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ORIGINAL PAPER

p63 Immunoexpression in lip carcinogenesis

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Abstract Actinic cheilitis (AC) is a potentially malignant disorder, which can present degrees of epithelial dysplasia, and may even evolve into lip squamous cell carcinoma (LSCC). Since p63 is a protein homologous to p53, which can be associated with tumorigenesis in epithelial tissues, this study aims to evaluate it in AC and LSCC, in the hopes to estimate the biological behavior of these lesions. Forty AC lesions and sixty-five cases of LSCC were quantitatively analyzed by immunohistochemistry, using anti-p63 antibody with ten cases of normal lip mucosa used as a control group. In all AC and LSCC cases studied, it was possible to detect the presence of the p63 protein. There was no statistically significant difference between immunostained cells and degree of epithelial dysplasias, nor between the LSCC grading malignancy. Nevertheless, p63 immunoexpression showed to be significantly correlated with AC and LSCC lesions as compared to normal lip epithelium. The results indicate that p63 protein is consistently expressed in AC and LSCC, and might be of help in the differential diagnosis between normal and dysplastic/neoplastic epithelium, although the evaluation using a primary antibody to all isotypes did not prove to be a risk biomarker during lip carcinogenesis. Thus, the production of antibodies for the six different p63 isotypes is urged, since in isolation they can have predictive value, mainly the ΔNp63 isoforms.

Keywords Actinic cheilitis · Squamous cell carcinoma · Lip neoplasms · Tumor biological markers · Immunohistochemistry

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Introduction

The development of lip squamous cell carcinoma (LSCC) refers to a model of photocarcinogenesis that can appear initially, or more frequently, from an actinic cheilitis (AC) lesion, due to regular and prolonged exposure to ultraviolet radiation, especially UVB rays (Fabbrocini et al. 2000). It is estimated that 95% of lower lip cancer cases originate from AC (Santos et al. 2003). Although there are no follow-studies of untreated AC that allow to the calculation of a precise malignant transformation rate, this has been considered to be a "miscellaneous" potentially malignant disorder (van der Waal 2009). This term is a new terminology for oral lesions with a predisposition to malignant transformation that was established in a World Health Organization (WHO) Workshop held in 2005 (van der Waal 2009). The malignant potential in AC might be explained by the inconstancy in histopathologic spectrum found, since the initial signs of lip damage caused by sunlight are subtle and the degree of observable, clinical damage may not coincide with the tissue damage, which, in AC, ranges from hyperkeratosis with or without epithelial dysplasia to early squamous cell carcinoma in the presence

of basophilic changes in the lamina propria (van der Waal 2009; Kaugars et al. 1999). Thus, microscopic evaluation is important to estimate the biological behavior of these alterations (Kaugars et al. 1999). However, the identification of the expression of biomarkers and their products has brought forth new prognostic perspectives for different groups of lesions (Girod et al. 1998).

The p63 protein is a member of the p53 family, located on human chromosome 3q27–29. The p63 protein expresses a minimum of six variants, three of which exhibit transactivational properties with a tumor suppressing function similar to that of p53, which favors cell differentiation and is known as TAp63 (TA-p63α, TA-p63β, TA-p63γ). The other three variants, known as ΔNp63 (ΔN-p63α, ΔN-p63β, ΔN-p63γ), lack the N-terminal acid domain required by p53 for transcriptional activation (Yang et al. 1998; Yang and Mckeon 2000). They are capable of blocking the activity of homologous variants and function as oncogenes thereby favoring cell proliferation (Yang et al. 1998; Foschini et al. 2004). The induction of the expression of TA-p63 isoforms and a rapid loss of ΔN-p63 isoforms can be observed after treatment of human keratinocytes with ultraviolet radiation, coinciding with p53 stabilization (Yang and Mckeon 2000). There is, however, a tendency to favor negative regulatory activity in cell growth, yet it is not known if this is due to p53 stabilization or if it is a coordinated response to DNA damage (Yang and Mckeon 2000; Liefer et al. 2000), highlighting the possibility of functional interactions between these genes (Yang et al. 1998). Since the lip is a transition tissue between skin and oral mucosa, expression of p53-regulated genes in the lip could differ from both intraoral and skin malignancies, as proven by Martínez et al. (2008), who showed alterations in the p53 pathway during early lip carcinogenesis, suggesting increased p53 expression to be a potential tool in the diagnosis of AC.

Despite the dual nature of the p63 protein, the transcriptional deregulation of the p63 gene, common in head and neck squamous cell carcinomas (Chen et al. 2004, 2005; Choi et al. 2002; Lo Muzio et al. 2005; Thurfjell et al. 2004), has enhanced its oncogenic function, and thus the proliferative capacity of neoplasic cells (Foschini et al. 2004). p63 overexpression has been associated with a lack of differentiation and an increase of proliferative activity in potentially malignant disorders and head and neck squamous cell carcinomas (Chen et al. 2005; Lo Muzio et al. 2005).

Since no reliable predictive marker exists, as yet, for the malignant transformation of potentially malignant disorders in the oral cavity, and taking into account the fact that alterations in gene p63 expression are present in the pathogenesis of benign and malignant lesions of the oral epithelium (Bortoluzzi et al. 2004; Nylander et al. 2000), we undertook to analyze p63 protein immunohistochemical expression in AC and LSCC lesions. To our knowledge, this is the first study demonstrating the presence of p63 in these lesions. We correlated p63 expression with the differing degrees of dysplasia and the histological grading of malignancy respectively established for these lesions.

Materials and methods

Case selection

Forty consecutive cases of AC and sixty-five consecutive cases of LSCC were retrieved from the Oral Pathology Laboratory archives at the Federal University of Bahia. Clinical data such as age, gender, race and occupation were not considered. Diagnostic confirmation of sequentially accessioned cases of AC and LSCC were independently reviewed by two of the authors (S. R. and L. R.). Additionally as a control group, ten cases of mucocele lesions were randomly selected to observe antibody immunolabelling patterns in normal lip epithelium.

Histopathological study

The AC cases were graded according to Banoczy and Csiba (1976) parameters, based on the eleven epithelial alterations defined by the Pindborg et al. (1997). The lesions were classified as mild dysplasia when up to two epithelial alterations were present, moderate when 2–4 epithelial alterations were detected, and severe when 5 or more were observed. The LSCC cases were classified according to the criteria used by Anneroth et al. (1987), later modified by Bryne et al. (1989). These criteria are used in a multi-factor histological grading system for squamous cell carcinoma based on morphological information such as tumor cell population as well as the tumor-host relationship. The mean total malignancy score values were calculated and split into two groups, low (a mean score range of 1.0–2.5 points) and high (a mean score range of 2.6–4.0).

Immunohistochemical staining of p63

For all cases, formalin-fixed, paraffin-embedded serial tissue sections were deparaffinized, cut at 4 μm, mounted on poly-L-lysine-coated glass slides, and stained using the streptavidin-biotin-peroxidase method with the monoclonal anti-p63 antibody, clone 4A4 (M7247, DakoCytomation, Glostrup, Denmark), which recognizes the six p63 isoforms, at a dilution of 1:200. Heat-induced antigen retrieval using a water bath (97°C) was performed, with the slides immersed in 10 mM citrate buffer (pH 6.0) and 0.2%

Tween for 30 min. Endogenous peroxidase activity was blocked by incubation in methanol with a concentration of 3% hydrogen peroxidase for 20 min. Nonspecific protein binding was attenuated by incubation for 1 h with 5% bovine serum albumin in phosphate-buffered saline (PBS). Specimens were incubated overnight with the primary antibody in a humidity-controlled chamber. The sections were washed twice with PBS and Tween 0.25% at room temperature. Immune complexes were subsequently treated with the secondary biotinylated antibody and after further washing were exposed to a streptavidin-biotin complex (K0690, LSAB + System-HRP, DakoCyomation), and both were subsequently incubated at room temperature for 30 min, respectively. The immunoreactivity was visualized by development for 30 min with the chromagen Amino-ethylcarbazole (K3461, AEC + High Sensitivity Substrate Chromogen Ready-to-Use, DakoCytomation). The sections were counterstained with Mayer's hematoxylin and a water-based mountant was used.

Positive controls consisted of tissue specimens of prostatic adenocarcinoma with known antigenic reactivity. A negative control was performed on the same tissue by substituting the primary antibody for normal serum, resulting in negative p63 immunoreactivity. Only nuclear staining of the epithelial cells was considered specific, without scoring the intensity of the stain. To quantitatively evaluate p63 expression, a mean percentage of positive cells was determined from the percentage of total positive and negative cells derived from 15 random areas at 400× magnification for both lesions and for normal lip epithelium.

Results

Histopathological findings

The hematoxylin-eosine stained sections of the 40 cases of AC presented 3 or more alterations of epithelial dysplasia. According to the Banoczy and Csiba (1976) parameters for grading dysplasia, nine cases were considered moderate, and 31 severe. Some histological alterations such as drop-shaped rete pegs, loss of basal layer cell polarity, nuclear polymorphism and enlarged nucleoli were statistically more prevalent in severe when compared to moderate dysplasias (Fisher's Exact Test, $P < 0.05$).

In the LSCC cases, basal membrane breakage was clearly visible and tumor cell invasions were present showing cellular abnormalities such as hyperchromatism, nuclear pleomorphism, changes in the nucleus-cytoplasmic ratio, abnormal mitoses and multiple nucleoli. The invasion of the underlying conjunctive tissue occurred in well-defined tumor margins or in small groups of cells, and in addition, variations in the quantity of dyskeratosis and peritumoral lymphoplasmacytic infiltrate were detected. Of the 65 cases studied, only four (6.15%) showed a high degree of malignancy, while the majority (93.85%) showed low-level malignancy. The epithelial tissue adjacent to the squamous cell carcinomas presented associated AC lesions in 37 of the 65 cases studied.

Immunohistochemical findings

p63 Expression in AC

All the specimens tested positive for the anti-p63 antibody, with a nuclear staining pattern expressed in the basal and suprabasal layers in all the samples. Nuclear p63 staining of the normal lip mucosa was scantly demonstrated in the basal and occasional suprabasal layers of the epithelium (Fig. 1a). Instead, in a great majority of the lesions, the level of reactivity gradually diminished moving from the basal to the suprabasal regions of the epithelial lining (Fig. 1b), but all AC lesions that showed expression of p63 in all cell layers presented severe epithelial dysplasia (Fig. 1c). After quantitative analysis, AC lesions showed significantly higher levels of p63 protein expression than normal lip epithelium (Mann–Whitney, $P = 0.001$; Table 1), however no statistically significant difference was observed in p63 expression when comparing moderate and severe dysplasias (Wilcoxon, $P > 0.05$; Table 2). AC in the immediate vicinity of LSCC also expressed p63 protein (Fig. 1d).

p63 Expression in LSCC

All LSCC cases showed reactivity for the anti-p63 antibody, restricted to the neoplasic cells, where diffuse immunostaining was noted in almost all of the neoplasic cells. In some cases, predominantly in the tumor's invasive margins, staining was found at the periphery of tumor nests in the immature/anaplastic cells (Fig. 1e), conversely terminally differentiated cells showing squamous maturation at the central zones lacked p63 expression (Fig. 1f). After quantitative analysis, LSCC lesions also showed significantly higher levels of p63 protein expression than normal lip epithelium (Mann–Whitney, $P = 0.001$; Table 1), but no statistic significance was found between the percentages of p63-positive cells in low-level malignancy carcinomas when compared to those with high level malignancy (Wilcoxon, $P > 0.05$; Table 2). Furthermore, the LSCC cases did not show any statistical association with regard to the percentage of p63-positive cells when compared to the dysplasias (Wilcoxon, $P > 0.05$).

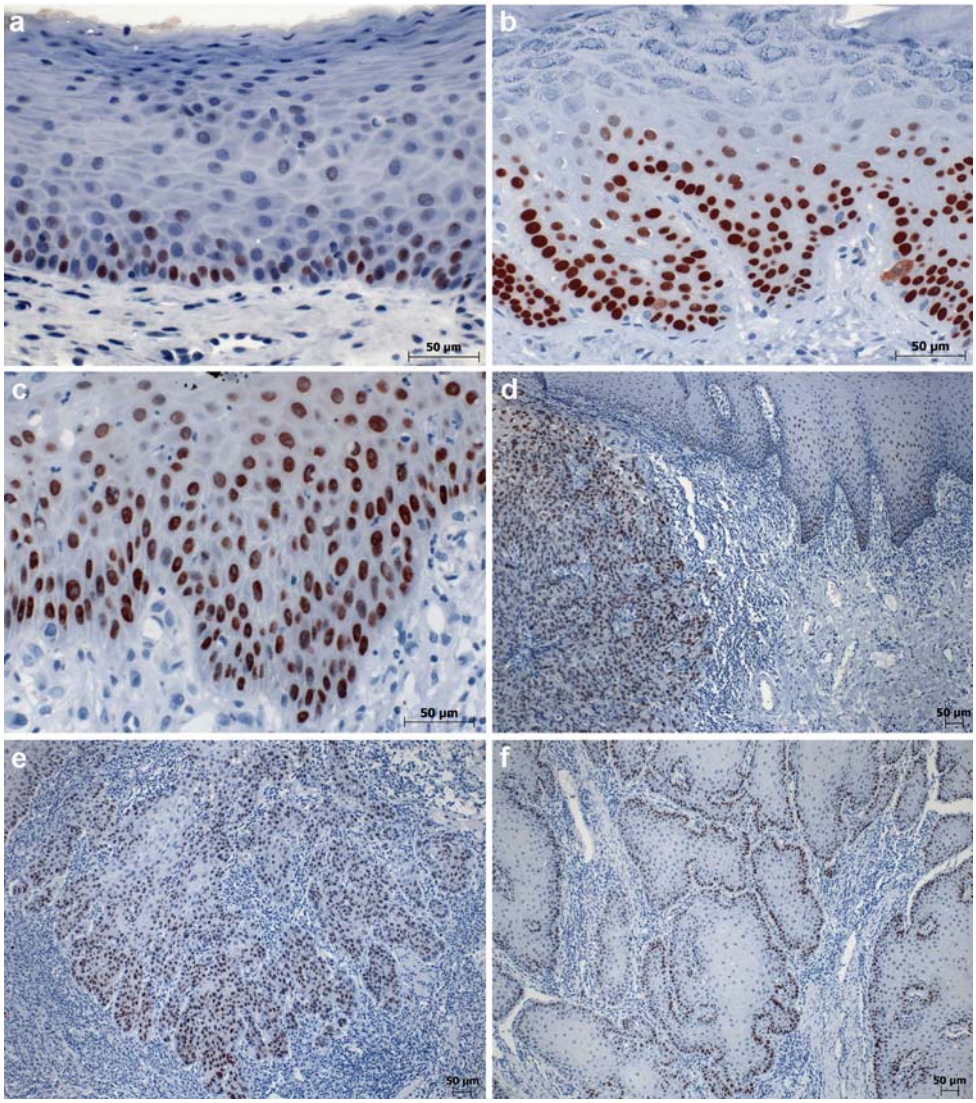

Fig. 1 p63 Immunostaining in normal lip (NL) mucosa, actinic cheilitis (AC) and lip squamous cell carcinoma (LSCC) samples. Scant p63 immunostaining in NL epithelium, especially in the basal and occasional suprabasal cells (**a**). In moderate epithelial dysplasia of AC lesion, p63 nuclear expression gradually diminished from the basal to the suprabasal layer cells (**b**), in contrast to nuclear p63 expression throughout all AC cell layers showing severe epithelial dysplasia (**c**). AC in the immediate vicinity of LSCC expressing p63 protein (**d**) could also be seen. Diffuse p63 nuclear immunostaining in neoplastic cells of LSCC's invasive margins (**e**), and p63 nuclear expression in LSCC, predominantly in the peripheral portion of the tumor island (**f**). Streptavidin-biotin, *scale bars* indicates 50 µm

Table 1 Percentage of total number of p63-positive cells (mean values) in the normal lip epithelium, AC and LSCC lesions (Mann–Whitney)

Lesion	*n*	p63 Mean ± SD	Value of *P**
Normal lip epithelium	10	38.94 ± 25.97	0.001
AC lesions	40	86.84 ± 11.66	
LSCC lesions	65	86.97 ± 14.58	

* 5% Significance level

Table 2 Percentage of total number of p63-positive cells (mean values) in the epithelial dysplasias present in cases of AC and LSCC (Wilcoxon)

Lesion	*n*	p63 Mean ± SD	Value of *P**
Moderate epithelial dysplasia	9	83.08 ± 14.37	0.3872
Severe epithelial dysplasia	31	87.93 ± 10.78	
Low-score squamous cell carcinoma	61	86.92 ± 14.90	0.9242
High-score squamous cell carcinoma	4	87.79 ± 9.72	

* 5% Significance level

Discussion

Actinic cheilitis has been associated with LSCC and both have been associated with exposure to solar radiation (Kaugars et al. 1999; de Rosa et al. 1999; Picascia and Robinson 1987), which provides support for the classification proposed by Cataldo and Doku (1981) that AC be considered a potentially malignant disorder. Some studies have been described in the literature attempting to quantify the percentages of malignant transformation of this lesion, whose values range from approximately 10–20% of the cases (Nicolau and Baelus 1964). Taking into account the fact that the basic mechanism to predict the evolution of

potentially malignant disorders has been the severity of the epithelial dysplasia, it was observed that the majority of our sample presented severe epithelial dysplasia, which is in agreement with studies carried out by Markopoulos et al. (2004), while Kaugars et al. (1999); Santos et al. (2003) found a lower ratio. Our data may indicate biopsies carried out in advanced stages of the lesion, since in the early stages of AC, due to its slow evolution, patients usually attribute the process to aging and neglect the lesion until it reaches advanced stages (Cavalcante et al. 2008). Furthermore, the lack of mild and small number of moderate epithelial dysplasia may also be explained by a more rigorous treatment due to the grading system used, which considered only the presence or absence of abnormalities regardless of their spatial distribution within the tissues. The presence of dysplasia in all AC cases also corroborates the fact that this lesion has potentially malignant biological behavior and may evolve into squamous cell carcinoma.

Squamous cell carcinoma is the most prevalent tumor in head and neck region (Rubira et al. 2007). With regard to the evaluation of the biological behavior of squamous cell carcinomas, lesions located on the lower lip are generally well defined, more easily diagnosed and present a better prognosis than squamous cell carcinomas in other oral locations (Beltrami et al. 1992). A majority of cases studied presented low-level malignancy corroborating the findings of other authors (Fabbrocini et al. 2000).

p63 is a reliable keratinocyte stem cell marker (Reis-Filho and Schmitt 2002) and seems to play a major role in ectodermal development, in the maintenance of a basal cell population of stratified epithelia, and also in the terminal differentiation of stratified epithelia (Parsa et al. 1999; de Laurenzi et al. 2000). Recent findings demonstrated that p63 was strongly expressed in epithelial cells with high clonogenic and proliferative capacity and that p63−/− epithelial stem cells undergo premature proliferation rundown, both in epidermis and in thymus, indicating that p63 is a key determinant of the proliferative capacity in epithelia stem cells (Senoo et al. 2007). It seems that p63 may induce expression of differential markers and that, shortly after, p63 levels are progressively reduced (Parsa et al. 1999; de Laurenzi et al. 2000). These findings are in accordance with previous observations describing a progressive reduction of p63 levels from basal to suprabasal epidermal cells (Reis-Filho and Schmitt 2002; Parsa et al. 1999; de Laurenzi et al. 2000). Similarly, our findings showed p63 expression pattern restricted to the basal and suprabasal layers of the epithelium in all the AC lesions, with majority of them lacking p63 staining in superficial epithelium layers, which could suggest that dysplasic cells have the capacity to assume a role similar to that of embryogenesis, producing an anti-differentiation effect and an enhanced proliferative capacity, also in accordance with observations carried out by Chen et al. (2005). Since AC is a potentially malignant disorder that presents considerable rates of malignant transformation, we believe that the expression of p63 immunostaining in our sample, especially spread in the entire stratified epithelium, together with the scant basal and suprabasal cell immunoexpression in the normal epithelium of the lip mucosa may be due to this potential. This has also been demonstrated by Foschini et al. (2004); Bortoluzzi et al. (2004); Chen et al. (2005) in the analysis of benign and malignant oral cavity lesions. However, we did not find a statistical association between the mean of p63-positive cells and the degree of severity of the dysplasic lesions, which was also demonstrated by Chen et al. (2005). Our findings are compatible with those of Bortoluzzi et al. (2004), probably due to the fact that only moderate and severe dysplasias were detected in our samples, which presented very similar means of p63-positive cells. Thus, a lack of sufficient sample number of moderate epithelial dysplasia and no cases of mild epithelial dysplasia in our sample probably mask this hypothetical relationship.

In the cases of LSCC, the high incidence of p63 immunostaining is in agreement with other studies on squamous cell carcinomas in the head and neck regions (Chen et al. 2004, 2005; Choi et al. 2002; Lo Muzio et al. 2005; Thurfjell et al. 2004), and in the skin; greater p63 expression was shown in cutaneous squamous cell carcinoma when compared with normal skin, actinic keratoses, and seborrheic keratoses (Wrone et al. 2004). This high level of p63 expression in these lesions is a reflection of its influence on neoplasic cell differentiation and tumorigenesis (Thurfjell et al. 2004; Wrone et al. 2004; Reis-Filho et al. 2002). Futhermore, Reis-Filho et al. (2002) demonstrated, as in normal epidermis, that terminally-differentiated cells observed in the squamous pearls of skin carcinomas lacked p63 expression; conversely, the immature cells of the squamous cell carcinoma showed strong p63 expression. These findings are in accordance with the observations of our study; strong immunostaining at the periphery of tumor nests in the immature/anaplastic cells, and terminally-differentiated cells showing squamous maturation at the central zones lacking p63 expression.

Some studies have even demonstrated the possibility of a positive correlation between p63 expression and the degree of malignancy of the neoplasia (Foschini et al. 2004; Lo Muzio et al. 2005). Additionally, the overexpression of p63 may be associated with a worse prognosis in oral squamous cell carcinomas (Lo Muzio et al. 2005). However, analysis of p63 expression in skin cancer failed to correlate protein immunostaining with the squamous cell carcinomas degree of differentiation and with other features associated with aggressive biological behavior (Reis-Filho et al. 2002). Similarly our findings did not detect a

statistical association between the mean of p63-positive cells and the histological grading of LSCC malignancy. Some authors (Reis-Filho et al. 2002) agree that p63 overexpression may play a role in the oncogenesis of squamous cell carcinomas, since p63 gene is frequently amplified in squamous cell carcinomas of the head and neck (Yamaguchi et al. 2000). This gene is expressed in specific isoforms, which have been demonstrated by studies using RT-PCR, and ΔNp63 mRNA expression has been found at significantly higher levels in tumors compared to matched normal tissues (Thurfjell et al. 2004; Nylander et al. 2000). In the present study, it was not possible to obtain RNA analysis by RT-PCR, since we did not employ fresh tissues. Additionally, the participation of other members of the p53 family and the different p63 isotypes should also be considered in future studies due to the higher genetic instability of the carcinomas.

Conclusions

p63 is present in all steps of lip carcinogenesis by its consistently expression in AC and LSCC, and might be of help in the differential diagnosis between normal and dysplastic/neoplastic epithelium. With regard to the gene that encodes the varied isoforms, which can either induce or inhibit the transcriptional activation of target genes, among other functions, the precise identification of p63's role in neoplasic transformation and tumor biology requires further clarification. Despite the fact that the clone 4A4 anti-p63 antibody used in this research did not confirm which isoprotein is involved in lip carcinogenesis, we believe that p63 expression in the lesions we studied is probably related to a specific isoform, since some studies have demonstrated the predominance of ΔN-p63 isoforms in squamous cell carcinomas in the head and neck regions (Thurfjell et al. 2004), and specifically in the oral cavity (Chen et al. 2004) as well as in oral dyspasias (Chen et al. 2005). In this context, subsequent studies on potentially malignant disorders and squamous cell carcinomas of the lip using p63 need to be developed in order to characterize the putative value of this gene as a risk biomarker in the cancer progression and to aid support in differential diagnosis of these lesions.

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