exon 8 resulted in stop codons, and one transversion in exon 9 was silent. Figure 3 shows a correlation between the percentage of the total number of mutations (transitions/transversions) found in the OSCCs from Sudanese toombak dippers in comparison with those found in the OSCCs from non-dippers in Sudan and Scandinavia. The mutations G:C to A:T, C:G to T:A and G:C to T:A, known to be associated with TSNAs, were found to be the most common in the OSCCs from toombak dippers. However, this was not found to be different from those observed in the OSCCs from non-dippers (Figure 3).

DISCUSSION

In the present work, a significantly higher incidence ($p = 0.04$; $p = 0.02$) of *p53*-gene mutations was found in OSCCs from Sudanese toombak dippers than in those from non-dippers in Sudan and Scandinavia together, and those in Scandinavia alone. Our results show that exon locations of the *p53*-gene mutations were related to exposure to toombak. These findings indicate that toombak use plays an important role in inducing increased incidence of *p53*-gene mutations with a wide spectrum of exons in OSCCs from toombak dippers. We found that the exon numbers and locations, but not the types of *p53*-gene mutations, differed in relation to exposure to toombak. In the OSCCs from toombak dippers, mutations were found in exons 5 to 9, while in those from non-dippers they were found in exons 5, 7, 8 and 9. However, no mutations were found in exon 8 in any of the OSCCs from non-dippers in Sudan. The types of mutations seen, in relation to those found in TSNAs in experimental animal models, were similar in toombak users and in the control groups from non-users in Sudan.

The most common types of mutations found in squamous-cell carcinoma of the head and neck are C:G to T:A transitions. These mutations are the second most common, after the G:C to T:A transversions found in cigarette-smoking-related lung tumor (Greenblatt *et al.*, 1994). G:C to T:A, non-CpG G:C to A:T, and A:T to G:C substitutions are known to be associated with tumors induced by smokeless tobacco, particularly *N*-nitrosamines, in experimental animal models (Greenblatt *et al.*, 1994). C:G to T:A transitions are the most common mutations observed in lung adenocarcinoma in rodents treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) (NNK) (Belinsky *et al.*, 1991; Oreffo *et al.*, 1993). In addition, G:C to T:A transitions are attributed to NNK in experimental animal models (Ronai *et al.*, 1993). These mutation types are similar to those we observed in the OSCCs from Sudanese toombak

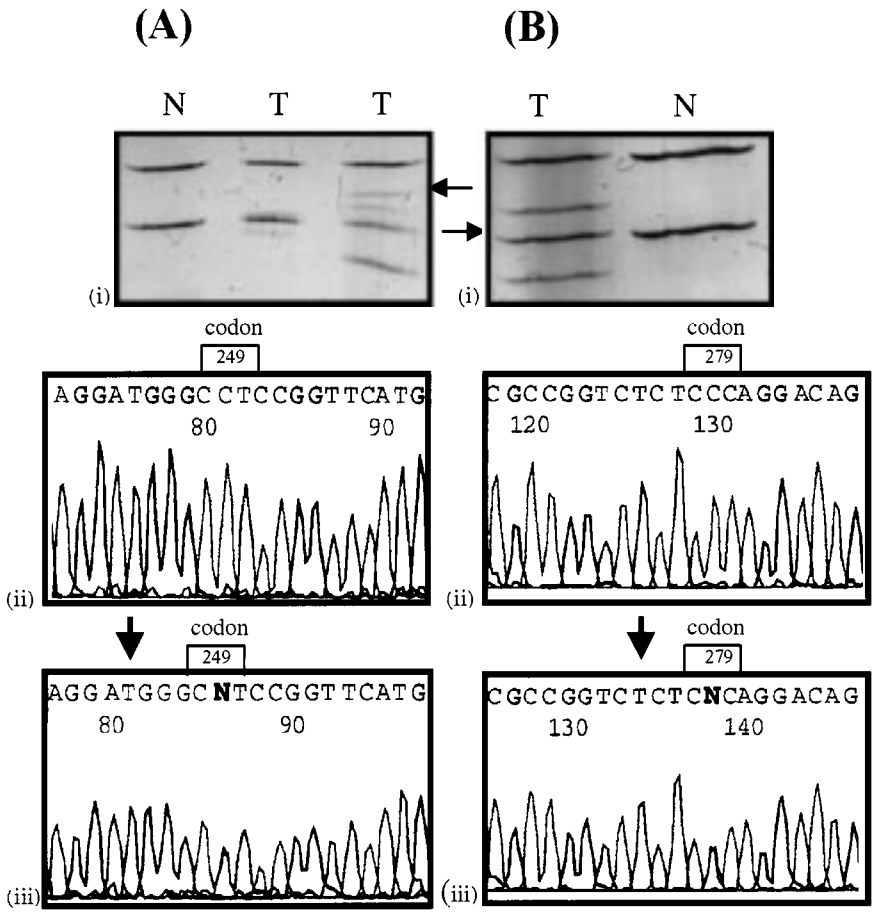

FIGURE 1 – An example of PCR-SSCP and sequence analysis of tumor DNA (T) and normal DNA (N) for exon 7 *(a)* in a carcinoma from a Sudanese toombak dipper) and for exon 8 *(b)* in a carcinoma from a non-dipper). (i) PCR-SSCP analysis illustrating mobility shifts found in T DNA (indicated by arrows) in comparison with that found in N DNA after sequencing. ABI PRISM electrophotograms of N DNA (ii) and T DNA (iii) are shown in 3′–5′ (anti-sense) directions. Multiple alignments of the 2 mutations have shown G-to-T transitions Arg-Lys (codon 249), and Gly-Glu (codon 279), respectively, for the 2 tumor DNA samples analyzed.

dippers (Table II), indicating that the increased incidence of mutations of these types are probably related to *N*-nitrosamines in tobacco (Belinsky *et al.,* 1991; Oreffo *et al.,* 1993; Greenblatt *et al.,* 1994). The possible role of TSNAs in the etiology of OSCCs in toombak dippers, through induction of *p53*-gene mutations, has been reported (Lazarus *et al.,* 1996*a*). Thus, our findings appear to support the suggested role of TSNAs, in particular the NNK, in induction of mutations in human tumors (Belinsky *et al.,* 1991; Oreffo *et al.,* 1993; Ronai *et al.,* 1993). It is therefore probable that TSNAs are involved in the etiology of OSCCs from Sudanese toombak dippers, through induction of *p53*-gene mutations (Lazarus *et al.,* 1996*a*).

It has been suggested that the TSNAs, particularly *N*-nitrosonornicotine (NNN) and NNK, are major contributors in the development of aerodigestive-tract tumors in tobacco chewers and snuff dippers (Hoffmann and Hecht, 1985). Total levels of NNN and NNK in cigarette smoke were found to be 2 to 10 times that of benzo[a]pyrene (BaP) (Adams *et al.,* 1987), and up to 1000 times that found in moist and dry North American snuff (Hoffmann *et al.,* 1987). NNN and NNK are the only tobacco constituents known to induce oral-cavity tumors in experimental animals (Hecht *et al.,* 1986). These findings strongly support the suggested role of NNN and NNK as causative agents in TSNA-related carcinogenesis (Brunnemann and Hoffmann, 1992). The Sudanese toombak has been found to contain exceptionally high levels of both, up to 560-fold higher than those reported in North American or European smokeless tobacco products (Idris *et al.,* 1991). According to these

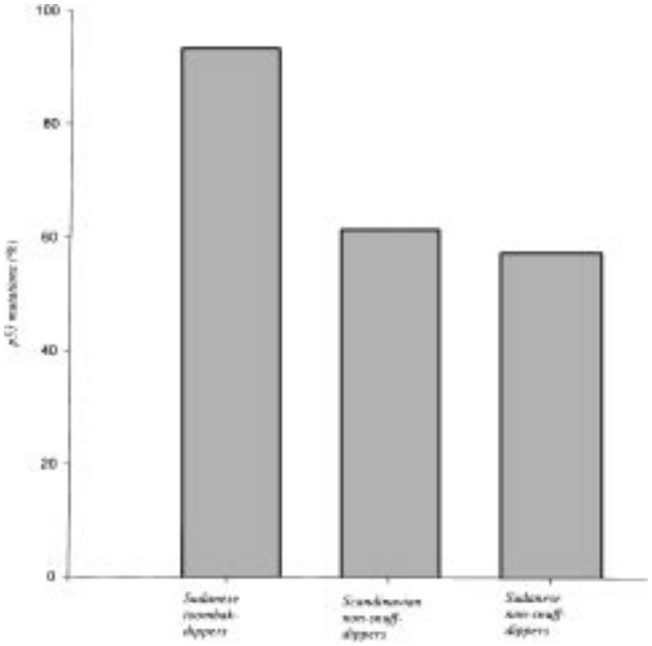

FIGURE 2 – The association of *p53*-gene mutations found with toombak dipping and non-dipping in oral carcinomas from Sudan and Scandinavia.

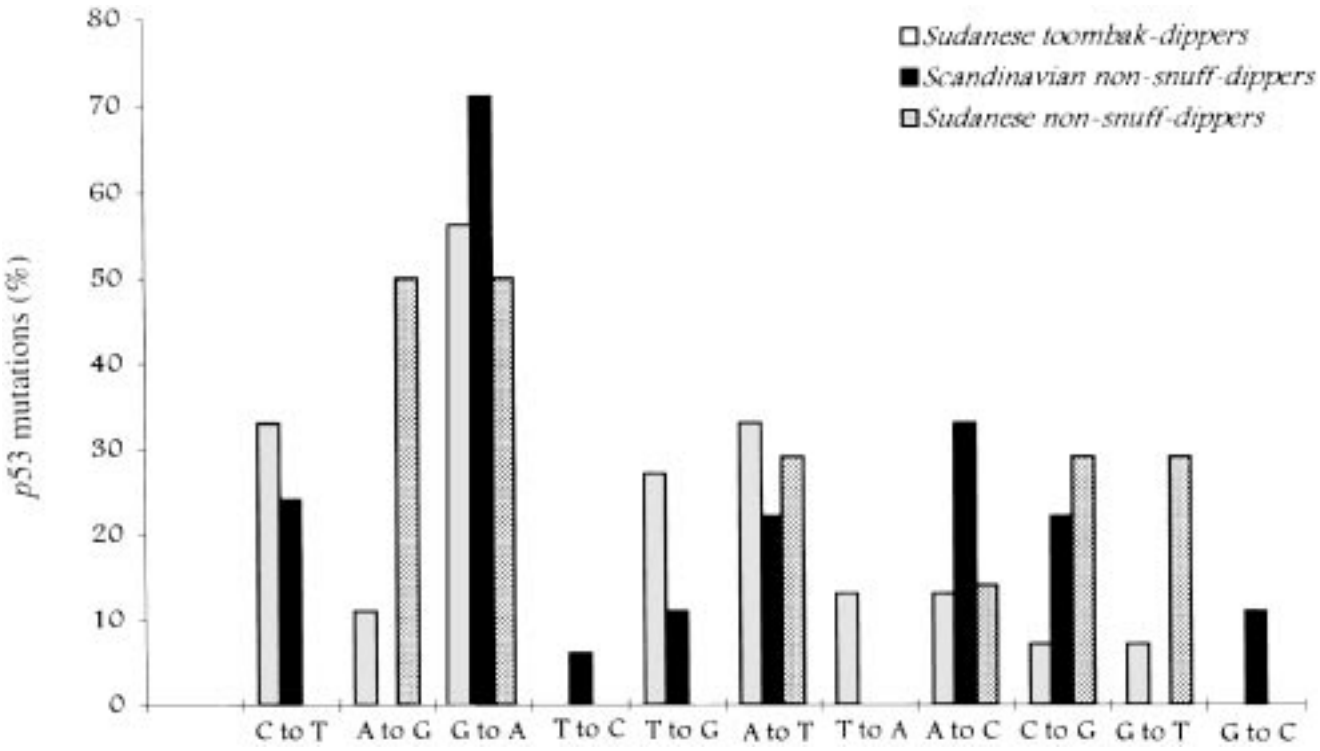

FIGURE 3 – The pattern of *p53*-gene substitution mutations (transitions/transversions) found in the carcinomas from Sudanese toombak dippers and in those from non-dippers in Sudan and Scandinavia, with no difference in the mutations known to be associated with TSNAs in toombak users and in control groups in Sudan.

observations, our findings of *p53*-gene mutations in the OSCCs from toombak dippers appear to confirm that NNN and/or NNK are strong human carcinogens. Thus, toombak use plays an important role in inducing *p53*-gene mutations with a wide spectrum of exons involved in OSCCs from toombak dippers. The differences in the spectrum and profile of the *p53*-gene mutations found in OSCCs from toombak dippers may also be attributed to the high levels of TSNAs found in toombak (Idris *et al.,* 1991). The high levels of NNN and of NNK found in toombak probably explain the increased incidence as well as the spectrum of mutational profile of the *p53* gene found in the OSCCs from Sudanese toombak-dippers as compared with those from non-dippers in Sudan and in Scandinavia.

In the present study, OSCCs from Sudanese non-toombak-dippers showed 2 G:C to T:A transversions, one G:C to A:T, and one A:T to G:C transitions. These findings are similar to those found in OSCCs from toombak dippers. OSCCs from Scandinavian non-dippers showed 12 G:C to A:T and 4 C:G to T:A. For the mutations observed in OSCCs from Sudanese non-dippers, there were 2 G:C to T:A transversions found at the CpG sites (codon 244), one non-CpG G:C to A:T (codon 240) and one A:T to G:C (codon 254), compared with 4 CpG G:C to A:T (at the hot spots of exon 7; codons 244, 248), and 6 mutations at the non-CpG G:C to A:T in the Scandinavian ones. In Western countries, the 2 major risk factors for the development of OSCCs are the use of tobacco and the consumption of alcohol (Johnson, 1991). In the OSCCs from Scandinavian non-dippers, 19 patients reported cigarette smoking, with no information on the amount smoked daily or weekly. The spectrum of *p53*-gene mutations found in OSCCs from cigarette smokers in Scandinavia is similar to that found in tobacco-related lung tumor and OSCCs (Greenblatt *et al.,* 1994). In addition, the spectrum of *p53*-gene mutations is in agreement with other studies on OSCCs reported from Europe and USA (Field *et al.,* 1991; 1994; Brennan *et al.,* 1995; Lazarus *et al.,* 1996*b*). Alcohol drinking is traditionally not considered a risk for the development of OSCCs in the Sudanese population (predominantly Muslim). Any similarities in the spectrum and profile of *p53*-gene mutations found in OSCCs from Sudanese non-dippers to those found in tobacco-related tumors in experimental animals and to those found in OSCCs from toombak dippers are probably a result of under-reported current or past history of cigarette smoking in the population. The involvement of other chemicals, habitual oral practices and/or environmental carcinogens, resulting in the observed mutations, is also possible.

We found lower levels of p53 protein expression in the same OSCCs from toombak dippers than in OSCCs from non-dippers in Sudan and Scandinavia (Ibrahim *et al.,* 1996). The present results indicate that the *p53* gene is mutated in these carcinomas. Mutations found included frameshifts, deletions and stop codons. These mutations would encode for a truncated p53 protein which can be difficult to detect by immunohistochemistry (Brennan *et al.,* 1995; Lazarus *et al.,* 1996*b*). The results of the present study support the view that more sensitive methods, such as PCR and sequencing of the gene, appear necessary to disclose *p53* mutations (Field *et al.,* 1991; Sasano *et al.,* 1992). Earlier, we found high expression of keratin 13, 14 and 19 in the same OSCCs from toombak-dippers (Ibrahim *et al.,* 1998). Our present findings support our earlier suggestion of cytoplasmic complexes between p53 protein and the keratins investigated. The high frequency of *p53*-gene mutations in the OSCCs from toombak dippers is also consistent with the absence of HPV in these carcinomas (Ibrahim *et al.,* 1998). In the present study, no *p53* mutations were found in pre-malignant oral lesions from Sudanese toombak dippers and non-dippers, compared to the *p53* mutations found in the OSCCs from toombak dippers and non-dippers. These data suggest that mutations of the *p53* gene play an integral role in the progression of OSCCs.

Some of the OSCCs investigated in this study showed multiple and silent mutations of the *p53* gene, in line with other studies (Kanjilal *et al.*, 1993, 1995). It has been suggested that multiple and silent mutations in the *p53* gene indicate that a special hypermutability process operates on the gene during the generation of tumors (Strauss, 1997). UV-induced murine skin tumors showed heterogeneity of *p53* mutations within individual samples, suggesting that these mutations may arise as a result of continuous exposure to environmental carcinogens (Kanjilal *et al.*, 1993). Multiple heterogeneous mutations may be detected by isolating individual mutant alleles from PCR products and cloning them into suitable vectors before sequencing. However, direct sequencing of the PCR products (Kanjilal *et al.*, 1995) may miss these mutations. We maintained stringent conditions during the two-round-PCR amplifications. No mutations were found in normal control and placental DNA or in the non-malignant oral-tissue specimens from Sudanese toombak dippers or non-dippers. The multiple mutations found in our study suggest a multicellular origin of the tumors examined, or that these mutations are due to continuous exposure to environmental carcinogens found in tobacco.

In conclusion, our results indicate that (*i*) toombak use plays a significant role in the induction of increased *p53*-gene mutations with a wide spectrum of exons involved, including exon 6, thus enhancing tumorigenesis of OSCCs among toombak dippers, (*ii*) the types of mutations observed were similar to those induced by TSNAs in rats and to those reported earlier in toombak dippers, (*iii*) there is no difference in the incidence of certain types of mutations associated with TSNAs found in the exposed and the control groups, (*iv*) a novel mutation in exon 6 was found in the OSCCs from toombak dippers, (*v*) mutation of exons 5 (codon 130), 6 (codons 190 and 216) and 7 (codons 229, 249 and 252) suggest that these are probable hot spots for TSNA-induced tumors in humans. In view of the similarities in the spectrum of mutations, the significant differences in the type and number of exons involved by mutations in the 2 populations may be explained by differences in the type of exposure rather than by the ethnic origin of patients. Further studies are needed to validate the *p53* gene as a biomarker of tobacco-induced tumor in humans, while differences in environmental exposures or inherited characteristics between the 2 populations, require detailed investigation.

ACKNOWLEDGEMENTS

We thank Professor N.W. Johnson and Dr. S. Warnakulasuriya, Department of Oral Medicine and Pathology, King's College School of Medicine and Dentistry, London, UK, for reading and commenting on the manuscript. We are grateful to Ms. I. Ottesen, Ms. G. Fjell and Ms. T. Sage for their very skilled technical assistance.

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