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FACULDADE DE ODONTOLOGIA
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COMPORTAMENTO DAS CÉLULAS EPITELIAIS DE LESÕES CÍSTICAS ODONTOGÊNICAS: UM ESTUDO IMUNOISTOQUÍMICO

MÁRCIA GAIGER DE OLIVEIRA

Tese apresentada como parte dos requisitos obrigatórios para a obtenção do título de Doutor em Patologia Bucal

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RESUMO

O propósito do presente estudo foi analisar as células epiteliais odontogênicas procurando um entendimento maior sobre a natureza e conseqüentemente o comportamento de algumas lesões odontogênicas. A expressão imunoistoquímica de p53 e PCNA foi analisada em cisto radicular, cisto dentígero, ceratocisto odontogênico e cisto odontogênico calcificante (Cisto de Gorlin) onde verificou-se que no cisto radicular e cisto dentígero a expressão dos marcadores está relacionado com proliferação e stress celular causado pelo estímulo inflamatório e em ceratocisto odontogênico e Cisto de Gorlin a expressão dos marcadores corresponde a proliferação celular não descartando também a presença de mutação no gene TP53. Também foi observada a expressão de Ki-67, EGFR e Survivin em folículo pericoronário, ceratocisto odontogênico e cisto dentígero que mostrou que as células epiteliais dos folículos pericoronários têm potencial proliferativo para formar lesões odontogênicas e que a proliferação das células do cisto dentígero é relacionada com o estímulo inflamatório. Todos os marcadores estudados comprovaram a natureza neoplásica do ceratocisto odontogênico.

PALAVRAS-CHAVE: Lesões Odontogênicas. Proteína p53. PCNA. Ki-67. EGFR. Survivin.

ABSTRACT

The purpose of this study was to analyze odontogenic epithelial cells to contribute to the knowledge about their nature and, consequently, about the behavior of certain odontogenic lesions. Immunohistochemical expressions of p53 and PCNA were analyzed in radicular cysts, dentigerous cysts, odontogenic keratocysts and calcifying odontogenic cysts (Gorlin cyst). In radicular and dentigerous cysts, the expression of these markers was associated with cell proliferation and stress caused by an inflammatory stimulus. In keratocysts and Gorlin cysts, the expression of markers corresponded to cell proliferation. Results also showed possible mutation in TP53 gene. Also, Ki-67, EGFR and Survivin were expressed in pericoronal follicles, odontogenic keratocysts and dentigerous cysts, which demonstrated that epithelial cells of pericoronal follicles may proliferate to form odontogenic lesions and that cell proliferation in dentigerous cysts was associated with an inflammatory stimulus. The analysis of all markers under study confirmed the neoplastic nature of odontogenic keratocysts.

KEY WORDS: Odontogenic Lesions. p53 protein. PCNA. Ki-67. EGFR. Survivin.

LISTA DE ABREVIATURAS E SÍMBOLOS

AFIP	Armed Forces Institute of Pathology
AgNOR	Regiões Organizadoras Nucleolares impregnadas pela Prata
ARF	Proteína ARF
Bcl-1	Proteína Bcl-1, regulador do ciclo celular
°C	Graus centígrados
DAB	Diaminobenzidina
DNA	Ácido Desoxiribonucleíco
EGF	Fator de crescimento Epidérmico
EGFR	Receptor do Fator de Crescimento Epidérmico
G1	Fase G1 do ciclo celular (Gap 1)
G2	Fase G2 do ciclo celular (Gap 2)
M	Fase M do ciclo celular (Mitose)
S	Fase S do ciclo celular (Sintese)
HE	Hematoxilina e Eosina
HPV	Papiloma Vírus Humano
IAP	Proteína Inibidora de Apoptose
Ki-67	Marcador de Proliferação celular
MCC	Proteína Humana MCC
MDM2	Proteína Humana MDM2
mRNA	Ácido Ribonucléico mensageiro
RNAr	Ácido Ribonucléico ribossomal

μm	Micrometro
N	Amostra
OMS	Organização Mundial da Saúde
PBS	Solução Tampão Fosfato
PCNA	Antígeno Nuclear de Proliferação Celular
pH	Potencial hidrogeniônico
PTCH	gene PTCH
p53	Proteína p53
RNAr	Ácido Ribonucleico ribossomal
SD	Standart deviation (Desvio Padrão)
TGFα	Fator de Crescimento Transformante α
TNF	Fator de Necrose Tumoral
TP53	Gene P53
WHO	World Health Organization
%	Por cento
<	Menor
≤	Menor ou igual
≥	Maior ou igual
>	Maior
+	Mais, positivo

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INTRODUÇÃO

Durante a odontogênese, existe uma interação entre epitélio e ectomesênquima e com isso as células proliferam e se diferenciam formando o germe dentário. Estas células ou seus remanescentes são consideradas como a origem dos cistos e tumores odontogênicos (KRAMER et al., 1992). O mecanismo exato de formação de uma lesão odontogênica muitas vezes não está bem esclarecido, porém, acredita-se que as células epiteliais odontogênicas sofram algum estímulo que as leva a proliferação e formação de lesões.

A Organização Mundial da Saúde (OMS) classifica as lesões císticas odontogênicas em lesões de natureza inflamatória ou lesões de desenvolvimento (KRAMER et al., 1992). Essas lesões císticas possuem características histopatológicas muitas vezes semelhantes mas comportamento clínico diverso. Um dos fatores que poderia explicar essa diversidade no comportamento é a origem dos cistos odontogênicos, pois sabe-se que derivados de restos epiteliais da odontogênese podem conservar em latência sua potencialidade tecidual de diferenciação podendo passar a uma atividade proliferativa frente a fatores desconhecidos, semelhante a que apresentavam na época de formação dentária (EBLING, 1977; SHEAR, 1994)

Estudos têm sido realizados para tentar entender as diferenças no comportamento destas lesões, analisando a origem das células em cada lesão, os fatores relacionados ao ciclo celular e apoptose das células odontogênicas, componentes da matriz extracelular e também estudos moleculares. Entretanto, o processo de formação e os diferentes potenciais ainda permanecem em discussão.

Um dos fatores mais estudados nestas lesões é a proliferação das células epiteliais odontogênicas. Em estudo prévio analisando AgNOR e PCNA em ceratocisto odontogênico

(OLIVEIRA et al., 2001), observou-se que a camada suprabasal do revestimento epitelial é o compartimento proliferativo desta lesão, mostrando que as células nesta camada possuem um padrão de proliferação e maturação diferenciado o que poderia explicar seu comportamento clínico compatível com uma lesão tumoral.

A partir deste estudo, resolveu-se investigar em células epiteliais de lesões císticas odontogênicas a expressão de marcadores que expressam proliferação celular e marcadores relacionados com o ciclo celular e apoptose com o objetivo de entender o comportamento biológico destas lesões.

Inicialmente analisou-se a expressão imunoistoquímica da proteína p53 em cisto dentígero, cisto radicular, ceratocisto odontogênico e cisto odontogênico calcificante (Cisto de Gorlin) e observamos que o cisto radicular apresentou o maior número de células p53 positivas. No cisto dentígero o maior número de células positivas foi encontrado em áreas de maior infiltrado inflamatório, o que sugere que o estímulo inflamatório aumenta a taxa de proliferação e com isso estabiliza a proteína p53 (wild-type). Como o ceratocisto e cisto de Gorlin apresentaram expressão semelhante sugerimos então que o ceratocisto odontogênico fosse classificado como uma lesão tumoral (OLIVEIRA et al., 2005).

Em 2005, a OMS classificou o ceratocisto odontogênico como um tumor odontogênico, passando a denominá-lo de tumor odontogênico ceratocístico. Esta modificação foi baseada em evidências moleculares de perda alélica em dois ou mais locus do cromossomo 9q22.3 levando a superexpressão de bcl-1 e TP53 em pacientes com Síndrome dos carcinomas nevóides basocelulares. Estas e também evidências que o gene PTCH contribui para o desenvolvimento de ceratocistos esporádicos suportariam o conceito que o ceratocisto odontogênico é um tumor (PHILIPSEN, 2005).

Como na literatura existe discussão sobre o significado da expressão imunoistoquímica da proteína p53 em tecidos normais ou não neoplásicos (CRUZ et al, 1998; NYLANDER et al 2000; PILLAI et al, 2003) e muitos estudos em lesões odontogênicas associam a p53 com proliferação celular (SLOOTWEG, 1995; LI et al, 1996; PIATELLI et al, 2001) decidiu-se então, analisar a expressão da p53 e PCNA nas mesmas lesões. Os resultados obtidos nos mostraram que a expressão da p53 em cisto radicular e cisto dentígero pode estar relacionada a uma resposta ao estímulo inflamatório e refletir tanto proliferação quanto stress celular, levando a sugerir que o cisto dentígero possui uma natureza inflamatória. Novamente o ceratocisto confirmou que a camada suprabasal possui um padrão de proliferação e maturação diferenciados e, como nesta nossa amostra a presença de infiltrado inflamatório não era marcante, os resultados nesta lesão e também no cisto de Gorlin refletem somente proliferação celular apesar de não estar descartada a presença da proteína mutada como já foi demonstrado em estudo de GONZÁLES-MOLES et al. (2006).

Baseados nestes resultados e em estudos que demonstram que os restos de células epiteliais odontogênicas encontrados nos folículos pericoronários de dentes não irrompidos têm potencial de proliferação para formar lesões odontogênicas (GLOSSER et al., 1999; ALDESPERGER et al, 2000; CURRAN et al, 2002; EDAMATSU et al., 2005; BAUMGART et al., 2006;), decidiu-se analisar a expressão do EGFR, Ki-67 e survivin nas células epiteliais de folículos pericoronários, cistos dentígeros e ceratocistos odontogênicos tentando com isso, estabelecer uma possível relação entre esses fatores e o desenvolvimento e comportamento destas lesões.

Observou-se neste último estudo que as células epiteliais dos folículos pericoronários são na sua maioria quiescentes, que quando estimuladas podem voltar a

proliferar em um ritmo fisiológico e que algumas delas têm potencial de formar lesões odontogênicas, principalmente o cisto dentígero. O ceratocisto odontogênico confirmou ter padrão de crescimento tumoral, células com potencial de proliferação independente.

A interpretação dos resultados obtidos do conjunto destes estudos nos proporcionaram uma visão mais ampla sobre as lesões odontogênicas analisadas e um entendimento maior sobre sua natureza e conseqüentemente seu comportamento.

p53 PROTEIN REACTIVITY IN ODONTOGENIC LESIONS: AN IMMUNOHISTOCHEMICAL STUDY

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Reatividade da proteína p53 em lesões odontogênicas: um estudo imunohistoquímico

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ABSTRACT

The purpose of this study was to investigate p53 immunoexpression in some odontogenic lesions. Fifty-seven odontogenic lesions were studied: 12 radicular cysts, 17 dentigerous cysts, 16 calcifying odontogenic cysts and 12 parakeratinized odontogenic keratocyst. All samples were stained for p53. The number of positive cells and staining intensity varied for the different types of lesions. Radicular cysts had the greatest number of positive cells, followed by calcifying odontogenic cysts. Most of the positive cells in the parakeratinized odontogenic keratocysts were in the suprabasal layer. Both parakeratinized odontogenic keratocysts and calcifying odontogenic cysts showed more intense and better defined staining than the other types of cysts. Considering the characteristics of odontogenic keratocysts and the fact that their p53 expression is similar to that of calcifying odontogenic cysts, we suggest that odontogenic keratocysts should be classified as tumors.

Keywords: p53. Odontogenic lesions. Immunohistochemistry.

INTRODUCTION

The p53 gene, located on chromosome 17p13, encodes a nuclear phosphoprotein that is believed to control cell growth at the G1/S checkpoint. The p53 gene and its protein product have become the focus of intensive study ever since it became clear that slightly more than 50% of all human cancers contain mutations in this gene (LEVINE, 1997; NYLANDER et al., 2000; SOUSSI, 2003).

In a cell, the p53 protein is usually kept at a low concentration by its relatively short half-life (about 20 minutes). In addition to this low protein concentration, the p53 protein is probably also present in some cells in a latent form, inactive for transcription. Under this condition, it must receive a signal or detect an alteration to be activated. The upstream events or signals that flow to p53 are mediated by several stressful situations. The p53 concentration increases when the protein half-life is lengthened, probably because the rate of translational initiation of p53 mRNA in the cells is enhanced (LEVINE, 1997).

Wild-type p53 can suppress or inhibit the transformation of culture cells by viral or cellular oncogenes, reduce or eliminate the tumorogenic potential of a cell line in culture, and arrest the cell cycle of a transformed cell at the G1 phase, whereas the mutant forms show dominant oncogene properties that promote cell proliferation and malignant transformation (LEVINE, 1997).

Expression of high levels of wild-type (but not mutant) p53 has two outcomes: cell cycle arrest or apoptosis. Although not required for viability, p53 acts as an “emergency brake” in response to genotoxic stress, inducing either arrest or apoptosis, and protecting the genome from accumulating excess mutations (KO; PRIVES, 2003).

According to Soussi (2003) the difficulties in understanding p53 have been exacerbated by the marked diversity of methodological approaches. One of the major misleading approaches is the comparison of immunohistochemical analysis used to evaluate the accumulation of stable p53 mutations in tumor cells with molecular analyses to demonstrate the nature of the mutational events that inactivate the p53 gene.

Immunohistochemical detection of p53 protein is frequently associated with the presence of p53 gene mutation, suggesting that the mutant product may be more stable than the wild-type product, which is not always detected by immunohistochemistry.

It is still unclear whether p53 immunohistochemical expression indicates p53 gene mutation or overexpression of the wild-type product due to stabilization by other gene products (LI et al, 1996).

Some antibodies used for immunohistochemical analysis of paraffin embedded material recognize wild-type as well as mutant p53 proteins. The wild-type usually has a very short half-life, and it is difficult to detect it with antibodies. However, under certain conditions, the wild-type protein may be retained in the tissue by some defect in the normal degradation pathway, and may therefore be detected by antibodies. It is very important to keep in mind that the “retained” wild-type protein is inactive, either due to blocking by another protein or to partial degradation (NYLANDER et al., 2000).

A study undertaken by Pillai et al. (2003) investigated the staining properties of two widely used antibodies to p53, DO-7 and 1801 (DAKO), in normal tissues. The results showed that both detect wild-type p53, but the DO-7 antibody detects it more reliably in normal tissues. Those authors believe that DO-7 is a more robust antibody for the detection of wild-type p53.

The epithelial lining of odontogenic cysts and tumors is derived from primitive oral epithelium of the oral mucosa, and contributes to tooth formation. Such epithelial cells are the common origin of odontogenic lesions, but the molecular mechanisms of the development of odontogenic lesions is not completely understood.

Odontogenic keratocysts behave differently from other types of cysts, such as dentigerous or radicular cysts, in that they have a more aggressive biological behavior with a marked tendency to recurrence. Some authors believe that odontogenic keratocysts should be classified as benign cystic tumors.

This study examines three types of odontogenic cysts - odontogenic keratocysts, dentigerous cysts and radicular cysts - and one type of odontogenic tumor, calcifying odontogenic cysts, and compares their immunohistochemical expression of p53.

MATERIALS AND METHODS

A total of 57 specimens were retrieved from the files of the Oral Pathology Laboratory, School of Dentistry, Universidade Federal do Rio Grande do Sul (Porto Alegre, RS, Brazil). The following formalin-fixed, paraffin-embedded tissues were used in this study: 12 radicular cysts (RC), 17 dentigerous cysts (DC), 16 calcifying odontogenic cyst (COC), and 12 parakeratinized odontogenic keratocyst (OKCp). All selected samples had been routinely fixed in 10% neutral buffered formalin, dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. The slides stained with hematoxylin-eosin were all reviewed and the diagnoses were confirmed on the basis of their clinical, radiological and histopathological features.

Immunohistochemical staining for p53 protein was performed at the Immunohistochemistry Laboratory, Armed Forces Institute of Pathology (Washington DC, USA) using a standardized EnVision™+ (DAKO Corporation, CA, USA) method. Sections (4 μm thick) were deparaffinized in 2 changes of xylene for 10 minutes each, rehydrated through graded alcohols, and immersed in 0.3% hydrogen peroxide in methanol for 5 minutes to block endogenous peroxidase. Sections were then washed in phosphate-buffered saline (PBS). Epitope retrieval was performed by using citrate buffer pH 6.0 (Dako Corporation, Carpinteria, CA, USA) and heated for 20 minutes in a steamer. Following this, the sections were allowed to cool at room temperature in a citrate buffer solution for 45 minutes. The p53 antibody (mouse monoclonal anti-human; DO-7, DAKO Cytomation, Carpinteria, CA; dilution 1:200) reactive to both wild-type and mutant p53 was used in a DAKO Autostainer.

Sections from a breast cancer were used as positive control, and a negative control was obtained by omitting the primary antibody.

The slides were washed in 2 changes of water and counterstained with Mayer's hematoxylin. The sections were then rehydrated, cleared in xylene and mounted in Permount (FisherScientific, Pittsburgh, PA).

For quantitative analysis of p53 positive cells, the slides were examined with a Nikon optical microscope, Eclipse E200 model, at 400x magnification. The full length of the lining epithelium was observed, and the image, under a fixed focus and with a clear field, was captured by a digital camera (Nikon Coolpix 995) and analyzed by ImageTool for Windows version 3.00 (UTHSCSA-University of Texas Health Science Center in San Antonio). The positivity to p53 protein was evaluated by counting the number of positive cells per 1000 epithelial cells for each case, 500 from the basal layer and 500 from the suprabasal layer. Only nuclear staining of epithelial cells was recorded, and the nuclei with brown color, regardless of staining intensity, were considered p53 positive. This procedure was applied for cells from the basal and suprabasal layers. The superficial cells were not counted.

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

Friedman, Mann-Whitney U and Kruskal-Wallis tests were used to evaluate statistically significant differences. Statistical significance was established at $p<0.05$.

RESULTS

The results show that all the lesions studied had p53 immunoreactivity (Table 1). The number of positive cells, the distribution of these cells, and the intensity of staining varied from lesion to lesion.

The radicular cysts had the greatest number of p53 positive cells, followed by the calcifying odontogenic cysts (Graph 1).

When comparing the basal layer with the suprabasal layer in each lesion, a significant difference was found for radicular cysts (P=.035), which had a greater number of positive cells in the basal layer, and for odontogenic keratocysts (P=.021), in which the greatest number of positive cells was in the suprabasal layer.

Comparing p53 positivity in the basal layer, a significant difference was found between radicular cysts and dentigerous cysts (P=.021), and between radicular cysts and odontogenic keratocysts (P=005); in both cases radicular cysts showed a greater number of positive cells in the basal layer. The comparison of results for the suprabasal layers did not reveal any significant differences.

Most odontogenic keratocysts and calcifying odontogenic cysts had more intense and better defined staining than radicular and dentigerous cysts.

DISCUSSION

The expression of p53 in odontogenic lesions has been investigated in several studies, but the results reported are quite conflicting (ODGEN et al.,1992; LOMBARDI et al., 1995; LI et al., 1996; CARVALHAIS et al., 1999; MUZIO et al., 1999; PIATELLI et al., 2002).

In this study, all lesions had immunoreactivity to p53. This may, to a certain extent, be explained not only by the analysis criteria adopted - all cells with brown nuclear staining, regardless of color intensity, were classified as positive –, but also by the immunohistochemical technique used, which was performed with the EnVision+ system (DAKO Corporation, Carpinteria, CA, USA), a very sensitive detection system.

Studies in the literature report that p53 immunohistochemical detection may be affected by many technical variables, such as the use of different antibody clones, antigen absolute concentration, antibody affinity, antibody dilution, incubation time, detection system sensitivity, fixation, and performance of antigen retrieval (WYNFORD-THOMAS, 1992; LAMBKIN et al., 1994; HALL; LANE, 1994; DOWELL; ODGEN, 1996).

Most of the studies associate protein p53 immunohistochemical expressivity with a variety of malignant tumors that show mutations in the p53 gene (ODGEN et al., 1992a; 1992b; TRIVEDY et al., 1998; COLETTA et al., 2001). It is important to point out that the DO-7 antibody (DAKO), used in this study, detects both the wild-type and mutated p53, and that Pillai et al. (2003), reported that this antibody can detect wild-type p53 more reliably in normal tissues.

Therefore, the concept that p53 immunoexpression occurs only due to p53 gene mutations should be reviewed. It is known that cell environment can affect the stability of

the p53 protein. Hudson et al. (1999) in an in vitro study, suggested that p53 may be inactivated by a pro-inflammatory cytokine released from T cells and macrophages. Interleukin-6, a cytokine that induces cell differentiation, is also a factor that inhibits the p53 apoptotic function (YONISH-ROUACH et al., 1991). Jarnbring et al. (2002) found p53 expression in gingival biopsies of patients with periodontitis and gingivitis, and observed dense inflammatory infiltrate in all sections.

Recent studies have shown that wild-type p53 stabilizes in the absence of mutation. Also, it becomes detectable by immunohistochemistry when forming a complex with the MDM2 gene protein, when degraded by HPV E6 protein, and when certain physiological mechanisms are active, such as DNA damage, hypoxia, or other relevant but not yet clearly defined mechanisms (NYLANDER et al., 2000). For these reasons, the overexpression of p53 protein detected by immunohistochemistry may or may not be indicative of p53 gene mutation.

Nylander et al. (2000), suggested that p53 expression in epithelial cells may be caused by gene mutation, by accumulation of wild-type protein as a result of a defect in normal degradation pathways, or by binding to other proteins. In our study, due to the antibody used, we were not able distinguish mutant p53 from wild-type p53 protein. However, the lesions studied and the number of stained cells led us to the conclusion that most of our results correspond to wild-type p53.

The description of differences in staining patterns in the different lesions analyzed, although not the purpose of this study, revealed important features. Calcifying odontogenic cysts and odontogenic keratocysts had more intense and better defined staining than dentigerous and radicular cysts.

According to Nylander et al. (2000) the evaluation of staining intensity is very difficult because intensity depends on several variables, such as pre-treatment, antibody concentration, and room temperature. Those authors point out that intensity can only be evaluated when the technique is carried out under reproducible conditions, such as when a programmable staining machine is used. In our study, all the staining was done following the same protocol and using a programmable machine and controlled room temperature, which allowed us to evaluate the intensity of staining.

The radicular cyst, an inflammatory lesion, has a high number of p53 positive cells, which may be attributed to the inflammatory stimulus. Most of the lesions analyzed had a dense inflammatory infiltrate, and the epithelium showed signs of reaction to this stimulus. Most of the p53 positive cells were located in the basal layer of the epithelium, indicating that the cells were active, probably by induction of the inflammatory stimulus. The distribution of the positive cells suggested that p53 expression in radicular cysts may be associated with cell proliferation. The intensity of staining in this type of lesion was usually weaker than in odontogenic keratocysts or calcifying odontogenic cysts (Figure 1a,b,c)

According Cruz et al. (1998) the p53 protective role is exerted during cell cycle, as the majority of studies performed so far indicate and that the proliferative compartment of the normal oral epithelium is the basal cell layer, one expects that p53 protein, when detectable in normal epithelium, will be restricted to the basal cell layer.

In dentigerous cysts, staining intensity was weak, and a larger number of positive cells were found in the basal layer (Figure 1d). The highest numbers of positive cells were usually found in areas of more intense inflammatory infiltrate.

Our results suggest that the inflammatory stimulus increases the proliferation rate of epithelial cells in dentigerous cysts and, more markedly, in radicular cysts. With

proliferation, more cells pass by the check point, and consequently more cells express p53. Furthermore, the inflammatory cytokine may be a stabilization factor of wild-type p53. This would explain the high number of p53 positive cells and the weak intensity of staining found mainly in the basal layer, probably because cells are undergoing proliferation and differentiation.

Recently, Kaplan and Hirshberg (2004) demonstrate a local increase in Ki-67 expression in the metaplastic epithelium of the odontogenic keratocyst in areas with moderate to severe inflammation. However, they consider that inflammation did not affect the overall proliferation activity of the epithelial lining of odontogenic keratocyst cases.

In the odontogenic keratocysts of our study, most of the p53 positive cells were in the suprabasal layer (Figure 1b), showing that this type of lesion has different characteristics. In this case, the positivity of cells is not associated with an inflammatory stimulus, which was demonstrated by the fact that the lesions analyzed did not have inflammatory infiltrates. Previous studies with AgNOR and PCNA as proliferation markers in odontogenic keratocysts revealed a high proliferation rate of epithelial cells in these cysts, and a distribution of the proliferative cells predominantly in the suprabasal layer (OLIVEIRA et al. 2001). These studies found an association between the cells in proliferation and p53 positive cells, which shows that, in this type of lesion, a number of epithelial cells are proliferating more than usual and that the control of the cell cycle may have been altered.

Piatelli et al. (2002) reported that p53 positivity in the suprabasal layer of epithelium of some oral lesions is usually found when there is some disorder in growth regulation or cell differentiation. Those authors found positivity stainings only in the basal

layer in the normal buccal epithelium, whereas p53 expression was found in the suprabasal and basal layers in leukoplakia, dysplasia and carcinoma.

Calcifying odontogenic cysts, which are neoplastic lesions, also had a high number of p53 positive cells both in the basal and suprabasal layers (Figure 1c). This should be expected in a tumoral lesion in which the cells have a growth regulation and differentiation disorder.

In odontogenic keratocysts and calcifying odontogenic cysts, p53 expression had different characteristics, such as a high number of positive cells in the suprabasal layer and intense staining. These findings suggested some alteration in cell regulation or differentiation, or even some DNA damage that stabilizes wild-type p53, which has a higher expression in these cells. Mutated cells may also be present and be expressed without function, and thus be unable to correct damage or take the cell to apoptosis. However, as this was an immunohistochemical study, we were not able to define whether p53 positive cells were mutated.

Studies have shown p53 positive staining in the odontogenic keratocyst epithelial lining using polyclonal and/or monoclonal antibodies for mutant and wild-type p53 (LI et al., 1996; ODGEN et al., 1992; LOMBARDI et al., 1995; SLOOTWEG et al., 1995). According to these studies, p53 overexpression in odontogenic keratocysts may indicate the physiological pattern of the wild-type protein instead of the gene mutation, and be associated with cell proliferation. The absence of gene mutation was reported by Li et al. (1996) in their molecular analysis of odontogenic keratocysts samples.

The literature presents abundant evidence to support the suggestion that odontogenic keratocyst is a benign cystic tumor (SHEAR, 2002a; 2002b; 2002c). Agaram et al. (2004), showed that there is a significant loss of heterozygosity of the tumoral suppressor genes,

mainly p16, p53, PTCH and MCC in odontogenic keratocysts. According to those authors, the presence of these allelic losses lends substantial support to the hypothesis that odontogenic keratocysts are neoplastic lesions.

We agree with Pillai et al. (2003) that further molecular studies will have to be undertaken to determine the exact mechanisms of p53 accumulation in normal tissues.

Considering the more aggressive clinical behavior of odontogenic keratocysts and their high recurrence rate, comparable to that of tumors, as well as previous cell proliferation studies with AgNOR, PCNA (OLIVEIRA et al., 2001) and p53 expression that showed them to be similar to calcifying odontogenic cysts, which are already classified as tumors, we suggest that odontogenic keratocysts be classified as tumors rather than as odontogenic cysts.

RESUMO

O objetivo deste estudo foi investigar a expressão da p53 em algumas lesões odontogênicas. Foram selecionadas 57 lesões odontogênicas: 12 cistos radiculares, 17 cistos dentígeros, 16 cistos odontogênicos calcificantes e 12 ceratocistos odontogênicos paraceratinizados. Todos os espécimes foram marcados, pela técnica da imunohistoquímica para a p53. O número de células positivas e a intensidade de marcação variaram nos diferentes tipos de lesão. Os cistos radiculares apresentaram o maior número de células positivas seguido pelo cisto odontogênico calcificante. A maioria das células positivas nos ceratocistos odontogênicos estavam localizadas na camada suprabasal. Tanto o ceratocisto odontogênico quanto o cisto odontogênico calcificante apresentaram marcação mais intensa e melhor definida que os outros tipos de cistos. Considerando as características do ceratocisto odontogênico e a expressão da p53 semelhante ao cisto odontogênico calcificante, os autores sugerem que o ceratocisto odontogênico seja classificado como uma lesão tumoral.

Palavras-chave: p53. Lesões odontogênicas. Imunohistoquímica.

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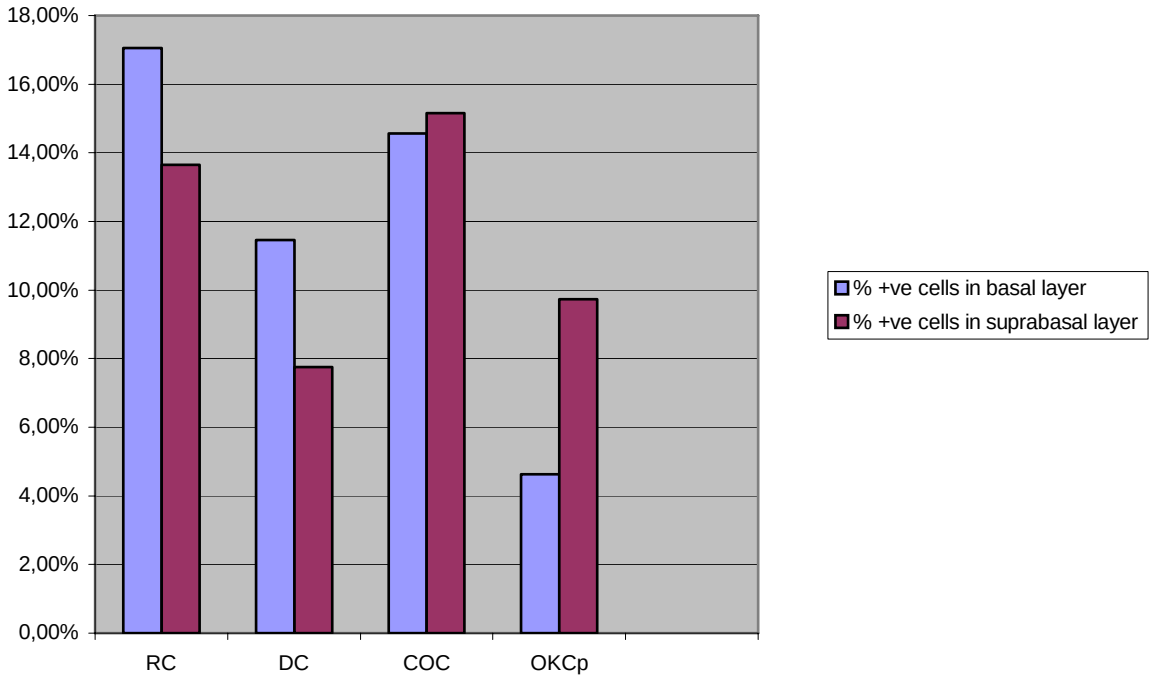
Table 1. Quantification of p53 immunoreactivity in odontogenic lesions.

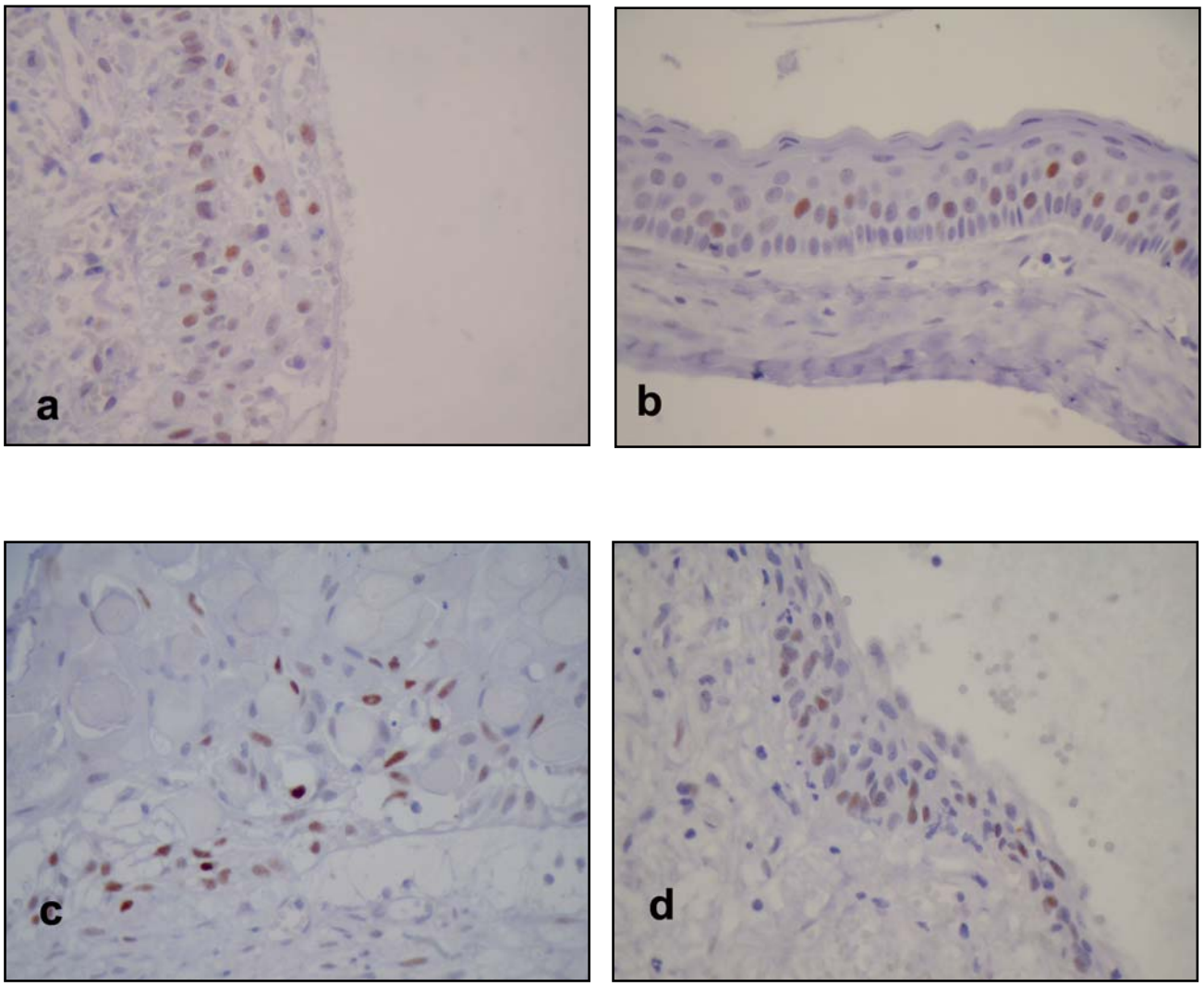
Diagnosis	n	+ve cells in basal layer	% +ve cells in basal layer	+ve cells in suprabasal layer	% +ve cells in suprabasal layer
RC	12	1023	17.05%	819	13.65%
DC	17	974	11.45%	657	7.73%
COC	16	1166	14.57%	1213	15.16%
OKCp	12	278	4.63%	584	9.73%

GRAPH 1. Percentage of p53 positive cells in the basal and suprabasal layers of odontogenic lesions.



LEGENDS

FIGURE 1 – Differences in p53 (DO7, DAKO) expression in odontogenic lesions. High number of p53+ cells with weak staining in radicular cyst (a); odontogenic keratocyst showing p53+ cells with intense and defined staining mainly in suprabasal layer (b); Calcifying odontogenic cyst showing basal and suprabasal layer with intense and defined staining of p53+ cells (c) and weak staining of p53+ cells located mainly in the basal layer of dentigerous cyst (d).


a
b
c
d

p53 AND PCNA IN ODONTOGENIC CYSTIC LESIONS: A NEW PERSPECTIVE ON THE NATURE OF THESE LESIONS.

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ABSTRACT

BACKGROUND: 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 PCNA expression in 4 different odontogenic lesions. METHODS: 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 expression of markers in dentigerous and radicular cysts was associated with the presence of an inflammatory infiltrate because of the inflammatory nature of these two lesions. In odontogenic keratocysts and Gorlin cysts, results indicate a pattern of tumor growth that is independent of inflammatory stimuli.

KEY WORDS: p53 protein; PCNA; etiology; 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 (1). 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 (2).

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. (1998) and Pillai et al. (2003) 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 (2003) 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 by damage to DNA when the cell cycle is arrested (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. However, a significant difference between layers was found only in keratocysts, which had greater values in 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 (figure 2), 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 cysts, in the basal layer of radicular cysts, and in the suprabasal layer of Gorlin 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 Mighel (1995), 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.

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.

PCNA and p53 expression may be a response to an inflammatory stimulus in radicular and dentigerous cysts. An inflammatory component was found in most dentigerous cyst samples (FIGURE 3), even in those classified as development cysts (21). Other studies have demonstrated that growth factors and cytokines (interleukin 1, interleukin 6 and tumoral necrosis factor (TNF) are released during inflammatory events. Inflammatory

stimuli increase cell proliferation, and inflammatory cytokines may also cause cell stress (22).

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 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 when the cell cycle is arrested.

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 (23, 24). Although inflammation may be a consequence of the formation of a dentigerous cyst, and not its cause, we believe that, similarly to what happens with radicular cysts, this inflammatory stimulus may make cells 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. 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 inflammation than with cell proliferation. Therefore, we suggest that dentigerous cysts should be classified as inflammatory lesions.

The association between inflammation and proliferation in odontogenic keratocysts was described by De Paula et al. (2000), 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 (2004), 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. 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.

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. (2006), who used a specific antibody for mutant p53.

The results of this study suggest that PCNA and p53 expression in radicular and dentigerous cysts have similar characteristics because of the inflammatory nature of these two lesions. The two tumoral lesions, odontogenic keratocysts and Gorlin cysts, however, did not share the same characteristics of marker expression. Results showed that their growth pattern is independent of inflammatory stimuli, but that their growth rates are different.

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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		84.75	9.82	
	Suprabasal	11	17.67	10.23	0.19	74.25	15.31	0.01*
Odontogenic Keratocyst	Basal	12	4.63	5.73		68.25	23.59	
	Suprabasal	12	9.73	4.22	0.01*	83.32	20.99	0.01*
Dentigerous cyst	Basal	15	8.41	10.38		60.89	31.75	
	Suprabasal	15	11.48	12.54	0.05	56.49	30.51	0.23
Gorlin cyst	Basal	10	14.96	16.12		71.48	33.28	
	Suprabasal	10	14.38	12.38	0.85	69.50	31.02	0.17

* 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*

* significant difference

LEGENDS

FIGURE 1. Odontogenic keratocyst lining showing proeminent suprabasal p53 (1A, arrows) and PCNA (1B, arrows) expression. (original magnification, X400)

FIGURE 2. Epithelial cells of radicular cyst showing mainly basal PCNA expression (arrows). (original magnification, X400)

FIGURE 3. Epithelial cells of dentigerous cyst showing mainly basal PCNA expression (arrows). Note the presence of inflammatory infiltrate in the connective tissue. (original magnification, X400)

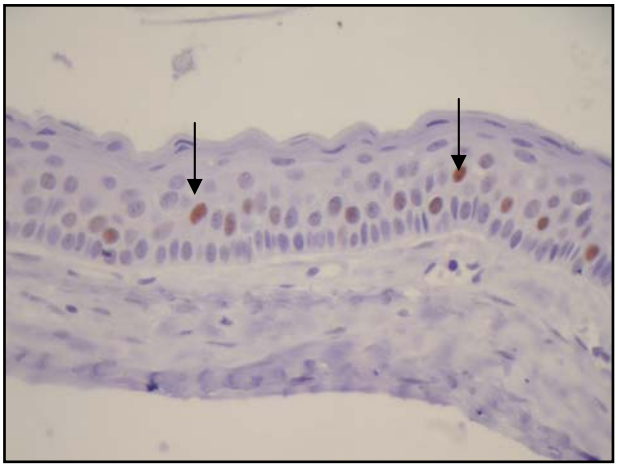

Figure 1A

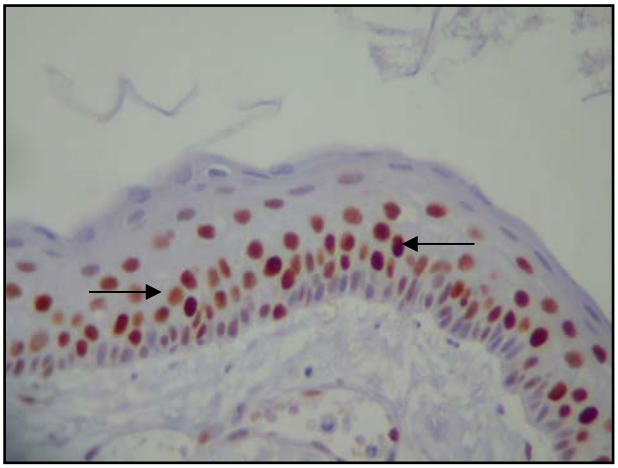

Figure 1B

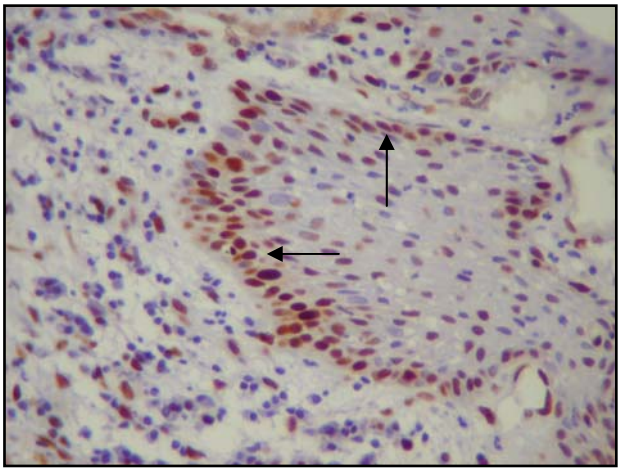

Figure 2

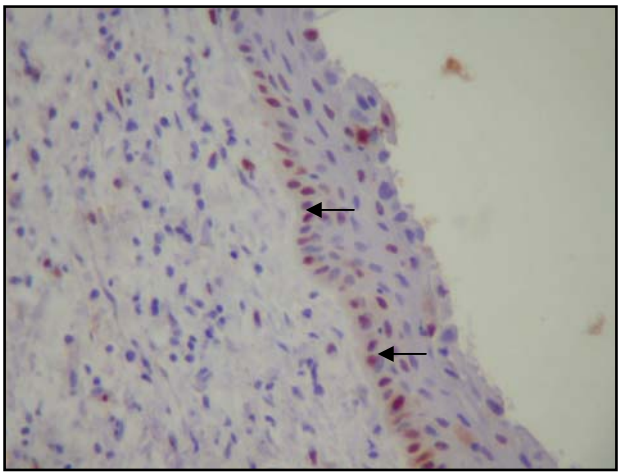

Figure 3

EXPRESSÃO DE EGFR, SURVIVIN E KI-67 EM EPITÉLIO ODONTOGÊNICO: RELAÇÃO COM A NATUREZA DAS LESÕES ODONTOGÊNICAS

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RESUMO

OBJETIVO: analisar fatores relacionados a proliferação celular e apoptose em células epiteliais odontogênicas para estabelecer uma possível relação entre esses fatores com a natureza e o comportamento de lesões odontogênicas. METODOLOGIA: análise imunoistoquímica de Ki-67, EGFR e Survivin em 13 ceratocistos Odontogênico (CO), 14 cistos dentígeros (CD) e 9 folículos pericoronários (FP). RESULTADOS: valores superiores de Ki-67 foram encontrados na suprabasal do CO e basal do CD, expressão maior de Survivin na camada suprabasal do CO e expressão de EGFR principalmente citoplasmática em CO e CD e em membrana e citoplasma e somente em membrana em FP. CONCLUSÕES: a proliferação celular no FP e CD depende do estímulo inflamatório, enquanto que as células do CO proliferam de maneira independente comprovando sua característica tumoral. O epitélio odontogênico dos FP são a origem dos cistos e tumores odontogênicos, justificando a remoção do tecido pericoronário de dentes não irrompidos.

INTRODUÇÃO

O epitélio que está envolvido na odontogênese pode dar origem a uma variedade de lesões odontogênicas que possuem comportamento clínico diverso. Estudos enfatizam as diferenças no potencial proliferativo das células epiteliais odontogênicas e sugerem que essas diferenças podem ser importantes na formação de cistos e tumores odontogênicos.[1-5] Sabe-se que a homeostase tecidual é mantida pelo balanço entre proliferação e morte celular e alterações na regulação destes mecanismos estão envolvidas na patogênese de uma variedade de lesões.

Um dos fatores associados com a proliferação celular em células epiteliais é a presença de fatores de crescimento como o Fator de Crescimento Epidérmico (EGF), o Fator de Crescimento Transformante α (TGFα) e seu receptor EGFR, um receptor que consiste de uma porção extracelular, uma região transmembrana e uma porção intracelular. A localização deste receptor na célula acredita-se estar associada ao modo que esta responde ao estímulo proliferativo: células em ritmo de proliferação fisiológico expressam o receptor na membrana e também no citoplasma, quando o receptor está localizado somente na membrana a resposta ao estímulo proliferativo parece ser mais rápida enquanto que a presença do receptor somente no citoplasma mostra que ele está internalizado ou inativo o que pode indicar uma resposta mais lenta.[6-8] A presença e localização do EGFR em epitélio odontogênico pode ter relação com a origem de cistos e tumores odontogênicos.[4]

Survivin pertence a uma família conhecida como Proteínas Inibidoras de Apoptose (IAP) que desempenham uma função na regulação da apoptose e da divisão celular. É uma proteína bifuncional que suprime a apoptose pela inibição da caspase –3 e –7 e regula a fase

G2/M do ciclo celular pela associação com microtúbulos do fuso mitótico. Encontrada em abundância em tecidos embrionários também pode estar presente em células em processo acelerado de divisão, como células basais epiteliais, estando quase ausente em células normais diferenciadas. Altos níveis da proteína são expressados na fase G2/M do ciclo celular enquanto que na fase G1/S o nível é muito baixo. [9-12]

O Ki-67 é um antígeno nuclear presente em todas as fases ativas do ciclo celular (G1, S, G2 e M) e ausente em G0. A detecção imunoistoquímica de Ki-67 é considerada como um indicador de proliferação celular e tem sido usada para avaliar o potencial proliferativo de células normais bem como de lesões pré neoplásicas e neoplásicas.[13]

O folículo pericoronário deriva do ectomesênquima odontogênico e histologicamente é caracterizado por tecido conjuntivo fibroso com quantidades variáveis de revestimento epitelial, o epitélio reduzido do órgão do esmalte, e restos epiteliais da lâmina dentária. Já o cisto dentígero é resultante do acúmulo de líquido entre o folículo pericoronário e a coroa do dente. Existe discussão sobre a capacidade proliferativa do epitélio odontogênico encontrado nos folículos pericoronários de dentes não irrompidos atribuindo-se à este epitélio a origem de algumas lesões odontogênicas. [1-5,14]

O ceratocisto odontogênico, agora classificado pela OMS como um tumor odontogênico, parece ter origem de células epiteliais odontogênicas da lâmina dentária e seus remanescentes ou até de extensões de células basais do epitélio bucal.[15] Esta lesão possui um comportamento clínico mais agressivo, o qual muitos estudos atribuem ao potencial proliferativo diferenciado das demais lesões odontogênicas.

O objetivo deste estudo foi analisar o comportamento de células epiteliais odontogênicas com relação a proliferação celular e apoptose em folículo pericoronário, cisto dentígero e ceratocisto odontogênico pela expressão de Ki-67, EGFR e Survivin para

estabelecer uma possível relação entre esses fatores com a natureza e o comportamento destas lesões odontogênicas.

METODOLOGIA

Foram selecionados 13 ceratocistos paraceratinizados, 14 cistos dentígeros e 9 folículos pericoronários. Todos os casos foram fixados em formalina neutra tamponada a 10%, desidratados em álcool, clareados em xilol e incluídos em parafina. Foram realizados cortes de 4µm de espessura e corados com hematoxilina e eosina (HE). As lâminas foram revisadas e o diagnóstico confirmado com base nas características clínicas, radiográficas e histopatológicas. Foram feitos três cortes histológicos de 4µm de espessura de cada bloco e cada um aderido a uma lâmina silanizada (DakoCytomation, USA) para marcação imunoistoquímica.

Imunoistoquímica:

Os cortes foram desparafinizados em xilol, reidratados em álcool e lavados em solução tampão fosfato (PBS), após foi realizado o bloqueio da peroxidase endógena com peróxido de hidrogênio 3% em metanol, duas trocas de 15 minutos cada. A recuperação antigênica foi realizada com solução de recuperação de baixo pH (ref. S1699, DakoCytomation, USA) previamente aquecida a 90°C, por 25 minutos em panela a vapor (Acqua Timer, ARNO, Brasil) a 96°C para o EGFR e survivin e no forno de microondas por 20 minutos (4 ciclos de 5 minutos) para o Ki-67. Os cortes foram incubados por 60 minutos em temperatura ambiente com Ki-67 (1:50, anticorpo monoclonal, anti-humano, clone MIB-1; DakoCytomation, Denmark A/S), EGFR (1:30, anticorpo monoclonal, anti-humano, clone EGFR113; Novocastra, UK) e Survivin (1:75, anticorpo monoclonal, anti-humano, clone DO-8, Santa Cruz Biotechnology, USA) e depois lavados em PBS. O sistema de detecção utilizado foi o Envision +® (DakoCytomation, USA) e o cromógeno

empregado foi o DAB líquido (DakoCytomation, USA). Os cortes foram lavados, contra-corados com hematoxilina de Harris, reidratados, clareados em xilol e as lâminas montadas com Entellan® (Merck, Germany). Como controle positivo foram utilizados cortes de carcinoma espinocelular de boca e no controle negativo foi feita a omissão do anti-corpo primário.

Análise das lâminas:

Em cada caso, a lâmina corada com hematoxilina e eosina foi examinada com microscópio óptico OLYMPUS modelo CX-40 sob aumento de 400X onde selecionou-se 5 campos significativos da lesão, estes mesmos campos foram identificados nas lâminas marcadas com Ki-67, EGFR e Survivin e as imagens foram fotografadas com uma câmera digital (NIKON Coolpix 995) com resolução de 2048 X 1536 pixels. A expressão dos marcadores foi analisada nas camadas basal e suprabasal do ceratocisto odontogênico e cisto dentígero e no epitélio odontogênico dos folículos pericoronários (ilhas de epitélio odontogênico ou epitélio reduzido do órgão do esmalte). O critério utilizado para identificar as células da camada suprabasal foi a morfologia nuclear, as células imediatamente acima da camada basal com o núcleo esférico foram classificadas como suprabasais e as células nas quais o núcleo começa a assumir uma morfologia mais achatada foram classificadas como superficiais e não foram analisadas. O critério de positividade na marcação imunoistoquímica foi a coloração marrom independente da intensidade.

Para análise do Ki-67, utilizou-se o programa ImageTool para Windows, versão 3.00 (UTHSCSA – University of Texas Health Science Center in San Antonio) onde quantificou-se o número total de células e o número de células positivas obtendo-se um percentual de células positivas. A expressão de EGFR foi descrita de acordo com a

localização na célula: somente membrana, somente citoplasma ou a combinação de membrana + citoplasma. A expressão de Survivin foi analisada de forma semi quantitativa e classificada em 4 categorias baseados no percentual de células positivas: 0 (< 5%), + (5 a 25%), ++ (26 a 50%) e +++ (> 50%).

Calibragem e análise estatítica:

A calibragem intra-examinador foi realizada durante o estudo, a cada 5 lâminas analisadas a primeira era revista. O índice de concordância Kappa entre 0,7 e 1,00 foi considerado aceitável.

Para análise estatística foram utilizados o teste não-parmétrico Kolmogorov-Smirnov para verificação da normalidade dos dados. Para comparação entre grupos das variáveis qualitativas foi utilizado o teste Qui-quadrado e nas comparações em que o tamanho da amostra foi menor foi realizado o teste Exato de Fisher. Para verificação do grau de correlação foi utilizada a análise de correlação de Spearman. Na comparação entre dois ou três grupos independentes foi utilizado o teste não paramétrico de Mann-Whitney e na comparação entre camada basal e suprabasal, por serem dados pareados utilizou-se o teste não paramétrico de Wilcoxon. Os dados foram analisados utilizando o software estatístico SPSS versão 10.0.

Este estudo foi aprovado pelo Comitê de Ética e Pesquisa da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, e pelo Comitê Nacional de Ética em Pesquisa (CONEP), parecer número 1166/2003.

RESULTADOS

A tabela 1 mostra os resultados encontrados para os três marcadores nos grupos estudados. Na expressão do Ki-67, verificou-se que no CO os valores foram significativamente maiores para suprabasal enquanto que no CD os valores foram maiores na camada basal (Teste de Wilcoxon; $p \leq 0,01$). Comparando-se as duas lesões, observou-se que a camada basal do CD apresenta valores significativamente maiores que o CO enquanto que na suprabasal os resultados são significativamente maiores para o CO (Teste Mann-Whitney, $p \leq 0,01$). Quando os três grupos foram comparados entre si, somou-se os resultados da camada basal e suprabasal do CO e CD para então compará-los com os resultados do FP e observou-se que o CO apresentou valores significativamente maiores que o CD e o FP (Teste Kruskal-Wallis, $p \leq 0,01$).

Quanto a expressão do EGFR observou-se que nas duas camadas do CO e CD a expressão mais frequente foi a citoplasmática enquanto que no FP houve uma expressão maior de membrana e citoplasma. Comparando-se CO e CD verificou-se que no CO o EGFR é mais frequente no citoplasma enquanto o CD tem uma maior frequência de expressão de membrana e citoplasma tanto para camada basal (Teste Qui-Quadrado; $\chi^2=12,20$, $p \leq 0,01$) quanto para suprabasal (Teste Qui-Quadrado; $\chi^2=14,04$, $p \leq 0,01$).

A expressão de Survivin na camada basal do ceratocisto foi classe ++ enquanto que na suprabasal foi predominantemente classe +++, no cisto dentígero ambas camadas apresentaram percentual maior para classe +++ e no folículo pericoronário os percentuais de classe +++ foram predominantes. Comparando-se o CO com o CD observou-se que na camada basal do CO são mais frequentes as classes + e ++ e no CD a classe +++ (Teste

Qui-Quadrado; χ^2=18,65, p≤0,01) enquanto que na camada suprabasal do CO é mais frequente a classe +++ e no CD classe + e ++ (Teste Qui-Quadrado; χ^2=25,77, p≤0,01).

Na correlação entre os marcadores verificou-se que existe uma correlação direta significativa entre os marcadores Ki-67 e Survivin apenas para o FP (Teste de Correlação de Spearman; r_s = 0,481; p≤0,01). Correlacionando EGFR e Survivin observou-se que na camada basal do CD existe associação significativa, a classe + está associada a marcação de citoplasma enquanto que classe +++ está associada a marcação de membrana e citoplasma (Teste Exato Fisher; χ^2=11,51, p≤0,01). Esta mesma associação foi encontrada para o FP (Teste Exato Fisher; χ^2=12,04, p≤0,01). Já na comparação do Ki-67 com EGFR verificou-se que os campos com marcação de EGFR em membrana e citoplasma apresentam resultados significativamente mais altos de KI-67 (Teste Kruskal-Wallis, p≤0,01). Nenhuma outra correlação ou associação entre os marcadores apresentou resultados estatísticamente significativos.

DISCUSSÃO

A literatura relata uma série de estudos em células epiteliais de lesões odontogênicas analisando proliferação celular, expressão de proteínas reguladoras de ciclo celular e apoptose. Entretanto, muitos dos resultados relatados são contraditórios e não relacionam esses fatores com o comportamento ou natureza das lesões. [16-20]

O folículo pericoronário mostrou que as células epiteliais têm pouca atividade proliferativa, porém a freqüência da expressão de EGFR em membrana e citoplasma foi maior e observou-se que neste grupo houve expressão somente de membrana em epitélio reduzido do órgão do esmalte e também em restos epiteliais da lâmina dentária encontrados na cápsula fibrosa. Isto nos sugere que as células epiteliais dos folículos pericoronários não estão proliferando mas possuem potencial de proliferação e nos casos onde observou-se expressão do EGFR só em membrana pode-se considerar que estas células, dependendo do estímulo que receberem, podem vir a proliferar e formar lesões odontogênicas. A expressão do survivin foi grau +++ em 55% dos casos.

No cisto dentígero, observa-se pela expressão do Ki-67 que a camada proliferativa é a camada basal, nesta camada a expressão do EGFR foi mais freqüente em citoplasma (57%) porém a expressão em membrana e citoplasma foi semelhante (42%) mostrando que essas células possuem capacidade de proliferação frente à estímulos, diferente da camada suprabasal onde na maioria dos casos (82,8%) a expressão foi citoplasmática. O Survivin, nas duas camadas mostrou uma freqüencia maior para o grau +++. Estes resultados indicam que no cisto dentígero as células da camada basal são as responsáveis pela proliferação e que estímulos externos podem levar as células epiteliais a aumentar a taxa de proliferação.

Considerando que membros da família do EGF são importantes e desempenham papel fundamental na iniciação e desenvolvimento dos cistos odontogênicos,[21-23] os resultados obtidos em folículo pericoronário e cisto dentígero nos sugerem que as células epiteliais do folículo pericoronário, na sua maioria, são células quiescentes que quando estimuladas retomar a um ritmo de proliferação fisiológico ou até mais acelerado. Acreditamos que as células do epitélio reduzido do órgão do esmalte que expressam o EGFR somente em membrana ainda estão sob o estímulo dos fatores de crescimento presentes na odontogênese e nestas células o estímulo inflamatório, que é o mais freqüente, leva a uma proliferação mais rápida e conseqüente acúmulo de líquido entre o folículo pericoronário e a coroa do dente, podendo formar o cisto dentígero, uma das lesões odontogênicas mais comuns. Alguns estudos relatam que mesmo sem imagem radiográfica de cisto dentígero o tecido pericoronário de dentes não erupcionados muitas vezes apresenta características císticas no epitélio reduzido do órgão do esmalte.[1,3]

Por outro lado, nos casos em que houve expressão do EGFR somente em membrana de restos epiteliais na cápsula fibrosa do folículo pericoronário, acreditamos que essas células podem dar origem a lesões odontogênicas mais agressivas uma vez que células com esse tipo de expressão parecem responder mais rápido à presença de fatores de crescimento no meio [7] e teriam potencial semelhante ao das células neoplásicas confirmando sua origem da lâmina dentária. Nossos resultados concordam com estudo de Baumgart et al. (2006) apesar da literatura referir que o desenvolvimento de lesões a partir do epitélio pericoronário de dentes não irrompidos é raro e não justificaria a remoção deste tecido. [14]

Já no Ceratocisto odontogênico, os resultados com o marcador Ki-67 concordam com a literatura quanto a taxa de proliferação diferenciada na camada suprabasal [14,19,24-26] além disso, expressão do EGFR principalmente citoplasmática sugere que estas células

estão com o receptor internalizado ou inativo e não estão proliferando pela presença de fatores de crescimento. A expressão do survivin na camada suprabasal é quase que exclusivamente grau +++ enquanto que na basal a maioria dos campos foi grau + ou ++. Isto mostra que as células epiteliais do ceratocisto odontogênico, principalmente as células da camada suprabasal têm menor diferenciação e possuem um potencial de proliferação independente, uma característica de células tumorais. Estes fatores, juntamente com as evidências moleculares de perda alélica em dois ou mais locus do cromossomo 9q22.3, que levam a superexpressão de bcl-1 e TP53, perda de heterozigosidade de genes supressores tumorais e da participação do gene PTCH desenvolvimento da lesão [27-31] poderiam explicar o comportamento recidivante característico do ceratocisto odontogênico.

Alguns estudos mostram que a expressão de Survivin ocorre durante o desenvolvimento embrionário e fetal, sendo indetectável na maioria dos tecidos adultos normais e superexpressada em vários tipos de neoplasias tendo sido considerado como um marcador de prognóstico negativo apesar de não estar esclarecido o mecanismo que leva à superexpressão.[10-12,32-35] Nossos resultados mostram que o Survivin está expresso em todos os grupos analisados com padrão de marcação citoplasmático ou citoplasmático e nuclear, sendo que o significado desses diferentes padrões ainda precisa ser esclarecido.[35-38] As bases moleculares para expressão de Survivin em tecidos normais, lesões pré-malignas e malignas não foram ainda completamente elucidadas [39] e apesar do gene Survivin estar expresso durante a mitose,[9] a expressão em neoplasias não está restrita somente à fração de células em mitose sugerindo que nas células neoplásicas existe uma falta de regulação global do gene Survivin.[39]

Kumanoto e Ooya (2004) demonstraram a expressão de Survivin em germes dentários e ameloblastomas sugerindo que esta proteína contribui tanto para regulação

celular durante o desenvolvimento dentário quanto a proliferação, diferenciação e sobrevivência celular em ameloblastomas. Em nossa amostra, acreditamos que a expressão de survivin em folículos pericoronários ainda está relacionada ao processo de odontogênese e no cisto dentígero e ceratocisto odontogênico está relacionado à proliferação e diferenciação celular.[40]

Baseados em nossos resultados, podemos sugerir que as células epiteliais do folículo pericoronário e do cisto dentígero são dependentes de um estímulo para desencadear a proliferação e a partir deste estímulo inicial existe o surgimento de uma cavidade cística onde o epitélio de revestimento deste cisto comporta-se como o epitélio de uma mucosa saudável quanto a proliferação celular, conforme verificamos na expressão do EGFR nesta amostra que foi semelhante a expressão em mucosa no estudo de Baumgart et al (2006). Por outro lado, as células do ceratocisto, que podem apresentar mutação, depois de um estímulo inicial, ainda desconhecido, assumem um padrão neoplásico de independência de proliferação, mostrados pela expressão dos marcadores utilizados, principalmente na camada suprabasal. Também observamos, pela expressão do EGFR, que restos epiteliais da lâmina dentária encontrados na cápsula fibrosa dos folículos pericoronários podem ter potencial de proliferação semelhante ao de células neoplásicas, sugerindo que esta seja a origem do ceratocisto odontogênico e não de células da camada basal do epitélio de revestimento bucal, justificando a remoção do tecido pericoronário de dentes não irrompidos.

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TABELA

Tabela 1. Expressão de Ki-67, EGFR e Survivin em ceratocisto odontogênico (CO), Cisto Dentígero (CD) e Folículo Pericoronário (FP)

			Ki-67		EGFR			SURVIVIN				
Lesão	nº campos	Camada	% médio	DP	M	C	M+C	-	0	+	++	+++
CO	65	Basal	11,82	8,97	0	84,6%	15,4%	0	0	32,3%	41,5%	26,1%
		Suprabasal	28,14	13,84	0	96,9%	0	3,1%	0	0	3,1%	96,9%
CD	70	Basal	16,06	9,12	0	57,1%	42,8%	0	1,4%	11,4%	27,1%	60%
		Suprabasal	3,28	3,11	0	82,8%	17,14%	0	0	21,4%	17,1%	61,4%
FP	45		7,88	6,94	22,2%	20%	57,8%	0	0	22,2%	22,2%	55,5%

Ki-67: % médio (percentual médio), DP (desvio-padrão) ;

EGFR: M (marcação de membrana), C (marcação de citoplasma), M+C (marcação de membrana e citoplasma), - (negativo);

SURVIVIN: 0 (< de 5% das células marcadas), + (5 a 25% das células marcadas), ++ (26 a 50% das células marcadas), +++ (mais de 50% das células marcadas).

LEGENDAS

FIGURA 1. Folículos pericoronários mostrando a expressão de EGFR somente em membrana em epitélio reduzido do órgão do esmalte (1A), e em restos epiteliais da lâmina dentária (1B) na cápsula fibrosa. (aumento de 400X).

FIGURA 2. Ceratocisto odontogênico mostrando expressão de Ki-67 predominante na camada suprabasal (2A), e expressão do survivin grau +++ na camada suprabasal (2B). (aumento de 400X)

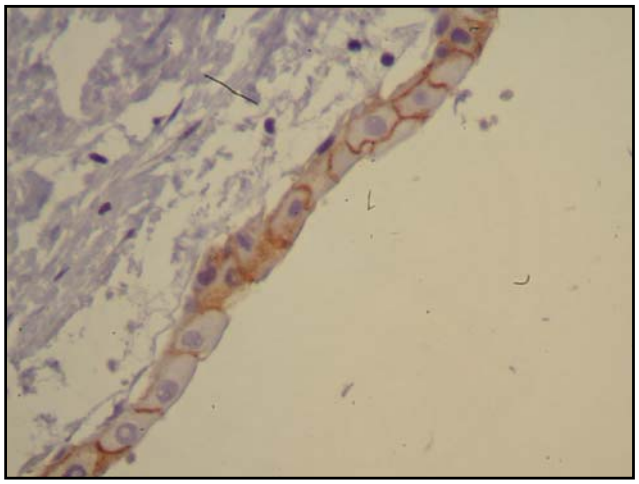

Figura 1A

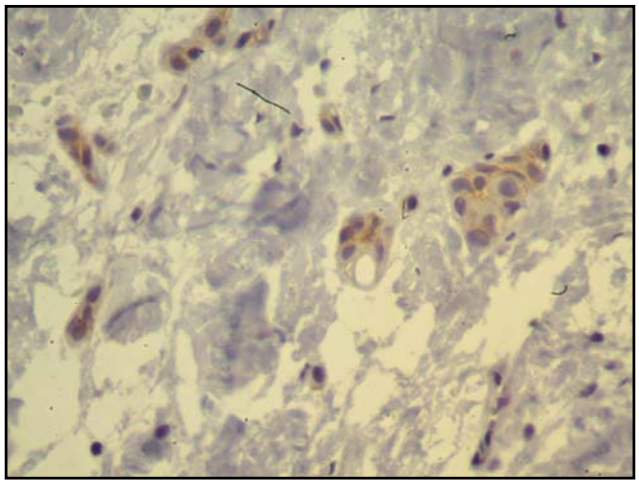

Figura 1B

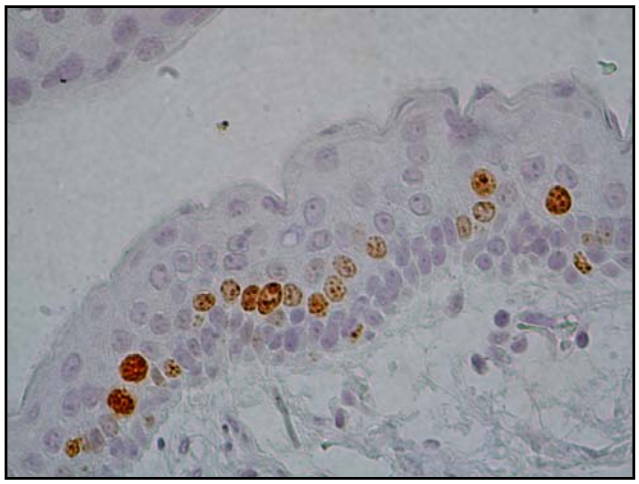

Figura 2A

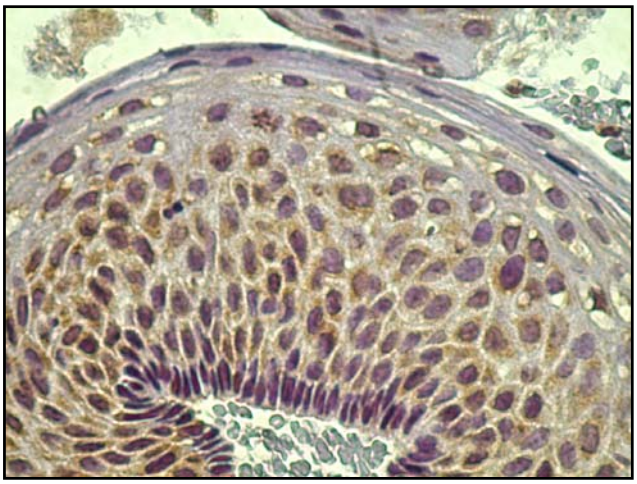

Figura 2B

CONCLUSÕES FINAIS

Acreditamos que vários métodos podem ainda ser utilizados para análise das lesões odontogênicas. Entretanto, no presente estudo, as relações descritas podem ser uma contribuição para o entendimento da natureza e o comportamento das lesões estudadas. Com isso, os resultados obtidos nos permitem concluir que:

- A expressão de p53, PCNA, EGFR, Survivn e Ki-67 no ceratocisto odontogênico comprova a natureza neoplásica da lesão;

- O ceratocisto mostrou maior proliferação celular e apoptose na camada suprabasal, sugerindo que está camada está envolvida no processo de crescimento e na patogênese desta lesão;

- A expressão dos marcadores utilizados nas células epiteliais do cisto dentígero sugere a natureza inflamatória desta lesão;

- As células epiteliais dos folículos pericoronários são a origem de alguns cistos, sendo que este tecido deve ser removido e examinado histopatológicamente.

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ANEXO 2: PARECER DO COMITÊ DE ÉTICA E PESQUISA DA FACULDADE DE ODONTOLOGIA DA UFRGS

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
COMITÊ DE ÉTICA EM PESQUISA

PARECER

O Comitê de Ética em Pesquisa, da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, avaliou e aprovou seu Projeto de Pesquisa.

Projeto: " COMPORTAMENTO BIOLÓGICO DAS CÉLULAS EPITELIAIS DE CISTOS ODONTOGÊNICOS"

Autores: CD Márcia Gaiger de Oliveira e Prof. Dr. Manoel Sant'Ana Filho
Relatora: Profa. Dra. Marisa Maltz

Parecer: O presente projeto baseia-se no uso de material de arquivo e integra um Banco de Tecidos Global do Laboratório. (Projeto Banco de Tecidos aprovado pelo CEP/ODONTO em 06/08/2001). Constitui-se de material enviado por Cirurgiões que indicaram o tratamento ou para exame diagnóstico. Por Lei esse material tem de ficar arquivado e este procedimento é de rotina. Não há interferência do pesquisador na coleta do material nem há indicação de qualquer procedimento. Este é um método consagrado na literatura mundial e o anonimato é garantido. O retorno ao paciente de qualquer descoberta é inútil, pois o mesmo já foi submetido ao tratamento convencional. Portanto o Parecer é favorável.

Porto Alegre, 1 de julho de 2003.

Profa. Marisa Maltz
Coordenadora do Comitê de Ética em Pesquisa

ANEXO 3: PARECER DO COMITÊ NACIONAL DE ÉTICA EM PESQUISA (CONEP)

MINISTERIO DA SAÚDE
Conselho Nacional de Saúde
Comissão Nacional de Ética em Pesquisa - CONEP

PARECER N.º 1166/2003

Registro CONEP: 7674 (Este n.º deve ser citado nas correspondências referentes a este projeto

Registro CEP: 04/03 Processo n.º 25000.041786/2003-05
Projeto de Pesquisa: " *Comportamento biológico das células epiteliais de cistos odontogênicos.* "
Pesquisador Responsável: Márcia Gaiger de Oliveira (pós graduanda)
Dr. Manoel Santana de Filho (orientador)
Instituição: Faculdade de Odontologia – UFRS
Área Temática Especial : Pesquisa com Cooperação Estrangeira

Ao se proceder à análise das respostas ao parecer CONEP nº 846/2003, relativo ao projeto em questão, considerou-se que:

a) foram atendidas as solicitações do referido parecer.

b) o projeto preenche, de modo geral, os requisitos fundamentais das Resoluções CNS 196/96 e 292/99, sobre Diretrizes e Normas Regulamentadoras de Pesquisas Envolvendo Seres Humanos;

c) o projeto foi aprovado pelo Comitê de Ética em Pesquisa da instituição supracitada

Diante do exposto, a Comissão Nacional de Ética em Pesquisa -CONEP, de acordo com as atribuições definidas na Resolução CNS 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto

Situação : Projeto aprovado

Brasília, 31 de julho de 2003

WILLIAM SSAD HOSSNE
Coordenador da CONEP /CNS/MS