UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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ANÁLISE DA IMUNOEXPRESSÃO E PERDA ALÉLICA DE *PTEN* EM LESÕES DISPLÁSICAS E CARCINOMAS EPIDERMÓIDES DE BOCA

ANALYSIS OF IMMUNOEXPRESSION AND *PTEN* ALLELIC LOSS IN DYSPLASTIC LESIONS AND ORAL SQUAMOUS CELL CARCINOMAS

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Estomatopatologia, na Área de Estomatologia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfilment of the requirements for the degree of Master in Pathology, in Stomatology area.

Orientador: Prof. Dr. Helder Antonio Rebêlo Pontes
Coorientador: Prof. Dr. Felipe Paiva Fonseca

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELA ALUNA LIGIA AKIKO NINOKATA MIYAHARA E ORIENTADA PELO PROF. DR. HELDER ANTONIO REBÊLO PONTES

Piracicaba
2017

Agência(s) de fomento e nº(s) de processo(s): CAPES, 33003033009P4

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

M699a

Miyahara, Ligia Akiko Ninokata, 1990-
Análise da imunoexpressão e perda alélica de *PTEN* em lesões displásicas e carcinomas epidermóides de boca / Ligia Akiko Ninokata Miyahara. – Piracicaba, SP : [s.n.], 2017.

Orientador: Helder Antonio Rebêlo Pontes.
Coorientador: Felipe Paiva Fonseca.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. *PTEN* Fosfo-hidrolase. 2. Leucoplasia bucal. 3. Neoplasias bucais. 4. Imuno-histoquímica. 5. Deleção de genes. I. Pontes, Helder Antonio Rebêlo. II. Fonseca, Felipe Paiva,1986-. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Analysis of immunoexpression and *PTEN* allelic loss in dysplastic lesions and oral squamous cell carcinomas
Palavras-chave em inglês:
PTEN Phosphohydrolase
Leukoplakia, oral
Mouth neoplasms
Immunohistochemistry
Gene deletion
Área de concentração: Estomatologia
Titulação: Mestra em Estomatopatologia
Banca examinadora:
Felipe Paiva Fonseca [Coorientador]
Fabio Luiz Coracin
Oslei Paes de Almeida
Data de defesa: 28-07-2017
Programa de Pós-Graduação: Estomatopatologia

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de Mestrado, em sessão pública realizada em 28 de Julho de 2017, considerou a candidata LIGIA AKIKO NINOKATA MIYAHARA aprovada.

PROF. DR. FELIPE PAIVA FONSECA

PROF. DR. FABIO LUIZ CORACIN

PROF. DR. OSLEI PAES DE ALMEIDA

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

DEDICATÓRIA

Dedico este trabalho aos meus pais, **Hitomi Ninokata Miyahara** e **Shunsuke Miyahara**, que sempre fizeram com que os estudos se sobressaíssem em minha vida, não sendo diferente em mais esta etapa.

AGRADECIMENTOS

À Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas. Privilegiada me sinto por ter feito parte desta instituição.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo apoio financeiro, através da concessão de bolsa, o que viabilizou dedicar-me aos estudos.

Aos Professores Doutores Alan Roger dos Santos Silva e Márcio Ajudarte Lopes, anterior e atual coordenadores do Programa de Pós-Graduação em Estomatopatologia, por todo o apoio, empatia na resolução de meus questionamentos e oportunidades cedidas.

A todos os professores integrantes do Programa de Pós-Graduação em Estomatopatologia, pela oportunidade de aprendizado.

Ao Professor Doutor Hélder Antônio Rebelo Pontes, pelo qual tenho inestimável admiração e respeito. Obrigada por mais uma orientação acadêmica e por todo o conhecimento proporcionado ao longo desses anos. Obrigada pelo apoio profissional e, principalmente, pessoal. Considero-o como alguém de minha família. Oro todos os dias pela sua mais breve recuperação.

Aos Professores Doutores Douglas Guimarães, Felipe Fonseca, Flávia Pontes, Nicolau Conte Neto e Rommel Burbano, por suas grandiosas colaborações na realização deste trabalho.

À família "patobucal" do Hospital Universitário João de Barros Barreto, profissionais, residentes, alunos e pacientes, por mais esses anos de bom convívio.

Aos colegas de mestrado, Camila Weissheimer, Gleyson Amaral, Jéssica Montenegro, Natália Palmier e Rodrigo Soares, que juntos entraram comigo nesta jornada, assim como aos colegas Bruno Mariz e Juliana Kern, por todo o apoio, sempre estando dispostos a ajudar e nunca negando conhecimento.

Ao amigo Lucas Lacerda, por toda a ajuda cedida no desenvolvimento desta dissertação.

Por fim, à minha família, pais e irmãos, por sempre darem o suporte necessário em minhas decisões pessoais e profissionais; e a todos que contribuíram direta ou indiretamente para a realização deste trabalho.

Muito obrigada.

RESUMO

O Carcinoma epidermoide é a neoplasia maligna mais comum de boca, representando mais de 95% dos casos diagnosticados neste sítio anatômico. Muitos casos de carcinomas epidermoides de boca (CEB) podem ser precedidos ou estão associados a lesões potencialmente malignas, especialmente as leucoplasias. O processo de carcinogênese envolve a aquisição progressiva de alterações irreversíveis de vários genes, com ativação de oncogenes e inativação de genes supressores tumorais e de genes envolvidos no reparo do DNA. A fosfatase homóloga à tensina deletada no cromossomo 10 (*PTEN*) é um gene supressor tumoral que exerce um importante mecanismo no controle da aquisição do fenótipo maligno ao inibir a expressão da proteína pAkt, sendo importante no controle da via de sinalização intracelular PI3K/AKT, responsável pelo controle da proliferação celular e apoptose. A perda ou inativação de *PTEN* ativa a via de sinalização PI3K/AKT, encontrada em diversos tipos de tumores. O objetivo deste estudo foi analisar a perda alélica do gene *PTEN* e avaliar a imunoexpressão da proteína nas lesões displásicas bucais e CEB. As amostras foram coletadas de 153 pacientes (20 casos controle – C, 30 leucoplasias com displasia leve – DL, 30 leucoplasias com displasia moderada a severa – DMS, e 73 amostras de CEB), provenientes do Serviço de Patologia Bucal do Hospital Universitário João de Barros Barreto (HUJBB). A expressão da proteína PTEN foi investigada através da técnica imuno-histoquímica e a perda alélica foi analisada pelo método de hibridização in situ por fluorescência (FISH). Diferenças entre os grupos foram avaliadas usando o teste Qui-quadrado. Os resultados mostraram que houve um aumento na expressão de PTEN, assim como uma maior perda alélica de acordo com a progressão para a malignidade, onde a expressão foi mais alta em DMS (p=0.002) e CEB (p=0.0259) comparados com o grupo C, além disso, foi observada uma maior expressão em DMS (p=0.0035) e CEB (p=0.049) do que em DL; em relação à análise de FISH, uma maior perda hemizigótica (cópia única) foi observada em CEB comparado com C (p=0.0467) e DL (p=0.0175), assim como uma maior deleção homozigótica em CEB comparado com C (p=0.0159) e DL (p=0.0145). Em conclusão, os resultados deste trabalho sugerem que a perda alélica de *PTEN* é um importante mecanismo nos estágios finais da aquisição do fenótipo maligno, onde a perda alélica foi observada em 34.25% das amostras de CEB.

Palavras-chave: PTEN Fosfo-Hidrolase. Leucoplasia. Câncer de Boca. Imuno-Histoquímica. Deleção de Genes.

ABSTRACT

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm in the oral cavity, accounting for more than 95% of the cases diagnosed in this anatomical site. Many cases of OSCC may be preceded by or associated with potentially malignant disorders, such as leukoplakias. The carcinogenesis process involves a progressive evaluation of irreversible changes of several genes, with activation of oncogenes and inactivation of tumour suppressor genes and genes involved in DNA repair. Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is a tumour suppressor gene that represents an important mechanism for the acquisition of a malignant phenotype by inhibiting the expression of the pAKT protein, which is important in the control of the intracellular signalling pathway PI3K/AKT, which is responsible for the control of cell proliferation and apoptosis. The loss or inactivation of *PTEN* activates the PI3K/AKT signalling pathway that is found in several types of tumours. The objective of this study was to analyse allelic loss of the *PTEN* gene and its protein immunoexpression in dysplastic oral lesions and OSCC. The samples were collected from 153 patients (20 ranulas used as a control – C, 30 leukoplakias with mild dysplasia – MD, 30 leukoplakias with moderate to severe dysplasia – MSD, and 73 OSCC) from the Department of Oral Pathology, João de Barros Barreto University Hospital (HUJBB). PTEN protein expression was investigated using immunohistochemistry, and allelic loss was analysed by fluorescence in situ hybridisation (FISH). Differences among groups were evaluated using the Chi-square test. The results showed that there was an increase in PTEN expression, as well as a greater allelic loss, according to progression to malignancy, where expression was higher in MSD ($p=0.002$) and OSCC ($p=0.0259$) compared with the C group, additionally, a higher expression was observed in MSD ($p=0.0035$) and OSCC ($p=0.049$) than MD; regarding FISH analysis, a higher hemizygous (single copy) loss was observed in OSCC than C ($p=0.0467$) and MD ($p=0.0175$), as well as a higher homozygous deletion in OSCC than C ($p=0.0159$) and MD ($p=0.0145$). In conclusion, the results of this work suggest that PTEN allelic loss is an important mechanism in the late stage of the development of oral potentially malignant lesions into oral cancer, where allelic loss was observed in 34.25% of OSCC samples.

Key Words: PTEN Phosphatase. Leukoplakia. Oral Cancer. Immunohistochemistry. Gene Deletion.

SUMÁRIO

1 INTRODUÇÃO

É inquestionável que o câncer é um problema de saúde pública, sendo refletido através do grande impacto gerado na população. No ano de 2016 foram estimados para o Brasil 11.140 casos novos de câncer da cavidade oral em homens e 4.350 em mulheres. Tais valores correspondem a um risco estimado de 11,27 casos novos a cada 100 mil homens e 4,21 a cada 100 mil mulheres (INCA, 2016). Uma maior exposição a fatores de risco, como o consumo de tabaco e álcool pode ser a causa de uma maior prevalência observada em homens, embora essa doença em mulheres tenha aumentado em muitas partes do mundo (Mifsud et al.,2017; Tota et al., 2017; Chen et al., 2017)

O carcinoma epidermoide de boca (CEB) representa cerca de 95% de todas as neoplasias malignas de boca. Essa doença constitui-se em um desafio a ser enfrentado na saúde pública mundial, particularmente nos países subdesenvolvidos, em razão, especialmente, da sua alta incidência, baixos índices de sobrevida e dos defeitos funcionais e estéticos acentuados que ocasiona (Lo et al., 2003; Pontes et al., 2011), visto que muitos destes tumores recebem diagnósticos tardios, em estadiamentos clínicos avançados da doença. Na América do Sul, as taxas de mortalidade variam entre 0.72 a 6.04/100,000 (Curado et al., 2016). E apesar do progresso nas estratégias de diagnóstico e tratamento, com avanço nos campos da quimioterapia e radioterapia, assim como das técnicas cirúrgicas, as taxas de morbidade e mortalidade ainda não melhoraram significativamente e a taxa de sobrevida em cinco anos ainda é baixa, variando em torno de 30 a 50% em várias partes do mundo (Warnakulasuriya, 2009; Pontes et al. 2015).

A presença de displasia epitelial parece ser o indicador prognóstico mais importante da transformação maligna (Lumerman et al., 1995), representando o fenótipo morfológico das diferentes etapas na progressão de um tecido normal ao neoplásico. No exame histopatológico, é a característica mais importante presente em lesões potencialmente malignas, pois auxilia no entendimento do comportamento biológico e orienta a conduta clínica (Liu et al., 2010; Vigneswaran, Williams, 2014).

O epitélio displásico é definido pela combinação de alterações arquiteturais somadas a anormalidades citológicas, sendo caracterizado pela presença de atipia celular e pela perda da maturação e estratificação normal (Pindborg et al., 1997). A displasia epitelial pode ser classificada em displasia leve, moderada e intensa, conforme a presença e severidade das alterações citológicas e estruturais do tecido afetado (WHO, 2005). A displasia epitelial leve ocorre quando as mudanças arquiteturais estão limitadas ao terço inferior do epitélio,

além de apresentar mínima atipia celular; na displasia moderada, as alterações estão no terço médio e observa-se maior atipia celular; por fim, a displasia epitelial intensa apresenta mais de dois terços do epitélio com distúrbios arquiteturais acompanhados de atipia celular (WHO, 2005).

Embora a maioria dos patologistas orais reconheça e aceite os critérios apresentados pela classificação da Organização Mundial de Saúde, ainda assim há variabilidade inter e intra-examinador quanto à avaliação da presença ou ausência e o grau de displasia epitelial. Desse modo, o uso de um sistema binário de classificação (sem/ questionável/ leve, como baixo risco; e moderada/ intensa, como alto risco) é relevante nesse contexto, já que a variabilidade reduzida para dois graus pode aumentar a probabilidade de acordo entre os patologistas (Warnakulasuriya et al., 2008; Speight et al., 2015).

A carcinogênese oral é um processo de múltiplos passos com alterações genéticas e epigenéticas em vias celulares, que envolve a aquisição progressiva de alterações e mutações na expressão de vários genes com ativação de oncogenes e inativação de genes supressores tumorais (Chen et al., 2008). Os oncogenes podem estimular a produção de uma excessiva quantidade de material genético novo por meio de amplificação ou superexpressão do gene envolvido, enquanto os genes supressores de tumor atuam indiretamente na produção do tumor quando se tornam inativados ou mutados (Neville et al., 2016). No entanto, o mecanismo molecular subjacente permanece largamente desconhecido, devido à complexidade biológica da doença.

O risco de progressão maligna das lesões displásicas é imprevisível. Para avaliar os riscos de transformação maligna das desordens epiteliais de forma mais precisa, o conhecimento fisiopatológico da lesão é necessário. O potencial desses processos patológicos ainda não está bem definido na literatura, sendo indispensável compreender e localizar alterações genéticas e epigenéticas nas células displásicas, de modo a contribuir para a melhor compreensão sobre a progressão para a malignidade e, com isso, melhorar fatores prognósticos dos pacientes (Nasser et al., 2011).

Esses fatos destacam a necessidade de identificação de marcadores moleculares que possam predizer a agressividade da doença e o curso clínico. Assim, é crucial entender os caminhos moleculares que governam a progressão da malignidade oral.

Um gene supressor tumoral muito importante durante o processo de tumorigênese é o gene *PTEN* (do inglês, phosphatase and tensin homolog deleted on chromosome 10), também denominado de MMAC1 (do inglês, mutated in multiple advanced cancers) e TEP1 (do inglês, TGFβ- regulated and epithelial cell enriched phosphatase). Este gene foi descrito

pela primeira vez em 1997 por Ramon Parsons e Jung Li, da Universidade de Columbia. É um gene de 200 kb e está localizado no cromossomo 10 (10q23), sendo composto por nove éxons e oito íntrons (Xu et al., 2014), e codifica a proteína PTEN, constituída por 403 aminoácidos, pertencente à família das proteínas tirosino-fosfatases (PTP) capaz de desfosforilar resíduos de serina, treonina e tirosina. A estrutura cristalina de *PTEN* revela uma sequência N-terminal associada ao domínio fosfatase e uma sequência C-terminal, associada ao domínio C2. A cauda C-terminal contém sítios de fosforilação que parecem ser responsáveis por manter a estabilidade da proteína PTEN e possui um sítio de ligação para proteínas que contém o domínio PDZ (PDZ é uma combinação das primeiras letras de três proteínas PSD95, DlgA e zo-1). Embora o domínio da fosfatase N-terminal seja responsável principalmente pela atividade fisiológica de *PTEN*, aproximadamente 40% das mutações tumorigênicas de *PTEN* ocorrem no domínio C2 C-terminal e na sequência da cauda, sugerindo um papel importante de C-terminal na manutenção da função de *PTEN* (Zhang, Yu, 2010).

Através da sua atividade fosfatase lipídica, PTEN converte fosfatidilinositol-3,4,5-trisfosfato (PIP3), no citoplasma, para fosfatidilinositol-4,5-bisfosfato (PIP2), antagonizando, assim, a atividade da proteína quinase PI3K, necessária para a ativação de Akt, uma proteína associada à proliferação celular, apoptose, invasão e metástase (Ye et al., 2012)

Recentemente tem-se mostrado a participação da proteína pAkt mostrando a maior marcação em lesões leucoplásicas mais avançadas e em CEB (Silva et al., 2012; Chaves et al. 2017), assim como a relação de uma alta expressão de pAkt pode contribuir para o crescimento, metástases para os linfonodos regionais e um menor tempo de sobrevida do paciente (Pontes et al., 2015). Sendo assim, Akt pode desempenhar um importante papel na conversão de uma lesão oral potencialmente maligna em carcinoma.

Além das mudanças específicas em oncogenes e genes supressores de tumor, as células malignas são geneticamente instáveis e apresentam mudanças cromossômicas extensas, incluindo amplificações, duplicações, deleções e translocações (Van Houten et al., 2000). A perda alélica em um locus específico gera instabilidade genética, resultando em alteração da expressão e função desses genes.

Neste contexto, nota-se a importância de *PTEN*, visto que a perda de função deste gene resulta na proliferação descontrolada e redução de apoptose, predispondo o indivíduo ao desenvolvimento neoplásico maligno. Mutações somáticas e supressão de *PTEN* estão sendo relatados em diferentes lesões malignas e potencialmente malignas nos seres humanos, como em mama, próstata, leucemia, do endométrio e, inclusive, o CEB (Alyasiri et al., 2012;

Snietura et al., 2012). As alterações genéticas ou deleções de PTEN, além de ocorrerem em uma variedade de tumores sólidos, também têm sido descritas frequentemente em carcinomas epidermoides de cabeça e pescoço (Poetsch et al, 2002; Chen et al.,2000).

Neste sentido, o presente estudo torna-se relevante, uma vez que a expressão de PTEN em lesões orais potencialmente malignas deve ser investigada, e sua importância para o processo de carcinogênese oral merece ser melhor compreendida. Nesta linha de pensamento, este trabalho teve como proposta avaliar a perda alélica de PTEN, assim como sua imunoexpressão em leucoplasias com diferentes graus de displasia oral e na lesão estabelecida de carcinoma epidermoide de boca.

2 ARTIGO

Este trabalho foi realizado no formato alternativo, conforme informação CCPG/001/2015, da Comissão Central de Pós-Graduação (CCPG) da Universidade Estadual de Campinas.

***PTEN* allelic loss is an important mechanism in the late stage of development of oral leukoplakia into oral squamous cell carcinoma.**

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ACKNOWLEDGMENTS

The authors acknowledge the Coordination for the Improvement of Higher Education Personnel (CAPES/PROEX), process number 33003033009P4, for financial resources for this research; and the Service of Oral Pathology of the João de Barros Barreto University Hospital for give us the samples used in this research.

ABSTRACT

OBJECTIVES: The aim of this study is to analyse allelic loss of the *PTEN* gene and its protein immunoexpression in dysplastic oral lesions and oral squamous cell carcinomas (OSCCs).

MATERIALS AND METHODS: Samples were collected from 153 patients (20 ranulas used as a control – C, 30 leukoplakias with mild dysplasia – MD, 30 leukoplakias with moderate to severe dysplasia – MSD, and 73 OSCC). PTEN protein expression was investigated using immunohistochemistry, and *PTEN* allelic loss was analysed by fluorescence in situ hybridisation (FISH). Differences among groups were evaluated using the Chi-square test.

RESULTS: PTEN expression was higher in MSD (p=0.002) and OSCC (p=0.0259) compared with the C group; additionally, a higher expression was observed in MSD (p=0.0035) and OSCC (p=0.049) than MD. Regarding FISH analysis, a higher hemizygous (single copy) loss was observed in OSCC than C (p=0.0467) and OSCC than MD (p=0.0175), as well as a higher homozygous deletion in OSCC compared with C (p=0.0159) and OSCC than MD (p=0.0145).

CONCLUSION: The results of this work suggest that *PTEN* allelic loss is an important mechanism in the late stage of the development of oral potentially malignant lesions into oral cancer.

Keywords: PTEN Phosphatase. Leukoplakia. Oral cancer. Immunohistochemistry. Gene Deletion

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most common types of cancer in the world[1], accounting for more than 95% of all malignant neoplasms in the oral cavity. It is a significant problem in many parts of the world, particularly in underdeveloped countries, owing to its high incidence and unsatisfactory five-year survival rate, and because treatment can result in severe functional and cosmetic defects[2]. Oral carcinogenesis is a multistep process that involves the activation of proto-oncogenes and inactivation of tumour suppressor genes. However, the underlying molecular mechanisms remain to be fully understood. A proportion of OSCCs develop from oral potentially malignant lesions, such as oral leukoplakia, oral submucous fibrosis, and oral erythroplakia.

The presence and degree of epithelial dysplasia are used to assess the risk of leukoplakia progression to malignancy; however, histological grading has limited value in predicting the individual risk of each case. Furthermore, the criteria reproducibility of microscopic grading of dysplastic lesions is very low and poses significant difficulties among pathologists[3-5]. Therefore, the understanding of the molecular mechanisms that govern the evolution of oral potentially malignant lesions into OSCC is highly relevant for clinical practice, potentially leading to the identification of genes and proteins suitable for therapeutic targeting, and predictors of prognostic determination[5-7].

Akt/protein kinase B is a major downstream target of growth factor receptor tyrosine kinase that signals via phosphatidylinositol-3 kinase (PI3K), and it is frequently activated in different human malignancies[6, 8-10]. Phospho-Akt (p-Akt) controls a variety of critical cellular pathways during the carcinogenic process, including those leading to apoptosis inhibition and increased cell proliferation, as well as enhanced tumour cell invasion, angiogenesis, and cell metabolism, through glucose metabolism[6, 11]. *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) is a tumour suppressor gene, and its major substrate is phosphatidylinositol - 3,4,5-triphosphate (PIP-3), which dephosphorylates PIP3 to regenerate PIP2, which antagonises PI3K function. Consequently, *PTEN* downregulates Akt activity by dephosphorylating and negatively regulating PI3K signalling[12]. Conversely, loss of *PTEN* expression results in increased Akt activity and continued cell survival and proliferation.

Recent studies have proposed that activation of Akt has shown to be associated with advanced stages and poor prognosis in OSCC[7, 13-15]; furthermore, studies using OSCC cell lines have showed that induced PTEN over-expression significantly down-regulated Akt

phosphorylation and induced apoptosis[16]. Regarding oral potentially malignant lesions, several studies have showed that p-Akt may play an important role in the conversion of these lesions to its malignant counterpart[6, 7, 15, 17].

The expression of PTEN in potentially malignant oral lesion remains to be investigated, and its importance to the oral carcinogenesis process deserves to be better understood. Therefore, the aim of this study is to determine the expression and the allelic loss of *PTEN* in oral leukoplakia with different degrees of epithelial dysplasia and in OSCC.

MATERIALS AND METHODS

Samples were collected from the Service of Oral Pathology of the João de Barros Barreto University Hospital (Belém, Brazil). The local ethics committee approved this study (process no. 1 426 757).

The specimens were obtained from 153 patients (20 samples of morphologically normal epithelium obtained from ranulas with inflammatory infiltrated located distant from the overlying epithelium to be used as a control group – group C; 30 oral leukoplakias with mild dysplasia – MD; 30 leukoplakias with moderate to severe dysplasia – MSD; and 73 OSCC). Histological sections (5 μm) of all samples were routinely stained with haematoxylin and eosin (H&E) and analysed under light microscopy. Two independent oral pathologists without prior knowledge of the clinical data assessed the stained sections to classify the histological grades of oral dysplasia according to previously proposed binary system criteria of classification[3]. In the cases of disagreement, pathologists discussed the findings and achieved a final agreement.

Immunohistochemistry

Immunohistochemical reactions were performed in 3μm sections that were dewaxed with xylene and rehydrated in ethanol series. Antigen retrieval was performed by immersing the slides in ethylenediamine tetraacetic acid (EDTA) solution (PH 8.0) for 15 min in a microwave oven. Peroxidase activity was blocked with 6% hydrogen peroxide and methanol (1:1) solution in two baths of 15 min each at room temperature. After washing in Tris buffer (pH 7.4), the slides were incubated with primary antibody (polyclonal anti-human PTEN, Invitrogen, CA, USA) in a dilution of 1:100 overnight for 18 h at 4°C. The slides were subsequently exposed to the avidin–biotin complex (LSAB-Kit + HRP; Dako Cytomation) and to the 3,3′-diaminobenzidine chromogen (DAB+; Dako Cytomation). The sections were

counterstained with Mayer's haematoxylin, dehydrated in ethanol, cleared in xylene, and mounted. Prostate cancer tissue was used as a positive control, whereas the negative control was obtained by omitting the primary specific antibody during the reaction. The results were considered positive when brown-coloured nuclear and cytoplasmaic stainings were observed, characterising the presence of DAB in the immunohistochemical reaction.

The scoring system used has previously been described in the literature[18]. Briefly, the analysis was based on both the intensity and distribution of staining. The distribution of stained cells was organised as follows: 0 (0% of cells stained), 1 (1–50% of cells stained), and 2 (51–100% of cells stained). The intensity of staining was rated as follows: 0 (no staining), 1 (mild staining), 2 (moderate staining), and 3 (strong staining). The final record for each case was defined by the sum of the values obtained as follows: FR0, FR2, FR3, FR4, and FR5. Finally, FR0 and FR2 were considered negative staining, whereas FR3, FR4, and FR5 were considered positive staining.

Fluorescence in situ hybridisation

For FISH analysis, 5 μm histological sections of 10% formalin-fixed tissues were dewaxed in xylene and rehydrated in ethanol series. The denaturation process was carried out on a hot plate at 95ºC for 10 min. Five μl of PTEN probe were applied on the samples according to the manufacturer's instructions (CAT# 07J74001, Vysis LSI PTEN SpectrumOrange/ CEP 10 SpectrumGreen Probes, Abbott Molecular Inc., USA). The samples were incubated in a humid chamber protected from light at 37°C for approximately 16 h. Sections were stained with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride), and the slides were incubated in a humid chamber protected from light for 10 min at 8°C.

The analysis was performed using an Olympus BX 41 fluorescence microscope, with the Case Data Manager 5.0 programme, where 100 cells/case were randomly counted regarding the probe signals in each allele of the gene investigated. Hemizygous (single copy) PTEN deletion was assigned when >50% of nuclei exhibited clonal loss of PTEN and adjacent probes. Homozygous PTEN deletion was defined by a simultaneous lack of both PTEN locus signals in 30% of scored nuclei[19].

Statistical analysis

To determine any significant difference regarding the allelic loss of PTEN and its immunoexpression among the groups investigated, a Chi-square test was applied.

GRAPHPAD PRISM 5.0 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis, and statistical significance was set at $p < 0.05$ with 95% confidence intervals.

RESULTS

The clinicopathological characteristics of 60 patients with leukoplakia and 73 patients with OSCC are summarised in Tables 1-3.

This study comprised 73 patients (44 male and 29 female) with OSCC. An average age of 61.13 years was observed, with a range of 33–87 years old. Fifty-one patients (69.86%) were smokers, and 22 (30.14%) were non-smokers. Tumors were mainly localised in the floor of the mouth (36.99%), tongue (31.50%), and alveolar ridge (19.18%). Tumors classified as T4 represented the majority of the cases (32.87%), followed by T3 (28.77%), T2 (23.29%), and T1 (15.07%). A large proportion of the patients had regional or locoregional involvement (52.05%), and 35/73 (47.95%) were free of lymph node involvement. Distant metastases were not observed in these samples. Following the AJCC criteria, patients were categorised as stage IV tumours in 46.57% of the cases, stage III in 27.40%, stage II in 15.07%, and stage I in 10.96%.

Regarding oral leukoplakia, 60 cases were retrieved (30 with MD and 30 with MSD). Of these samples, 28 (46.67%) were male and 32 (53.33%) female. An average age of 55.63 years was observed, with a range of 14–84 years old. The proportion of smokers/non-smokers was 25/33 (ratio 1:1.3). The majority of the cases affected the tongue (53.33%), followed by the floor of the mouth (16.67%).

Immunohistochemical staining

PTEN immunoreaction showed, predominantly, a cytoplasmatic staining. However, nuclear staining was sometimes observed. In normal oral mucosa and dysplastic tissues, the immunoreaction was predominantly found in the parabasal and basal layers, whereas OSCC showed a diffuse distribution in neoplastic islands (Fig. 1).

In the C group, 35% (7 cases) were positive for PTEN immunoexpression, whereas 65% (13 cases) were negative. In the oral leukoplakia group, MD showed that 43.33% (13 cases) were positive and 56.67% (17 cases) were negative, whereas MSD showed positivity in 80% of the cases (24 cases) and 20% (6 cases) demonstrated negativity. Regarding OSCC samples, 63.01% (46 cases) were positive and 36.99% (27 cases) were negative.

PTEN expression was higher in MSD (p=0.002) and OSCC (p=0.0259) compared with the C group. Additionally, a higher expression was observed in MSD (p=0.0035) and OSCC (p=0.049) than MD (Table 4).

Fluorescence in situ hybridisation analysis

All samples of normal epithelium did not demonstrate allelic loss. In MD, only 3.33% (1/30) showed homozygous deletion; in MSD, 3.33% (1/30) showed PTEN hemizygous (single copy) deletion, whereas 10% (3/30) showed homozygous deletion. In OSCC samples, single copy loss was observed in 13.70% (10/73) of the cases, and biallelic loss occurred in 20.55% (15/73) (Fig. 2). Statistically, a higher hemizygous loss was observed in OSCC than C (p=0.0467) and OSCC than MD (p=0.0175), as well as a higher homozygous deletion in OSCC than C (p=0.0159) and OSCC than MD (p=0.0145) (tables 5 and 6).

DISCUSSION

Despite the advances in therapeutic approaches, the morbidity and mortality associated with OSCC have not significantly improved in the last 30 years, with the five-year overall survival rate varying between 30% and 50%[13]. OSCC may be preceded by a potentially malignant lesion, and the presence of epithelial dysplasia seems to be the most important prognostic indicator of its potential for malignant transformation[20]; however, the incidence of malignant change varies in different studies, possibly as a result of differences in ethnic and environmental factors[6]. Moreover, the low reproducibility of current histological criteria for grading epithelial dysplasia is largely recognised and remains to be improved. These facts highlight the necessity of identifying molecular markers that could better predict oral leukoplakia aggressiveness and clinical course. For this reason, it is crucial to understand the deregulated molecular pathways that govern the progression of oral carcinogenesis[6, 21, 22.]

PTEN is a tumour suppressor that opposes PI3K function by dephosphorylating PIP3 to regenerate PIP2. It is believed that downregulation of PTEN leads to an accumulation of PIP3, leading to increased activation of PI3K and pAkt. pAkt participates in the regulation of the cell cycle, proliferation, apoptosis, cell adhesion, migration, invasion, and metastasis during cancer progression by regulating various downstream substrates.

Therefore, in this study, we investigated the allelic loss of PTEN and its immunoexpression in dysplastic oral lesions and in OSCC. We employed the binary system to graduate dysplasia because this system has been proven to be a reliable tool, reducing

variability and enhancing the reproducibility of epithelial dysplasia[3, 23]. Our results revealed that PTEN expression was higher in MSD (p=0.002) and in OSCC (p=0.0259) when both compared with the C group; moreover, a higher expression was observed in MSD (p=0.0035) and OSCC (p=0.049) than MD. In addition, a higher hemizygous loss was observed in OSCC than C (p=0.0467) and OSCC than MD (p=0.0175), as well as a higher homozygous deletion in OSCC when compared with C (p=0.0159) and in OSCC when compared with MD (p=0.0145). To the best of our knowledge, this is the first study analysing the expression and PTEN allelic loss in oral leukoplakias and in OSCC together.

The molecular mechanism underlying malignant transformation of leukoplakias has not been elucidated yet. Now, it is widely accepted that there are divergent molecules and pathways that sequentially affect the process of transformation from leukoplakia to oral squamous cell carcinoma[24, 25]. Recently, the tumor microenvironment showed to carry out an important role in oral leukoplakia and oral cancer progression[26, 27]. In addition, several factors including age, sex, smoking, oral subsite, as well as dysplasia grade have been suggested as predictors of the risk of malignant transformation of oral leukoplakia[26, 27, 28]. These lines of evidence suggest that carcinogenic transformation of a leukoplakia is multifactorial and is patient specific[29]. Then, it is possible that inter-individual and inter-population differences in risk could be partially explained by different distributions of genetic variants that may cause variation in the ability to metabolize carcinogens and/ or effective repair of the damage caused by them[30]. The above observations contribute to understanding of 20% of negative expression of PTEN in MSD group and 37% of negative expression in OSSC group. We must keep in mind also that some oral cancers can to develop from lesions that lack dysplastic changes[31, 32]. On this basis, we can explain, in some measure, the proportion of cases in the C group with PTEN expression positive.

Recently, Chaves et al. studied p-Akt function in oral epithelial dysplasia and in OSCC, observing that p-Akt immunostaining was significantly higher in the malignancy than in dysplasias and controls[15]. Other studies investigated p-Akt in the process of malignant transformation of oral epithelium, suggesting that activation of the PI3K-AKT signal pathway is involved in the malignant phenotype acquisition process since its early stages[6, 7, 33]. In addition, Akt expression was shown to be a prognostic determinant for patients affected by OSCC[13, 34]. In this regard, in our work, the increase in PTEN expression observed in the evolution of dysplastic lesions can be understood as an attempt to control the increased expression of Akt described in several studies of oral dysplastic lesions[6, 7, 15, 33]. In addition, a decrease in PTEN expression was observed in OSCC. Reduced PTEN protein expression in

OSCC has also been observed in previous studies[21, 35, 36] suggesting a PTEN functional inactivation in this malignancy.

Here we also investigated the loss of PTEN copy number in oral dysplastic lesions. Our results showed that the loss of PTEN copy number in oral leukoplakias can represent an important mechanism for the development of oral potentially malignant lesions into progression to oral cancer when the loss of PTEN copy number occurred in 34.25% of our sample (10 hemizygous deletion and 15 homozygous deletion). As PTEN is a tumour suppressor gene, for its complete inactivation there is a need for both copies to be deleted. In the cases of OSCC in which there was hemizygous deletion, the evaluation of the mutation or hypermethylation of the PTEN promoter region in the remaining alleles may elucidate whether the loss of PTEN expression in OSCC samples would be occurring through genetic or epigenetic events. Some authors have already addressed this issue, demonstrating that mutation in PTEN is a rare event in HNSCC[36-41], whereas others have shown that hypermethylation of the PTEN promoter region is frequent in this tumour[36, 42], which is an important mechanism of epigenetic silencing that may contribute to cancer.

Some papers highlight that homozygous deletion is unlikely to play a key role in the PTEN inactivation process in OSCC[43, 44]. However, in this study biallelic loss occurred in 20.55% of the OSCC group. This homozygous loss of PTEN alleles was statistically higher in OSCC samples when compared with lesions that presented MD and the C group. An important increase in loss of both alleles was observed in OSSC when compared with MSD, although there was no statistically significant difference.

In conclusion, taking into consideration the fact that longitudinal studies are more indicated to attribute predictive value for malignant transformation, this cross sectional study to permit only to speculate, to some extent, that PTEN biallelic loss can be an important mechanism in the late stage of development of oral potentially malignant lesions into oral cancer. Moreover, we observed an increase in the expression of PTEN in the dysplastic lesions, suggesting an attempt of PTEN to control the increased expression of Akt. After the acquisition of the malignant phenotype, a decrease in PTEN expression was observed, which may be explained by the increased deletion in this group.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest

The authors declare that they have no conflict of interest.

Funding

Coordination for the Improvement of Higher Education Personnel (CAPES/PROEX) process number 33003033009P4

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

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TABLES

Table 1. Clinicopathological profile of OSCC samples (n=73).

Characteristics	Data (%)
Age (y)	
Average	61.13
Range	33- 87
Sex	
Male/ female	44/ 29 (60.27%/ 39.73%)
Tobacco consumption	
Smoker/nonsmoker	51/ 22 (69.86%/ 30.14%)
TNM classification	
T classification	
T1	11 (15.07%)
T2	17 (23.29%)
T3	21 (28.77%)
T4	24 (32.87%)
N classification	
N0/ pN+	35/ 38 (47.95%/ 52.05%)
N1	16
N2	19
N3	3
M classification	
M0	73 (100%)
M1	0 (0%)
Staging	
I	8 (10.96%)
II	11 (15.07%)
III	20 (27.40%)
IV	34 (46.57%)
Anatomic location	
Floor of the mouth	27 (36.99%)
Tongue	23 (31.50%)
Alveolar ridge	14 (19.18%)
Palate	5 (6.85%)
Jugal Mucosa	3 (4.11%)
Retromolar region	1 (1.37%)

Table 2. Clinicopathological profile of Leukoplakia samples (n=60).

Characteristics	Data (%)
Age (y)	
Average	55.63
Range	14- 84
Sex	
Male/ female	28/32 (46.67%/ 53.33%)
Tobacco consumption	
Smoker/nonsmoker	25/ 33 (41.67%/ 55%)
Not specified	2 (3.33%)
Anatomic location	
Tongue	32 (53.33%)
Floor of the mouth	10 (16.67%)
Others (Jugal mucosa, retromolar region, palate, attached gingiva, alveolar ridge	18 (30%)

Table 3. Clinicopathological profile of control samples (n=20).

Characteristics	Data (%)
Age (y)	
Average	18.4
Range	3- 45
Sex	
Male/ female	9/11 (45%/55%)
Tobacco consumption	
Nonsmoker	20 (100%)
Anatomic location	
Floor of the mouth	20 (100%)

Table 4: Chi-square test. p-value combination among groups at immunohistochemistry. Abbreviations: C (control group), MD (Mild dysplasia), MSD (Moderate/severe dysplasia), OSCC (Oral Squamous Cell Carcinoma), NS (not significant).

	C	**MD**	**MSD**	**OSCC**
C	---	NS	0.002	0.0259
MD	NS	---	0.0035	0.049
MSD	NS	NS	---	NS
OSCC	NS	NS	NS	---

Table 5: Chi-square test. p-value combination among groups at hemizygous deletion. Abbreviations: C (control group), MD (Mild dysplasia), MSD (Moderate/severe dysplasia), OSCC (Oral Squamous Cell Carcinoma), NS (not significant).

	C	**MD**	**MSD**	**OSCC**
C	---	NS	NS	0.0467
MD	NS	---	NS	0.0175
MSD	NS	NS	---	NS
OSCC	NS	NS	NS	---

Table 6: Chi-square test. p-value combination among groups at homozygous deletion. Abbreviations: C (control group), MD (Mild dysplasia), MSD (Moderate/severe dysplasia), OSCC (Oral Squamous Cell Carcinoma), NS (not significant).

	C	**MD**	**MSD**	**OSCC**
C	---	NS	NS	0.0159
MD	NS	---	NS	0.0145
MSD	NS	NS	---	NS
OSCC	NS	NS	NS	---

FIGURES

Figure 1: Immunoexpression of PTEN in normal, dysplastic, and malignant epithelium (streptavidin-biotin, x200 original magnification). (A) Normal oral epithelium, (B) epithelium with mild dysplasia, (C) epithelium with moderate to severe dysplasia, (D) oral squamous cell carcinoma.

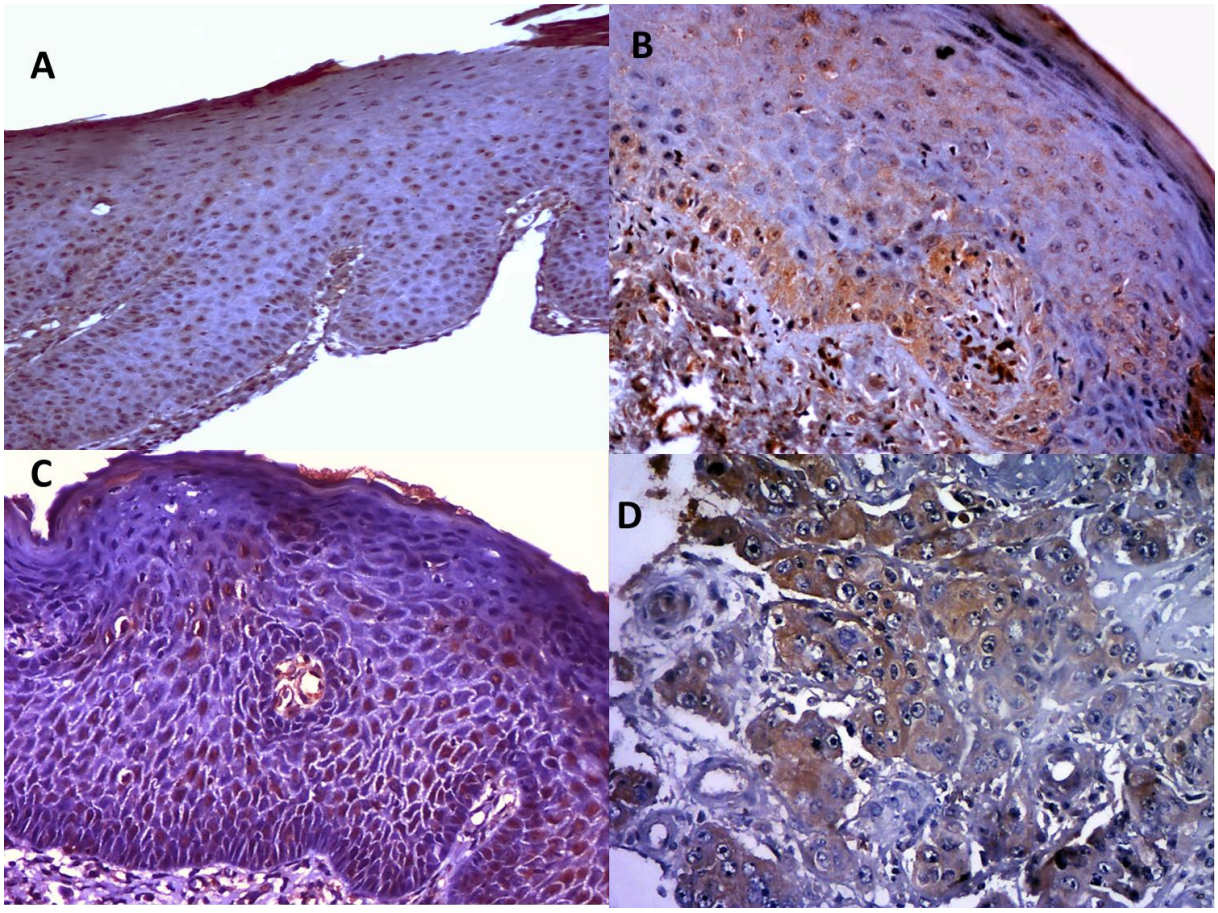
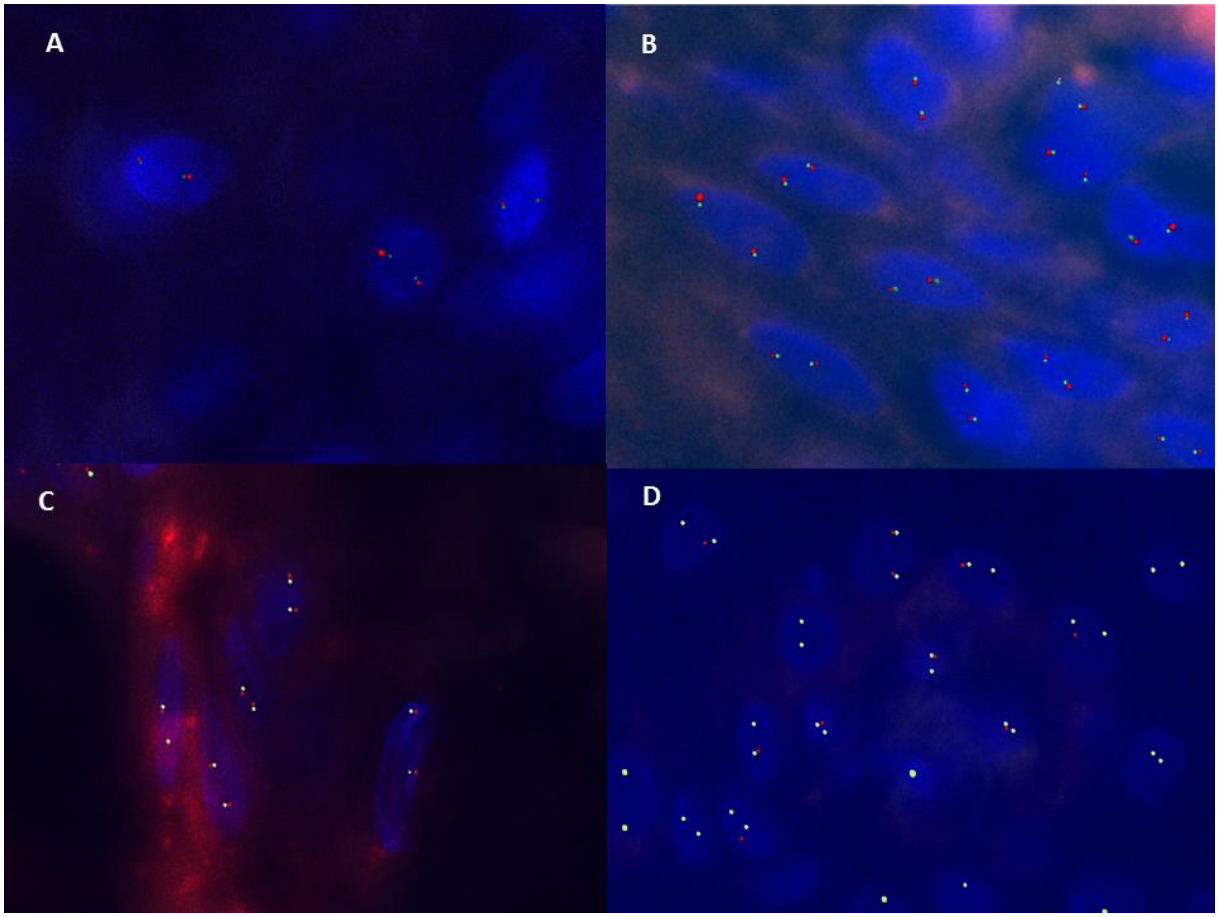


Figure 2: Vysis LSI *PTEN* SpectrumOrange/CEP 10 SpectrumGreen Probes hybridise to band 10q23 (SpectrumOrange LSI *PTEN*) and to the centromere, band region 10p11.1–q11.1 (SpectrumGreen CEP 10) of human chromosome 10. FISH image of *PTEN* in normal, dysplastic, and malignant epithelium (x100 original magnification). (A) Normal oral epithelium, (B) epithelium with mild dysplasia, (C) epithelium with moderate to severe dysplasia, (D) oral squamous cell carcinoma.



3 CONCLUSÕES

✓ O aumento da expressão de PTEN nas lesões displásicas sugere uma tentativa de PTEN em controlar o aumento da expressão de Akt, visando o reparo do DNA ou de conter a progressão do processo de carcinogênese.

✓ A diminuição da expressão de PTEN após a aquisição do fenótipo maligno pode ser explicada pelo aumento na quantidade de deleções observado nesse grupo.

✓ A perda bialélica de *PTEN* pode ser um importante mecanismo nos estágios finais de desenvolvimento na progressão de lesões orais potencialmente malignas ao câncer bucal.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed

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ANEXOS

Anexo 1 – Parecer Consubstanciado do CEP

HOSPITAL UNIVERSITÁRIO JOÃO DE BARROS BARRETO - UFPA

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ANÁLISE DA IMUNOEXPRESSÃO DA PROTEÍNA PTEN E DA PRESENÇA DE MUTAÇÃO NO GENE PTEN PELO MÉTODO DE FISH EM AMOSTRAS DE CARCINOMAS EPIDERMOIDES DE BOCA E DE LEUCOPLASIAS COM DIFERENTES GRAUS DE DISPLASIA

Pesquisador: HÉLDER ANTÔNIO REBELO PONTES

Área Temática:

Versão: 1

CAAE: 52465315.8.0000.0017

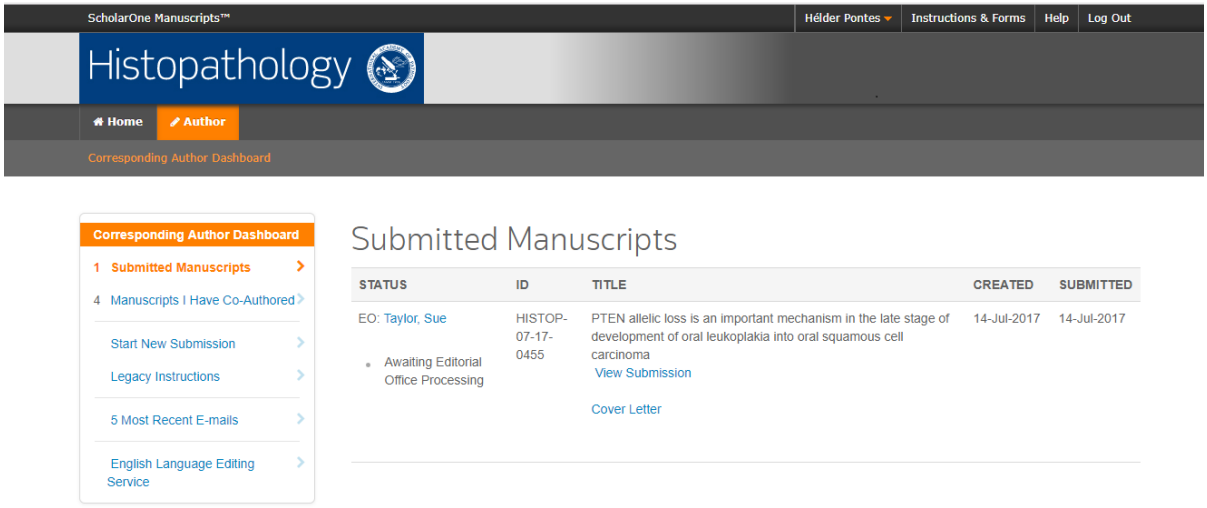
Instituição Proponente: Hospital Universitário João de Barros Barreto - UFPA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.426.757

Anexo 2 - Certificado de submissão ao periódico



Histopathology. 2017 Aug 31. doi: 10.1111/his.13381. [Epub ahead of print]

PTEN allelic loss is an important mechanism in the late stage of development of oral leukoplakia into oral squamous cell carcinoma.

Miyahara LAN[1], Pontes FSC[2], Burbano RMR[2], Conte Neto N[2], Guimarães DM[2], Fonseca FP[3], Pontes HAR[1,2].

Author information

Abstract

OBJECTIVES: The aim of this study is to analyse allelic loss of the PTEN gene and its protein immunoexpression in dysplastic oral lesions and oral squamous cell carcinomas (OSCCs).

MATERIALS AND METHODS: Samples were collected from 153 patients (20 ranulas used as a control - C, 30 leukoplakias with mild dysplasia - MD, 30 leukoplakias with moderate to severe dysplasia - MSD, and 73 OSCC). PTEN protein expression was investigated using immunohistochemistry, and PTEN allelic loss was analysed by fluorescence in situ hybridisation (FISH). Differences among groups were evaluated using the Chi-square test.

RESULTS: PTEN expression was higher in MSD (p=0.002) and OSCC (p=0.0259) compared with the C group; additionally, a higher expression was observed in MSD (p=0.0035) and OSCC (p=0.049) than MD. Regarding FISH analysis, a higher hemizygous (single copy) loss was observed in OSCC than C (p=0.0467) and OSCC than MD (p=0.0175), as well as a higher homozygous deletion in OSCC compared with C (p=0.0159) and OSCC than MD (p=0.0145).

CONCLUSION: The results of this work suggest that PTEN allelic loss is an important mechanism in the late stage of the development of oral potentially malignant lesions into oral cancer. This article is protected by copyright. All rights reserved.

KEYWORDS: Gene Deletion; Immunohistochemistry; Leukoplakia; Oral cancer; PTEN Phosphatase