Immunohistochemical analysis of the patterns of p53 and PCNA expression in odontogenic cystic lesions

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Abstract

Objective: the role of p53 expression in odontogenic lesions has not been fully determined, but has been associated with cell proliferation. The purpose of this study was to analyze p53 and proliferating cell nuclear antigen (PCNA) expression in 4 different odontogenic lesions. Design: expression of p53 and PCNA was analyzed in radicular and dentigerous cysts, odontogenic keratocysts, and calcifying odontogenic cysts (Gorlin cysts) using monoclonal antibodies for detection of p53 and PCNA. Results: PCNA expression was significantly greater in the basal layer of radicular cysts and in the suprabasal layer of odontogenic keratocysts; the percentage of p53 positive cells was significantly greater in the suprabasal layer of odontogenic keratocysts. Conclusions: The patterns of p53 and PCNA expression in dentigerous and radicular cysts were similar although the two lesions are of different origin. In odontogenic keratocysts and Gorlin cysts, results indicate a different pattern of tumor growth.

Key words: *p53 protein, PCNA, odontogenic cysts, odontogenic tumors, immunohistochemistry.*

Introduction

Of the oral lesions that affect maxillary bones, the highly prevalent odontogenic cysts have been the focus of several studies that adopted different analytic approaches. The interest in these lesions is high because of their similar radiographic and histopathologic features but different clinical behavior.

The p53 protein, a product of the TP53 tumor suppressor gene, is expressed in the G1 phase of the cell cycle to allow the repair of possible damage to DNA and to arrest cell cycle progression to the S phase, or, alternatively, to induce apoptosis of cells that cannot be repaired. A low concentration of wild type p53 is usually found in cells because of its relatively short half-life, about 20 minutes. Its concentration increases as its half-life is extended, which may occur due to TP53 gene mutation, association of wild type p53 with other proteins, or disruption of its degradation pathway (1-3).

Immunohistochemical detection of the p53 protein is often associated with TP53 gene mutations, which suggests that the mutant product is more stable than the wild type. However, the p53 clones most frequently used in immunohistochemical analyses recognize both wild type and mutant p53. Therefore, it is unclear whether expression indicates gene mutation or overexpression of the wild type protein due to stabilization. Overexpression of p53 is found in lesions without TP53 gene mutation or even in normal tissue, as Cruz et al. (4) and Pillai et al. (5) have demonstrated. In these tissues, positive results are associated with the presence of the wild type protein that, for some reason, is stabilized and highly concentrated, and is, therefore, detected by immunohistochemical methods.

The p53 protein may accumulate in cells as a response to stress, which changes the balance between synthesis and degradation. Under normal conditions, the p53 protein is synthesized continuously. In the nucleus, it binds to the MDM2 protein, and the MDM2/p53 complex is exported to the cytoplasm, where it is degraded by proteosomes. This process keeps the cell concentration of p53 low. Protein concentration increases do not depend on gene activation, transcription and translation, but, rather, on inhibition of its degradation. Under stress, the ARF protein, which regulates MDM2, is released from the nucleolus to the nucleoplasm, where it binds to MDM2 or to the MDM2/p53 complex, blocks p53 export to the cytoplasm and later prevents its degradation, which results in the accumulation of the p53 protein in the nucleus (6,7).

Rubbi and Milner (8) suggested that the nucleolus is the main stress sensor in the cell, and that any disruption in nucleolar functions affects p53 degradation. Mammalian cells do not have a nucleolus during mitosis. Their full nucleolar functionality, in the form of a maximal level of RNAr synthesis, is only reached in the end of the G1 phase. This period of recovery of the nucleolar functionality is the time window when p53 levels increase during the cell cycle.

Results of immunohistochemical studies of p53 expression in odontogenic lesions are contradictory. A number of studies showed p53 expression in cysts (9-12) and odontogenic tumors (13,14), and associated these positive findings with the overexpression or stabilization of the wild type protein, which may be associated with cell proliferation. Other studies reported p53 positive findings in odontogenic cysts but not in dentigerous or radicular cysts (15), or only in cases of keratocysts associated with the nevoid basal cell carcinoma syndrome (16). Still another study found no p53 expression in odontogenic cysts and tumors (17) and assigned these findings to the absence of mutation, to mutation that did not lead to protein stabilization, or to deletion of the TP53 gene.

PCNA is a nuclear nonhistone protein necessary for DNA synthesis, and is an accessory protein for DNA polymerase-alpha, which is elevated during the G1/S phase of the cell cycle. Quiescent and senescent cells have a very low level of PCNA mRNA (18). PCNA expression may be used as a marker of cell proliferation because cells remain a longer time in the G1/S phase when proliferating. Also, this protein has an essential role in nucleic acid metabolism as a component of DNA replication and repair mechanism. An increase in PCNA levels may be induced by growth factors or as a result of DNA damage in the absence of cell cycling (19,20).

To understand the behavior of epithelial cells in odontogenic cysts, this study analyzed p53 and PCNA expression in 4 different lesions: radicular cysts, which are inflammatory lesions; dentigerous cysts, classified as development cysts; odontogenic keratocysts and Gorlin cysts, which are tumoral lesions with cystic features.

Material and Methods

Forty-eight samples were included in the study: 11 radicular cysts, 12 odontogenic keratocysts; 15 dentigerous cysts, and 10 calcifying odontogenic cysts (Gorlin cyst). All samples were fixed in 10% neutral buffered formalin, dehydrated in alcohol, cleared in xylene and embedded in paraffin. After that, 4-µm sections were obtained and stained with hematoxylin-eosin. All slides were reviewed, and the diagnosis was confirmed according to clinical, radiologic and histopathologic features. Two other 4-µm sections were obtained from each block for immunohistochemical staining.

- *Immunohistochemistry:*

Immunohistochemical staining was performed at the Immunohistochemistry Laboratory of the Armed Forces Institute of Pathology (AFIP), Washington, DC. The sections were deparaffinized in xylene, rehydrated through graded alcohols, and immersed in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase. Sections were then washed in phosphate buffered saline (PBS). Antigen retrieval was performed in a 20-min water bath (ISOTEMP 210, Fisher Scientific, USA) using a low-pH retrieval solution (ref. S1699, DakoCytomation, USA). After that, the sections were kept in the same solution for 45 minutes to reach room temperature. Monoclonal p53 (DO-7, 1:200, DakoCytomation, USA) and PCNA (PC-10, 1:50 , DakoCytomation, USA) antibodies were used for p53 and PCNA labeling using the Autostainer Dako. The detection system was Envision +® (DakoCytomation, USA). Sections were rinsed, counterstained with Mayer hematoxylin, rehydrated, and cleared in xylene; slides were mounted in Permount (Fisher Scientific, USA). Sections of breast carcinoma were used as positive control, and omission of the primary antibody, as negative control.

- *Analysis:*

For the quantitative analysis of p53 and PCNA positive cells, slides were examined under a Nikon optical microscope, model Eclipse E2000, at 400x magnification. All epithelial extension was analyzed; images of selected fields were captured using a digital camera (Nikon Coolpix 995, USA) and analyzed using ImageTool for Windows 3.00 (UTHSCSA- University of Texas Health Science Center in San Antonio). For the analysis of p53 and PCNA positivity, positive cells were counted in 1000 cells of each sample: 500 cells in the basal layer and 500 cells in the suprabasal layer. All brown-stained nuclei of epithelial cells were classified as positive regardless of staining intensity. Cells in the superficial layer were not counted.

The criterion used to identify cells in the suprabasal layer was nuclear morphology: cells immediately above the basal layer and with a spherical nucleus were classified as suprabasal; cells with flattened nuclei were classified as superficial and were not counted.

- *Calibration and statistical analysis:*

Intra-and interobserver calibrations were performed during the study. Kappa coefficient ranged from 0.8 to

1.0, and agreement was excellent. The Student t test for paired samples was used to compare mean percentages of positive cells in the different layers, and the Pearson coefficient was used to analyze the correlation of values for the two markers in each lesion.

The study was approved by the Ethics and Research Committee of the School of Dentistry, Universidade Federal do Rio Grande do Sul, and by the Brazilian National Ethics in Research Committee (CONEP), under number 1166/2003.

Results

PCNA and p53 expression was found in all the 48 odontogenic cyst samples. Mean percentages of p53 positive cells showed that radicular cysts had the greatest number of positive cells, followed by Gorlin cysts, dentigerous cysts and odontogenic keratocysts, both in the basal and in the suprabasal layers (Figure 1A,B,C,D,E,F,G,H).

However, a significant difference between layers was found only in keratocysts, which had greater values in

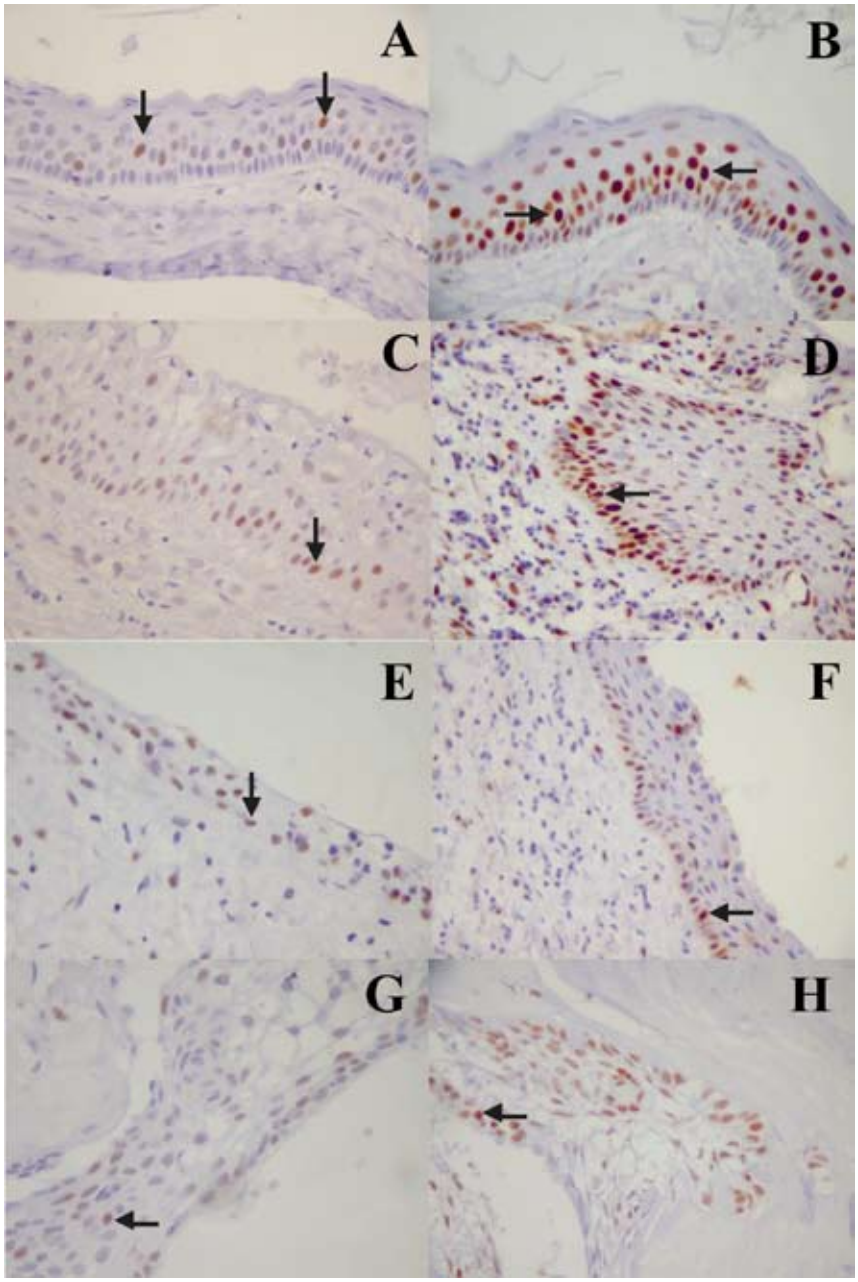

Fig. 1. Odontogenic keratocyst lining showing prominent suprabasal p53 (A, arrows) and PCNA (B, arrows) expression. Epithelial cells of radicular cyst showing p53 (C, arrows) and PCNA (D, arrows) expression. Epithelial cells of dentigerous cyst showing p53 (E, arrow) and PCNA (F, arrows) expression. Note the presence of inflammatory infiltrate in the connective tissue. Epithelial cells of Gorlin cyst showing p53 (G, arrow) and PCNA (H, arrow) expression. (original magnification, X400)

the suprabasal layer (Figure 1A). In the analysis of p53 expression, standard deviation values were high, which revealed greater dispersion around the mean and suggested that data were heterogeneous (Table 1).

The greatest mean percentage of positive PCNA cells in the basal layer was found in radicular cysts, followed by Gorlin cysts, keratocysts and dentigerous cysts. In the suprabasal layer, the greatest mean percentage was found in keratocysts, followed by radicular cysts, Gorlin cysts, and dentigerous cysts. There was a significant difference between values in the basal and suprabasal layers of keratocysts and radicular cysts; in keratocysts, values were greater in the suprabasal layer (Figure 1B), whereas in the radicular cysts, greater values were found in the basal layer (Table 1).

The correlation of mean percentages of positive p53 and PCNA cells in each layer revealed a direct significant correlation in the basal and suprabasal layers of dentigerous and Gorlin cysts, and in the basal layer of radicular cysts (Table 2).

Discussion

Expression of p53 and PCNA was found in all lesions analyzed in this study. Results of studies about p53 and PCNA expression in odontogenic lesions are contradictory (9-17). According to Mighell (20), the interpretation of immunohistochemical studies of PCNA and p53 should take into consideration the complex biology of PCNA and p53, the effect of histologic processing, and the protocol used for immunohistochemical labeling. The lesion under study, its etiology and its clinical behavior should be taken into consideration because all these factors contribute to the accuracy of results.

Results of cell proliferation in our study revealed that radicular, dentigerous and Gorlin cysts had greater mean percentages of positive PCNA cells in the basal layer. In these 3 lesions, the basal layer was the proliferation compartment; in odontogenic keratocysts, the suprabasal layer had greater PCNA values. These unique findings of odontogenic keratocysts suggest that the proliferation compartment in these lesions is formed by the basal and suprabasal layers. We believe that this is a process specific

Table 1. Comparison of mean percentages of positive p53 and PCNA cells in basal and suprabasal layers in each lesion.

LESION	*LAYER*	*N*	**P53+**			**PCNA+**		
			Mean %	*SD*	*p*	*Mean %*	*SD*	*p*
Radicular cyst	Basal	11	17.96	12.84	0.19	84.75	9.82	0.01*
	Suprabasal	11	17.67	10.23		74.25	15.31	
OdontogenicKeratocyst	Basal	12	4.63	5.73	0.01*	68.25	23.59	0.01*
	Suprabasal	12	9.73	4.22		83.32	20.99	
Dentigerous cyst	Basal	15	8.41	10.38	0.05	60.89	31.75	0.23
	Suprabasal	15	11.48	12.54		56.49	30.51	
Gorlin cyst	Basal	10	14.96	16.12	0.85	71.48	33.28	0.17
	Suprabasal	10	14.38	12.38		69.50	31.02	

* significant difference (Student t test for paired samples)

Table 2. Correlation between p53 and PCNA percentages in the basal and suprabasal layers in each lesion.

LESION	LAYER	Pearson correlation coefficient	P
Radicular cyst	Basal	r = 0.829	0.01*
	Suprabasal	r = 0.195	0.57
Odontogenic Keratocyst	Basal	r = 0.469	0.24
	Suprabasal	r = 0.435	0.16
Dentigerous cyst	Basal	r = 0.756	0.01*
	Suprabasal	r = 0.784	0.01*
Gorlin cyst	Basal	r = 0.646	0.04*
	Suprabasal	r = 0.777	0.01*

to the behavior of this type of lesion, in which the cells in the suprabasal layer have an increased proliferative potential in comparison with healthy epithelial tissue.

Several studies about odontogenic lesions have found an association between p53 expression and cell proliferation (9, 10, 13). We believe that the association of PCNA and p53 expression in odontogenic lesions should be analyzed according to each type of lesion. The p53 protein is expressed by proliferating cells, but its accumulation in the cell may be caused by several factors. Cell stress is one of these factors since p53 is a primary mediator of cell response to stress.

The analysis of PCNA and p53 expression in each type of lesion suggests that, in radicular and dentigerous cysts, it may result from a response to cell stress generated by the inflammatory stimulus, even in those classified as development cysts. Studies have demonstrated that growth factors and cytokines (interleukin 1, interleukin 6 and tumoral necrosis factor) are released during inflammatory events. Inflammatory stimuli increase cell proliferation, and inflammatory cytokines may also cause cell stress (21).

In radicular cysts, the inflammatory stimulus originates from the continuous aggression caused by the bacterial contamination of the root canal, which makes epithelial cells, particularly those in the basal layer, increase and maintain proliferation. Therefore, the high expression of p53 and PCNA in radicular cysts (Figure 1C,D) reflects both cell stress and cell proliferation caused by inflammatory stimuli, which may inhibit degradation of the p53 protein and increase the level of PCNA even in the absence of cell cycling.

The initial stimulus for the formation of dentigerous cysts has not been clearly identified, but an inflammatory infiltrate in the cystic capsule has often been described (22). Similarly to what happens in radicular cysts, the inflammatory stimulus may induce epithelial cells to initiate proliferation. Therefore, we interpret our results as responses to inflammatory stimuli that may be the result of the eruptive process, may make cells proliferate, but may be inconstant and present for only short periods of time. This may explain the lower PCNA and p53 percentages found in dentigerous cysts in this study Figure 1E, F). Their rate of proliferation is slower than that of radicular cysts, and the expression of markers may be more closely associated with cell stress caused by the inflammatory stimulus that led to the formation of the cyst cavity. This cavity, once formed, initiates a feedback process due to the physiology of the epithelial tissue, which proliferates in the basal layer and desquamates into the cavity. The cyst becomes denser and thus attracts fluids to maintain its osmotic balance. Therefore, once the process initiates, it becomes independent of new inflammatory stimuli.

The association between inflammation and proliferation in odontogenic keratocysts was described by De Paula et al. (23), who used Ki67, PCNA and AgNOR for their analyses and found that cell proliferation was greater in cysts that had a moderate to dense inflammatory infiltrate than in cysts without inflammation. They reported that growth factors and cytokines released by the inflammatory infiltrate induced the increase of the number of proliferating cells, and that this increase was also associated with the disruption of the typical cyst structure. Kaplan and Hirshberg (24), however, analyzed areas with and without inflammation in the same lesion, compared results of fields in the same and in different samples, and did not find a significant difference. They concluded that inflammatory infiltrates in odontogenic keratocysts do not affect their proliferative potential.

Gorlin cysts had elevated values for both PCNA and p53 expression in our study, and there was a direct significant correlation between the 2 markers in the 2 layers under analysis (Figure 1G,H). Neoplastic cells may have an increased proliferative potential regardless of physiological conditions. Therefore, p53 may be associated with the rate of proliferation in these lesions. However, Gorlin cysts are tumoral lesions, and the possibility of the presence of a mutant protein should also be taken into consideration (25,26).

In odontogenic keratocysts, now classified by the WHO as tumors (27), results reflected their unique characteristics. The inflammatory infiltrate was not a remarkable finding: although found in some areas, it was not always predominant. The expression of markers in this type of lesion may indicate a cell proliferation pattern compatible with neoplastic cells, which is independent of inflammatory stimuli. Both p53 and PCNA were higher in the suprabasal layer, which demonstrated that this lesion has proliferation and maturation patterns that differ from those found in the other lesions studied. Such findings may explain its clinical behavior and its tendency to recurrence. The presence of mutant p53 in odontogenic keratocysts should also be taken into consideration, as demonstrated by Gonzáles-Moles et al. (28), who used a specific antibody for mutant p53.

The results of this study show that PCNA and p53 expression in radicular and dentigerous cysts have similar characteristics although these two types of cyst are classified as lesions of different origin. The two tumoral lesions, odontogenic keratocysts and Gorlin cysts, however, did not share the same characteristics of marker expression, and results showed that their growth pattern is different. Further studies should be conducted to investigate the inflammatory characteristics of these four types of lesion, in particular in dentigerous cysts, to better understand the role of the inflammatory stimulus in their development.

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