PROJETO

"ANÁLISE MOLECULAR E IMUNO-HISTOQUÍMICA DA VIA RELACIONADA AO GENE PTCH EM CERATOCISTOS ODONTOGÊNICOS E NEOPLASIAS"

RELATÓRIO FINAL

Coordenador: Ricardo Santiago

Relatório apresentado a Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG em cumprimento ao processo CDS 99/01 de 03 de dezembro de 2001 a 02 de dezembro de 2003.
PROJETO

“ANÁLISE MOLECULAR E IMUNO-HISTOQUÍMICA DA VIA RELACIONADA AO GENE PTCII EM CERATOCISTOS ODONTOGÊNICOS E NEOPLASIAS”

RELATÓRIO FINAL

Belo Horizonte, MG.
FUNDEP
agosto 2003
Immunolocalization of PTCH Protein in Odontogenic Cysts and Tumors

INTRODUCTION

The human homologue of the Drosophila segment polarity gene Patched encodes the transmembrane protein PTCH, which is a receptor for the morphogen Sonic Hedgehog (Stone et al., 1996). Sonic Hedgehog/patched signaling controls cell fates, patterning, and growth in numerous tissues, including teeth (Biggood and McMahon, 1995). Sonic Hedgehog signaling regulates growth and determines the shape of the tooth, but it is not essential for differentiation of ameloblasts or odontoblasts (Dassule et al., 2000). Hardcastle et al. (1998) demonstrated that addition of exogenous Sonic Hedgehog protein directly to early tooth progenitors and also adjacent to tooth progenitors results in abnormal epithelial invagination. Analysis of these data reinforces the role of Sonic Hedgehog signaling in epithelial cell proliferation during tooth development.

Mutations in the PTCH gene were identified as the underlying genetic event in nevoid basal cell carcinoma syndrome (Shah et al., 1996). The demonstration of frequent loss of heterozygosity within the region containing the PTCH gene in sporadic and hereditary odontogenic keratocysts (Lench et al., 1996; Levant et al., 1996), and the subsequent finding of PTCH mutations in sporadic keratocysts have sparked intense interest in the role of this gene in odontogenic disorders (Barreto et al., 2000).

Mutational inactivation of PTCH leads to overexpression of the mutant transcript owing to failure of a negative feedback mechanism (Undén et al., 1997; Nagano et al., 1999). Expression studies with in situ hybridization and reverse-transcription/polymerase chain-reaction have shown PTCH overexpression in basal cell carcinomas compared with normal skin, a finding not seen in other types of skin cancer (Gailani et al., 1996; Undén et al., 1997; Nagano et al., 1999). The paucity of data concerning the localization of PTCH protein in many lesions, coupled with the evidence of PTCH gene mutations in odontogenic keratocysts and the importance of the Hedgehog signaling pathway during tooth formation, prompted us to investigate PTCH protein expression and localization in various odontogenic cysts and tumors.

MATERIALS & METHODS

Tissue Samples of Odontogenic Lesions

Sixty-eight odontogenic lesions, all from unrelated patients, were retrieved from the files of the Oral Pathology Laboratory, School of Dentistry, Universidade Federal de Minas Gerais. This study followed an informed consent protocol that was approved by the University's Ethics Committee. The odontogenic lesions consisted of 15 radicular cysts, 29 odontogenic keratocysts, 1 glandular odontogenic cyst, 6 dentigerous cysts, 3 odontogenic myxomas, 6 calcifying odontogenic cysts, and 8 ameloblastomas. In 7 out of 29 odontogenic keratocysts included in the present study, sequencing analysis of the PTCH gene was previously performed (Table) (Barreto et al., 2000). The single glandular
Figure 1. Positive PTCH staining in intermediate and superficial layers of nodular cyst (A), odontogenic keratocyst (B), glandular odontogenic cyst (C), and dentigerous cyst (D). Note positive immunostaining of the hyaline bodies.

odontogenic cyst included in this study did not present a PTCH gene mutation (Barreto et al., 2001).

The majority of previous PTCH expression studies have been done with RNA-based methods in basal cell carcinomas (Galliani et al., 1996; Undén et al., 1997; Nagano et al., 1999). To compare the immunolocalization of PTCH protein present in the odontogenic epithelium with that of other types of epithelial cells, we also studied 11 basal cell carcinomas and 10 samples of normal oral mucosa. All tissues had been fixed in formalin and embedded in paraffin.

Antibody Production
Polyclonal antibody was raised in rabbit against a human patched peptide from the carboxy-terminal region of the protein (RLPTPSPEPPSYVRFAMP). Validation of this antibody was previously described (Karpen et al., 2001).

Immunohistochemical Method
PTCH protein staining was performed by the streptavidin-biotin method. Briefly, 3-μm sections were de-waxed in xylene and hydrated with graded ethanol. Removal of formolite pigment was performed. Endogenous peroxidase was blocked by the incubation of sections in 6% (v/v) H2O2/methanol. Slides were subjected to microwave pre-treatment (Shi et al., 1997) and incubated with the primary antibody (anti-PTCH) for 18 hrs at 4°C. After being washed in 20 mmol/L Tris-HCl buffer (pH 7.4) containing 0.05% NaCl, sections were incubated for 30 min at room temperature with biotinylated rabbit-antibody swine anti-goat, mouse, and rabbit immunoglobulin (DAKO, Carpinteria, CA, USA). Sections were washed and incubated for 30 min at room temperature with 1:100 horse radish-peroxidase-conjugated streptavidin. The peroxidase activity was visualized by the application of 0.01% diaminobenzidine tetrahydrochloride and 0.03% H2O2. Sections were counterstained with Meyer’s hematoxylin and mounted in Permount®. Negative controls consisted of omission of the primary or the secondary antibody or primary incubation in the presence of non-immune rabbit serum instead of the primary antibody. Immunoreactions were independently analyzed by two investigators unaware of the clinical data. Staining was qualitatively analyzed as negative or positive and graded semi-quantitatively.

RESULTS
All tissue examined showed positive intra-cytoplasmic staining of PTCH (Fig. 1A, 1B), notwithstanding variable intensity. Positive staining in the intermediate and superficial layers of the cystic epithelium was detected in 14/15 of radicular cysts (Fig. 1A), with the basal layer also staining in two of them. PTCH staining was found in the intermediate and superficial cells of the epithelium in all odontogenic keratocysts (Fig. 1B). In addition, staining of the basal cell layer with loss of the characteristic features of a keratocyst and a dense inflammatory infiltrate was detected in two keratocysts (1 and 3, Table). Immunoreactivity was present in the superficial cells of the epithelium of an odontogenic glandular cyst (Fig. 1C). Similarly, immunostaining was found in the intermediate and superficial epithelial cells of dentigerous cysts (Fig. 1D), two of them showing weak staining of basal cells. In addition, hyaline bodies present in two dentigerous cysts showed a strong positivity (Fig. 1D). Ghost and superficial epithelial cells of calcifying odontogenic cysts had positive immunolabeling, as well as the central polyhedral and loosely connected angular cells of ameloblastoma (Fig. 2A). Only one ameloblastoma showed no staining. Immunopositivity for PTCH was also noticed in the mesenchymal cells of odontogenic myxoma (Fig. 2B).

Figure 2. Positive PTCH staining in central polyhedral and loosely connected angular cells of ameloblastoma (A) and mesenchymal cells of odontogenic myxoma (B). Fragment of normal oral mucosa shows weak immuno-positivity in the epithelium (C). While the epidermis overlying the basal cell carcinoma preserved a strong PTCH staining, the vast majority of neoplastic cells were negative for PTCH (D).

The photomicrographs (Fig. 1A, 1B, 1C, 1D, 2A, 2B, 2C) illustrate the results of the immunohistochemical study.
the stroma were immuno-positive (Fig. 2D).

**DISCUSSION**

Patched is both a member of the hedgehog pathway and a target of the pathway. *PTCH* mRNA expression is a marker of activation of the hedgehog pathway. High levels of *PTCH* mRNA are seen in virtually all basal cell carcinomas, which have either inactivating mutations of *PTCH* (a negative regulator of the hedgehog pathway) or activating mutations of *SMOF* (a positive regulator of the hedgehog pathway) (Gailani *et al.* 1996; Undén *et al.* 1997; Nagano *et al.* 1999; Tojo *et al.* 1999). There are relatively few data on PTCH protein expression in tumors and other lesions (Zedan *et al.* 2001).

The purpose of the current study was to examine expression of *PTCH* in odontogenic tumors and cysts, which also can arise with mutations in *PTCH*. We used immunohistochemical methods to assess *PTCH* expression at the protein level. As a means of validating this technique, we examined PTCH protein in basal cell carcinomas. As expected, there was virtual complete lack of immunostaining of PTCH in the peripheral and central cells of tumor sheets. Although the tumor cells overexpress *PTCH* mRNA, PTCH protein would not be expected to be present in basal cell carcinomas, because the vast majority of these tumors have truncating *PTCH* mutations4 to the region encoding the peptide against which our antibody was made.

Our findings of marked staining in normal epidermis are in contrast with those of previous studies showing very little *PTCH* mRNA in skin (Gailani *et al.* 1996; Undén *et al.* 1997). The presence of PTCH protein in skin may reflect higher sensitivity of immunostaining than the *in situ* hybridization methods used by those authors. *PTCH* mRNA is detected in normal epidermis by RT-PCR (Hahn *et al.* 1996). In addition, it is possible that PTCH protein accumulates in skin cell due to high stability compared with PTCH mRNA.

Immunostaining of odontogenic lesions revealed the presence of PTCH protein in virtually all cysts and tumors. In epithelial lesions, PTCH was commonly observed in all superficial layers but not basal cells. Increased *PTCH* mRNA levels may reflect a clonal genetic change, resulting in loss of autoregulation, causing mRNA overexpression (Undén *et al.* 1997). However, loss of *PTCH* regulation could also result from activation of the hedgehog signaling pathway by mutations in other genes (Undén *et al.* 1997). The finding of PTCH staining in normal epidermis could reflect a differentiation process occurring in the epithelia of these odontogenic lesions.

The dentigerous cyst, a developmental odontogenic cyst, can also be caused by the functional loss of PTCH (Levanat *et al.*, 2000). We demonstrated positive staining in the epithelium and an intense immunolabeling in hyaline bodies in two dentigerous cysts. Hyaline bodies are globulous structures seen within or below the epithelial lining of odontogenic cysts (Yamaguchi, 1980), but their origin remains unknown (Philippon *et al.*, 1990). The intense immunolocalization of the PTCH in these hyaline bodies suggests that their formation is associated with PTCH accumulation in epithelial cells of the lesion. However, the importance of PTCH in the pathogenesis of hyaline bodies remains to be established.

The positive labeling for PTCH in epithelial lesions (radicular cyst, glandular odontogenic cyst, calcifying epithelial odontogenic cyst, and ameloblastoma) and in a mesenchymal tumor (myxoma) is in agreement with both cell types expressing this protein during early odontogenesis (Hardcastle *et al.*, 1998). The staining in all lesions was more intense and evident than in the epithelium of normal oral mucosa, consistent with a model whereby the Hedgehog pathway is activated in these lesions. It is unlikely that PTCH itself is mutated in all of these lesions, because most inactivating PTCH mutations would be expected to result in low levels of PTCH protein (as in basal cell carcinomas). More studies are needed to determine how this pathway is switched on.

Loss of heterozygosity in *PTCH* was previously reported in 7 sporadic odontogenic keratocysts (Lench *et al.*, 1996; Levanat *et al.*, 1996). Two of the keratocysts reported in this study have PTCH mutations predicted to result in a truncated protein. One would expect no immunostaining of the epithelial cells of these lesions. Surprisingly, an immunoreactivity was detected, indicating that the epithelial cells may be heterozygous for the *PTCH* mutation. Therefore, these results suggest that odontogenic keratocyst may arise with haplo-insufficiency of *PTCH*. Consistent with this model, retention of one normal copy of *PTCH* in a mouse medulloblastoma with a heterozygous *PTCH* mutation was demonstrated (Zurawel *et al.*, 2000).

**ACKNOWLEDGMENTS**

This investigation was supported in part by grants from FAPESPI, PRONEX, and CNPq, (Brazil) and by NIH ROI-CA57605 (AEB).

**REFERENCES**


Gailani MR, Stahl-Backdahl M, Leffell DJ, Glynn M, Zaphiropoulos...


A Novel Mutation of the Cathepsin C Gene in Papillon-Lefèvre Syndrome

Vanessa F. Cury,* José E. Costa,* Ricardo S. Gomez,* Wolfgan L. Boson,† Cyro G. Loures,‡ and Luiz De Marco†

Background: Papillon-Lefèvre syndrome (PLS) is a disorder that involves destruction of the periodontium and abnormal hyperkeratosis of the palms of the hands and soles of the feet. Mutations of the lysosomal protease cathepsin C gene (CTSC) have been associated with PLS. However, genotypic and phenotypic correlation has not been established. In the present study we investigated the CTSC gene in a Brazilian cohort affected by PLS.

Methods: Eight consanguineous members of a kindred with PLS were studied. DNA was extracted and all exons of the gene amplified by the polymerase chain reaction (PCR) using specific primers. Mutations were identified by DNA sequencing of the coding region and introns of the CTSC gene.

Results: Sequence analysis of CTSC from subjects affected by PLS identified a novel mutation (587T → C) in exon 4, predicted to cause a Leu196Pro amino acid substitution. Three of 3 subjects were homozygous for cathepsin C mutations inherited from a common ancestor. One patient was heterozygous and showed plantar hyperkeratosis without periodontal disease. Two other family members were also heterozygous but did not present palmoplantar hyperkeratosis and/or periodontal disease.


KEY WORDS
Cathepsin C; keratoderma, palmoplantar; Papillon-Lefèvre syndrome; periodontitis, early-onset/etiology; genetic factors.

Papillon-Lefèvre syndrome (PLS) is a rare autosomal recessive disorder characterized by severe aggressive periodontitis associated with palmoplantar hyperkeratosis. It has an estimated prevalence of 1 in 4 million people and consanguinity demonstrated in one-third of the cases. Both genders are equally affected and no racial predominance appears to exist.

PLS can manifest itself as early as 2 months of age with the appearance of hyperkeratotic lesions of the hands and feet. Both the deciduous and permanent dentitions are affected, resulting in premature tooth loss. The patient is usually totally edentulous by age 15.

Severe periodontitis involving the teeth in order of their appearance usually starts at the beginning of the primary dentition. After the loss of the primary dentition, the gingival inflammation subsides until secondary dentition eruption, when the process resumes. An increased susceptibility to infection chiefly leading to pyoderma or furunculosis has been reported in a number of families.

The PLS locus has been mapped to chromosome 11q14-q21.5-7 Correlation of physical and genetic maps of this interval indicate it includes at least 40 ESTs (expressed sequence tags) and 6 known genes including the lysosomal protease cathepsin C gene (CTSC).8

Cathepsin C, or dipeptidyl amino peptidase I, is a lysosomal protease capable of removing dipeptides from the amino terminus of protein substrates. It is expressed at high levels in the lungs, kidney, placenta, polymorphonuclear leukocytes, alveolar macrophages, and their precursors. The

* Department of Oral Surgery and Pathology, School of Dentistry, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil.
† Department of Pharmacology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais.
‡ Private clinic, Belo Horizonte, Brazil.
CTSC message is expressed at high levels in a variety of immune cells including polymorphonuclear leukocytes, macrophages, and their precursors. The 4.7 kb CTSC gene consists of 7 exons.

The genomic structure of the CTSC gene has been defined and mutations in families affected by PLS described. In addition, mutations in this gene were also found in a Jewish-Hindu family with Haim-Munk syndrome and prepubertal periodontitis. The majority of mutations so far described are localized to exons 5 to 7, indicating that this region is important for enzyme activity. Although phenotypic variations exist among patients affected by PLS, the association between such variations and the genetic abnormalities of the CTSC gene is not known. The aim of the present study was to investigate the CTSC gene in a Brazilian kindred affected by PLS.

MATERIALS AND METHODS

Patients
Eight consanguineous members of a kindred with PLS were studied (Fig. 1). Patients were examined by a dermatologist and a dentist who performed a complete periodontal examination, and the diagnosis of PLS was made following previously established criteria. All family members provided written consent for this study, which was approved by the University’s Ethics Committee. Figures 2 to 6 illustrate clinical manifestations of PLS seen in patient IV.5.

DNA Extraction
DNA was extracted from whole blood as previously described. Blood samples were obtained from patients III.2, IV.2, IV.3, IV.5, IV.6, IV.8, IV.9, and IV.10. Briefly, leukocytes were resuspended in 900 μl lyses buffer (970 mg/ml guanidine HCl; 0.1 mol/L Tris, pH 6.4; 0.2 mol/L EDTA, pH 8.0; 26 mg/ml Triton X-100) plus 40 μl silica suspension and incubated at 56°C for 10 minutes. After 2 washings with buffer (970 mg/ml guanidine; 0.1 mol/L Tris, pH 6.4), the precipitate was dried in 70% ethanol and acetone. DNA was then extracted from silica by TE buffer (10 mmol/L Tris, pH 8.0; 1 mmol/L EDTA, pH 7.4) at 56°C for 10 minutes.

Polymerase Chain Reaction
The CTSC gene comprises 7 exons that were amplified by the polymerase chain reaction (PCR) using specific primers as previously reported. PCR reactions were performed in a final volume of 50 μl containing 100 ng of template DNA, 200 μmol/l dNTPs, 10 pmol/L of each primer, and 1.25 μL Taq polymerase. Thirty-five cycles of amplification were done in a PTC-100-60 thermocycler at 94°C for 40 seconds; 59°C for 60 seconds; 72°C for 90 seconds; and a final extension for 5 minutes at 72°C.

Sequence Analysis
After amplification, the single band generated by the specific PCR product was excised from the 1.5% agarose gel and the DNA fragment extracted accord-
Figure 3.
Solos of the feet with well demarcated hyperkeratotic lesions (Subject IV.S).

Figure 4.
Dorsal view of hands, showing diffuse hyperkeratotic lesions (Subject IV.S).

Figure 5.
Panoramic radiograph showing severe alveolar bone loss of permanent incisors (Subject IV.S).

Figure 6.
Panoramic radiographs following the progression of the periodontal disease in the propositus (subject IV.S). A. The subject at 10 years of age with severe periodontal destruction. B. The same individual at age 12 and C. at the age of 14, showing severe bone loss, having lost almost all permanent teeth.
RESULTS
Eight of 11 members of the fourth generation of this kindred were clinically examined and 3 (IV.5, IV.8, and IV.9) were diagnosed with PLS. IV.8 and IV.9 were totally edentulous while IV.5 presented only the teeth 1, 16, 17, and 32 at age 15 years old when first examined by one of the authors. All 3 exhibited palmoplantar hyperkeratosis. Another family member (IV.6) had only plantar hyperkeratosis. No other family members had aggressive periodontitis or a history of palmoplantar hyperkeratosis.

Sequencing Analysis
Sequence analysis of exonic, intronic, and the 5' regulatory regions of the cathepsin C gene showed that PLS affected subjects were homozygous for a mutation in codon 196 of exon 4 (587 T → C), that altered the original CTT codon to a CCT codon (Fig. 7). This mutation results in substitution of a conserved leucine residue at position 196 by a proline (Leu196Pro). The mother of the affected subjects (III.2) was a heterozygous carrier of the Leu196Pro mutant. The sister who presented only with palmoplantar hyperkeratosis (IV.6) was also a heterozygous carrier. No other family members who were heterozygous (IV.2 and IV.10) manifested clinically identifiable characteristics of palmoplantar hyperkeratosis nor had a history of severe, early onset periodontitis. One member (IV.3) was homozygous for the normal CTSC gene and did not show periodontal or dermatological abnormality. Analyses of the CTSC coding region was also performed in DNAs extracted from 20 normal volunteers and the sequences obtained were not different from published wild type.9

DISCUSSION
PLS is an autosomal recessive disorder characterized by palmoplantar hyperkeratosis and severe aggressive periodontitis. Several case reports in the literature showed phenotypic variations among patients affected by this syndrome. In some patients, primary teeth were not affected either by periodontitis or by premature loss.16,17,20,21 The nature of the periodontal disease affecting the permanent teeth can vary from an acute fulminating type infection on their eruption, resulting in their early loss in spite of treatment22,23 to a disease recognized only later in life (third decade) that has a more subdued clinical presentation20,21

The finding that distinguishes PLS from other palmoplantar keratoderma is severe periodontal destruction. In the classical report, the periodontal disease component of the syndrome affects the deciduous dentition causing destruction of the periodontium and early tooth loss followed by a rapid destruction of the support of the permanent dentition and edentulism by age 15. Destruction of the alveolar bone is usually severe, complicating dental therapy. Of the 3 affected patients in this report, 2 were totally edentulous while the third affected patient presented with only teeth 1, 16, 17, and 32.

It has been suggested that the susceptibility to peri-
odontal disease results from interactions of genetic alterations and polymorphisms with numerous environmental agents. Several diseases are associated with aggressive periodontitis, including Papillon-Lefèvre syndrome, Down syndrome, leukocyte adhesion deficiency, and others. In contrast to adult forms of periodontitis, there is evidence that genetic factors are important in aggressive periodontitis. It has been suggested that juvenile periodontitis is genetically heterogeneous and, although the gene(s) responsible have not been identified, a major gene for a localized form was mapped to chromosome 4q12-21.

In the present study we demonstrated a novel mutation of the CTSC gene responsible for Papillon-Lefèvre syndrome in a consanguineous kindred. The 3 patients with PLS were homozygous for the mutated allele. The substitution of leucine to proline, both neutral and hydrophobic amino acids, may cause a conformational alteration, as a proline residue can disrupt the usual organization of the backbone of the polypeptide and may decrease or cease activity. The patients' mother (III.2) is a heterozygous carrier of one mutated allele. Although edentulous, she did not present with palmo-plantar hyperkeratosis at the time of examination, therefore, it is somewhat difficult to conclude whether tooth loss has any relationship with genetic findings. One patient (IV.6) who showed only plantar hyperkeratosis also was heterozygous for the CTSC gene alteration. However, other heterozygous individuals had neither periodontal nor skin alterations. Therefore, the impact of the mutated plus wild type allele on periodontal or dermatological picture remains to be established.

The association between CTSC gene alterations and PLS patients' susceptibility to periodontal disease could be explained by two mechanisms. First, CTSC protein is implicated in the activation of proteases related to phagocytosis, antigen presentation, local activation, and deactivation of cytokines and other inflammatory mediators. Therefore, CTSC is involved in a wide variety of immune and inflammatory responses that include the activation of phagocytic cells and T-lymphocytes, leading to the final elimination of the pathogen. Second, CTSC might influence periodontal disease progression through its role in epithelial differentiation or desquamation. As the sulcular and junctional epithelium represent the first line of defense, their aberrant differentiation may alter the mechanical barrier to periodontal pathogens.

It has been suggested that genetic factors affect inflammatory periodontal disease progression. This is demonstrated by the strong familial aggregation seen in aggressive periodontitis. However, efforts to dissect the specific genetic factors involved in the chronic inflammatory periodontal disease pathogenesis have not succeeded. The complex interactions between the environmental and host factors may result in different clinical forms of the disease. It is difficult to ascertain the specific role of CTSC on aggressive periodontitis. A recent report demonstrated molecular alterations on this gene on prepubertal periodontitis. The possibility of polymorphism, heterozygosity of the CTSC gene leading to partial activity needs to be established. In conclusion, we here describe a novel mutation responsible for Papillon-Lefèvre syndrome in a Brazilian kindred.

ACKNOWLEDGMENTS
The authors are grateful to Dr. Diele C. Barreto for comments and Ms. Adriana Moreira for technical assistance. This study was supported in part by grants from PRONEX and CNPq (Brazilia, Brazil) and FAPEMIG (Belo Horizonte, Brazil).

REFERENCES
1. Papillon MN, Lefèvre B. Two cases of familial symmetric palmo plantar keratosis (Méda's disease) in a brother and his sister. Alterations in both cases (in French). Bull Soc Francaise Dermatologie Syphiligraphie 1924;31:81-84.


Send reprint requests to: Dr. Luiz De Marco, Department of Pharmacology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, Belo Horizonte 31270-901, Brazil. Fax: 55 31 3499-2695; e-mail: ldemarco@icb.ufmg.br.

Accepted for publication October 1, 2001.
Immunolocalization of the p53 Protein in a Case of Ameloblastic Fibrosarcoma

Alfredo Maurício Battista de Paula, DDS, MS,*
José Quaresma da Costa Neto, MD,†
Edson da Silva Gusmão, MD,‡
Francis Balduíno Guimarães Santos, MD,§
and Ricardo Santiago Gómez, DDS, PhD‖

Malignant mixed odontogenic tumors comprise a rare group of odontogenic neoplasms. These tumors include ameloblastic fibrosarcoma, ameloblastic fibrodentinoma, ameloblastic fibro-odontosarcoma, and odontogenic carcinoma. The ameloblastic fibrosarcoma exhibits a microarchitecture that resembles that of ameloblastic fibroma, in which the mesenchymal component shows evidence of malignancy. When a jaw tumor presents an ameloblastic fibroma-like pattern with cytologic evidence of malignancy involving both the epithelial and connective tissue components, the designation odontogenic carcinoma is used.

Because of the rarity of malignant odontogenic tumors, little is known about the immunolocalization of cell cycle-associated proteins in them. p53 is a tumor suppressor gene that is associated with many kinds of malignant tumors. Given the paucity of information about its immunolocalization in epithelial or mesenchymal components of odontogenic tumors, we decided to investigate the immunolocalization of p53 in a single case of ameloblastic fibrosarcoma.

Report of a Case

A 25-year-old man was referred to a surgeon for evaluation of a swelling in the left mandibular molar region that had been present for 3 months. Oral examination revealed that the swelling was covered with normal mucosa; the patient was asymptomatic. Radiographs revealed a well-defined radiolucency that extended from the distal root of the second molar to the second premolar region (Fig 1). The medical history was unremarkable. A needle aspiration produced no fluid. An incisional biopsy was performed, and microscopic findings were interpreted as *ameloblastoma.* Subsequently, the patient underwent a partial mandibulectomy. Microscopic examination of the surgical mass disclosed a neoplasm composed of loosely cellular mesenchymal stroma that resembled dental papilla that contained strands and islands of ameloblastic epithelium (Fig 2). The epithelial components were characterized by peripherally palisading columnar cells. The mesenchymal component was characterized by increased cellularity in some regions with polygonal and spindle-shaped cells showing pleomorphic, hyperchromatic nuclei (Figs 2, 3). Some mitotic figures were also recognized. Because of the benign appearing odontogenic epithelium and the malignant mesenchymal component, the diagnosis was revised to "ameloblastic fibrosarcoma." There has been no evidence of recurrence at 16 months after surgery.

Paraffin-embedded tissue blocks of the tumor were cut at 3-μm sections and subjected to the biotin-streptavidin amplified system for the immunolocalization of the p53 protein. Microwave stimulation was performed to unmask the epitope. The slides were subjected to a microwave (700 W/vacuum) treatment for 15 minutes (3 × 5 minutes) and then left to cool for 50 minutes. The primary antibody used was anti-p53 suppressor gene product (Clone DO7, 1:50 dilution; Biogenex, San Ramon, CA).
Samples from cases of oral squamous cell carcinoma and of normal oral mucosa with known immunoreactivity for anti-p53 antibody were used as positive controls. Negative controls included parallel sections from which either the primary or secondary antibodies were excluded.

The immunohistochemical reactions showed a generalized immunolocalization of the p53 protein to the nucleus of the mesenchymal tumor cells (Fig 4). The epithelial cells failed to show nuclear positivity (Fig 4).

Discussion

Ameloblastic fibrosarcoma is a rare malignant odontogenic tumor considered to be the malignant counterpart of the ameloblastic fibroma. It is found in the mandible more often than in the maxilla. Histologically, the tumor consists of 2 components: strands and anastomosing cords of mainly columnar ameloblastic elements and a mesenchymal component that resembles dental papilla but with cytologic atypia, increased cellularity, pleomorphism, and mitotic figures. Immunohistochemistry has shown higher Ki-67 immunolocalization in the mesenchymal component of ameloblastic fibrosarcoma than in the epithelial component. In poorly differentiated mesenchymal foci, there may be a diminished epithelial component. After local recurrences, the epithelial component may become less prominent or disappear.

Some cases of ameloblastic fibrosarcoma have been reported to have arisen de novo, whereas others appear to have originated from a preexisting benign ameloblastic fibroma. The finding that ameloblastic fibrosarcomas occur at a later age compared with their benign counterparts has been used to support a theory of stepwise malignant transformation of a benign to a malignant tumor, as opposed to de novo malignancy.

Despite the clearly malignant histologic features of the ameloblastic fibrosarcoma, metastases rarely occur. Although some fatal cases of the tumor have been reported, they are usually associated with
uncontrollable local tumor infiltration after numerous recurrences. 17 The treatment of choice for ameloblastic fibrosarcoma is surgical resection with wide surgical margins. Radiotherapy and chemotherapy may be used in cases where there are multiple recurrences.18

p53 is a tumor suppressor gene with an important role in the normal cell cycle. It is a transcription factor that upregulates the expression of target genes (p21) in response to DNA damage. This in turn blocks cell proliferation in the G1 phase of the cell cycle.19 21

Mutations of the p53 gene usually result in a product with an increased half-life that can sometimes be shown immunohistochemically. Under certain conditions, the normal p53 protein can also be retained in the tissue by, for example, binding to other proteins or through some defect in the normal degradation pathway. In this way, it can be detected with immunohistochemical examination. In the latter instances, this "retained" wild-type protein is inactive, either due to blocking by another protein or due to partial degradation.2 Alterations of the p53 gene are the most common genetic changes found in human malignant tumors. Abnormalities of p53 in human sarcomas has been reported.*

In the case reported here, p53 immunolocalization was observed in the connective tissue components of the tumor exclusively. This together with the cytologic atypia of the mesenchymal component supports the diagnosis of "ameloblastic fibrosarcoma" rather than "odontogenic carcinoma." Although no molecular analysis was performed in this case, the findings suggest that p53 alteration may be a step in malignant odontogenic tumorigenesis. Altered p53 immunolocalization has not been reported in benign odontogenic tumors.22 Additional immunohistochemical and molecular studies may help to delineate the role, if any, of p53 in the pathogenesis of malignant odontogenic tumors.

References

TP53 Codon 72 Polymorphism in Oral Squamous Cell Carcinoma

SÉRGIO NEVES DRUMMOND1, LUIZ DE MARCO2, ISABELA DE ALMEIDA PORDEUS3, ALVIMAR AFONSO BARBOSA4 and RICARDO SANTIAGO GOMEZ1

1Department of Oral Surgery and Pathology, 2Department of Pharmacology, 3Department of Pediatric Dentistry, Universidade Federal de Minas Gerais and 4Luxemburgo Hospital, Belo Horizonte, 31270-901, Brazil

Abstract. Background: Oral squamous cell carcinoma (OSCC) is a multifactorial neoplasm related to tobacco smoke, alcohol consumption and molecular genetic changes such as p53 mutations. The human tumor suppressor gene TP53 contains single nucleotide polymorphism that encodes either arginine (Arg) or proline (Pro) at amino acid codon 72 of the p53 protein. The relationship between human cancer susceptibility and p53 polymorphism at codon 72 is controversial. The aim of this study was to investigate the association between this polymorphism and OSCC development in a Brazilian population. Materials and Methods: Eighty-two patients with OSCC and 82 age-sex-matched controls were included in the study. DNA was extracted from all subjects’ normal oral mucosa and the polymerase chain reaction amplification (PCR) was performed for detection of the TP53 genotypes. PCR products were analyzed in a 6.5% polyacrylamide gel and silver-stained. Statistical analyses were performed with the χ2 test and Fisher’s exact test. Results: The frequencies of the genotypes Arg/Arg, Arg/Pro and Pro/Pro, were 37.8%, 54.8% and 7.4%, respectively, for OSCC cases and 40.2%, 54.8% and 4.9% for controls. No significant differences in the distribution of TP53 genotypes were seen between the groups (p = 0.794). Conclusion: The present study does not support the hypothesis that this TP53 polymorphism is associated with oral cancer susceptibility.

Squamous cell carcinoma is the commonest cancer of the oral cavity and its pathogenesis is related to tobacco smoke, alcohol consumption and molecular genetic changes (1). Among these genetic alterations, somatic mutations of TP53 have been demonstrated in almost all human cancers. The TP53 gene is a tumor suppressor gene located on the short arm of chromosome 17, consisting of 11 exons and encoding the p53 nuclear phosphoprotein with 393 amino acids (2). p53 acts as a “molecular policeman” monitoring the integrity of the genome through cell cycle control, DNA repair and apoptosis (3). Loss of p53 genomic stabilization properties predisposes a cell to acceleration in the rate of genetic damage and greatly increases the likelihood of neoplastic transformation and/or malignant progression (4). The human tumor suppressor gene TP53 contains a single nucleotide polymorphism that encodes either arginine (Arg) or proline (Pro) at amino acid codon 72 (5). Biochemical and biological differences related to these polymorphisms have been reported (6). While the p53-Pro is more efficient on transcription, the p53-Arg is more efficient in inducing apoptosis and also tumor growth.

The relationship between TP53 polymorphism and cancer susceptibility is controversial (7). The association between the genotype Arg/Arg and laryngeal cancer has been previously shown (8). However, this association was not confirmed by other authors (9, 10). In addition, it has also been suggested that the frequencies of this TP53 polymorphism is associated with geographical latitude (11).

Considering that TP53 codon 72 polymorphism has an impact on p53 activity, together with the fact that the frequencies of these TP53 alleles are correlated with latitude and have not been evaluated in tropical countries, the aim of this study was to investigate the association between this TP53 polymorphism and oral squamous cell carcinoma (OSCC) development in a Brazilian population.

Materials and Methods

Subjects. Oral swabs were collected from the oral mucosa of 82 smokers with oral squamous cell carcinoma and from 82 age-sex-matched control smokers. Sterile plastic spatulas containing swabs were immediately placed in Eppendorf microtubes containing 150 µl of Krebs buffer (NaCl 20%, KCl 2%, CaCl2·2H2O 2%, MgSO4, KH2PO4, CaH2O4). The pellet obtained after 10 minutes of centrifugation at 6,000 x g was stored at -20°C until further processing. All samples from the patients with carcinoma were obtained from the lesions' contralateral mucosa.

TP53 polymorphism analysis. The DNA extraction was carried out as described by Boon et al. (12). Polymerase chain reaction was performed for detection of the TP53 genotypes. The primers used have been previously described (13). The amplification was carried out in a total volume of 25 µL containing 3.0 µL of genomic DNA, 1.5 mM MgCl2, 40 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.1% Triton X-100, 13.125 H2O, 4.0

Correspondence to: Ricardo Santiago Gomez, Department of Oral Surgery and Pathology, School of Dentistry, Av. Antônio Carlos, 6627, Belo Horizonte - 31270-901, Brazil. Tel: +55 (31) 34992477, Fax: +55 (31) 34992472, e-mail: rsgomez@mail.odonto.ufmg.br

Key Words: Oral cancer, p53, squamous carcinoma, polymorphism.
The bands revealing either proline or arginine homozygotes and arginine proline heterozygotes are shown in Figure 1. Genotype frequencies of the TP53 codon 72 polymorphism in oral cancer patients and controls are presented in Table 1. No significant differences in the distribution of TP53 genotypes between both groups were seen. The frequency of the arginine and proline-coding alleles in the group with OSSC (65.2% and 34.7%, respectively) was not statistically different from the control (67.7% and 32.3%, respectively). The subjects were also stratified by sex and age. Again, there was no difference between groups in the distribution of polymorphism (data not shown).

Discussion

In the present report, we evaluated the association between the TP53 codon 72 polymorphism and OSSC. Available data in literature suggest that TP53 codon 72 variants are a risk factor for some human cancers such as lung, colorectal, breast and cervical cancer (14, 8). However, other studies concluded that codon 72 allelicism does not have an impact on human cancer risk (15, 9, 10).

The use of DNA from tumor samples for polymorphism analysis may lead to inaccurate results as allelic loss (loss of heterozygosity) can be present in some neoplastic cells (16). The analysis of DNA derived from tumor specimens is a potential source of bias and it is possible that cells could have undergone somatic mutations and therefore misrepresent host genotype. To overcome this problem, in our study genomic DNA was extracted from desquamated mucosal cells of a site without neoplastic transformation.

The codon 72 germline polymorphism of the TRS3 gene participates in genetically-determined susceptible to smoking-induced lung cancer since individuals with Pro/Pro genotype are more susceptible to smoking-induced lung cancer than other haplotypes (17, 18). Women genotypically homozygous for arginine had a 7-fold higher risk for HPV-related cervical cancer development. The p53 protein containing arginine at codon 72 was more efficiently degraded by E6 HPV oncoprotein (13).

Our results showing that there is no relationship between TP53 polymorphism and the risk for OSSC in our Brazilian population confirm the data obtained in case-control studies with other populations (9, 19, 10). Taiwanese women with the Pro/Pro genotype showed an increased chance of having lung adenocarcinoma (20). In addition, the proline homozygous genotype has been related to a higher risk of lung cancer in
Table I. Genotype frequencies of TP53 codon 72 in oral squamous cell carcinoma (OSCC) cases and controls.

<table>
<thead>
<tr>
<th></th>
<th>A/A</th>
<th>A/P</th>
<th>P/P</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSCC</td>
<td>31 (37.8%)</td>
<td>45 (54.8%)</td>
<td>6 (7.4%)</td>
</tr>
<tr>
<td>(n = 82)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 82)</td>
<td>33 (40.2%)</td>
<td>45 (54.9%)</td>
<td>4 (4.9%)</td>
</tr>
<tr>
<td>Total</td>
<td>64 (39.0%)</td>
<td>90 (54.9%)</td>
<td>10 (6.1%)</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 0.462; df = 2; p = 0.794. \]

American individuals younger than 53 years old (18). In the present study, when we stratified the groups by sex and age, the results showed no association of TP53 polymorphism and oral cancer. These data are in accordance with previous studies with gastric cancer and acute myeloid leukemia (21, 22).

The frequencies of the arginine and proline-coding alleles in this study were 65.2% and 34.7% in cases and 67.7% and 32.3% in controls. There are significant differences between human racial groups with respect to frequencies of TP53 alleles and a strong correlation with latitude has been shown. The frequency of the Arg allele increases and that of the Pro allele decreases with latitude (11). The frequency of the arginine-coding allele (67.7%) observed in our control group in the population of Belo Horizonte (latitude 20°) was different from the frequency (55.9%) reported in a Brazilian population from Joao Pessoa (latitude 7°), which suggests an allele frequency-latitude correlation (23).

In conclusion, the present study does not support the hypothesis that TP53 codon 72 polymorphism is associated with oral cancer susceptibility. We also demonstrated a higher frequency of the Arg allele in our population. Further studies are necessary to delineate the implications of geographic variation of TP53 polymorphism at this codon with human cancer.

Acknowledgements

This investigation was supported in part by grants from FAPEMIG, PRONEX and CNPq, Brazil. Dr. De Marco L., Podeaes IA and Gomes RS are research fellows of CNPq.

References


Received May 24, 2002
Accepted July 15, 2002

3381