

Mutations of the Cell Cycle Arrest Gene $p21^{WAF1}$, but not the Metastasis-inducing Gene S100A4, are Frequent in Oral Squamous Cell Carcinomas from Sudanese *toombak* Dippers and Non-snuff-dippers from the Sudan, Scandinavia, USA and UK

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Abstract. PCR and direct DNA sequencing methods were used to analyse the prevalence of mutations in exon 2 of the $p21^{WAF1}$ gene in 14 oral squamous cell carcinomas (OSCCs) and 8 non-malignant oral mucosal lesions from Sudanese *toombak* dippers. For comparison, OSCCs (14 from the Sudan, 16 from Norway, 11 from Sweden, 21 from the USA and 14 from the UK) and non-malignant oral mucosal lesions (3 from the Sudan) from non-snuff-dippers were included. The prevalence of mutations in exons 2 & 3 of the S100A4 gene were analysed in the 14 OSCCs from *toombak*-dippers and in 25 cases of OSCCs from the control non-snuff-dippers. Of the 14 OSCCs investigated from *toombak*-dippers, mutations in the $p21^{WAF1}$ exon 2 were found in 43% (6 out of 14), compared to 14% (2 out of 14), 22% (6 out of 27) and 14% (5 out of 35) found in those from non-snuff-dippers from the Sudan, Scandinavia and the USA/UK, respectively. OSCCs from *toombak*-dippers showed 13 different mutations distributed as 10 (77%) transitions and 3 (23%) transversions. OSCCs from non-snuff-dippers from the Sudan, Scandinavia, the USA and the UK showed 33 different mutations distributed as 14 (42%) transitions and 19 (58%) transversions. In the OSCCs examined, cases with mutations in the $p21^{WAF1}$ also had p53 gene mutations. Only exon 2 of the S100A4 gene was found mutated in 3 cases of OSCCs (one from a *toombak*-dipper and two from the non-snuff-dippers). The *toombak*-dipper OSCC had 4 mutations (one transition, 3

transversions), compared to the OSCCs from non-snuff-dippers which showed 3 mutations each (one transition, 2 transversions). All these 3 cases were negative for mutations in the $p21^{WAF1}$ and p53 genes. No mutations of $p21^{WAF1}$ or S100A4 were found in the non-malignant oral mucosal lesions from the snuff-dippers/non-dippers. These findings suggest that; (i) $p21^{WAF1}$, together with p53, is a target gene of oral carcinogenesis in OSCCs from *toombak*-dippers, with the tobacco specific nitrosamines present in *toombak* possibly acting as principal carcinogens in these OSCCs; (ii) findings of $p21^{WAF1}$ exon 2 mutations in the OSCCs unrelated to snuff use further demonstrate that this gene may play an important role during the pathogenesis of OSCCs caused by smoked tobacco use; (iii) mutations in the S100A4 gene are rare in OSCCs, but appears to be complementary to $p21^{WAF1}$ and p53 mutations. Since molecular analysis of OSCCs can provide clues to endogenous or environmental factors contributing to the high risk of OSCCs, further analysis of the role of the $p21^{WAF1}$ gene mutations as a biomarker of malignant transformation, which is linked to the p53 gene, is necessary, especially in habitual users of *toombak* from the Sudan.

In many parts of the world where social habits of tobacco use and alcohol consumption are high, oral squamous cell carcinoma (OSCC) represents a major health problem (1-3). Epidemiological studies have shown that the incidence of this disease varies world-wide, with a relative frequency of less than 1% to over 40% of all malignancies (1-3). In general, the incidence rates of this disease are higher in developing countries compared to developed ones, with males being more affected than females (1-3). However, the incidence of OSCC is increasing in many parts of developed countries, in particular in Southern and Eastern Europe (4), Scandinavia (5) and US blacks (1-3, 6, 7), but not in US whites (1-3, 6, 7). This increase in the incidence involves in particular tumours

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of the larynx and oral cavity, with the increase of risk being seen in younger people (1-7). In 1998, the incidence rates of OSCC (ASR per 100,000 population) in countries chosen for this investigation were in Sweden 4.91 for males and 2.55 for females, in Norway 6.12 for males and 2.51 for females, in the US (both white and blacks taken together) 7.67 for males and 3.85 for females, in the UK 4.12 for males and 1.93 in females and in the Sudan 11.60 for males and 6.91 for females (8). These observations suggest that the disease is less prevalent in Nordic countries, in Europe (9, 10), in the UK and the USA, compared with the Sudan (11). American blacks are also more susceptible to OSCCs compared to white Americans with an age-adjusted incidence rate of 20.4 per 100,000 (12). Despite major advances in the diagnosis and therapy, five-year survival rates (35-50%) remain poor in patients with OSCCs, mainly because of the increased risk of developing second primary carcinomas within the upper aerodigestive tract (13). With better loco-regional control, a growing incidence of metastatic disease is also noted (13).

Progression of cells through the cell cycle is under the control of genes encoding proteins which transmit positive [like activated cyclin and cyclin-dependent kinases (cdks)] and negative signals (like inhibitors of cdk) (14, 15). Any state of cellular imbalance that affects any of these genes can lead to premature entry of the cells into the next phase of the cell cycle prior to completion of critical macromolecular events, including repair of DNA damage, and generate genomic instability and neoplastic transformation (14, 15). $p21^{waf1}$, the first cdk inhibitor to be identified and located on chromosome 6p21.2, is a cell cycle protein of 21 kDa that regulates and can arrest the cell cycle in the G1 or the S-phase either dependently or independently of the p53 protein (16-19). $p21^{waf1}$ is thought to be a "universal" cdk inhibitor, that inhibits the activity of both Cyclin D-cdk4/6 and Cyclin E-cdk2 complexes (16-19). The $p21^{waf1}$ coding sequence is composed of 3 exons, with a 2.4 kb sequence encoding a p53-binding site at the 5'-part of the gene (16-19). The metastasis-associated gene S100A4 (also known as mts1, p9Ka, pEL98, 18A2, 42A, calvasculin, fspl, cap1), is a member of the S100 family of the calcium-binding proteins (20, 21). The gene is known to be capable of regulating cell cycle progression, modulating intercellular adhesion and the invasive and metastatic properties of cancer cells (20, 21). The S100A4 protein has been suggested to sequester and disable the p53 protein (20). It has recently been demonstrated that the S100A4 gene binds to the extreme end of the C terminal regulatory domain of the p53 gene (at amino acid residues 360-393), illustrating both a physical and functional interaction between these two proteins (22). The S100A4 gene is located on chromosome 1q21 (20-22), and is composed of 2 introns and 3 exons (23).

Although the p53 tumour suppressor gene has been extensively studied in human tumours in many body sites (24), including OSCCs (see review 25), the possible implications of the expression levels of other genes that mediate the ability of

p53 to induce cell cycle arrest or apoptosis remain unclear (24, 25). $p21^{waf1}$ has been suggested to be involved in a wide variety of tumour cell lines and in some primary tumours (reviewed in 18, 19). To our knowledge, a few studies have examined the expression and possible mutations of the $p21^{waf1}$ gene, but none the S100A4 gene, in OSCCs (18, 19). In this study, our hypothesis states that abrogation of both $p21^{waf1}$ and S100A4, besides the p53 tumour suppressor gene, may be a contributor to development of OSCCs. We therefore wanted to determine the prevalence of $p21^{waf1}$ and S100A4 point mutations in the OSCCs and non-malignant oral mucosal lesions from our previously well-characterised and published Sudanese case-series (26, 27), together with comparison OSCCs and non-malignant oral mucosal lesions from non-snuff-dippers in the Sudan, Sweden, Norway, the USA and the UK. Polymerase chain reaction and direct DNA sequencing techniques were used for the molecular analysis and the findings were correlated with the habitual oral use of snuff and/or any other form of tobacco.

Materials and Methods

Patients, oral tissue specimens and DNA extraction. Formalin-fixed, paraffin-embedded tissue specimens of oral SCCs: 14 from Sudanese tobacco-dippers and 76 from non-snuff-dippers including 14 from the Sudan, 16 from Norway, 11 from Sweden, 21 from the USA and 14 from the UK, were included in the study (Table I). Eleven other non-malignant oral lesions were included: 8 from Sudanese snuff-dippers and 3 from non-snuff-dippers. The majority of the non-snuff-dippers diagnosed with oral SCC from Europe and USA were regular tobacco smokers (Table I), but their alcohol habits were unavailable to the authors. DNA from all the cases included in this study was extracted using a standard protocol as described in detail elsewhere (27). Purified DNA was quantified spectrophotometrically (Beckman, Fullerton, CA, USA DU® 530, Life Science).

Polymerase chain reaction (PCR). The nucleotide sequences of the $p21^{waf1}$ and the S100A4 genes were derived from and numbered according to El-Deiry *et al.* (16), Abbaturnian *et al.* and Tulchinsky *et al.* (23, 28), respectively. For the *in vitro* amplification of the $p21^{waf1}$ from each tumour, we used the primer pairs WU2/WL2 with the sequences and the PCR fragment sizes given in Table II, which brackets exon 2, or 89.6% of the $p21^{waf1}$ coding sequence (16). For the *in vitro* amplification of the S100A4 gene fragments from each tumour, the primers for exons 2 and 3 were chosen with the assistance of the software computer package programmes Vector NTI 5.1, Align X 1.0 (InforMax BioSuite™) Version 5.0 for Windows™ (InforMax, Inc. North Bethesda, USA). The primer pairs sequences and the PCR fragment sizes for S100A4 exons 2 and 3 are presented in Table II. All primers used in this study were purchased from (MWG-Biotech AG, Germany). The PCR was carried out in the GeneAmp® PCR System 9700 (PE Applied Biosystems). The 50 µl PCR-mixture consisted of 1 µl of the genomic DNA solution, 200 µM of each of the deoxynucleotide triphosphates (dNTPs), 0.20 U of AmpliTaq® Gold DNA polymerase (5 U/µl, PE Applied Biosystems, Foster City, CA, USA), 2 (for $p21^{waf1}$ primer exon 2), 2.5 and 3.5 (for S100A4 primer exon 2 and exon 3) mM MgCl₂, respectively, and 10 pmol of each of the primers. 5% dimethyl sulfoxide (5% DMSO) was present in the PCR mixture for amplification of the S100A4 exons 2 and 3. A "hot start" at 95°C for 10 minutes was followed by 40 cycles of amplification, where each cycle consisted of 90 seconds denaturation at 95°C, 90 second annealing at 65°C (for $p21^{waf1}$

Table I. Summary of the tissue samples selected for the study. Distribution according to country of origin, snuff dipping and type of lesion.

Country/Habit	Snuff dipper*		Non-snuff dippers**	
	OSCC	Other lesion [#]	OSCC	Other lesion [#]
Sudan	14	8	14	3
Norway	0	0	16	0
Sweden	0	0	11	0
USA	0	0	21	0
UK	0	0	14	0
All	14	8	76	3

*Snuff use in the Sudan is called *toombak*.

**The majority of non-snuff users with OSCCs were cigarette smokers: 11 out of 16 in Norway, 8 out of 11 in Sweden, all 14 in the UK.

[#]Other lesions included were epithelial dysplasia ($n=8$) and one carcinoma *in situ*.

primer exon 2) and at 67°C and 64°C (for S100A4 primer exons 2 and 3, respectively), and 2 minutes extension at 72°C. The last PCR cycle was followed by a final extension at 72°C for 10 minutes. For mutation analysis in the $p21^{waf1}$ gene, we used primers that brackets exon 2, or about 90% of the $p21^{waf1}$ coding sequence. For the S100A4 gene, only the coding sequences of the S100A4 gene (exons 2 and 3) were analysed. As negative controls for the amplification reactions, PCR reactions without DNA as a template were used. Human placental DNA was used as a wild-type control. Size determination of the PCR amplification product was done by electrophoresis and staining with 0.5 µg/ml ethidium bromide in a 3% agarose gel, and the amplified samples were recorded. Before further analysis, PCR amplification products were purified using either GenElute™ Agarose Spin Columns (Sigma, Saint Louis, Missouri USA) protocol, the QIAquick Gel Extraction Kit and/or the QIAquick PCR Purification Kit Protocols using a microcentrifuge as described in QIAquick Spin Handbook (QIAGEN®, QIAquick™, QTAvac).

Direct DNA sequencing. Following the initial PCR amplification analysis of all the tumours, the purified product of only the tumours that showed amplification reaction with PCR were subjected to direct sequencing using the ABI PRISM™ BigDye Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). Sequencing was done on the automated ABI PRISM™ 377 DNA Sequencer (PE Applied Biosystems, Foster City, CA, USA). The software packages Vector NTI 5.1 and Align X 1.0 (InforMax BioSuite™) Version 5.0 for Windows™ (InforMax, Inc. North Bethesda, USA) and the Chromas scientific software programme (Technelysium Pty Ltd) were used to analyse the sequencing results.

Statistical analysis. Either Chi-square or two-tailed Fischer's exact tests (at $p < 0.05$ significance levels) were used to compare the analysis of $p21^{waf1}$ and S100A4 mutations in the OSCCs from the Sudan, Scandinavia, the USA and from the UK. When appropriate, $p21^{waf1}$ and S100A4 gene mutations were correlated with snuff dipping, alcohol drinking, cigarette smoking and/or previous status of $p53$ gene mutations.

Table II. Primers used for amplification of the $p21^{waf1}$ exon 2 and the S100A4 exons 2 and 3.

Name	Sequence 5'-3'	Size
$p21^{waf1}$		
*Exon 2		
Sense (WU2)	GGC GCC ATG TCA GAA CCG GCT	(447 bp)
Antisense (WL2)	CAT GCT GGT CTG CCG CCG TTT T	
S100A4		
Exon 2		
Sense (F)	GCC TGG CAT TCT GAA CAC ATC T	(333 bp)
Antisense (R)	CCA CCC ACT GAT AGA TGC CCT C	
Exon 3		
Sense (F)	TGC TCC ACC CAC TGG GCT T	(240 bp)
Antisense (R)	CCC CAA CCA CAT CAG AGG AGT	

* cDNA positions 70-91 and 495-516 (16), which brackets exon 2, or 89.6% of the $p21^{waf1}$ coding sequence.

Results

DNA sequencing analysis for $p21^{waf1}$. A summary of the substitution mutations (transitions and transversions) of the $p21^{waf1}$ gene exon 2 detected from the OSCCs from Sudanese *toombak*-dippers and in those from non-snuff-dippers from the Sudan, Scandinavia, the USA and the UK is shown in Table III. No mutations were detected in the non-malignant oral mucosal lesions from *toombak*-dippers or non-*toombak*-dippers from the Sudan. In Figures 1A (a carcinoma from a Sudanese *toombak*-dipper) and 1B (a carcinoma from a non-snuff-dipper), examples of the sequencing results of the $p21^{waf1}$ gene exon 2 are illustrated. Of the 14 OSCCs investigated from *toombak*-dippers, mutations in $p21^{waf1}$ exon 2 were found in 43% (6 out of 14), compared to 14% (2 out of 14), 22% (6 out of 27) and 14% (5 out of 35) found in those from non-snuff-dippers from the Sudan, Scandinavia and the USA/UK, respectively. The difference in the mutation frequencies of the $p21^{waf1}$ gene exon 2 was not statistically significant in the OSCCs from Sudanese *toombak*-dippers when compared to either those from non-snuff-dippers from the Sudan, Scandinavia, or the USA and UK combined ($p = 0.07$), or only to those from Sudan ($p = 0.21$), Scandinavia ($p = 0.27$), and the USA/UK combined ($p = 0.05$). The difference in mutation frequencies of the $p21^{waf1}$ gene exon 2 was also not statistically significant in the OSCCs from Sudanese non-snuff-dippers when compared to those from

Table III. Mutations of the p21^{waf1} exon 2 and the S100A4 exon 2 found in the OSCCs from the Sudan, Sweden, Norway, the USA and the UK.

Tumour number	p21 ^{waf1} exon 2 codon/ mutation	S100A4 exon 2 codon/ mutation	Previous p53 status	Tumour number	p21 ^{waf1} exon 2 codon/ mutation	S100A4 exon 2 codon/ mutation	Previous p53 status
Sudanese toombak-dippers				Scandinavian			
	(6/14; 43%)	(1/14; 7%)			(6/27; 22%)	(1/10; 10%)	
1.	6/GGG → GAG 7/GAT → GGT 35/GAT → TGA ^{a,*}	WT	M	2.	138/CAG → TAG ^a 31/AGC → AGA* 31/AGC → AGA*	WT	M
5.	30/CTG → TTG (S)	WT	M	7.	8/GTC → ATC	WT	M
9.	10/CAG → CAA (S)	WT	M		62/GAC → AAC		
10.	68/GTG → GTA (S) 83/CGG → CGA (S)	WT	M		117/TGT → TAT 137/TCT → TGT*		
28.	138/CAG → TAG ^a 144/CAG → TAG ^a	WT	M	7.	WT	3/TGC → GGC* 8/GCC → GAC* 11/GTG → GTA	WT
31.	84/CGA → TGA ^a 98/TCA → TAA ^{a,*} 106/GCA → GTA 107/GAG → CAG*	WT	M	9.	126/CAG → CAC* 140/CGA → CGC* (S) 142/CGG → ACG*	WT	M
40.	WT	6/GAG → AAG 9/CTG → CAG* 14/TCC → TGC* 16/TTC → TAA ^{a,*}	WT	15.	29/CAG → CAC* 52/GAC → CAC* 88/GAG → TGA ^{a,*}	WT	M
Non-snuff-dippers				USMUK			
Sudanese				USMUK			
	(2/14; 14%)	(1/5; 20%)			(5/35; 14%)		
23.	107/GAG → CAG*	WT	M	4.	32/CGC → ACGC* (1) 35/GAT → TGA ^{a,*}	WT	n.d.
	114/TCA → CCA 116/TCT → CCT 125/GAG → AGA			9.	19/CGC → CGA* (S) 20/CGC → CGT (S) 64/GCC → GTC 79/CCC → ACC*	WT	n.d.
34.	20/CGC → CGT (S) 92/GGC → GGT (S) 94/CGG → CGTG* (1) 95/CCT → GTC* 117/TGT → TAG ^a	WT	M	11.	95/CCT → ACT* 98/CGC → TGA ^a	WT	n.d.
				19.	95/CCT → TCT 98/TCA → TAA ^{a,*}	WT	n.d.
37.	WT 12/ATG → TTG* 18/AAG → ACG*	10/GAT → GGT	WT	3.	31/AGC → AGA*	WT	n.d.

^a Stop codon; S, silent mutation; * Transversion; 1, insertion leading to frameshift resulting in stop codon; WT, wild-type or not mutated; M, mutated for p53 gene; n.d., not done.

non-snuff-dippers from Scandinavia combined (p=0.69)/or USA/UK combined (p = 0.91).

The p21^{waf1} gene exon 2 mutated OSCCs from toombak dippers (Table III) showed 13 different mutations, 10 (77%) transitions [(4 G→A; A→G; 5 C→T), four of these were silent mutations and three have resulted in a stop codon], and 3 (23%) transversions [(G→T; C→A; G→C), two of these have resulted in a stop codon]. The OSCCs from non-snuff-

dippers from the Sudan, Scandinavia, the USA and the UK (Table III) showed 33 individual mutations distributed as 14 (42%) transitions [(2 T→C; 5 G→A; 7 C→T), three of these were silent mutations, while three have resulted in a stop codon], and 19 (58%) transversions [(4 G→C; 3 G→T; 2 C→G; 9 C→A; one A→C, two were silent mutations, and three have resulted in a stop codon, and two with insertion of one base pair leading to frameshift resulting in a stop codon].

DNA sequencing analysis for the S100A4 exons 2 & 3. All the 14 OSCCs from Sudanese *toombak*-dippers and 25 cases of OSCCs (5 each from the Sudan, Sweden, Norway, USA and UK) from non-snuff-dippers were analysed for mutations in exons 2 & 3 of the S100A4 gene. Of the 14 OSCCs investigated from the *toombak*-dippers, mutations of the S100A4 gene were only found in exon 2 in 7% (1 out of 4), compared to 20% (1 out of 5) and 10% (1 out of 10) of the OSCCs from non-snuff-dippers from the Sudan and Scandinavia, respectively (Table III). No mutations were found in any of the cases investigated from the USA and UK. The difference in mutation frequencies of the S100A4 exon 2 was not statistically significant in the OSCCs from Sudanese *toombak*-dippers when compared to those from non-snuff-dippers from the Sudan ($p = 0.46$) or Scandinavia ($p = 0.97$) alone, or to those from the Sudan/Scandinavia together ($p = 0.95$). The OSCC from one Sudanese *toombak*-dipper (No. 40) showed 4 mutations, one G→A transition and 3 transversions (2 T→A, where one has resulted in a stop codon, and one C→G). The OSCC from one Sudanese non-snuff-dipper (No. 37) showed 3 mutations (one A→G transition, one A→T and one A→C transversions), compared to the OSCC from one Swedish non-snuff-dipper (No. 7) which showed 3 mutations (one G→A transition, one T→G and one C→A transversions). All these 3 cases of OSCCs were found negative for mutations in both $p53$ exons 5 to 9 and $p21^{waf1}$ exon 2 genes (Table III). No mutations were found in exon 3 of the S100A4 gene in any of the OSCCs investigated.

Relationship between $p21^{waf1}$, S100A4, $p53$ DNA sequencing analysis and *toombak*-dipping. All the OSCCs from Sudanese *toombak*-dippers that showed mutations in the $p21^{waf1}$ exon 2 (Table III), had previously shown mutations in exons 5 to 9 of the $p53$ tumour suppressor gene. Contrary, the only case of OSCC from a *toombak*-dipper that has shown mutations in the S100A4, was found negative for mutations in both $p21^{waf1}$ exon 2 and $p53$ exons 5 to 9.

Discussion

In the present study, mutations in exon 2 of the $p21^{waf1}$ gene were frequent (43%) in OSCCs of Sudanese *toombak*-dippers, compared to OSCCs from non-snuff-dippers from the Sudan (14%), Scandinavia (22%) and the USA/UK (14%), respectively. In the appropriate OSCCs, the presence of $p21^{waf1}$ exon 2 mutations coincided with the detection of a mutation in the $p53$ gene exons 5 to 9 previously published (27). It has been reported that, mutations within the coding region of the $p21^{waf1}$ were undetectable in a large series of human tumours, many of which had a normal $p53$ gene, suggesting that $p21^{waf1}$ alterations are generally caused indirectly, through $p53$ mutations rather than through intragenic mutation of the $p21^{waf1}$ itself (29, 30). It is known that the $p21^{waf1}$ gene is a general inhibitor of the cdk's, a

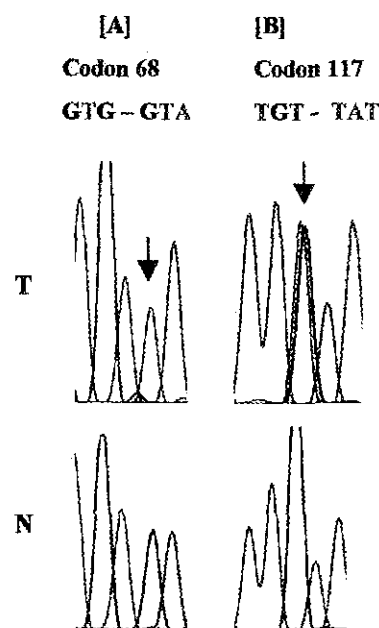


Figure 1. An example of the PCR-DNA sequence analysis of tumour DNA (T) and normal DNA (N) for $p21^{waf1}$ exon 2 in A (a carcinoma from a Sudanese *toombak*-dipper) and B (a carcinoma from a non-snuff-dipper). The ABI PRISMTM electrophotograms of T DNA and N DNA are shown in 5' - 3' (sense) directions. Multiple alignments of the two mutations have shown G to A transitions (silent) Val-Val (codon 68), and Cys-Tyr (codon 117), respectively for the two tumour DNAs analysed.

mediator of the $p53$ tumour suppression function, and its expression is transcriptionally induced by the $p53$ protein (16-19). The results of the current work indicate that both $p21^{waf1}$ and $p53$ are target genes of oral carcinogenesis in OSCCs from *toombak*-dippers. The tobacco specific nitrosamines, present at unusually high levels in *toombak*, may act as the principal carcinogens in these OSCCs. In addition, the findings that mutations in the $p21^{waf1}$ gene are present in the same OSCCs that also carry $p53$ mutations (27), further demonstrate that $p21^{waf1}$ may play an important role during the pathogenesis of OSCCs. Since $p53$ regulates $p21^{waf1}$ gene expression, our results might further suggest that mutations resulting in functional deficiencies or loss of the $p21$ protein might give rise to similar phenotypes as mutations in the $p53$ gene. The fact that $p21^{waf1}$ and $p53$ mutations exist in the same tumour would strengthen the loss of cell cycle control and a more aggressive tumour type would therefore be expected. In breast carcinoma, results from different studies have questioned whether $p21^{waf1}$ expression is dependent or independent of the $p53$ status (31-34). However, and in view of the results published recently on the influence of the $p53$ gene mutations and chemoresistance in breast carcinoma (35), our findings of OSCCs with both $p21^{waf1}$ and $p53$ mutations would be predicted to be strongly chemoresistant. Our results might further suggest that loss of the $p21^{waf1}$ function might often be a sequence of inactivation of the $p53$ tumour suppressor gene resulting in genetic

alterations in its own structure. Furthermore, loss of the $p21^{waf1}$ gene pathway in the $p53$ gene response may contribute in some cases to neoplastic transformation; the frequent inactivation of the $p53$ gene found in OSCCs might reflect simultaneous loss of multiple regulatory pathways as suggested by others (16-18, 24, 25).

Previous studies have observed nucleotide change in codons 31, 91 and 94 of the $p21^{waf1}$ gene exon 2 (36-41). These changes however, were shown to be polymorphisms rather than acquired somatic mutations (36-41). In our study, we found genetic changes in codon 94 of the $p21$ exon 2 in one case of OSCC from a Sudanese non-snuff-dipper and genetic aberrations in codon 31 in two cases of OSCC from non-snuff-dippers from Norway, and in one case of oral squamous cell papilloma from a non-snuff-dipper from the UK. It is noteworthy that changes in codons 31, 91 or 94 were not observed in any of the OSCCs from Sudanese *toombak*-dippers. Nevertheless, tumours from non-snuff-dippers with genetic changes in codons 31 and 94 have previously shown mutations in the $p53$ gene exons 5 to 9 (27). In 3 (21%) out of the 14 OSCCs investigated from *toombak*-dippers, we observed nucleotide change in codons 10, 30, 68 and 83. However, in 6 (8%) of the 76 controls from non-snuff-dippers, we observed nucleotide change in codons 19, 20, 92 and 140. These observations may suggest that certain $p21^{waf1}$ exon 2 somatic mutations, and possibly certain types of polymorphisms, are specific for OSCCs from Sudanese *toombak*-dippers, while others are specific for OSCCs from non-snuff-dippers. To our knowledge, only one study has reported multiple somatic $p21^{waf1}$ mutations in human cancers, in particular prostate cancer (42). Most of the mutations reported in that work (found at codons 31, 32 & 33) were found in prostate cancer from black individuals (42). In this study, and by grouping the total number of cases investigated into Sudanese blacks (*toombak*-users/non-users) and white Caucasians (Scandinavian, USA/UK non-snuff-users), no significant difference in mutation frequencies of the $p21^{waf1}$ exon 2 was observed between the two groups (8 out of 48; 29% for the Sudanese blacks vs. 11 out of 62; 18% for the white Caucasians). This might suggest that mutations of the $p21^{waf1}$ exon 2 are frequent in OSCCs from both African blacks and white Caucasians. The observation however, of frequent mutations of the $p21^{waf1}$ exon 2 in OSCCs of *toombak*-dippers warrants further analysis to better understand the role of the $p21^{waf1}$ gene in development of OSCCs from Sudanese populations.

In the current study, only in 3 cases of OSCCs (one from a *toombak* dipper, 2 from non-snuff-dippers, one from a Sudanese and one from a Swede), did we observe mutations in the S100A4 gene. All these mutations were located to exon 2. All 3 cases were found negative for mutations in both $p21^{waf1}$ and $p53$ genes. To our knowledge, this is the first report of this kind of mutation in the S100A4 gene in human

cancers, in particular OSCCs. It has been suggested that the S100A4 protein is able to sequester and disable the $p53$ protein, which controls the G1-S transition of cells as well as the exit of cells from the S-phase into the mitosis C2-M-transition phase (20). Recently, it has further been demonstrated that the S100A4 protein binds to the extreme end of the C-terminal regulatory domain of the $p53$ gene, in particular at the amino acid residues 360-393, demonstrating both a physical and functional interaction between the two genes (22). This binding area is, however, located outside the area of the $p53$ gene (exons 5-8), where most of the mutations in human cancers have been found (24, 25). Our observation that mutations in the S100A4 gene are not present in tumours bearing mutations in the $p21^{waf1}$ and $p53$ genes, may suggest that S100A4 mutations are complementary to the later genes in tumour development.

To conclude, mutations of the $p21^{waf1}$ gene exon 2 are frequent in OSCCs from Sudanese *toombak*-dippers. This finding suggests that; (i) $p21^{waf1}$, together with $p53$, is a target gene of oral carcinogenesis in OSCCs from *toombak*-dippers, with the tobacco-specific nitrosamines present in *toombak* possibly acting as principal carcinogens in these OSCCs; (ii) findings of $p21^{waf1}$ mutations in the OSCCs unrelated to snuff use further demonstrate that this gene may play an important role during the pathogenesis of OSCCs caused by smoked tobacco use; (iii) mutations in the S100A4 gene are rare in the OSCCs, but appears to be complementary to $p21^{waf1}$ and $p53$ mutations. Mutations of the $p21^{waf1}$ together with the previous $p53$ gene mutations, found in the OSCCs from Sudanese *toombak*-dippers, may explain the high burden of OSCCs in the Sudan, which do not appear to be related to tobacco smoking and alcohol consumption, the two major risk factors identified in Europe and North America. Since molecular analysis of OSCCs can provide clues to endogenous or environmental factors contributing to the high risk of OSCCs, further analysis of the role of the $p21^{waf1}$ gene mutations as a biomarker of malignant transformation, which is linked to the $p53$ gene, is necessary, especially in habitual users of *toombak* from the Sudan.

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