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Expression of p53 family members and CD44 in oral squamous cell carcinoma (OSCC) in relation to tumorigenesis

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Summary. Oral squamous cell carcinomas (OSCCs) are described as the result of a multistep tumorigenesis process. In order to develop useful diagnosis of pre-malignant lesions, expression of p53 family members and the cancer stem cell (CSCs) marker, CD44v6, were studied in histologically normal oral epithelium, precancerous lesions and succeeding invasive OSCCs. p53 was expressed focally in normal epithelium adjacent to tumors, while expression was high in intra-epithelial neoplasia and moderate in OSCC. p63 nuclear staining was important in basal and suprabasal layers of histologically normal oral mucosa and in immature compartments of premalignant lesions and cancer. In epithelium without neoplasia, intense p73 staining was observed in the basal layer, while focal expression was present in suprabasal layers. Most immature dysplastic areas showed either high or moderate staining, whereas those in OSCCs expressed low and moderate p73 level expression. CD44v6 was only expressed in poorly differentiated areas of epithelium, altered or not. p53, p63 and p73 positive stainings were statistically related in intra-epithelial neoplasia to tumours. Analysis of *TP53* mutations in 17% of tumours principally revealed G>A and A>G transitions. No relation was observed between this mutational profile and different immunostainings. In conclusion, our results support that immunostaining of p53 family members might be helpful in diagnosis and monitoring of high-risk pre-malignant lesions of oral epithelium. The combination of staining patterns of p63, p73α and CD44v6 enabled us to isolate phenotypic undifferentiated or transient amplifying areas, reflecting the immaturity of the tumour cell lineage. While CD44v6 expression is an interesting marker of such epithelial cells, it is not specific enough to be useful alone and other phenotypic markers are needed.

Key words: OSCC, p53, p63, p73, CD44

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Introduction

Oral Squamous Cell Carcinoma (OSCC) is the most common head and neck cancer with a severe impact on the quality of life of patients and survivors. Despite recent therapeutic advances, prognosis has not been improved over the last decades and the 5-year survival rate remains within 35-45% (Lacy et al., 2000; Remontet et al., 2003). The major reasons for poor survival are the recurrence and the emergence of second primary tumours in the same or adjacent anatomical area, including the oesophageal mucosal lining (Braakhuis et al., 2005). It is common for patients with OSCC to develop a series of pre-malignant (intra-epithelial neoplasia, IEN) and malignant lesions at several sites within the upper digestive mucosa (Lumerman et al., 1995). Therefore, the development of efficient prevention and early diagnosis of high-risk pre-malignant lesions is a major challenge for reducing mortality due to OSCC.

Numerous molecular pathology studies have led to consider OSCC carcinogenesis as a multiple step process arising from "fields of cancerisation" (Braakhuis et al., 2005). These fields contain genetically altered cells and

are presumed to develop independently at multiple sites in the upper digestive epithelia. Among the key genes involved in early expanding oral malignancy, the tumour suppressor gene *TP53* is the most targeted by genetic changes. In opposition to other suppressor genes, the *TP53* mutational pattern predominantly displays missense mutations which are located, in particular, within the DNA-binding domain. These mutations lead to a loss of transcriptional activity or acquisition of oncogenic activities, which may facilitate clonal expansion in the initial stages of tumorigenesis (Dittmer et al., 1993). The p53 protein can be detected by immunohistochemistry. Although a significant relationship between p53 expression (mutant or not) and the presence of mutations has not yet been established (Ries et al., 1998; Shahnavaz et al., 2000), the abnormal accumulation of p53 protein appears to be a common step in the development of oral carcinomas. Thus, the use of immuno-detection of p53, associated with mutation analysis as a tumour-specific marker of residual tumour cells in molecular diagnosis, has already been investigated and could be a useful tool for OSCC diagnosis (Braakhuis et al., 2005).

The transcriptional factors p63 and p73, members of the p53 family, share functional homologies with the p53 tumour suppressor gene (Yang et al., 1998). p63 and p73 have specific biological functions illustrated by p53-family transgenic knockout mice (Mills et al., 1999; Yang et al., 1999, 2000). They play an important role in the development and differentiation of limbs and epithelial structures, as well as in sensory, infectious and inflammatory pathways. They express many isoforms due to several promoter utilisations (TA full-length forms and N truncated forms) and alternative splicing in C-terminal region (α ß, γ, …). Generally, the full-length isoform possesses transactivating properties, while the truncated one exerts dominant-negative effects (Benard et al., 2003).

Unlike *TP53, p63* and *p73* genes are rarely mutated in human tumours. Molecular studies of individual isoforms have associated up-regulations of TA and ΔN p63 and p73 with the histopathologic features of pre-malignant lesions and Head and Neck Squamous Cell Carcinoma (HNSCC) (Faridoni-Laurens et al., 2001, 2008). Studies on p63 and p73 have observed differential expressions in normal and malignancy states of HNSCC, so, over-expressions of ΔNp63 and ΔNp73 proteins, dominant-negative isoforms, were found to be a common oncogenic event in pre-malignant and primary HNSCC (Thurfjell et al., 2004; Partridge et al., 2007).

OSCC contains cellular heterogeneity, some of which is accounted for by cells occurring from changes linked to genetic instability and environmental factors. Recently, a small population of so-called cancer stem cells (CSCs), isolated from most solid tumours and haematological malignancies, has been defined as only a subpopulation of cells within cancer, having the capacity to sustain tumour growth. They possess two main stem cell properties: self renewal and differentiation (Dalerba et al., 2007). Prince et al., (2007) have recently reported evidence in support of the existence of a cell population with stem cell functions in HNSCC. The HNSCC CSCs, present in low percentages, are characterised by the expression of the cell surface marker CD44. CD44 is a receptor of hyaluronic acid, implicated in cell-to-cell adhesion, cell-to-matrix adhesion, cell migration and proliferation. Only CD44-positive cells, transplanted into immunodeficient mice, phenotypically duplicate the original patient tumour and this phenomenon was repeated in secondary and in tertiary recipient mice. The remaining cells within the tumour (CD44-negative cells) do not have this capacity.

As described previously, in several squamous epithelia, p63 could be a CSCs marker of epithelial tissue (Pellegrini et al., 2001). The p63 staining pattern is strongest in immature zones, including basal and progenitor layers, and declines progressively through transit amplifying cells to differentiated squamous layers. Expression of ΔNp63 in normal, pre-malignant and invasive OSCC lesions has essentially been reported in undifferentiated areas, such as basal cell layers (Thurfjell et al., 2004). These findings offer a new histological domain that may assist cancer diagnosis. The specific targeting of CSCs could offer another strategy for detecting early OSCC.

The aim of this retrospective study was to develop a useful diagnosis strategy of early pre-malignant lesions and small oral tumours (T1N0 stage) through the expression of p53 family members and the CD44 HNSCC CSCs surface marker, by using conventional histopathological techniques and a direct sequencing method for the research of *TP53* mutations.

Material and methods

Tissue samples

Thirty-six patients presenting with primary OSCC, 31 men and 5 women, surgically treated between 1990 and 2000 in two French regional cancer hospitals (F. Baclesse Center in Caen, Lower Normandy and H. Becquerel Center in Rouen, Upper Normandy) were selected for the retrospective study. No patient received either radiotherapy or chemotherapy before surgery. Mean age was 61.4 years (40-97 years). All haematoxylin-eosin-stained slides were reviewed and classified by a pathologist using the WHO criteria for oral lesions. All 36 selected tumours were classified as T1N0. These blocks firstly were used for the molecular analysis of *TP53* mutations. As a result of this first step, only 34 OSCC samples were available for the immunohistochemistry. For the two remaining patients, immunostainings were performed on pre-malignant lesions. The quality of each section was assessed and selected for quantitative evaluation based on successful fixation, proper orientation and the presence of a

representative lesion. Histological criteria used for the classification of pre-malignant lesions were carried out as previously described (Mandard et al., 1997): a total of 34 invasive carcinoma, 30 IEN and 35 histologically normal epithelium were identified.

Immunohistochemical staining

Tissue specimens were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4µm thickness. The sections were dewaxed in toluene and rehydrated in a graded serie of alcohol solutions. At the moment, there are only a few antibodies against p53 family members both validated for immunohistochemistry (including paraffin-embedded sections) and available on the market. So, immunostaining was investigated with antibodies against the following antigens: 1) p53 protein (monoclonal mouse anti-human, clone DO-7, DAKO). 2) p63 protein all isoforms (monoclonal mouse anti-human, clone 4A4, DAKO). 3) p73 protein alpha (monoclonal mouse anti-human, clone 24, Novocastra). 4) CD44 variant 6 (mouse monoclonal antibody, clone VFF-7, Lab Vision).

Antigen retrieval was performed using cycled microwave irradiation with a 10mM citrate buffer (pH 6.0). Immunostaining was performed using a DakoCytomation Autostainer (DAKO) according to the manufacture's instructions. Antibody binding was visualised using a diaminobenzidine chromogen. The sections were counterstained with Mayer's hematoxylin, dehydrated by a graded alcohol serie, cleared with toluene and mounted.

Negative controls were prepared by replacing the primary antibody by non-immune mouse serum.

Evaluation of immunohistochemical findings

Immunostainings were performed independently of patient status and for each sample, staining was evaluated in different layers of histologically normal epithelium thickness: basal and suprabasal layers. For IEN and invasive carcinoma, only p53 family members nuclear staining in tumoral cells was considered. The slides were viewed twice without clinicopathologic data by the pathologist. Immunostaining was evaluated using a semi-quantitative scale: 0, negative; +, little staining (<25%); ++, intermediate staining (25-60%) and +++, strong staining (more than 60% of the cells in tissue sections).

As the result of recent advances in HNSCC CSCs (Prince et al., 2007), we decided to assess CD44v6 immunoreactivity in representative lesions remaining embedded in paraffin after the complete analysis of p53 family members, including *TP53* mutations. A total of 16 normal epithelia, 4 IEN and 15 OSCC were available. The CD44v6 expression analysis was carried out according to the state of epithelial differentiation and not according to the positivity level as described above.

DNA preparation

Total DNA was extracted from tumoral tissue using a DNA mini kit (QIAGEN), according to the manufacturer's instructions. A supplementary step: DNA partial restoration was added, according to Imyanitov's publication (Imyanitov et al., 2001), with the aim of repairing damaged DNA.

Polymerase Chain Reaction (PCR)

For amplification of specific regions of *TP53* gene, the primers sequences used were previously described (Breton et al., 2003). PCR of exons 5, 6, 7 and 8 was carried out in a volume of 50 µl containing 100 ng of DNA solution, 2 mM of $MgCl_2$, 5 µl of GeneAmp 10X PCR buffer (Applied Biosystems), 200 µM of each deoxynucleotide triphosphate, 200 nM of each primer (Eurogentec) and 1.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR was performed in a Mastercycler gradient thermocycler (Eppendorf), using a standard PCR technique: 94°C, 10 min; 94°C, 30 sec; primers specific hybridization temperatures 62°C for exons 5, 6, 7 and 55°C for exon 8, 1 min; 72°C, 1 min 30; for 35 cycles; 72°C, 7 min; 98°C, 10 min and primer hybridization temperature, 30 min.

Direct sequencing of TP53 mutations

Exon amplifications were verified on a 2% agarose gel containing ethidium bromide. After electrophoresis in a TBE buffer (35 min, 100 V), correctly amplified samples were selected and DNA was isolated on GFX columns (GE Healthcare), according to the manufacturer's instructions. Sequencing was performed on both strands using DTCS Quick start kit (Beckman) on an automatic sequencer CEQ 8000 (Beckman).

Mutation analysis

To analyse sequences and mutations of *TP53*, we used the Thierry Soussi database (http://p53.free.fr) and the Mut-TP53 matrix tool (Soussi et al., 2006). In order to avoid the inclusion of artifactual mutations in our data, we only kept mutations showing a loss of transcriptional activity and previously described as frequent in human tumours, as advised by authors.

Statistical analysis of data

Statistical analysis was done using the SAS Software. A non-parametric correlation Spearman was used to study correlations between each protein expression in different compartments of mucosa (normal adjacent to lesions, IEN and invasive carcinoma).

To evaluate the different expressions and the presence of *TP53* mutations, we used bilateral Wilcoxon's test.

Results

Immunohistochemical patterns of p53, p63 and p73α

For all immunoreactivities, only nuclear staining of epithelial cells was observed.

For p53 protein in tumours neighbouring normal tissue, 8 cases did not express this protein in basal layers and 9 in suprabasal layers (Table 1).

All other samples showed p53 expression in basal and/or suprabasal layers. Furthermore, we observed focal p53 immunostaining (Fig. 1a). Twenty-eight out of 30 IEN specimens expressed p53 throughout the entire epithelium depth (Fig. 1b), with important staining for half. Of the 34 OSCC, 14 showed moderate immunoreactivity for p53, while 3 specimens showed none (Table 1).

In table 2, all of the thirty-five samples taken from histologically normal oral mucosa adjacent to lesions showed p63 immunoreactivity. Of the 30 positive intra-epithelial neoplasia, 24 (80%) were identified as having a strong expression (Fig. 1c), the same applying to 76% (26/34) of tumoral tissue. With the exception of one case, all OSCC displayed p63 positive-cells within

Table 1. p53 expression in oral squamous cell carcinoma.

	Neighbouring normal tissues								Intra-epithelial neoplasia				Tumour tissues			
	Basal layer				Suprabasal layer											
Number of cases	35				35				30				34			
Immunostaining quantification	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++
Number of patients	8	18	5	4	9	18	8	0	2	5	9	14	3	9	14	8

Table 2. p63 expression in oral squamous cell carcinoma.

	Neighbouring normal tissues								Intra-epithelial neoplasia				Tumour tissues			
	Basal layer				Suprabasal layer											
Number of cases	35				35				30				34			
Immunostaining quantification	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++
Number of patients	0	2	1	32	0	5	14	16	0	0	6	24	0	0	8	26

Table 3. p73α expression in oral squamous cell carcinoma.

	Neighbouring normal tissues								Intra-epithelial neoplasia				Tumour tissues			
	Basal layer				Suprabasal layer											
Number of cases	35				35				30				34			
Immunostaining quantification	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++
Number of patients	0	4	4	27	21	11	3	0	0	3	11	16	1	8	15	10

Table 4. *TP53* mutational analysis in oral squamous cell carcinoma.

Case	Exon	Codon	Change	Amino-acid Change	Mutation name	Activity*
31	7	236	TAC→TGC	Tyr→Cys	Y236C	0.7
5	7	239	AAC→AGC	Asp→Ser	N239S	14.86
13	7	246	ATG→GTG	Met→Val	M246V	0
4	7	246	ATG→ATA	Met→Ile	M246I	0.28
4	7	250	CCC→CTC	Pro→Leu	P250L	0
2	7	250	CCC→CTC	Pro→Leu	P250L	0
4	7	258	GAA→AAA	Glu→Lys	E258K	0.31
11	9	331	CAG→TAG	Glu→Stop	Q331X	0

* p53 mutation verification test 2.0 by T. Soussi and J.M. Rubio-Nevado.

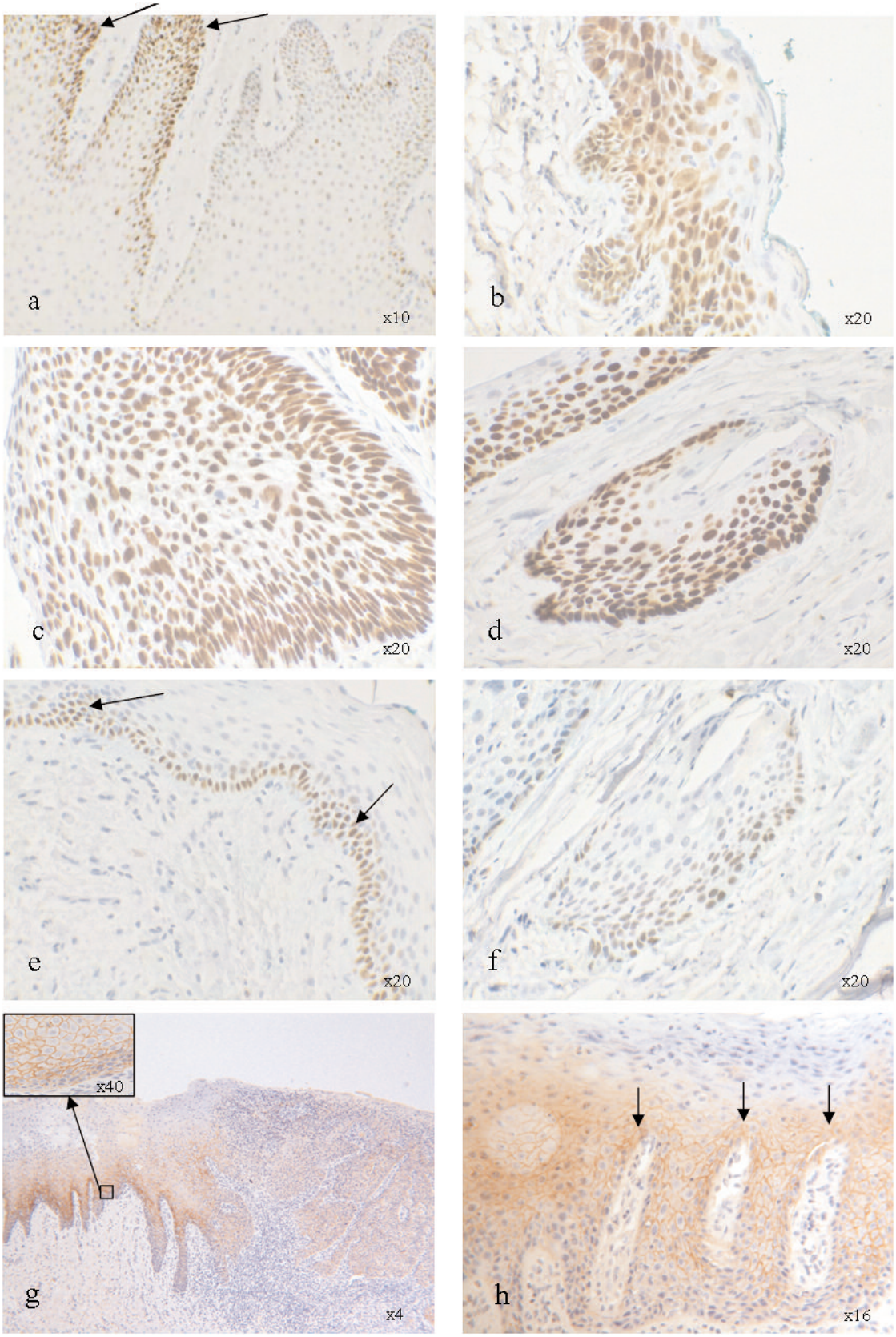

Fig. 1. Immunohistochemical patterns of p53, p63, p73α and CD44v6 in different areas of oral mucosa. **a.** Focal p53 expression in histologically normal oral mucosa (arrows). **b.** p53 expression in IEN. **c.** Intense p63 expression in IEN. **d.** p63 expression in OSCC disappeared with cell maturation **e.** p73α expression in normal epithelium adjacent to tumour, focal expression in suprabasal layers (arrows). **f.** p73α expression in immature compartment of OSCC. **g.** CD44v6 expression in weakly undifferentiated areas of IEN and normal epithelium adjacent to tumour, at the top left: a magnification of membrane staining. **h.** CD44v6 expression concentrated at the top of papillae in histologically normal area (arrows).

undifferentiated regions (Fig. 1d).

p73α protein was highly expressed in basal layers (Table 3, Fig. 1e) and focally expressed in suprabasal cells in histologically normal epithelium. Within IEN, high staining was observed in 16 out of 30 cases, moderately in 11 and weakly in 3. In tumours, separation is less clear since percentages of p73-positive cells were comprised between 25 to 60% for 15 out of 34 cases and exceeded 60% for 10 specimens. All expressions were localised only in immature compartments (Fig. 1f).

CD44v6 expression

In histologically normal oral mucosa, CD44v6 immunoreactivity was either undetectable or very faint in the basal cell layer (Fig. 1g). CD44v6-positive cells were concentrated at the top of papillae (Fig. 1h) and spread out of transient amplifying cells. In IEN and OSCC, where a clear hierarchy of cell differentiation is present within tumours, CD44v6 expression was restricted both to weakly undifferentiated areas and a particular state of maturing cells (Fig. 1g). This cell surface marker was absent in well-differentiated epithelial regions.

TP53 gene mutations

Direct sequencing enabled us to characterise 8 mutations in 6 out of 36 tumors, corresponding to 17% (Table 4). One mutation (P250L) was found in two different patients. Only one tumour harboured more than one abnormality, showing 3 mutations (case 4). All mutations, except one, occurred in exon 7. Five out of 8 were G>A transitions, and 3 were A>G transitions. Seven mutations were missense, the remaining one being nonsense. Among these mutations, 6/7 were described as showing no transcriptional activity at all, only one retaining a very low activity (N239S).

Statistical analysis

We observed several correlations between the expressions of studied proteins. For p53, p63 and p73α proteins, expression in IEN was interrelated with the expression of the same protein in tumours (respectively r=0.53, p=0.0033; r=0.44, p=0.019 and r=0.53, p=0.0039). These correlations evolved in the same manner: when the expression within IEN was important, immunohistochemical staining in tumours was also high, and vice versa.

To assess the efficacy of p53, p63 and/or p73α proteins as an immunohistochemical marker for the pre-malignant diagnosis of OSCC, we evaluated the different expressions and the presence of *TP53* mutation by means of a bilateral Wilcoxon's test. We therefore compared the subset of 30 cases without mutations versus the one of 6 mutated patients. We compared IHC data for p53, p63 and p73α in the two groups for each histological compartment: basal and suprabasal layers of normal mucosa adjacent to lesions, IEN and invasive carcinoma. No significant correlations were found.

Discussion

To develop novel early diagnostic strategies for the detection of high risk pre-malignant lesions, we set up a retrospective study, the aim of which was to evaluate the expression of p53-family members and CD44v6 as phenotypic biomarkers of pre-malignant lesions. Paraffin-embedded specimens of histologically normal oral mucosa neighbouring to lesions, IEN and invasive carcinoma were obtained from 36 patients who had undergone a routine oral surgical procedure. Through the analysis of p53 family member expression using immunohistochemistry and *TP53* mutations screening, we demonstrated the following points: (a) specific staining patterns characterised expression of each p53-family member within oral squamous mucosa, especially for p63 and p73α, which are restricted to undifferentiated cells, (b) a statistically significant relationship between p53, p63 and p73α positive cells was noted in IEN related to tumours, reinforcing the role of this gene family in oral carcinogenesis and considering the possibility of identifying high-risk neoplasia, (c) *TP53* mutations were not related to a specific pattern of p63 and p73α expression.

Specific pattern of p63, p73α and p53 linked to the immaturity of normal or neoplastic cells

Using an available monoclonal antibody to p63 (4A4), which recognises all six p63 isoforms, we found the protein p63, being uniformly expressed throughout the basal and progenitor layers of histologically normal oral mucosa and in undifferentiated areas of pre-malignant lesions and carcinoma. Our results are consistent with those previously reported in HNSCC and other tumours of lining squamous epithelium (Nylander et al., 2000; Faridoni-Laurens et al., 2001). Discrimination of TA and ΔNp63 isoforms by immunohistochemistry or Reverse-Transcriptional Polymerase Chain Reaction has been investigated in oral epithelium and tumours. In this manner, Chen et al., observed an over-expression of ΔNp63 and a somewhat under-expression of TA isoform in human primary oral carcinomas (Chen et al., 2004). Isotypes p63α/Np63· were over-expressed in head and neck tumours (Nylander et al., 2000). In normal tissue, expression of ΔNp63/TAp63 was preferentially observed in basal and suprabasal layers (Nylander et al., 2000; Thurfjell et al., 2004). In normal stratified epithelium, several studies have demonstrated that the p63 network is involved in fate specification, cell proliferation, adhesion or senescence (Koster et al., 2004; Senoo et al., 2007). In this study, high-level expression of p63 was clearly observed in IEN, which is classically described by pathologists as an immature state. This may represent either maintained expression by the basal cells from

which the tumours arose or a contribution by p63 towards cell invasion and migration (Gu et al., 2008). The specific role of each p63 isoform during oral tumour development may warrant thorough investigation.

The over-expression of ΔNp63 in normal stem cells and in pre-malignant and neoplastic tissue suggests that this isoform may contribute to maintaining a stem cell-like phenotype in the corresponding CSC. Recently, Prince et al. identified, by a xenograft technique in immunodeficient mice and by purification with a cell-surface marker CD44+, a subpopulation of cells with cancer stem cell properties in HNSCC (Prince et al., 2007). It was also demonstrated that this cancer stem cell marker, CD44, can be regulated by ΔNp63 in HNSCC (Boldrup et al., 2007). Our findings were similar to the prior study, with immunostaining present in undifferentiated areas and in the transient amplifying cells, whatever the lesions. Consequently, CD44v6 staining pattern combined with that of p63 reflects the immaturity of the tumour cell lineage, but it does not allow the identification of the tumour initiating subpopulation.

Although p73 has been much less studied than its counterpart, few studies have investigated its role in OSCC (Faridoni-Laurens et al., 2001, 2008; Choi et al., 2002). In our study, we used a monoclonal antibody recognizing the carboxy-terminal domain of p73α isotypes. As previously observed, p73α expression was preferentially related to immature cells of squamous lineage and not to proliferating cells (Faridoni-Laurens et al., 2001). However, our analysis indicates that p73α and p53 expression patterns are similar in basal and suprabasal cells of histologically normal epithelium. Concerning p53 expression, the patchy staining in proliferating cells suggests a clonal unit of p53 mutated cells preceding the expanding cancer fields (Braakhuis et al., 2005). With regard to our findings on p73α, all data, including immunostaining within undifferentiated areas of IEN and carcinoma, may reflect a key role of p73α in the initiation of oral tumours.

A statistically significant relationship between p53, p63 and p73α positive cells was noted in IEN related to tumours, reinforcing the role of this gene family in oral carcinogenesis and considering the possibility of identifying high-risk neoplasia

In this study, we observed a significant correlation between p53, p63 and p73α expression respectively in IEN and invasive carcinoma. Firstly, this supports the view that the expression of p53 family members is an early event in OSCC tumorigenesis, in accordance with other studies (Choi et al., 2002; Moll and Slade, 2004). In addition, these significant data reinforce the role of these proteins in the malignant transformation of oral epithelium. Further molecular studies of the specific expression of isoforms in pre-malignant lesions and carcinomas will be required for better understanding of the involvement of one or more obvious candidate(s). Larger studies should be designed to evaluate the significance of p53, p63 and/or p73 variants as diagnostic and/or prognostic markers of high-risk pre-malignant lesions of oral cavity.

In contrast with a previous study (Choi et al., 2002), we found no correlation between each protein expression in different compartments of oral mucosa. This lack of correlation between p73α, p63 or/and p53 suggests an independent or/and compensatory functional role. Indeed, many retro-control loops exist between p73 isoforms (Marabese et al., 2007). ΔNp73 forms exert a dominant-negative effect on p53 and TAp73 by blocking their transactivation activity and hence their ability to induce apoptosis and growth suppression. This suggests that p53 and p73 are highly connected to cancer cells as previously demonstrated in ovarian tumours, in which p73α can inhibit p53 wild-type transcriptional activity by competing for the p53 binding site (Vikhanskaya et al., 2000). The interrelationship between the two partners p63 and p73 has been investigated in oral tumorigenesis and may be involved in the blockage of cell cycle control and apoptosis (Melino et al., 2003). This finding may also indicate the potential role of this interaction in the maintenance of pre-malignant lesions in an undifferentiated status. Collectively, these results highlight the need for specific antibodies enabling discrimination between each isoform.

TP53 mutations

The rate of *TP53* mutations in our sample was rather low (17 %) compared to those reported in several studies (40-55%) (Moura et al., 2005; Tunca et al., 2007). This result could be explained by the method used and by the "clean-up" of artifactual mutations using the Mut-TP53 matrix tool (Soussi et al., 2006). Indeed, the direct sequencing of genomic DNA extracted from paraffin embedded tissues is known to be a lowly sensitive method (Breton et al., 2006). Our pattern of *TP53* mutations was compared to that found in oral tumours (http://www-p53.iarc.fr). The most prevalent mutations in the IARC database are G>A, G>T and A>G. The G>A (5/8) and A>G transitions were also predominant in our tumour specimens. This result is in accordance with recent experimental data suggesting that G>A could be linked to alcohol consumption (Paget et al., 2008). Tobacco and alcohol are the two major risk factors of OSCC (Pelucchi et al., 2008). Exposure information given in pathology reports have revealed that all patients bearing this type of mutation were smokers and alcohol drinkers. Contrary to expectation, no G>T transversion was observed, despite the fact that these genetic changes are classically found in tobacco-related tumours. This absence may be explained by the low number of samples. Although in our study no mutations were hot-spots for oral tumours, all mutated codons, except one (codon 331), were already described in oral tumours in the IARC database.

No correlation between *TP53* mutations and p53,

p63 and p73α expression were found. These results should be considered with caution due to the lack of sensitivity of *TP53* mutation detection in paraffin embedded samples. Nevertheless, several experimental models have observed that deleted or mutated p53 could interact with p63 and p73 networks; e.g. it was demonstrated that ΔNp63 is over-expressed in oral and oesophageal squamous epithelium of p53-deficient mice (Suliman et al., 2001). Conversely, Ratovitski et al. have shown lost or maintained ΔNp63 functions according to the type of *TP53* mutants (Ratovitski et al., 2001). In the same manner, p73 can interact with p53 mutants (Strano et al., 2000). Such interactions are far more difficult to demonstrate in human tumours. In oral tumours, our results suggest that *TP53* mutations do not affect p63 and p73α expression. These data support that the reductionnist analysis of *TP53* gene mutations is insufficient and should be combined with molecular characterization of p53, p63 and p73 isoforms.

In conclusion, the combination of staining patterns of p63, p73α and CD44v6 enabled us to isolate phenotypic undifferentiated or transient amplifying areas, reflecting the immaturity of the tumour cell lineage. However, we were unable to precisely localise the tumour initiating subpopulation. While CD44v6 expression is an interesting marker of such epithelial cells, it is not specific enough to be useful alone and other phenotypic markers are needed. In perspective, our results highlight the need to study the p53 family network at the messenger level in frozen samples in order to accurately identify each isoform and to better understand their involvement in malignant transformation.

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References

Benard J., Douc-Rasy S. and Ahomadegbe J.C. (2003). TP53 family members and human cancers. Hum. Mutat. 21, 182-191.

Boldrup L., Coates P.J., Gu X. and Nylander K. (2007). DeltaNp63 isoforms regulate CD44 and keratins 4, 6, 14 and 19 in squamous cell carcinoma of head and neck. J. Pathol. 213, 384-391.

Braakhuis B.J., Brakenhoff R.H. and Leemans C.R. (2005). Second field tumors: a new opportunity for cancer prevention? Oncologist 10, 493-500.

Breton J., Sichel F., Abbas A., Marnay J., Arsene D. and Lechevrel M. (2003). Simultaneous use of DGGE and DHPLC to screen TP53 mutations in cancers of the esophagus and cardia from a European high incidence area (Lower Normandy, France). Mutagenesis 18, 299-306.

Breton J., Lechevrel M. and Sichel F. (2006). Specificity of TP53 mutation screening methods in cancerous tissues. Int. J. Cancer 118, 254-255.

Chen Y.K., Hsue S.S. and Lin L.M. (2004). Expression of p63 (TA and deltaN isoforms) in human primary well differentiated buccal carcinomas. Int. J. Oral Maxillofac. Surg. 33, 493-497.

Choi H.R., Batsakis J.G., Zhan F., Sturgis E., Luna M.A. and El Naggar A.K. (2002). Differential expression of p53 gene family members p63 and p73 in head and neck squamous tumorigenesis. Hum. Pathol. 33, 158-164.

Dalerba P., Cho R.W. and Clarke M.F. (2007). Cancer stem cells: models and concepts. Annu. Rev. Med. 58, 267-284.

Dittmer D., Pati S., Zambetti G., Chu S., Teresky A.K., Moore M., Finlay C. and Levine A.J. (1993). Gain of function mutations in p53. Nat. Genet. 4, 42-46.

Faridoni-Laurens L., Bosq J., Janot F., Vayssade M., Le Bihan M.L., Kaghad M., Caput D., Bénard J. and Ahomadegbe J.C. (2001). P73 expression in basal layers of head and neck squamous epithelium: a role in differentiation and carcinogenesis in concert with p53 and p63? Oncogene 20, 5302-5312.

Faridoni-Laurens L., Tourpin S., Alsafadi S., Barrois M., Temam S., Janot F., Koscielny S., Bosq J., Bénard J. and Ahomadegbe J.C. (2008). Involvement of N-terminally truncated variants of p73, deltaTAp73, in head and neck squamous cell cancer: a comparison with p53 mutations. Cell Cycle 7, 1587-1596.

Gu X., Coates P.J., Boldrup L. and Nylander K. (2008). p63 contributes to cell invasion and migration in squamous cell carcinoma of the head and neck. Cancer Lett. 263, 26-34.

Imyanitov E.N., Grigoriev M.Y., Gorodinskaya V.M., Kuligina E.S., Pozharisski K.M., Togo A.V. and Hanson K.P. (2001). Partial restoration of degraded DNA from archival paraffin-embedded tissues. Biotechniques 31, 1000-1002.

Koster M.I., Kim S., Mills A.A., DeMayo F.J. and Roop D.R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev. 18, 126-131.

Lacy P.D., Piccirillo J.F., Merritt M.G. and Zequeira M.R. (2000). Head and neck squamous cell carcinoma: better to be young. Otolaryngol. Head Neck Surg. 122, 253-258.

Lumerman H., Freedman P. and Kerpel S. (1995). Oral epithelial dysplasia and the development of invasive squamous cell carcinoma. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 79, 321-329.

Mandard A.M., Marnay J., Lebeau C., Benard S. and Mandard J.C. (1997). Expression of p53 in oesophageal squamous epithelium from surgical specimens resected for squamous cell carcinoma of the oesophagus, with special reference to uninvolved mucosa. J. Pathol. 181, 153-157.

Marabese M., Vikhanskaya F. and Broggini M. (2007). p73: a chiaroscuro gene in cancer. Eur. J. Cancer 43, 1361-1372.

Melino G., Lu X., Gasco M., Crook T. and Knight R.A. (2003). Functional regulation of p73 and p63: development and cancer. Trends Biochem. Sci. 28, 663-670.

Mills A.A., Zheng B., Wang X.J., Vogel H., Roop D.R. and Bradley A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature 398, 708-713.

Moll U.M. and Slade N. (2004). p63 and p73: roles in development and tumor formation. Mol. Cancer Res. 2, 371-386.

Moura Gallo C.V., Azevedo E Silva Mendonca, de Moraes E., Olivier M.

and Hainaut P. (2005). TP53 mutations as biomarkers for cancer epidemiology in Latin America: current knowledge and perspectives. Mutat. Res. 589, 192-207.

Nylander K., Coates P.J. and Hall P.A. (2000). Characterization of the expression pattern of p63 alpha and delta Np63 alpha in benign and malignant oral epithelial lesions. Int. J. Cancer 87, 368-372.

Paget V., Lechevrel M. and Sichel F. (2008). Acetaldehyde-induced mutational pattern in the tumour suppressor gene TP53 analysed by use of a functional assay, the FASAY (functional analysis of separated alleles in yeast). Mutat. Res. 652, 12-19.

Partridge M., Costea D.E. and Huang X. (2007). The changing face of p53 in head and neck cancer. Int. J. Oral Maxillofac. Surg. 36, 1123-1138.

Pellegrini G. and others (2001). p63 identifies keratinocyte stem cells. Proc. Natl. Acad. Sci. USA 98, 3156-3161.

Pelucchi C., Gallus S., Garavello W., Bosetti C. and La Vecchia C. (2008). Alcohol and tobacco use, and cancer risk for upper aerodigestive tract and liver. Eur. J. Cancer Prev. 17, 340-344.

Prince M.E., Sivanandan R., Kaczorowski A., Wolf G.T., Kaplan M.J., Dalerba P., Weissman I.L., Clarke M.F. and Ailles L.E. (2007). Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc. Natl. Acad. Sci. U. S. A. 104, 973-978.

Ratovitski E.A., Patturajan M., Hibi K., Trink B., Yamaguchi K. and Sidransky D. (2001). p53 associates with and targets Delta Np63 into a protein degradation pathway. Proc. Natl. Acad. Sci. USA 98, 1817-1822.

Remontet L., Estève J., Bouvier A.M., Grosclaude P., Launoy G., Menegoz F., Exbrayat C., Tretare B., Carli P.M., Guizard A.V., Troussard X., Bercelli P., Colonna M., Halna J.M., Hedelin G., Macé-Lesec'h J., Peng J., Buemi A., Velten M., Jougla E., Arveux P., Le Bodic L., Michel E., Sauvage M., Schvartz C. and Faivre J. (2003). Cancer incidence and mortality in France over the period 1978-2000. Rev. Epidemiol. Santé Publique 51, 3-30.

Ries J.C., Schreiner D., Steininger H. and Girod S.C. (1998). p53 mutation and detection of p53 protein expression in oral leukoplakia and oral squamous cell carcinoma. Anticancer Res. 18, 2031-2036.

Senoo M., Pinto F., Crum C.P. and McKeon F. (2007). p63 Is essential for the proliferative potential of stem cells in stratified epithelia. Cell 129, 523-536.

Shahnavaz S.A., Regezi J.A., Bradley G., Dube I.D. and Jordan R.C. (2000). p53 gene mutations in sequential oral epithelial dysplasias and squamous cell carcinomas. J. Pathol. 190, 417-422.

Soussi T., Rubio-Nevado J.M. and Ishioka C. (2006). MUT-TP53: a versatile matrix for TP53 mutation verification and publication. Hum. Mutat. 27, 1151-1154.

Strano S., Munarriz E., Rossi M., Cristofanelli B., Shaul Y., Castagnoli L., Levine A.J., Sacchi A., Cesareni G., Oren M. and Blandino G. (2000). Physical and functional interaction between p53 mutants and different isoforms of p73. J. Biol. Chem. 275, 29503-29512.

Suliman Y., Opitz O.G., Avadhani A., Burns T.C., El Deiry W., Wong D.T. and Rustgi A.K. (2001). p63 expression is associated with p53 loss in oral-esophageal epithelia of p53-deficient mice. Cancer Res. 61, 6467-6473.

Thurfjell N., Coates P.J., Uusitalo T., Mahani D., Dabelsteen E., Dahlqvist A., Sjöström B., Roos G. and Nylander K. (2004). Complex p63 mRNA isoform expression patterns in squamous cell carcinoma of the head and neck. Int. J. Oncol. 25, 27-35.

Tunca B., Erisen L., Coskun H., Cecener G., Ozuysal S. and Egeli U. (2007). P53 gene mutations in surgical margins and primary tumor tissues of patients with squamous cell carcinoma of the head and neck. Tumori 93, 182-188.

Vikhanskaya F., D'Incalci M. and Broggini M. (2000). p73 competes with p53 and attenuates its response in a human ovarian cancer cell line. Nucleic Acids Res. 28, 513-519.

Yang A., Kaghad M., Wang Y., Gillett E., Fleming M.D., Dötsch V., Andrews N.C., Caput D. and McKeon F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Molecular Cell 2, 305-316.

Yang A., Schweitzer R., Sun D., Kaghad M., Walker N., Bronson R.T., Tabin C., Sharpe A., Caput D., Crum C. and McKeon F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature 398, 714-718.

Yang A., Walker N., Bronson R., Kaghad M., Oosterwegel M., Bonnin J., Vagner C., Bonnet H., Dikkes P., Sharpe A., McKeon F. and Caput D. (2000). p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 404, 99-103.

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