Differential Expression of Apoptosis, Cell Cycle Regulation and Intermediate Filament Genes in Oral Squamous Cell Carcinomas Associated with *Toombak* Use in Sudan

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Abstract. *Previously we used microarray genomic hybridization technology to explore genome-wide profiles of chromosomal aberrations in samples of oral squamous cell carcinomas (OSCCs) and paired normal controls. Based on these findings, 9 genes related to apoptosis, cell cycle regulation and intermediate filament proteins were selected and their differential expression status was examined by real-time quantitative RT-PCR in 26 samples of Sudanese OSCCs and their matched normal controls. The findings were correlated with the habit of toombak use. The mRNA levels of Bcl2, keratin 1, keratin 13 and p53 were significantly lower and the level of survivin was significantly higher in the OSCC samples of the toombak users compared to their paired control samples. A significant down-regulation in keratin 1 and keratin 13 expression levels was found in the OSCC samples of the non-toombak users compared to their normal control samples. The differential expression of genes related to apoptosis, cell cycle regulation and types I and II keratin could be useful diagnostic markers and provide valuable information for the understanding of oral malignancy in relation to toombak use.*

Oral squamous cell carcinoma (OSCC) is a common malignancy characterized by poor prognosis and low survival rate (1). Important risk factors for OSCC development are habits of tobacco and alcohol use (2). OSCC is one of the major health problems in the Sudan, ascribed to the extensive use of the Sudanese snuff, *toombak*. *Toombak* contains high levels of potent carcinogenic substances, the tobacco-specific N-nitrosamines (TSNAs) (3).

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Direct contact of *toombak* with the adjacent tissue of the lower lip, lower vestibule and floor of the mouth has been demonstrated as an important factor in carcinogenesis in *toombak* users (4). In Sudan, over one-third of adult males use *toombak* regularly, and the prevalence among older men in rural areas is close to 50% (5). A close relationship between the use of *toombak* and the development of OSCC has been reported (6). In comparison with other forms of snuff, *toombak* has been found in numerous studies to be associated with the highest risk for developing oral cancer (7). However the exact molecular changes that are critical in the pathogenesis of this disease remain largely unknown (8). Previously we used microarray genomic hybridization technology to explore genome-wide profiles of chromosomal aberrations in patients from Sudan and Norway with OSCC. Regions of copy number increase were identified at 6p21 (p21), 7p12 (EGFR: Epidermal growth factor receptor), 17p13 (p53) and 19p13.2 ($p19^{INK4d}$: Cyclin-dependent kinase 4 inhibitor D), while regions showing deletion included, among others, 3p25.2 (RAF1: proto oncogene) and 9p21 (p15, p16) (9). Based on these findings the aim of the present study was to examine the differential expression of nine selected genes related to apoptosis (survivin, Bcl2), cell cycle regulation (p53, $p16^{INK4a}$, $p21^{WAF1/CIPI}$), and intermediate filament proteins (keratin 1, 13, 14 and 19) by real-time quantitative RT-PCR in 26 samples from Sudanese patients with OSCCs and their matched normal controls. The possible correlation of the findings to habit of *toombak* use was also investigated.

Patients and Methods

Patients and tissue specimens. Specimens of primary OSCCs from 26 patients (23 male, 3 female, age range 23-85, mean age 54.9, ±SD 17 years) were obtained from the Department of Oral and Maxillofacial Surgery at the Khartoum University Dental Teaching Hospital, Khartoum, Sudan. Immediately after surgery, the biopsies were submerged in the tissue storage and RNA stabilization solution, RNA *Later* (Ambion, Applied Biosystems, Foster City, CA, USA) and

Table I. *Clinicopathological parameters of the 26 oral squamous cell carcinomas (OSCCs).*

Age/Gender	Toombak dipper*	Cigarette smoker*	Alcohol drinker*	Tumour site	Differentiation
50/M	+	+	ex-user	Labial sulcus	W
49/M	–	+	–	Tongue	M
85/M	ex-user	–	–	Labial sulcus	M
63/M	+	–	–	Gingiva	W
65/M	+	–	–	Buccal mucosa	W
60/M	+	–	+	Floor of mouth	W
57/F	+	+	–	Labial sulcus	W
65/M	+	–	+	Labial sulcus	W
40/M	–	–	–	Hard palate	M
65/M	+	–	–	Tongue	W
35/M	+	–	–	Labial sulcus	W
35/M	+	–	–	Labial sulcus	W
42/M	–	ex-user	–	Buccal mucosa	W
75/M	–	ex-user	–	Maxilla	W
45/M	+	+	–	Tongue	W
63/M	+	–	–	Gingiva	W
46/M	–	+	–	Buccal mucosa	W
23/M	–	–	–	Maxillary sinus	W
70/M	ex-user	+	–	Labial vestibule	W
36/M	+	+	–	Gingiva	W
85/F	+	–	–	Buccal mucosa	W
52/F	–	–	–	Tongue	W
32/M	–	–	–	Palate	W
70/M	ex-user	–	+	Retromolar area	W
40/M	–	+	–	Buccal mucosa	W
79/M	–	–	–	Gingiva	W

M: Male; F: female; *user: +, non-user. –; W: well-differentiated OSCC; M: moderately differentiated OSCC.

stored at –20˚C until RNA extraction. Clinicopathological information including age, gender, *toombak* use, smoking and alcohol intake, tumour site and histopathological differentiation are shown in Table I. Clinically normal control samples were obtained pair-wise from the site opposite the tumour and were microscopically verified as normal.

RNA extraction and cDNA synthesis. RNA was isolated with combined TRIzol® reagent (Gibco BRL, Carlsbad, CA, USA) and an E.Z.N.A.™ Tissue RNA kit (Omega Bio-tek, Doraville, USA) as recommended by the manufacturer. DNase I digestion was performed on the extracted RNA to ensure removal of residual genomic DNA (20 U/μl RNase-Free DNase I, E.Z.N.A.™ Tissue RNA kit). The RNA quantity was measured by obtaining an A260/A280 nm wavelength ratio using a NanoDrop spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The quality of the RNA was checked by a denaturing Flash gel® system (Cambrex Bio Science, Rockland, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A high-Capacity cDNA Archive Kit system supplied by Applied Biosystems, Foster City, CA, USA was used to generate PCR-ready cDNA from 300 ng total RNA. Finally the cDNA was dissolved in 50 μl reaction volume and 5 μl separated on a 1.5 % agarose gel.

Quantitative real-time PCR. Real-time quantitative RT-PCR was performed for nine genes: survivin (Hs00977611_g1), Bcl2 (Hs00608023_m1), p53 (Hs00153340_g1), $p16^{INK4a}$ (Hs00923894_m1), $p21^{WAF1/CIP1}$ (Hs00355782), keratin1 (Hs00196158_m1), keratin13 (Hs00999762), keratin14 (Hs00559328), keratin 19 (Hs00372324_m1) and β-actin (Hs99999903_m1), using the ABI 7900 HT and 384 well optical plates (Applied Biosystems). Each individual reaction contained 1μl cDNA, 5μl 2xTaqMan Universal Master mix (Applied Biosystems), 0.5 μl Taqman probe and H_2O to a final volume of 10 μl, and was run in triplicate. The PCR reaction consisted of an initial enzyme activation step at 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. The comparative threshold cycle (Ct) method $2^{-\Delta\Delta Ct}$ was used to determine the relative gene expression levels for each target gene. Ct indicates the PCR cycle number at which the amount of amplified target reaches a fixed threshold. The β-actin gene was used as a housekeeping gene control. Briefly, the mean Ct value of the target genes in each sample was normalized to that samples averaged housekeeping gene ΔΔCt value to give a Ct value. This was then normalized to the control samples (Ct), and finally the $2^{-\Delta\Delta Ct}$ value was obtained. For the unknown samples, evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the reference sample.

Statistical analyses. All the data were analyzed with SPSS version 15.0 (Statistical Package for the Social Sciences, IBM, Chicago IL, USA). The Wilcoxon signed-rank test was used to compare gene expression between OSCCs and their corresponding control samples. $P<0.05$ was considered as statistical significance.

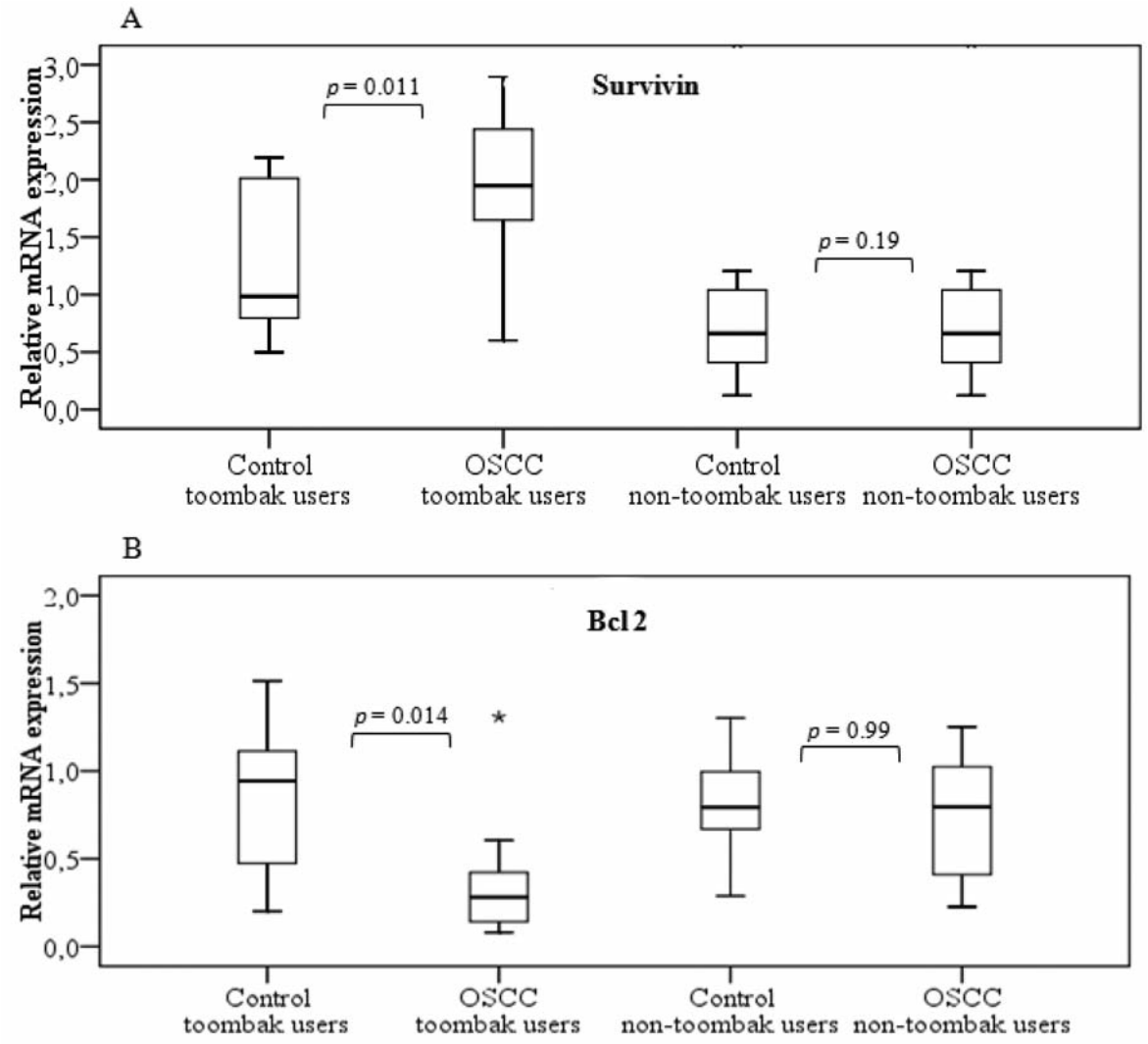

Figure 1. *Expression levels of apoptosis related genes survivin (A) and Bcl2 (B) in OSCC tumours compared with their paired normal controls. Lines in the boxes represent median values. The vertical lines indicate the 10th and 90th percentiles, the circles represent outliers and stars represent extreme value. Each individual reaction was run in triplicate. β-actin was used as housekeeping gene.*

Results

Study subjects. There was a predominance of male subjects (88%). The tumour samples were obtained from different sites of the oral cavity: labial sulcus, 6 (23%); buccal mucosa, 5 (19%); tongue and gingiva, 4 (15%) each; palate and maxillary mucosa, 2 (8%) each; floor of mouth, retromolar region and upper labial vestibule, 1 (4%) each (Table I). There were 13 (50%) *toombak* dippers, 8 (31%) cigarette smokers and 3 (12%) alcohol drinkers. Three of the OSCC were moderately and 23 (88%) well-differentiated tumours.

Quantitative real-time PCR. The level of differential expression of survivin in the OSCC of the *toombak* users was significantly higher than in their normal control samples (p=0.01, 0.6-fold), while significant down-regulation of Bcl2 was observed in the *toombak* users OSCC samples compared to their normal controls (p=0.014, 3-fold) (Figure 1). A significant down-regulation of keratin 1 (p=0.007, 13-fold) and keratin 13 (p=0.039, 2.7-fold) was found in the *toombak* users OSCC compared to their normal control samples (Figure 3). Keratin 1 (p=0.049, 7.4-fold), and keratin 13 (p=0.046, 2.9-fold) also showed significantly lower expression level in the *non-toombak* users OSCC compared to their normal control samples. No significant differences were detected in keratin 14 and keratin 19 levels in the OSCC and control samples in relation to *toombak* use or non use. The level among the *toombak* users OSCC was significantly

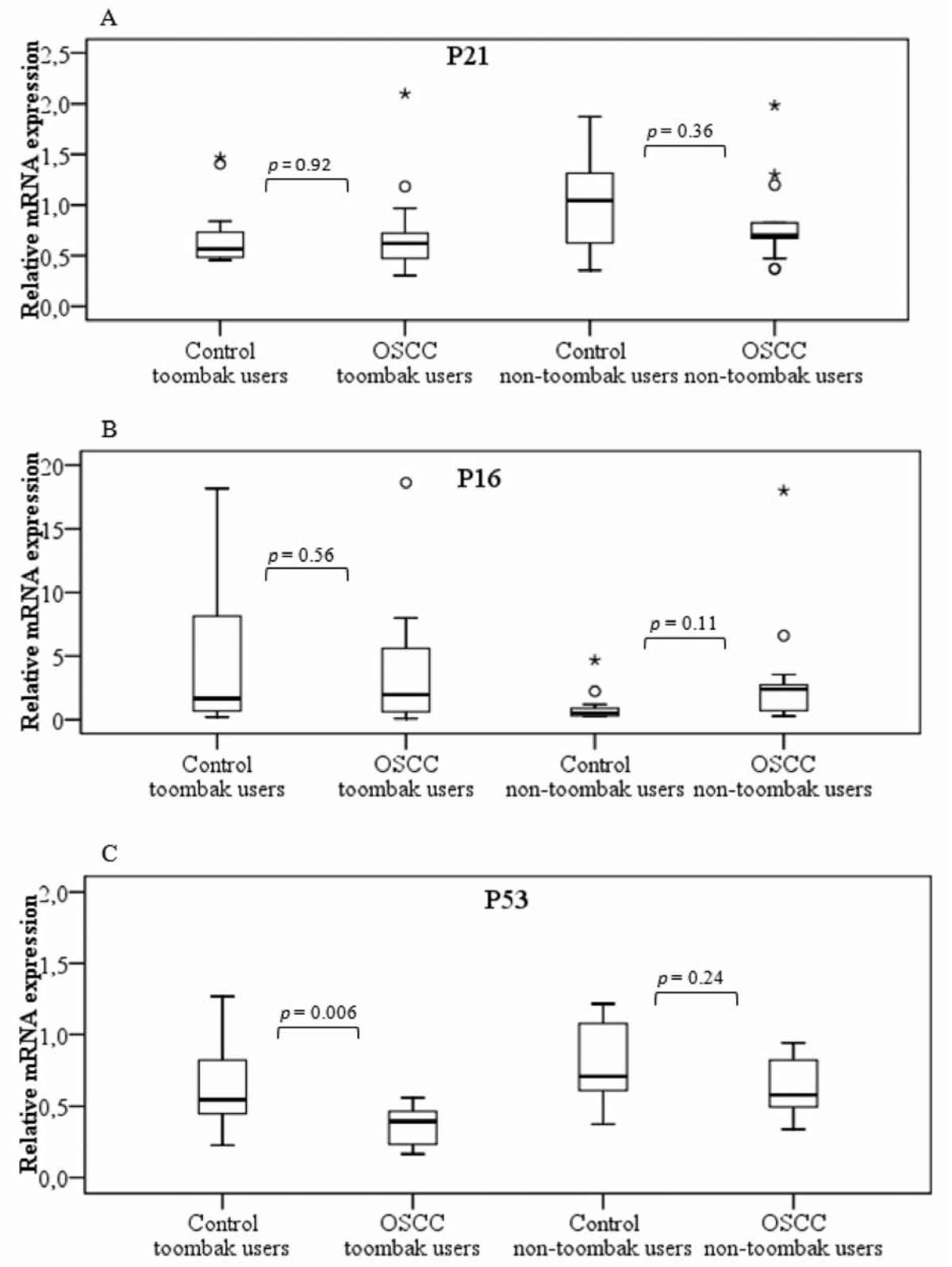

Figure 2. *Expression levels of cell cycle regulatory genes p21$^{WAF1/CIP1}$ (A), p16^{INK4a} (B) and p53(C) in OSCC tumours compared with paired normal samples. Lines in the boxes represent median values. The vertical lines indicate the 10th and 90th percentiles, the circles represent outliers and stars represents extreme values. Each individual reaction was run in triplicate. β-actin was used as housekeeping gene.*

down-regulated p53 (p=0.006; 1.7-fold) compared to the *toombak* users normal control samples. No significant differences in the level of expression were detected in $p16^{INK4a}$ and $p21^{WAF1/CIP1}$ in the OSCC and control samples in relation to *toombak* use and non use as shown in Figure 2.

Discussion

Survivin is the smallest mammalian member of the inhibitor of apoptosis (IAP) gene family and plays a pivotal role in determining cell survival. The overexpression of survivin has been demonstrated in many types of tumour (10-11). The up-regulation of survivin in oral premalignant lesions and in OSCC has also been demonstrated previously (12). In argument with the present study where significantly higher survivin expression (p=0.011) was found in the *toombak* users OSCCs samples compared to their normal controls. So far the mechanisms of IAP overexpression in cancer are largely unknown, although amplification of the survivin locus on chromosome 17 and DNA demethylation of its promoter region have been reported as possible mechanisms of survivin up-regulation in some carcinomas (13). The differential expression of survivin found in human cancer suggests that survivin is an attractive target for cancer therapy (14).

In the present study the Bcl-2 mRNA levels were down-regulated significantly (p=0.014) in the *toombak* users OSCC samples compared to their normal controls. Previously it has been reported that Bcl-2 mRNA and protein levels decrease progressively with increasing degree of dysplasia (15). Other studies have reported sporadic Bcl-2 expression (16) or lack of expression (17). The mechanism underlying *toombak* dependent down-regulation of Bcl-2 is unknown, thus suggesting that the mechanism(s), by which *toombak* regulate Bcl-2 expression may involve indirect biological pathway(s).

P53 is a tumour suppressor gene involved in cell cycle regulation and apoptosis (18). In the present study the p53 levels were 1.7-fold significantly (p=0.006) down-regulated in the *toombak* users OSCC samples compared to their normal controls. Previously, we showed a higher frequency of p53 gene mutations in *toombak*-associated OSCC from the Sudan versus OSCC in non-users (19), but a significantly lower relative frequency of p53-protein expression in OSCCs from *toombak*-dippers compared with those from non-dippers in Sudan and Scandinavia (20), indicating that *toombak* use could play a important role in increased p53 mutation and possibly tumour development.

Loss of keratin 13 related to the invasive nature and ability to form metastases of the cancer cell line, has previously been observed in tongue OSCC (21). In the present study keratin 1 and keratin 13 expression was down-regulated significantly in the OSCC samples from the *toombak* users (p=0.007; 13-fold and p=0.039; 2.7-fold) and the non-users (p=0.049, 7.4-fold change and p=0.046, 2.9-fold change) compared to control normal samples respectively. These results suggested that tumour cells are unable to synthesize keratin 1 and 13, a finding which may be valuable for cancer therapy.

Conclusion

Differential expression of genes related to apoptosis, cell cycle regulation and type I and II keratin could be useful diagnostic markers providing valuable information for the understanding of oral malignancy in relation to *toombak* use. The expression profile of the genes could also provide a valuable screening tool for patients at risk of developing OSCCs in Sudan. Such screening could also provide important information concerning the molecular mechanisms underlying the development of OSCC and, thus, help in the elaboration of new forms of treatment.

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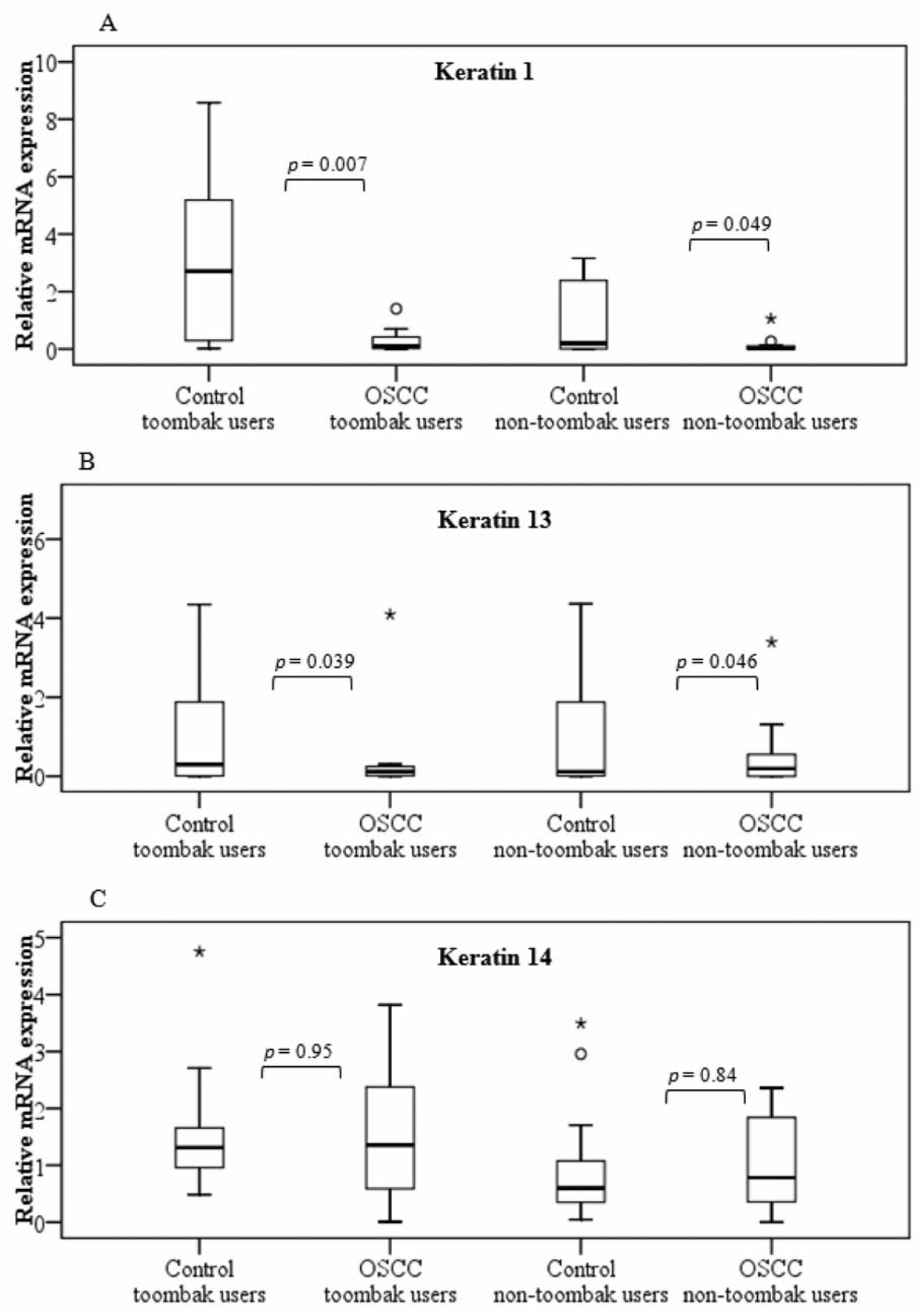

Figure 3. *Continued*

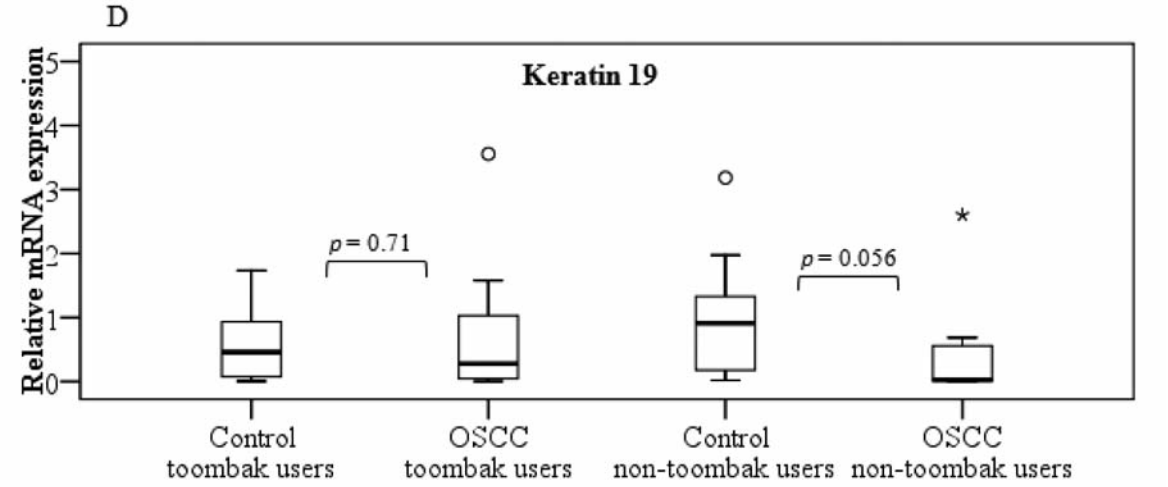

Figure 3. *Expression levels of intermediate filament genes keratin1 (A), keratin 13 (B), keratin 14 (C) and keratin 19 (D) in OSCC tumours compared with paired normal samples. Lines in the boxes represent median values. The vertical lines indicate the 10th and 90th percentiles; the circles represent outliers and stars represents extreme values. Each individual reaction was run in triplicate. β-actin was used as housekeeping gene.*

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