ALINE CRISTINA BATISTA RODRIGUES JOHANN

IMUNO-EXPRESSÃO DA METALOTIONEÍNA EM CISTOS E TUMORES ODONTOGÊNICOS

Metallothionein immunoexpression in odontogenic cysts and in odontogenic tumours

BELO HORIZONTE

FACULDADE DE ODONTOLOGIA DA UNIVERSIDADE FEDERAL DE MINAS GERAIS

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Tese apresentada ao Colegiado de Pós-graduação da Faculdade de Odontologia da Universidade Federal de Minas Gerais, como requisito parcial para obtenção do titulo de Doutor em Odontologia.

Área de concentração: Patologia Bucal.

Orientador: Prof. Dr. Ricardo Alves Mesquita

Co-orientadora: Profa. Dra. Maria Cássia Ferreira de Aguiar

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Tese intitulada **"Imuno-expressão da metalotioneína em cistos e tumores odontogênicos"**, área de concentração Patologia Bucal, apresentada pela candidata **Aline Cristina Batista Rodrigues Johann**, para obtenção do grau de **Doutor em Odontologia**, **APROVADA** pela Comissão Examinadora constituída pelos seguintes professores:

Prof. Dr. Ricardo Alves de Mesquita
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Profa. Dra. Maria Cássia Ferreira de Aguiar
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Prof. Dr. Saul Martins de Paiva
Coordenador do Colegiado do
Programa de Pós-Graduação em Odontologia

Belo Horizonte, 21 de dezembro de 2009

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Ata da Comissão Examinadora para julgamento da Tese de Doutorado em Odontologia, área de concentração em Patologia Bucal, da candidata **Aline Cristina Batista Rodrigues Johann**. Aos 21 (vinte e um) dias do mês de dezembro de 2009, às 14h00min, na sala de Pós-Graduação (3403) da Faculdade de Odontologia, reuniu-se a Comissão Examinadora, composta pelos professores Prof. Dr. Ricardo Alves de Mesquita – Orientador – FO-UFMG, Profa. Dra. Maria Cássia Ferreira de Aguiar – Co-Orientadora – FO-UFMG, Profa. Dra. Diele Carine Barreto Arantes – Instituto Cultural Newton Paiva Ferreira Ltda, Prof. Dr. Martinho Campolina Rebello Horta – PUC-Minas, Prof. Dr. Marcelo Vidigal Caliari – ICB-UFMG e Prof. Dr. Ricardo Santiago Gomez – FO-UFMG. A Profa. Dra. Maria Cássia Ferreira de Aguiar, Sub-Coordenadora do CPGO abriu os trabalhos, apresentou a Comissão Examinadora e passou a presidência da sessão ao orientador da Tese, Prof. Ricardo Alves de Mesquita. A candidata foi dado o tempo de até 50 (cinquenta) minutos para fazer a exposição oral sobre o seu trabalho **"Imuno-expressão da metalotioneína em cistos e tumores odontogênicos".** Encerrada a exposição, foi iniciada a argüição, dentro do limite de tempo de 30 (trinta) minutos, pelos Professores Diele Carine Barreto Arantes, Martinho Campolina Rebello Horta, Marcelo Vidigal Caliari, Ricardo Santiago Gomez, Ricardo Alves de Mesquita e Maria Cássia Ferreira de Aguiar, com limite de 30 (trinta) minutos para a resposta. Terminadas as argüições, o presidente suspendeu os trabalhos por 10 minutos para que os examinadores pudessem decidir pelo resultado a ser dado à candidata. A Comissão Examinadora opta pela aprovação da candidata. Para constar, lavrou-se a presente ata, que vai assinada por mim e pela comissão Examinadora. Belo Horizonte, 21 de dezembro de 2009. Elizabeth Soares Teles Noronha, Secretária do Colegiado do Programa de Pós-Graduação em Odontologia.

Prof. Dr. Ricardo Alves de Mesquita
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DEDICATÓRIA

Ao Ricardo Johann, meu amor, por iluminar a vida com seu sorriso.

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À Deus por guiar o meu caminho.

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PREFÁCIO

O trabalho foi estruturado em duas partes, sendo que a primeira compreende uma introdução, uma revisão de literatura objetiva contendo os dados mais relevantes da literatura, justificativa, objetivos, metodologia e referências bibliográficas. Na segunda parte são expostos três artigos e as considerações finais. As normas das revistas selecionadas para a submissão dos artigos 1 e 2 (Anexo B, página 119) e do artigo 3 (Anexo C, página 133) encontram-se nos anexos.

ABREVIATURAS E SIGLAS

AB	-Ameloblastoma
ABS	-Ameloblastoma sólido
CAPES	-Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior
CD	-Cisto dentígero
CNPq	-Conselho Nacional de Desenvolvimento Científico e Tecnológico
COEP	-Comitê de Ética em Pesquisa
COO	-Cisto odontogênico ortoceratinizado
CR	-Cisto radicular
DAB	-Diaminobenzidina
DNA	-Ácido Desoxirribonucléico
EUA	-Estados Unidos da América
FO	-Faculdade de Odontologia
HE	-Hematoxilina e eosina
IK	-Índice de Ki-67
IMT	-Índice de metalotioneína
IP	-Índice de PCNA
MCF7	-Células do carcinoma de mama humano
mRNA	-Ácido ribonucleico mensageiro
MT	-Metalotioneína
MT-I	-Metalotioneína do tipo 1
MT-I+II	-Expressão simultânea da MT-I e MT-II
MT-II	-Metalotioneína do tipo 2
MT-III	-Metalotioneína do tipo 3
MT-IV	-Metalotioneína do tipo 4
NF-κB	-Fator nuclear Kappa B
OMS	-Organização Mundial de Saúde
PCNA	-Antígeno nuclear de proliferação celular
SCBN	-Síndrome do Carcinoma Basocelular Nevóide
TOA	-Tumor odontogênico adenomatóide
TOC	-Tumor odontogênico ceratocístico
TOCC	-Tumor odontogênico cístico calcificante
TOE	-Tumor odontogênico escamoso
TOEC	-Tumor odontogênico epitelial calcificante
TRIS-HCL	-Hidroximetil amino metano e cloreto de sódio
TUNEL	-Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UFMG	-Universidade Federal de Minas Gerais

ÍNDICE

RESUMO

Cistos e tumores odontogênicos são lesões originadas dos tecidos que formam os dentes e apresentam diferentes comportamentos biológicos. A metalotioneína (MT) é relacionada à homeostase de metais, regulação da diferenciação e proliferação celular e inibição da apoptose. Com relação aos cistos e tumores odontogênicos, a MT poderia ter um papel na regulação da diferenciação e proliferação celular e na inibição da apoptose, refletindo no comportamento biológico. Os objetivos são avaliar e comparar a expressão da MT entre: 1) cistos odontogênicos e tumor odontogênico ceratocístico (TOC); 2) TOC associados à Síndrome do Carcinoma Basocelular Nevóide (SCBN) e não associados; e 3) tumores odontogênicos benignos. Objetivou-se também correlacionar a imuno-expressão da MT com a proliferação celular e com a inflamação.

A amostra incluiu cisto radicular (CR), cisto dentígero (CD), TOC (primário associado ou não à SCBN), cisto odontogênico ortoceratinizado (COO), ameloblastoma sólido (ABS), tumor odontogênico escamoso (TOE), tumor odontogênico adenomatóide (TOA), tumor odontogênico cístico calcificante (TOCC) e tumor odontogênico epitelial calcificante (TOEC). Foi realizada imunoistoquímica para MT, Ki-67 e PCNA. Os índices de MT (IMT), Ki-67 (IK) e PCNA (IP) foram obtidos. Células inflamatórias foram contadas nos cistos odontogênicos, nos TOCs e nos ABSs, sendo os primeiros agrupados em grau: discreto a moderado (grupo A) e intenso (grupo B). A análise estatística foi realizada com o programa BioEstat® 4.0.

O IMT foi mais alto no CR, seguido pelo CD, TOC e COO; e as diferenças foram significantes, exceto entre o CR e o CD. O IK foi maior no TOC, seguido pelo COO, CR e CD; e as diferenças foram significantes entre o TOC e as demais lesões. O IMT foi inversamente correlacionado com o IK no TOC, e no COO, mas positivamente no CR e no CD. Nenhuma diferença no IMT foi observada entre o grupo A e B. O IMT foi variável entre as lesões e isto pode ser devido ao seu papel na diferenciação celular e na inibição da apoptose. A correlação da MT com a proliferação celular parece ser inversa no TOC no COO, mas direta no CR e CD. O IMT parece não ser influenciado pela inflamação. O TOC não associado à SCBN apresentou um IMT mais alto do que o associado, já o IK foi similar.

Uma inversa correlação foi verificada entre o IMT e IK. Os grupos A e B mostraram IMT similar. Os casos de TOC associados à SCBN apresentam um diferente imuno-fenótipo com menor MT comparado com os não associados, que pode ser contribuitório na apoptose celular. O IMT parece não ser influenciado pela inflamação. Nos tumores odontogênicos benignos, o IMT foi mais alto no ABS, seguido pelo TOCC, TOE, TOA e ausente no TOEC. Diferenças significantes foram vistas entre o ABS e o TOE, TOA e TOCC. O IK foi mais alto no ABS, seguido pelo TOE, TOA, TOEC e TOCC. O IP foi mais alto no ABS, seguido pelo TOE, TOEC, TOCC e TOA. Tanto para o IK quanto para o IP, diferenças significantes foram observadas entre o ABS e o TOA, e ABS e TOCC. Foi observada correlação positiva entre IMT e IK, e IMT e IP no ABS, TOE e TOCC, e inversa no TOA. No ABS foi identificada uma correlação positiva entre a inflamação e o IMT. O variável IMT entre as lesões pode ser possivelmente devido ao seu papel na diferenciação celular e/ou comportamento biológico. A correlação da MT com a proliferação celular parece ser inversa no TOA, mas direta no ABS, TOE e TOCC. No ABS, o IMT parece sofrer influência da inflamação.

O presente estudo revela diferenças na expressão da MT nas diversas lesões odontogênicas, que foram associadas com diferenças na diferenciação e apoptose celular. Além disso, a MT foi positivamente correlacionada com a proliferação nos cistos e tumores odontogênicos, com exceção do TOC, COO e TOA. A inflamação pode influenciar a MT no ABS, mas não nos cistos odontogênicos e no TOC.

Palavras chave: ameloblastoma, antígeno Ki-67, cisto odontogênico, imuno-histoquímica, metalotioneína, Síndrome do carcinoma basocelular nevóide, tumor odontogênico.

ABSTRACT

Odontogenic cysts and tumours are lesions which originate from tooth-forming tissues and present different biological behaviors. Metallothionein (MT) is related to homeostasis of metals, regulation of cellular differentiation and proliferation, and inhibition of apoptosis. In odontogenic cysts and tumours, MT could have a role in the regulation of cellular proliferation and differentiation, and in inhibition of apoptosis, though interfering in their biological behavior. The aims are to evaluate and to compare MT expression among: 1) odontogenic cysts and keratocystic odontogenic tumour (KOT); 2) KOT associated with nevoid basal cell carcinoma syndrome (NBCCS) and not associated one; and 3) benign odontogenic tumours. Also, the correlation of MT immunoexpression with cellular proliferation and inflammation was assessed.

Cases of radicular cyst (RC), dentigerous cyst (DC), KOT (primary, associated or not with NBCCS), orthokeratinized odontogenic cyst (OOC), solid ameloblastoma (SAB – plexiforme and follicular types), squamous odontogenic tumour (SOT), adenomatoid odontogenic tumour (AOT), calcifying cystic odontogenic tumour (CCOT) and calcifying epithelial odontogenic tumour (CEOT) were submitted to immunohistochemistry for MT, Ki-67 and PCNA. The index of MT (IMT), Ki-67 (IK) and PCNA (IP) was calculated. Counting of inflammatory cells was also performed in odontogenic cysts, KOT and SAB. Odontogenic cysts and KOT were grouped into group A (weak to moderate inflammation) or group B (strong). BioEstat® 4.0 was used for statistical analysis.

The highest IMT was observed in RC followed by DC, KOT and OOC. Differences were not significant only between RC and DC. The highest IK was observed in KOT followed by OOC, RC, and DC. Differences were significant between KOT and all other lesions. IMT was inversely correlated with IK in KOT and OOC, but positively in RC and DC. No difference of IMT was observed between groups A and B. IMT was variable among lesions and this can be attributed to its role in cellular differentiation and inhibition of apoptosis. The correlation of MT with cellular proliferation seems to be inverse in KOT and OOC, but direct in RC and DC. IMT do not seem to be modified by inflammation. Non-syndromic KOT showed a higher IMT

than syndromic ones, but IK was similar. An inverse correlation between IMT and IK was noted. No difference in IMT was observed between groups A and B. Syndromic KOT showed a different immunofenotype with lower MT than non-syndromic ones, which may contribute more in apoptosis than in cellular proliferation. Besides, IMT did not seem to be influenced by inflammation. In benign odontogenic tumours, the highest IMT was observed in SAB, followed by CCOT, SOT, AOT, and it was absent in CEOT. Significant differences were observed between SAB and the lesions SOT, AOT and CCOT. The highest IK was observed in SAB, followed by SOT, AOT, CEOT, and CCOT. The highest IP was observed in SAB, followed by SOT, CEOT, CCOT and AOT. For IK and IP, significant differences were observed between SAB and AOT, SAB and CCOT. A positive correlation between IMT and IK, and IMT and IP was observed in SAB, SOT and CCOT, but the correlation was inverse in AOT. In SAB, a positive correlation between inflammation and IMT was noticed. This variable IMT among lesions may possibly be due to its role in cellular differentiation and/or biological behavior. The correlation between MT and cellular proliferation seems to be inverse in AOT, but direct in SAB, SOT, and CCOT. In SAB, IMT seems to be influenced by inflammation.

The present study reveals differences in MT immunoexpression among the odontogenic lesions evaluated. This finding can be associated with differences in cellular differentiation and apoptosis. Correlation of IMT and cellular proliferation did seem to be positive in odontogenic cysts and tumours, except in KOT, OOC and AOT. Besides, inflammation did not modify MT immunoexpression in odontogenic cysts and KOT. A positive correlation was observed between inflammation and MT expression in SAB.

Key-words: ameloblastoma, Ki-67 antigen, odontogenic cyst, immunohistochemistry, metallothionein, nevoid basal cell carcinoma syndrome, odontogenic tumour.

1 INTRODUÇÃO

Cistos e tumores odontogênicos são lesões originadas dos tecidos que formam os dentes. Cistos odontogênicos são lesões freqüentes na boca e correspondem a cavidades císticas revestidas por epitélio odontogênico e apresentam baixa taxa de recorrência e comportamento clínico não agressivo. Tumores odontogênicos são lesões encontradas em menor freqüência na cavidade bucal e correspondem a hamartomas, neoplasias benignas e malignas, que apresentam diferentes comportamentos biológicos (Regezi *et al.*, 1978; Kramer *et al.*, 1992; Barnes *et al.*, 2005). A metalotioneína (MT) é uma proteína cujas principais funções são: 1) regulação da homeostase e detoxificação de metais, 2) regulação da diferenciação e divisão celular e 3) inibição da apoptose celular (Hamer, 1986; Cherian *et al.*, 1993; Cherian *et al.*, 2003; Vasák, 2005). Na cavidade bucal, a imuno-expressão da MT tem sido avaliada em lesões malignas (Szelachowska *et al.*, 2008; Cardoso *et al.*, 2009), lesões potencialmente malignas (Johann *et al*, 2008; Pontes *et al*, 2009), periodontite (Katsuragi *et al.*, 1997) e tatuagem por amálgama (Leite *et al.*, 2004). Com relação aos cistos e tumores odontogênicos, a MT poderia ter um papel na regulação da diferenciação e proliferação celular e na inibição da apoptose, refletindo no comportamento biológico dessas lesões. Entretanto, não existem estudos que avaliem a expressão da MT nessas lesões. Além disso, a proteína Ki-67 e o antígeno nuclear de proliferação celular (PCNA) são indicadores de proliferação celular que têm sido utilizados na avaliação de cistos e tumores odontogênicos (Kichi *et al.*, 2005; Barboza *et al.*, 2005; Mateus *et al.*, 2008; Gadbail *et al.*, 2009; Rizzardi *et al.*, 2009). A expressão do Ki-67 e do PCNA tem sido correlacionada com a da MT em lesões malignas e benignas (Ioachim *et al.*, 1999**a;** Hishikawa *et al.* 1999; Zagorianakou *et al.,* 2006; Athanassiadou *et al.*, 2007), no entanto, não existem estudos que façam esta correlação nos cistos e tumores odontogênicos. Os objetivos desse estudo são avaliar e comparar a expressão da MT entre: 1) os cistos odontogênicos e o tumor odontogênico ceratocístico (TOC), 2) casos de TOC associados à Síndrome do Carcinoma Nevóide Basocelular (SCNB) e não associados e 3) tumores odontogênicos benignos.

Objetivou-se ainda correlacionar a imuno-expressão da MT com a proliferação celular e com a inflamação.

2 REVISÃO DE LITERATURA

2.1 Cistos odontogênicos

Segundo a Organização Mundial de Saúde (OMS - 1992), os cistos odontogênicos são cavidades císticas revestidas por epitélio, podendo ser subclassificados em: 1) inflamatório que incluem os cistos radiculares (CR) e o paradentário; e 2) de desenvolvimento - cisto dentígero (CD), cisto de erupção, cisto gengival do recém nascido, cisto gengival do adulto, cisto periodontal lateral, cisto odontogênico glandular e ceratocisto odontogênico (Kramer *et al.*, 1992). O ceratocisto odontogênico foi reclassificado pela OMS em 2005 como um tumor odontogênico, sendo denominado de tumor odontogênico ceratocístico (TOC). Essa mudança reflete o seu comportamento clínico agressivo, a alta taxa de recorrência, o padrão destrutivo de crescimento e a sua associação com a Síndrome do Carcinoma Basocelular Nevóide (SCBN), uma rara condição autossômica dominante caracterizada por múltiplos carcinomas de células basais, alterações esqueletais como costelas bífidas ou fusionada, múltiplos TOCs e disqueratose palmo-plantar. Há também o cisto odontogênico ortoceratinizado (COO) que foi classificado (OMS, 2005) como uma entidade distinta do TOC (Philipsen, 2005). Dentre os cistos odontogênicos, serão de interesse para o presente estudo o CR, CD e COO. Mesmo após a classificação da OMS (2005), estudos imunoistoquímicos permaneceram comparando o TOC com os cistos odontogênicos (de Oliveira *et al.*, 2008; Mateus *et al.*, 2008; Rangiani & Motahhary, 2009).

O COO é uma rara lesão possivelmente oriunda da lâmina dentária e de seus remanescentes (Li *et al.*, 1998), que apresenta comportamento clínico menos agressivo comparado ao TOC, além de potencial de crescimento limitado e menor taxa de recorrência (Wright, 1981). O achado histológico é cavidade cística revestida por epitélio pavimentoso estratificado ortoceratinizado, de poucas camadas de células, com camada granular bem desenvolvida e camada basal composta por células pavimentosas ou cuboidais, mostrando

pouca tendência à polarização ou organização em paliçada, além da cápsula fibrosa (Wright, 1981).

Os CRs são cistos inflamatórios oriundos de remanescentes epiteliais do ligamento periodontal (remanescentes epiteliais de *Malassez*), representando a lesão cística mais freqüente nos maxilares (Kramer *et al.*, 1992). A lesão pode se desenvolver a partir de uma necrose pulpar ocasionando inflamação no periápice ou na porção lateral do dente. Esta inflamação pode estimular os remanescentes epiteliais de *Malassez* a se proliferarem. Três teorias foram propostas para explicar a formação da cavidade cística a partir dessa proliferação epitelial: 1) o ninho epitelial cresce de forma que a porção central degenera devido à deficiência de nutrientes, 2) um abscesso ocorre no tecido conjuntivo, o qual é posteriormente revestido por epitélio, ou 3) cordões epiteliais crescem aprisionando o tecido conjuntivo que se degenera (Lin *et al.*, 2007). Histopatologicamente, o CR corresponde a uma cavidade cística revestida por epitélio pavimentoso estratificado não ceratinizado. A cápsula da lesão é constituída por tecido conjuntivo fibroso denso, com quantidades variáveis de vasos sangüíneos e de infiltrado inflamatório (Kramer *et al.*, 1992).

O CD compreende de 14 a 33% dos cistos maxilares (Kreidler *et al.*, 1993; Ochsenius *et al.*, 2007) e apresenta-se sempre associado à coroa de um dente incluso. Várias patogêneses têm sido propostas: 1) a pressão exercida por um dente em erupção em um folículo impactado pode obstruir o retorno venoso, induzindo transudação do soro e causar um acúmulo de fluido entre o epitélio reduzido do esmalte ou entre as camadas do órgão do esmalte, culminando na formação do cisto; 2) a inflamação do ápice radicular de um dente decíduo pode levar ao desenvolvimento de um cisto ao redor do dente permanente; 3) a coroa do dente permanente erupciona dentro de um CR oriundo do dente decíduo (Benn & Altini, 1996). Histopatologicamente observa-se cavidade cística revestida por epitélio estratificado pavimentoso não ceratinizado de espessura variável apresentando áreas semelhantes ao epitélio reduzido do esmalte. Na cápsula da lesão, observam-se graus variáveis de inflamação (Shear, 1994). O prognóstico é bom e são raras as recorrências (Motamedi & Talesh, 2005).

2.2 Tumores odontogênicos benignos

Segundo a OMS (2005), tumores odontogênicos compreendem um grupo de lesões raras que derivam dos tecidos que formam os dentes. Essas lesões apresentam taxas de recorrência e potencial destrutivo variáveis. O conhecimento do comportamento clínico dessas lesões é de fundamental importância para o tratamento (Barnes *et al.*, 2005). Os tumores odontogênicos benignos podem ser subclassificados de acordo com o tipo de tecido odontogênico que compõe a lesão: 1) *Tumores de epitélio odontogênico com estroma maduro e fibroso sem ectomesênquima odontogênico* – ameloblastoma (AB), tumor odontogênico escamoso (TOE), tumor odontogênico epitelial calcificante (TOEC), tumor odontogênico adenomatóide (TOA) e TOC, 2) *Tumores de epitélio odontogênico com ectomesênquima odontogênico, com ou sem formação de tecido duro* - fibroma ameloblástico, fibrodentinoma ameloblástico, fibro-odontoma ameloblástico, odontoma, odontoameloblastoma, tumor odontogênico cístico calcificante (TOCC), tumor dentinogênico de células fantasmas e 3) *Tumores de ectomesênquima odontogênico e/ ou mesênquima com ou sem epitélio odontogênico* - fibroma odontogênico, mixoma odontogênico e cementoblastoma (Barnes *et al.*, 2005). Serão avaliados no presente estudo os seguintes tumores odontogênicos: TOC, AB, TOE, TOA, TOCC e TOEC.

O TOC é uma lesão possivelmente oriunda da lâmina dentária e de seus remanescentes (Li *et al.*, 1998; Zhang *et al.*, 2006), e apresenta um comportamento clínico agressivo, com propensão a recorrência. Essa lesão pode apresentar-se solitariamente ou na forma de lesões múltiplas, quando pode estar associado à SCNB (Philipsen, 2005)

Histopatologicamente o tumor é caracterizado por cavidade cística revestida por epitélio estratificado pavimentoso paraceratinizado de poucas camadas de células (5 a 8 camadas) e superfície corrugada. As células da camada basal são colunares ou cúbicas e apresentam organização em paliçada, polarização nuclear invertida e hipercromatismo nuclear. A interface epitélio conjuntivo é plana (Philipsen, 2005). Tem sido demonstrado que o gene supressor de tumor PTCH possui um papel importante na patogênese do TOC, tanto nos casos esporádicos quanto nos relacionados à SCBN (Barreto *et al.*, 2000 e 2002). A

expansão cística desta lesão tem sido atribuída principalmente à atividade proliferativa do epitélio (El-Murtadi *et al.*, 1996; Shear, 2002). A taxa de recorrência varia de 0 a 62% (Bataineh & Qudah, 1998). Devido ao seu potencial agressivo, é necessário acompanhamento prolongado dos pacientes, uma vez que podem ocorrer cistos satélites e lesões múltiplas (Philipsen, 2005).

O AB é um tumor que possivelmente origina-se da lâmina dental (Crivelini *et al.*, 2003), e apresenta crescimento lento, sendo localmente destrutivo, com alta taxa de recorrência e com rara capacidade de metastatizar (Ueda *et al.*, 1989; Gardner *et al.*, 2005). A freqüência identificada dessa lesão tem sido de 10 a 25% dos tumores odontogênicos (Regezi *et al.*, 1978; Ochsenius *et al.*, 2002). AB é subdividido em: 1) *sólido/ multicístico*, 2) *periférico/ extra-ósseo*, 3) *desmoplásico* e 4) *unicístico* (Gardner *et al.*, 2005).

O *AB sólido ou multicístico* (ABS), também denominado AB convencional ou AB clássico, apresenta histopatologicamente dois padrões básicos de organização: folicular e plexiforme. O padrão folicular é o mais comum e consiste em ilhas de epitélio odontogênico em um estroma fibroso. As ilhas são caracterizadas por células periféricas colunares e hipercoradas, exibindo organização em paliçada e polarização invertida. As células centrais são organizadas frouxamente, semelhantes ao retículo estrelado. Essas áreas centrais podem sofrer degeneração cística. As células centrais podem ainda se apresentar basalóides, granulares ou exibirem diferenciação escamosa, caracterizando o AB como AB de células basais, de células granulares e acantomatoso, respectivamente. O padrão plexiforme é constituído por cordões anastomosantes, os quais exibem células basais organizadas em paliçada com polarização invertida e poucas células centrais organizadas frouxamente (Reichart *et al*., 1995; Gardner *et al.*, 2005). Serão de interesse no presente estudo o AB folicular e o plexiforme por serem os subtipos mais comuns.

O *AB periférico* é a contraparte extra-óssea do AB sólido/multicístico. O *AB desmoplásico* é caracterizado, histopatologicamente, por ninhos e cordões epiteliais de formas irregulares ou bizarras, exibindo células periféricas cúbicas com ocasionais núcleos hipercromáticos e células centrais fusiformes ou escamosas, em um estroma conjuntivo

fibroso denso, rico em fibras colágenas espessas (Gardner *et al.*, 2005). O *AB unicístico* é uma variante do AB que se apresenta radiograficamente, clinicamente (no momento cirúrgico) e histologicamente como um cisto. Os *ABs periférico, desmoplásico e unicístico* não serão alvos do presente estudo, pois representam poucos casos no arquivo pesquisado.

O TOE é uma lesão possívelmente oriunda dos remanescentes epiteliais de Malassez do ligamento periodontal (Ochsenius *et al.*, 2002; Ladeinde *et al.*, 2005), sendo localmente destrutiva e de baixo índice de recorrência. Histopatologicamente é caracterizada pela proliferação de ninhos de epitélio odontogênico bem diferenciado, de diversos tamanhos e formas. Individualmente os ninhos revelam uma camada superficial composta por células cuboidais ou células pavimentosas e uma região central de células escamosas que pode sofrer degeneração microcística ou conter material calcificado (Philipsen & Reichart, 1996; Reichart, 2005).

O TOA é um tumor de crescimento lento, porém progressivo, de rara recorrência (Regezi *et al.*, 1978; Philipsen *et al.*, 1991; Philipsen & Nikai, 2005). As possíveis origens dessa lesão são o epitélio reduzido do esmalte (Crivelini *et al.*, 2003) e remanescentes da lâmina dentária (Batra *et al.*, 2005). Geralmente a lesão está associada a um dente impactado (Philipsen *et al.*, 1991; Philipsen & Reichart, 1999). Histopatologicamente o TOA apresenta uma cápsula fibrosa bem definida. A lesão é composta por células colunares a cuboidais formando nódulos sólidos e estruturas semelhantes a rosetas e a ductos que, por sua vez, são delimitadas por uma única camada de células epiteliais colunares com polarização contrária ao lúmen. Centralmente a essas estruturas ductiformes, observa-se um material eosinofílico, restos celulares ou um espaço vazio. Esses nódulos são conectados entre si por cordões epiteliais arranjados em um padrão cribiforme. O estroma da lesão é pouco estruturado e contém vasos congestos de paredes finas (Philipsen *et al.*, 1991; Philipsen & Nikai, 2005).

TOEC é uma lesão cuja origem possivelmente está nos elementos do órgão dentário, no entanto existem divergências acerca de qual região do órgão ela deriva: do epitélio externo do esmalte, do extrato intermediário (Belmonte-Caro *et al.*, 2002), da bainha epitelial

de Hertwing (Crivelini *et al.*, 2003), do epitélio reduzido do esmalte, ou de remanescentes da lâmina dentária (Deboni *et al.*, 2006). Essa lesão é localmente destrutiva, com taxa de recorrência de 14%, sendo de 22% na variante de células claras (Takata & Slootweg, 2005). Esse tumor é também chamado de tumor de Pindborg, adamantoblastoma adenóide, AB atípico e odontoma cístico. Histopatologicamente é caracterizado por ninhos e cordões de células epiteliais poliédricas com citoplasma eosinofílico, de limites precisos e pontes intercelulares bem desenvolvidas. Os núcleos dessas células apresentam-se pleomórficos com raras figuras de mitose. Internamente aos cordões pode–se observar deposição de material hialino eosinofílico e homogêneo, por vezes calcificado, no qual foi identificado proteínas da matriz extracelular como a fibronectina e o colágeno do tipo I e III (Mesquita *et al.*, 2003) e fragmentos de proteína associada ao ameloblasto (Murphy *et al.,* 2008). Em alguns casos, podem-se observar células claras nos ninhos epiteliais (Mesquita *et al.*, 2003), as quais apresentam acúmulo de glicogêneo (Kumamoto *et al.*, 1999). O estroma da lesão é composto por tecido conjuntivo fibroso.

O TOCC é uma neoplasia benigna cística possivelmente oriunda da lâmina dentária e de seus remanescentes (Crivelini *et al.*, 2009). Essa lesão tem sido também denominada de cisto odontogênico calcificante e ceratinizante, cisto odontogênico calcificante, cisto de Gorlin, tumor odontogênico calcificante de células fantasmas, tumor odontogênico calcificante cístico, tumor epitelial odontogênico de células fantasmas e tumor odontogênico de células fantasmas (Toida, 1998; Mosqueda-Taylor, 2005; Praetorius & Ledesma-Montes, 2005). Essa lesão é localmente destrutiva e apresenta baixo índice de recorrência. Histopatologicamente, o TOCC é caracterizado por cavidade cística delimitada por epitélio de espessura variável, apresentando células da camada basal cuboidais a colunares, polarizadas, com núcleo hipercromático, e células suprabasais dispostas dispersamente, à semelhança do retículo estrelado do órgão do esmalte, onde se observa a presença de células fantasmas. As células fantasmas apresentam-se pálidas, eosinofílicas, intumescidas, sem núcleo, com preservação das membranas celular e nuclear (Mosqueda-Taylor *et al.*, 2005; Praetorius & Ledesma-Montes, 2005). Possivelmente tais caracterísicas celulares são

decorrentes de necrose por coagulação ou uma forma de ceratinização normal ou aberrante do epitélio odontogênico (Regezi *et al.*, 1978; Hong *et al.* 1991). Ocasionalmente, dentina displásica ou odontoma pode ser observada no tecido conjuntivo adjascente.

2.3 Metalotioneína

A MT é uma proteína de baixo peso molecular de aproximadamente 6 a 7 KDa, que contêm altos níveis de resíduo de cisteína (30%) além de tióis sulfúricos e de metais, contendo 7 a 12 átomos de metal por molécula (Vasák, 2005). MT tem a forma de um haltere com dois grupos: um grupo é composto por 9 cisteínas ligadas ao domínio N-terminal β que alojam de 3 a 6 átomos de metal. O outro é composto por 11 cisteínas ligadas ao domínio C- terminal-α que alojam 4 a 6 átomos de metal (Fischer & Davie, 1998; Pedersen *et al.,* 2009).

Em tecidos normais a MT é imunodetectável principalmente em células epiteliais (pele, mucosa bucal, rins, fígado, estômago, colo do útero, próstata e células basais ductais das glândulas salivares) e mioepiteliais (ao redor das glândulas sudoríparas, mamárias e salivares) (van den Oord *et al.*, 1993; van den Oord *et al.*, 1994; Sundelin *et al.*, 1997). Quatro isoformas da MT podem ser expressas: MT-I e MT-II que são estruturalmente similares e observadas em vários tecidos, inclusive no epitélio bucal; MT-III encontrada principalmente no sistema nervoso central; e MT-IV que é expressa nas células diferenciadas do epitélio estratificado escamoso (Quaife *et al.*, 1994; Vasák, 2005). Na cavidade bucal, a avaliação simultânea da expressão de MT-I e MT-II (MT-I+II) pelo anticorpo E9 foi realizada em lesões malignas (Sundelin *et al.*, 1997; Cardoso *et al.*, 2002; Cardoso *et al.*, 2009; Szelachowska *et al.*, 2008), lesões potencialmente malignas (Johann *et al,* 2008; Pontes *et al.*, 2009), periodontite (Katsuragi *et al.*, 1997) e tatuagem por amálgama (Leite *et al.*,2004). Na mucosa bucal normal a expressão da MT-I+II foi identificada somente nas células das camadas basal e parabasal do epitélio (Sundelin *et al.* 1997; Johann *et al.*, 2008; Pontes *et al.*, 2009).

A principal função da MT é regular a homeostase (cobre e zinco) e detoxificar metais

(cádmio e mercúrio) funcionando como protetora das células (Hamer, 1986; Cherian *et al.*, 1993; Klaassen *et al.*, 1999; Cherian *et al.*, 2003). Essa proteína está também relacionada com: 1) proteção contra o estresse oxidativo causado por radicais livres e quimioterápicos; 2) regulação do desenvolvimento embrionário; 3) regulação da diferenciação e da proliferação celular; e 4) inibição da apoptose celular (Kagi & Shäffer, 1988; Cherian *et al.*,1993; Sundelin *et al.*, 1997; Lazo *et al.*,1998; Klaassen *et al.*, 1999; Hishikawa *et al.*, 1999; Davis & Cousins, 2000; Coyle *et al.*, 2002; Zagorianakou *et al.*, 2005; Athanassiadou *et al.*, 2007; Pedersen *et al.*, 2009). Essa proteína é encontrada principalmente no citoplasma celular, mas também pode ser observada no núcleo. A MT pode atravessar a membrana nuclear por difusão passiva, entretanto a sua permanência no núcleo requer energia (Woo *et al.*, 1996). O significado da localização nuclear é uma maior proteção contra o estresse oxidativo e dano genômico, além de afetar a regulação genômica de outras proteínas ligadas ao DNA (Cherian *et al.*,2003; Chubatsu & Meneghini, 1993).

A MT regula a diferenciação celular afetando diretamente os fatores transcricionais por doar zinco. *In vitro,* MT tem sido relacionada com a diferenciação celular de mioblastos em miotubulos (Apostolova *et al.*, 1999) e na maturação de células acinares de glândulas salivares (Hecht *et al.*, 2002). Em mioblastos, MT foi localizada, através da imunofluorescência, principalmente no citoplasma, porém foi translocada para o núcleo de miotúbulos recém-formados durante o estágio inicial de diferenciação. Nos miotúbulos completamente diferenciados o conteúdo de MT diminuiu, sendo localizado no citoplasma (Apostolova *et al.*, 1999). Superexpressão do gene MT-1 em células da linhagem de glândulas salivares levam a uma diferenciação morfológica dessas células e aumenta o tamanho do ácino formado (Hecht *et al.*, 2002). A relação da MT com o desenvolvimento dos tecidos também tem sido verificada *in vivo* em rim, fígado e mucosa lingual (Nishimura *et al.*, 1989; Quaife *et al.*, 1994). No rim de neonatos e fetos, a MT foi identificada, utilizando imuno-histoquímica, no epitélio do túbulo renal, mudando de localização para o córtex renal durante o desenvolvimento. Já no fígado de fetos e neonatos, a expressão da MT nos hepatócitos é forte e localizada no citoplasma e núcleo. Essa expressão diminui com o

desenvolvimento hepático, acompanhado pelo desaparecimento da marcação nuclear. Desta forma, a imuno-expressão da MT diminui à medida que o tecido se desenvolve, de forma que tecidos bem diferenciados expressam menos MT (Nishimura *et al.*, 1989). Também através da imuno-histoquímica, altos níveis de MT foram observados no fígado de neonatos e fetos, ao passo que baixos níveis foram identificados no fígado de adultos (Panemangalore *et al.*, 1983**;** Nartey *et al.*, 1987; Cherian *et al.*,1987; Nishimura *et al.*, 1989). Em epitélio da língua de ratos, a hibridização *in situ* mostrou abundante MT-I mRNA na camada basal e pouca na camada suprabasal. Já o MT-IV mRNA foi indetectável na camada basal e mostrou altos níveis na camada espinhosa, indicando a existência de uma mudança na síntese de isoformas da MT durante a diferenciação desse epitélio (Quaife *et al.*, 1994). Em ratos com oito a nove semanas de idade, a ausência de imuno-marcação da MT-I+II foi observada na polpa dentária por Tamura *et al.* (1999), Sogawa *et al.* (2001) e Izumi *et al.* (2007). Izumi *et al.* (2007) notaram que quando a polpa foi submetida a uma injúria, o índice de MT no odontoblasto foi de 37.2%, retornando a zero cinco dias após a injúria. Esses autores sugeriram que a expressão da MT é diretamente relacionada à diferenciação de novos odontoblastos durante o processo de reparo.

Inibição da apoptose é outra função atribuída à MT (Apostolova *et al.*, 1999; Miles *et al.*, 2000; Shimoda *et al.*, 2003; Formigari *et al.*, 2007). Apoptose, também conhecida como morte celular programada ou morte celular fisiológica, apresenta diversos papéis na embriogênese e na homeostase normal, bem como em uma variedade de condições patológicas (Matalová *et al.*, 2006; Kumamoto, 1997; Setkova *et al.*, 2006). Dois possíveis papéis da metalotioneína na apoptose são: 1) regulando a concentração de zinco celular, estimulando o fator de transcrição antiapoptótico zinco dependente, o fator nuclear Kappa B (NF-κB) (Abdel-Mageed & Agrawal, 1998; Butcher *et al.*, 2004); e 2) interagindo com proteínas envolvidas com a apoptose, inibindo a apoptose por indução de oncogenes antiapoptóticos como Bcl-2 e c-myc, ou por inibição de proteínas supressoras tumorais proapoptóticas como a caspase-1 e -3 ou a liberação do citocromo c (Shimoda *et al.*, 2003; Pedersen *et al.*, 2009). Alguns estudos, utilizando a técnica de detecção terminal

deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), mostraram uma correlação inversa entre a imuno-expressão da MT e a apoptose em neoplasias malignas (Sundelin *et al.*, 1997; Deng *et al.*, 1998; Cai *et al.*, 1998; Jayasurya *et al.*, 2000; Li *et al.*, 2003). Entretanto, outros estudos revelaram correlação positiva (Zhang & Takenaka, 1998; Dziegiel *et al.*, 2004; Dumańska *et al.*, 2004) e um estudo não mostrou correlação (Jin *et al.*, 2002). *In vitro,* foi demonstrado que durante o estágio inicial de diferenciação de miotúbulos recém-formados, a MT é translocada para o núcleo e quando esses se diferenciam completamente, o conteúdo de metalotioneína diminuiu, sendo localizada no citoplasma. Essas mudanças na localização subcelular dos miotúbulos foram acompanhadas pelo aumento da apoptose nos mesmos (Apostolova *et al.*, 1999). Abdel-Mageed & Agrawal (1997) demonstraram, também *in vitro,* em células MCF7 (carcinoma de mama humano) que a diminuição de MT bloqueia a proliferação celular e induz a apoptose. Em células imaturas da linhagem T, a detecção imuno-histoquímica da MT seguida pela avaliação por TUNEL mostrou que todas as células TUNEL positivas foram negativas para MT, sugerindo que a expressão da MT previne a apoptose (Tsangaris & Tzortzatou-Stathopoulou, 1998).

O papel da MT na proliferação celular também tem sido relatado na literatura. A localização celular da MT parece ser ciclo específica, atingindo um pico de marcação nuclear durante a fase S e G_2/M e máxima expressão no citoplasma durante G_0 e G_1 (Nagel & Vallee, 1995; Cherian & Apostolova, 2000). Elevados níveis de MT têm sido identificados em tecidos que proliferam rapidamente como neoplasias e em células em processo de regeneração (Nagel & Vallee, 1995; Moffatt & Denizeau, 1997; Cardoso *et al*., 2002; Cardoso *et al*., 2009). A imuno-expressão da MT foi relacionada à proliferação de odontoblastos e angiogênese durante o processo de reparo da polpa dentária (Izumi *et al.,* 2007).

2.4 Marcadores de proliferação celular

O Ki-67 e o PCNA são indicadores de proliferação celular. O PCNA é uma proteína nuclear de 36kD que participa diretamente da síntese de DNA, sendo auxiliar da DNA

polimerase δ. A distribuição do PCNA no ciclo aumenta durante a fase G1, apresentando um pico de expressão em G1/S e diminui durante a fase G2 (Gerdes *et al*., 1984). PCNA pode ser detectado também no processo de reparo de DNA (Toschi & Bravo, 1988) e apresenta uma meia-vida longa (20 h) (Bravo & MacDonald-Bravo, 1987). Já o Ki-67 é um antígeno que aparece do meio para o final da fase G1, elevando durante as fases S e G2, alcança um pico máximo na mitose e diminui rapidamente após a mesma. Ki-67 tem uma meia vida de 60 a 90 minutos (Gerdes *et al*. 1984; Heidebrecht at al., 1996). Devido às diferenças de meia-vida dessas proteínas e também ao fato do PCNA estar envolvido não somente com a proliferação, mas também com o processo de reparo do DNA, é sugerido que o Ki-67 seja mais sensível que o PCNA para refletir a proliferação celular (Gong *et al*., 2008).

Um maior índice de Ki-67 (IK) e de PCNA (IP), calculado pela porcentagem de células marcadas, tem sido relatado para o TOC quando comparado com o CR, CD (Li *et al*., 1994; Piatelli *et al*., 1998; Kichi *et al*., 2005; Mateus *et al*., 2008; Tsuneki *et al*., 2008; Gadbail *et al*., 2009) e COO (Li *et al*., 1998). Similar IK ou IP entre os TOC associados ou não à SCNB têm sido relatados (Lo Muzio *et al*., 1999; Kimi *et al*., 2000; Mateus *et al*., 2008; Gurgel *et al*., 2008). Entretanto, outros estudos relatam um maior IK nos casos associados à SCNB quando comparados aos não associados, porém o desenho desses estudos é baseado em cálculos do número de células positivas por comprimento de membrana basal, não podendo então ser comparados com os estudos anteriores (Li *et al*., 1995; Pan & Li, 2009). El Murtadi *et al*. (1996) também observaram um maior IP nos casos associados à SCNB. Um maior IK e IP têm sido relatados para ABS folicular quando comparado com o plexiforme (Funaoka *et al*., 1996; Ong'uti *et al*., 1997; Bologna-Molina *et al*., 2008). Entretanto, Rizzardi *et al*. (2009) relatou um IK mais alto no tipo plexiforme que no folicular. Além disso, uma expressão similar do Ki-67 ou do PCNA entre esses subtipos também tem sido relatada (Kim & Yook, 1994; Slootweg *et al*., 1995; Takahashi *et al*., 1998; Kumamoto, 1997; Sandra *et al*., 2001; Barboza *et al*., 2005). A razão para esses resultados diferentes pode estar relacionada com diferenças na metodologia utilizada (Meer *et al*., 2003; Bologna-Molina *et al*., 2008). Um maior IP foi relatado para o ABS quando comparado com o TOA

(Barboza *et al*., 2005). O IK ou IP foi maior no ABS que no TOCC (Takata *et al*., 1998; Gong *et al*., 2009); entretanto, quando comparado o ABS com a variante proliferativa do TOCC, o IMT é similar (Takata *et al*., 1998). Nenhum estudo compara IK ou IP no ABS com o TOE, ou com o TOEC.

A correlação entre a MT e a proliferação cellular varia de acordo com o tipo de lesão estudada. Uma correlação positiva tem sido observada entre: a) MT e Ki-67 em lesões malignas (Siu *et al.*, 1998; Hiura *et al.,* 1998; Ioachim *et al.*, 2000; Jayasurya *et al*., 2000; Jin *et al.*, 2002; Diziegiel *et al.*, 2003; Diziegiel *et al.*, 2004; Dumańska *et al.*, 2004; Zagorianakou *et al.,* 2006; Athanassiadou *et al.*, 2007); b) MT e PCNA em lesões benignas, potencialmente malignas e malignas (Ioachim *et al.*, 1999**a;** Hishikawa *et al.* 1999). Entretanto, no endométrio normal a imuno-expressão de MT foi inversamente correlacionada com o PCNA e o Ki-67 (Ioachim *et al.*, 2000). Alguns estudos não relatam correlação entre MT e Ki-67 em lesões malignas (Ioachim *et al.*, 1999**b**; Ioachim *et al.*, 2001; Cardoso *et al.*, 2002; Surowiak *et al.*, 2004; Tao *et al.* 2007; Pastuszewski *et al.*, 2007; Szelachowska *et al.*, 2008) e em lesões benignas ou *boderline* (Zagorianakou *et al.,* 2006). Nenhuma correlação entre o PCNA e MT também foi descrita em lesões malignas (Ioachim *et al.*, 1999**b;** Joseph *et al.*, 2001; Ioachim *et al.*, 2001) e em lesões benignas ou *boderlines* (Zagorianakou *et al.,* 2006).

Diversos fatores afetam a expressão tecidual da MT: metais; glicocorticóides; inflamação; condições de estresse como calor, frio e privação de nutrientes; e luz ultravioleta (Oh *et al.*, 1978; Webb & Cain 1982; Hamer, 1986; Cherian *et al*., 1993; Fischer & Davie, 1998; Davis & Cousins, 2000; Cherian *et al.*, 2003; Yamada *et al.* 2004). No entanto, o mecanismo de ação desses agentes é desconhecido.

Não existem estudos que avaliem e comparem a expressão dessa proteína entre: 1) os cistos odontogênicos e o TOC, 2) casos de TOC associados à SCNB e não associados; 3) os tumores odontogênicos benignos. Não existem também estudos que correlacionem a imuno-expressão da MT e marcadores de proliferação celular nos cistos e tumores odontogênicos e nem que avaliem a influência da inflamação na expressão da MT no ABS.

3 JUSTIFICATIVA

Cistos e tumores odontogênicos são lesões originadas dos tecidos que formam os dentes. Apesar da origem semelhante, essas lesões apresentam diferentes comportamentos biológicos. Cistos odontogênicos são lesões freqüentes na boca e correspondem a cavidades císticas revestidas por epitélio odontogênico, apresentam baixa taxa de recorrência e um comportamento clínico não agressivo. Tumores odontogênicos são lesões encontradas em menor freqüência na cavidade bucal, apresentam variáveis taxas de recorrência e potencial destrutivo, podendo ser agressivas com altas taxas de recidiva ou apresentar crescimento limitado e baixa recidiva. A MT é relacionada a eventos de diferenciação e proliferação celular e inibição da apoptose. Com relação aos cistos e tumores odontogênicos, a MT poderia ter um papel na regulação da diferenciação e proliferação celular e na inibição da apoptose, refletindo no comportamento biológico dessas lesões. Entretanto, não existem estudos que avaliem a expressão dessa proteína nessas lesões. A identificação da MT nos cistos e tumores odontogênicos é contribuitória na avaliação dessas lesões com diferentes comportamentos biológicos.

4 OBJETIVOS

4.1 Objetivo geral

Avaliar a imuno-expressão da proteína MT em cistos odontogênicos e tumores odontogênicos benignos.

4.2 Objetivos específicos

- Comparar a imuno-expressão da proteína MT entre CR, CD, COO e o TOC;
- Investigar a provável correlação entre a imuno-expressão da MT e do Ki-67 no CR, CD, COO e TOC;
- Investigar a provável correlação entre a imuno-expressão da MT e a inflamação no CR, CD e TOC;
- Comparar a imuno-expressão da proteína MT entre o TOC associado à SCBN e casos não associados;
- Investigar a provável correlação entre a imuno-expressão da MT e do Ki-67 no TOC associado à SCBN e nos casos não associados;
- Comparar a imuno-expressão da proteína MT entre o ABS, TOE, TOA, TOCC e TOEC;
- Investigar a provável correlação entre a imuno-expressão da MT, do Ki-67 e do PCNA no ABS, TOE, TOA e TOCC;
- Investigar provável correlação entre a imuno-expressão da MT e a inflamação no ABS.

5 METODOLOGIA

Foi realizado um estudo retrospectivo através da pesquisa documental e observacional de cistos e tumores odontogênicos. Esse estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais (COEP-UFMG) no dia 2 de abril de 2008, sob o número 15/08 (anexo A).

5.1 Amostra

A amostra foi selecionada dos arquivos do Serviço de Patologia Bucal da Faculdade de Odontologia da UFMG (FO-UFMG), no período de 1976 a 2007. Para a seleção foram separadas: fichas de biópsia, lâminas (cortes histológicos de 4μm corados em HE) e blocos de inclusão em parafina com os seguintes diagnósticos: CR, CD, COO, TOC, AB, TOE, TOA, TOCC e TOEC. Uma avaliação histopatológica dos casos foi realizada por dois observadores calibrados, através da visualização de lâminas coradas em HE em um microscópio de luz (Carl Zeiss – Axiostar 1122-100), seguindo os critérios da OMS (1992) para os cistos odontogênicos (Kramer *et al.*, 1992) e da OMS (2005) para os tumores odontogênicos (Barnes *et al.*, 2005). Foram excluídos os casos com fichas de biópsia de preenchimento incompleto, blocos de inclusão em parafina ausentes ou com material insuficiente, além de casos de TOC previamente marsupializados ou casos recidivantes. Não foi adotado nenhum critério de exclusão relativo aos pacientes como sexo, cor, idade, nacionalidade, procedência, profissão ou outros. A amostra foi composta por casos de CR (9 casos), CD (9), TOC (8 primários não associados à SCBN e 6 primários associados à SCNB), COO (4), ABS (5 do tipo plexiforme, 5 do tipo folicular), TOE (4), TOA (5), TOCC (4) e TOEC (3). Os casos de CD apresentavam epitélio estratificado pavimentoso não ceratinizado de poucas camadas. Os CR e o CD não apresentavam áreas de metaplasia escamosa ou espessamento. Os subtipos de ABS plexiforme e folicular foram os escolhidos por serem os mais comuns. Todos os TOCC foram do tipo cístico, não associado ao odontoma e eram do padrão não proliferativo: uma estrutura unicística delimitada por um epitélio fino (Takata *et al.*, 1998). Em um caso de TOEC observou-se, além do lençol de

epitélio poliédrico clássico com abundante citoplasma eosinofílico e pleomorfismo nuclear, a presença de células volumosas com citoplasma claro e espumoso e bordas celulares distintas, sendo classificado como variante de células claras. Todas as lesões foram intra-ósseas, exceto um caso de TOCC que foi extra-ósseo. Somente um caso de ABS era recorrente, todas as outras lesões eram primárias.

5.2 Análise da inflamação

Nos cistos odontogênicos e no TOC, a análise da inflamação foi realizada por um único observador, utilizando-se lâminas coradas em HE, através de um retículo de contagem para delimitação de campos, ajustado a um microscópio de luz (Carl Zeiss – Axiostar 1122-100), com aumento final de 400X, seguindo o critério de Hirshberg *et al.* (2007). O índice inflamatório foi determinado pela contagem de células inflamatórias adjacentes ao epitélio em 20 campos consecutivos na profundidade de um campo a partir da membrana basal. As lesões foram graduadas em: Grau 0 – sem inflamação, Grau 1 – <15 células por campo, Grau 2 – 15–50 células por campo e Grau 3 – >50 células por campo. O índice inflamatório foi calculado como a média de todos os campos examinados. As lesões foram então divididas em dois grupos: grupo A – Grau 0–2 (discreto a moderado), e grupo B – Grau 3 (intenso). No CR e no CD, 5 casos pretenciam ao grupo A e 4 ao grupo B. Os casos de TOC não associados à SCNB, 4 casos pertenciam ao grupo A e 4 ao grupo B. Todos os casos de TOC associados à SCNB pertenceram ao grupo A. Três casos de COO pertenciam ao grupo A e um ao grupo B, mas os casos de COO não foram incluídos na análise de inflamação devido ao número restrito de casos.

Nos tumores odontogênicos, a análise de inflamação foi realizada somente nos ABS, devido ao maior número de casos. Essa análise foi feita da mesma forma que a anterior , porém foram contadas células inflamatórias no tecido conjuntivo adjacente ao epitélio em 10 campos consecutivos obtendo-se número de células inflamatórias.

5.3 Reação imuno-histoquímica para metalotioneína, PCNA e Ki-67

As reações de imuno-histoquímica para a marcação da proteína MT, PCNA e Ki-67 foram realizadas pela técnica manual da streptavidina-biotina no Serviço de Patologia Bucal da FO-UFMG.

As lâminas foram lavadas em água corrente por 2 horas e banhadas por 4 horas em uma mistura de 30 gramas de gelatina (Vetec Química Fina, Rio de Janeiro, Brasil, código 628) com 3 gramas de sulfato de cromo e potássio (Vetec Química Fina, Rio de Janeiro, Brasil, código 728) diluídos em três litros de água fervente aquecida por 30 minutos em microondas na potência 9. Em seguida as lâminas foram secas à temperatura ambiente por 24 horas. Foram obtidos, a partir de blocos de parafina, cortes histológicos de 4 µm de espessura, sendo estes estendidos sobre a lâmina previamente preparada e colocados em estufa a 55 ºC durante 24 horas. Os cortes foram desparafinizados em dois banhos de xilol: um de 30 minutos em estufa a 55 ºC e outro de 15 minutos à temperatura ambiente. Em seguida, os cortes foram hidratados em banhos decrescentes de etanol (100%, 90% e 70%) por 5 minutos cada. Para remoção do pigmento de formol, os cortes foram imersos por dez minutos em solução de hidróxido de amônio a 10% em etanol a 95% e lavados em cinco banhos de água destilada.

Para a recuperação antigênica, as lâminas foram imersas nas soluções apresentadas na tabela 1 e incubadas em panela de pressão (Manttra Inc, Índia) por 20 minutos. Após a recuperação, essas lâminas foram mantidas por 20 minutos em temperatura ambiente para resfriar, sendo lavadas em cinco banhos de água destilada.

Tabela 1- Anticorpos primários utilizados, diluição, temperatura e tempo de incubação, agente de recuperação antigênica.

Anticorpo (Clone)	***Diluição***	***Temperatura de incubação/ tempo***	***Agente de recuperação / pH***
MT-I+II (E9 / M0639)	1:100	4ºC/ 18 horas	EDTA*/ 8,0
PCNA (PC10 / MO879)	1:1000	Ambiente/ 18 horas	Ácido cítrico ** / 6,0
Ki-67 (MIB-1/ M7240)	1:100	Ambiente / 18 horas	Ácido cítrico ** / 6,0

*1 mM ácido etileno diamino tetraacético (Vetec Química Fina, Rio de Janeiro, Brazil, 114); **10 mM ácido cítrico (Lab Synth, Diadema, Brazil, A2270).

Para os casos submetidos ao anti-MT foi feito um bloqueio da atividade de ligação à avidina endógena segundo o protocolo de Miller *et al.* (1999). Primeiramente, incuba-se em solução de ovo (filtrando a mistura de 2 claras em 200 ml de água) por 10 minutos, seguido de cinco banhos de água destilada e reincuba-se por 15 minutos com leite em pó desnatado (15 g em 90 ml de água) lavando, em seguida, com cinco banhos de água destilada.

Em todos os casos, a fim de inibir a peroxidase endógena, os cortes foram incubados em dois banhos de peróxido de hidrogênio a 10 volumes por 15 minutos cada, sendo em seguida, lavados em cinco banhos de água destilada. Seguiu-se então a incubação em três banhos em solução tampão com 2,42g de TRIS hidroximetil amino metano PA (Nuclear, São Paulo, Brasil, código 318768) e 8,5g de cloreto de sódio PA (Vetec Química Fina, Rio de Janeiro, Brasil, código 106) em 1000mL de água destilada (TRIS-HCL), pH 7,4 por cinco minutos. Os cortes foram então incubados com os anticorpos primários monoclonais (Dako, Carpinteria, CA, EUA), cujas diluições, temperaturas e tempo de incubação encontram-se dispostos na tabela 1. Após a incubação, os cortes foram submetidos a três banhos de solução tampão TRIS-HCL, pH 7,4. Em seguida foi realizada a incubação com anticorpo secundário, pertencente ao Kit LSAB+ Peroxidase (LSAB® System, Streptavidin-HRP, Dako Corporation, Carpinteria, CA, EUA, código K0675), à temperatura ambiente em câmara úmida por 30 minutos e lavagem em três banhos em solução tampão TRIS-HCL, pH 7,4. As lâminas foram então incubadas em complexo terciário também pertencente ao Kit LSAB+ Peroxidase (LSAB® System, Streptavidin-HRP, Dako Corporation, Carpinteria, CA, EUA, código K0675), à temperatura ambiente em câmara úmida por 30 minutos, sendo lavadas em três banhos em solução tampão TRIS-HCL, pH 7,4. A revelação da reação foi executada com a solução cromógena contendo 25mg de 3,3' diaminobenzidina (DAB - Sigma Chemical, St. Louis, EUA, código D5637) diluídos em 100 ml de tampão TRIS-HCL pH 7.4, acrescido de 1,20 ml de peróxido de hidrogênio 10 volumes por 5 minutos. Seguiram-se 5 banhos de água destilada. A contra-coloração foi feita com solução de hematoxilina de Mayer por 25 segundos. Em seguida realizou-se um banho de água destilada e um banho de solução aquosa de hidróxido de

amônio a 10%, sendo imediatamente lavadas em cinco banhos de água destilada. Seguiu-se a desidratação em soluções de etanol em concentrações crescentes: 70%, 90% e 100% por cinco minutos e mais duas vezes a 100% por dez minutos, diafanizadas em um banho de cinco e outro de dez minutos em xilol. As lâminas foram montadas com lamínulas de vidro e Permount (Fisher Scientific®, Fair Lawn, EUA, código SP15-500).

5.4 Análise da imuno-expressão

As análises da imuno-expressão das proteínas estudadas foram descritas separadamente: 1) para os cistos odontogênicos e o TOC e 2) para os tumores odontogênicos benignos.

Na análise dos cistos odontogênicos e do TOC, foram digitalizadas 20 imagens através de um microscópio acoplado a uma microcâmera JVC TK-1270/RGB (Tokyo, Japan), com aumento final de 400X. Todas as imagens foram analisadas no software KS300 contido no analisador de imagens Carl Zeiss (Oberkochen, Germany). De cada imagem foi obtido o número de células positivas e negativas (separadamente nas camadas basal e suprabasal) para os marcadores MT e Ki-67. A camada basal foi composta por células colunares ou cuboidais localizadas em uma ou três camadas a partir ou próximo da camada basal. A camada suprabasal foi composta por células relativamente grandes e redondas, acima da camada basal (Kichi *et al.*, 2005). Para MT foram consideradas células positivas aquelas que possuíam marcação somente citoplasmática ou somente nuclear, ou em ambos os compartimentos. Já as células positivas para Ki-67 apresentaram marcação somente nuclear. Os índices de células marcadas para MT (IMT) e Ki-67 (IK) foram obtidos dividindo as células positivas pelo total de células epiteliais e multiplicando por 100.

Na análise dos tumores odontogênicos, o epitélio lesional foi avaliado em cada caso, através de um microscópio óptico (Carl Zeiss – Axiostar 1122-100), com aumento final de 400X, sendo contadas 500 células (Cardoso *et al.*, 2002). A imuno-expressão da MT, do Ki-67 e do PCNA foi estratificada em células periféricas e centrais nos casos de ABS, TOE, TOA e TOCC. Essa estratificação não foi realizada para o TOEC, por ser uma lesão que se

apresentou no padrão de lençol. No ABS, as células colunares ou cuboidais localizadas adjacentes à lâmina basal foram consideradas as células periféricas, e as células arranjadas frouxamente lembrando o retículo estrelado do órgão do esmalte compunham as células centrais. Já no TOE, as células cuboidais ou achatadas localizadas adjacentes a lâmina basal corresponderam às células periféricas, e as células escamosas as centrais. No TOA, a porção periférica do tumor em contato com o tecido conjuntivo foi composta por células do tipo II: células alongadas e células fusiforformes formando cordões em padrão cribiforme. Essas células foram consideradas as células periféricas. Adjacentes às células do tipo II, foram observadas as células do tipo I: células colunares a cuboidais formando nódulos sólidos e estruturas semelhantes a rosetas e a ductos. Essas células compunham as células centrais (Takahashi *et al*., 2001). No TOCC, as células cuboidais basais correspenderam às células periféricas e as células das camadas superiores que se assemelhavam ao retículo estrelado do órgão do esmalte corresponderam às centrais. Para MT foram consideradas células positivas aquelas que possuíam marcação somente citoplasmática ou somente nuclear, ou em ambos os compartimentos. Já as células positivas para Ki-67 e PCNA apresentaram marcação somente nuclear. Em cada lâmina foi obtido o número de células positivas e negativas para MT, Ki-67 e PCNA. Os índices de células marcadas para MT (IMT), Ki-67 (IK) e PCNA (IP) foram obtidos dividindo as células positivas pelo total de células epiteliais e multiplicando por 100.

5.5 Análise estatística

Na análise estatística dos cistos odontogênicos e do TOC foi utilizado o programa BioEstat® 4.0 (BioEstat, Brasil). Como serão apresentados dois artigos sobre essas lesões, a análise foi dividida em duas partes: 1) comparação de cistos odontogênicos e do TOC, e 2) TOC em pacientes sem associação com a SCNB comparados com os casos associados. Na primeira parte da análise foram consideradas amostras com distribuição não normal pelo teste de Shapiro-Wilk: IMT total, IMT nuclear, IMT citoplasmática e IK. Nesses casos utilizou-se os testes Kruskal-Wallis e Mann-Whitney U, sendo realizado em seguida um ajuste para

seis post hoc testes com correção de Bonferroni, com o nível de significância estatística de 5% atingido com p<0,008. Foram consideradas amostras com distribuição normal no teste de Shapiro-Wilk: IMT basal, IMT suprabasal, IMT nuclear e citoplasmática. Para estes casos o teste ANOVA (com o teste post hoc de Tukey) foi usado, com significância estatística de p<0,05. Além disso, a correlação de Spearman foi realizada para correlacionar o IMT com o IK no CR, uma vez que o IMT não apresentou distribuição normal nesta lesão. Já a correlação de Pearson foi utilizada para comparar o IMT e o IK no TOC, COO e CD, uma vez que estes índices apresentaram distribuição normal. A correlação foi graduada em: fraca – <0,30; moderada – 0,30-0,50; e forte – >0,50 (Cohen *et al.*, 1988). Para a análise de inflamação, já que IMT (em todas as lesões agrupadas e no CR) e IK (em todas as lesões agrupadas) não mostraram uma distribuição normal pelo teste de Shapiro-Wilk, o teste de Mann-Whitney foi usado. Como IMT (nos casos de TOC e CD) e IK (nos casos de TOC, CR e CD) mostraram uma distribuição normal pelo teste de Shapiro-Wilk, o teste T de Student foi realizado, com significância estatística de p<0,05.

Na segunda parte da análise foram considerados não normais no teste de Shapiro-Wilk: IMT nuclear e IMT citoplasmático. Para estes casos, foi usado o teste de Mann-Whitney. A significância estatística aceita foi p<0,05. Entretanto, foram considerados normais no teste de Shapiro-Wilk: IK, IMT total, IMT citoplasmático e nuclear, IMT basal, IMT suprabasal, IMT grupo A e B e IK grupo A e B. Para esses casos o teste empregado foi o teste Student t. A significância estatística aceita foi p<0,05. A correlação de Pearson foi usada para avaliar a correlação entre IMT e IK nos TOC não associados à SCNB e nos casos de TOC agrupados. A correlação foi graduada em: fraca – <0,30; moderada – 0,30-0,50; e forte – > 0,50 (Cohen *et al.*, 1988).

Na análise estatística dos tumores odontogênicos também foi utilizado o programa BioEstat® 4.0 (BioEstat, Brasil). O IMT, IP e IK não apresentaram uma distribuição normal no teste de Shapiro-Wilk; portanto os testes Kruskal-Wallis e Mann-Whitney U foram utilizados, sendo realizado em seguida um ajuste para dez post hoc testes com correção de Bonferroni, com o nível de significância estatística de 5% atingido com p<0,005, quando

todos os tumores foram comparados. Por outro lado, os valores de IMT nas células centrais e periféricas mostraram uma distribuição normal no teste de Shapiro-Wilk, então, utilizou-se o teste T pareado, com significância estatística de $p<0,05$. Nos ABS plexiforme e folicular, desde que IMT, IP e IK tiveram uma distribuição normal no teste de Shapiro-Wilk, o teste T de Student foi aplicado para comparar esses subtipos, com significância estatística de $p<0,05$. Nos casos de ABS, TOE, TOA e TOCC avaliados individualmente, o IMT, IK e IP tiveram uma distribuição normal, sendo a correlação de Pearson usada para correlacionar estes índices. Já nos casos de ABS, a correlação de Spearman foi usada para correlacionar a inflamação (que não teve uma distribuição normal) com IMT, IK e IP. A correlação foi graduada em: fraca – <0,30; moderada – 0,30-0,50; e forte – > 0,50 (Cohen *et al.*, 1988).

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7 RESULTADOS

ARTIGO 1 - Metallothionein immunoexpression in radicular, dentigerous, orthokeratinized odontogenic cysts and in keratocystic odontogenic tumour

TITLE: Metallothionein immunoexpression in radicular, dentigerous, orthokeratinized odontogenic cysts and in keratocystic odontogenic tumour

RUNNING TITLE: Metallothionein in odontogenic cysts and keratocystic odontogenic tumour

SUMMARY

Radicular (RC), dentigerous (DC) and orthokeratinized odontogenic cysts (OOC) and keratocystic odontogenic tumour (KOT) are lesions derived from tooth-forming epithelium, with different histopathological features and clinical behavior. Metallothionein (MT) is a protein correlated with differentiation and proliferation cellular, and inhibition of apoptosis. It was hypothesized that these lesions show differences in MT expression. The aims were: 1) to compare the MT immunoexpression in RC, DC, KOT, and OOC; 2) to correlate the MT immunoexpression with cellular proliferation; and 3) to evaluate the influence of the inflammation in MT immunoexpression. Nine cases of RC, 9 DC, 8 KOT, and 4 OOC were submitted to immunohistochemistry using anti-MT and anti-Ki-67. Indexes of MT (IMT) and Ki-67 (IK) were obtained. Lesions were grouped in inflammation: mild-to-moderate (group A), and intense (group B). BioEstat® 4.0 was used in statistical analysis. In RC and DC, MT staining was observed in all epithelial layers. In KOT, MT staining was present in basal layer and in the inferior half of the suprabasal layer, and absent or rare in the superior half. In OOC, MT staining was visualized in basal and absent or scarce in suprabasal layer. IMT was highest in RC (91%), followed by DC (89%), KOT (78%) and OOC (63%); and the differences were significant, except between RC and DC. IK was highest in KOT (12%), followed by OOC (4%), RC (3%) and DC (2%); and the difference was significant among KOT and the other lesions. IMT was inversely correlated with IK in KOT (Pearson correlation [r]= -0.5126), and OOC (r= -0.6140), but positively in RC (Spearman correlation [r_s]= 0.6178) and in DC (r= 0.4763). No differences in IMT and in IK were observed between group A and group B. IMT was variable among the lesions and this may be due to its role in the cellular differentiation and inhibition of apoptosis. Correlation of IMT and cellular proliferation did seem to be inverse in KOT and OOC, but direct in RC and DC. IMT and IK did not seem to be modified by inflammation.

Keywords: odontogenic cyst, dentigerous cyst, radicular cyst, neoplasm, metallothionein, Ki-67 Antigen, MIB-1 antibody, immunohistochemistry.

Introduction

Odontogenic cysts are cavities lined by odontogenic-derived epithelium. Radicular (RC) and dentigerous cysts (DC) are the most prevalent odontogenic cysts and show a non-aggressive clinical behavior with rare recurrences.[1] Odontogenic keratocyst was re-classified as an odontogenic neoplasm and it was re-named to keratocystic odontogenic tumour (KOT) by the World Health Organization (WHO, 2005).[2] This change reflects KOT's aggressive clinical behavior, high recurrence rate, destructive growth, and association with Nevoid Basal Cell Carcinoma Syndrome (NBCCS).[2] In the same classification,[2] orthokeratinized odontogenic cyst (OOC) was not considered a part of the spectrum of KOT, as it shows a less aggressive clinical behavior, lower proliferative activity, and low tendency to recur.[2,3] After KOT reclassification, recent immunohistochemical studies compared it with odontogenic cysts.[4,5,6] In spite of their high prevalence, the pathogenesis and factors related to their biological behavior still need be elucidated.[4]

Metallothionein (MT) is a protein correlated with important processes in the cell as homeostasis of essential metals, regulation of cellular differentiation and proliferation, and inhibition of cellular apoptosis. These processes are involved not only in normal cellular process, but may reflect in the biological behavior of many lesions.[7-9] MT is immunodetectable in myoepithelial and epithelial cells, and four isoforms can be identified: MT-I and MT-II isoforms are similar and they are observed in many tissues inclusive in oral epithelium; MT-III is found mainly in the central nervous system; and MT-IV is expressed in differentiated cells of stratified squamous epithelium.[10,11] In oral cavity, the simultaneous immunoexpression of MT-I and MT-II (MTI+II) evaluated by antibody E9 was performed in oral malignant[7,12,13] and premalignant lesions,[14] periodontitis,[15] and amalgam tattoos.[16] It was hypothesized that odontogenic cysts and KOT show differences in MT expression, and this was not investigated yet.

Ki-67 is a nuclear antigen present in all active phases of the cell cycle and absent in G0,[17] and it is a reliable marker of cellular proliferation that has been used in odontogenic cysts.[5,18,19]

The aims of this study were: 1) to report and to compare the MT immunoexpression in RC, DC, KOT, and OOC; 2) to correlate the MT immunoexpression with cellular proliferation in these lesions; and 3) to evaluate the influence of inflammation in MT immunoexpression.

Materials and methods

Specimens

Samples of RC (9 cases - Figure 1A), DC (9 cases - Figure 1C), KOT (8 cases - Figure 1E), and OOC (4 cases - Figure 1G) were retrieved from files of Oral Pathology Service of Universidade Federal de Minas Gerais (Brazil). Histological slides stained with haematoxilin and eosin (HE) were reviewed in accordance to the WHO classifications (1992, 2005).[1,2] Cases of KOT associated with NBCCS, recurrent KOT and biopsies of KOT following decompression treatment were excluded. The study was approved by the Committee of Ethics in Research of Universidade Federal de Minas Gerais (UFMG/COEP - 15/08).

Inflammatory assessment

Using an optical microscope (Axiolab Zeiss, Germany) at 400x magnification, the inflammatory score was determined by counting total of inflammatory cells adjacent to the epithelium in 20 consecutive fields, in one high power field (HPF)-depth from the basement membrane (BM). It was graded on: Grade 0 – no inflammation, Grade 1 – <15 cells/field, Grade 2 – 15–50 cells/field, and Grade 3 – >50 cells/field. Inflammatory score was calculated as the average of all HPFs examined. RC, DC and KOT cases were divided into two groups

according to the inflammatory score: group A – grades 0–2 (mild-to-moderate), and group B – grade 3 (intense).[20] In RC and DC, 5 cases belonged to Group A and 4 cases to Group B. In KOT, 4 cases belonged to Group A and 4 cases to Group B. Three cases of OOC belonged to Group A and 1 case to Group B, but OOC was not included in analysis of inflammation.

Immunohistochemistry

Immunohistochemical reaction was performed using streptavidin-biotin (SB) standard protocol. Sections of 4μm from routinely processed paraffin-embedded blocks were deparaffinized and dehydrated. Specimens were immersed in antigen retrieval buffer for 20 minutes at 98ºC (Table 1). For MT, endogenous avidin was blocked according to Miller *et al.*[21]. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Sections were incubated with primary monoclonal antibodies (Dako, Carpinteria, CA, USA) listed in Table 1. Detection was performed using LSAB®+system, HRP Peroxidase Kit (Dako Corporation, Carpinteria, CA, USA, K0690) and 3.3'-diaminobenzidine tetra hydrochloride chromogen (DAB, Sigma Chemical, St. Louis, USA, D5637). Mayer's haematoxilin was used for counter staining. Squamous cell carcinoma was the positive control.

Immunohistochemical assessment

Twenty digital images were captured with a digital micro camera (JVC TK-1270/RGB, Tokyo, Japan), at 400x magnification and analyzed using KS300 software coupled to a Carl Zeiss Image Analyzer (Oberkochen, Germany). MT and Ki-67 immunoexpression was stratified by epithelium layer into basal and suprabasal. Basal layer was composed of columnar or cuboidal cells located from one to three rows at or near the BM.[18] Suprabasal layer was composed of relatively large round cells above basal layer. Cell compartment with MT staining was registered: cytoplasmatic and nuclear, cytoplasmatic only or nuclear only,

whereas the Ki-67 staining was nuclear. Indexes of labeled cells for MT (IMT) and Ki-67 (IK) were obtained dividing the positive cells per total epithelial cells and multiplying by 100.

Statistical analysis

BioEstat® 4.0 software (BioEstat, Brazil) was used. Since IMT total, IMT cytoplasmatic, IMT nuclear, and IK total did not have a normal distribution in Shapiro-Wilk tests, Kruskal-Wallis and Mann-Whitney U-test were used. An adjustment for six post hoc tests was done using Bonferroni procedure, with 5% level of statistical significance attained at $p<0.008$. Conversely, values for IMT basal, IMT suprabasal, IMT nuclear and cytoplasmatic showed a normal distribution in Shapiro-Wilk tests, though one-way analysis of variance (ANOVA) test (with Tukey post hoc test) was used, with statistical significance of $p<0.05$. Spearman's correlation was performed to correlate IMT and IK in RC (It did not have a normal distribution). Conversely, IMT DC, IMT KOT, IMT OOC and IK CD, IK KOT, IK OOC showed a normal distribution, though Pearson's correlation was performed to correlate IMT and IK in DC, KOT and OOC. Correlation was graded on: weak – <0.30; moderate – 0.30-0.50; and Strong – >0.50.[22]

For inflammation analysis, since IMT (in all lesions grouped, and in RC) and IK (in all lesions grouped) did not have a normal distribution, Mann-Whitney U-test was used. Conversely, IMT (in KOT and DC) and IK (in RC, DC and KOT) showed a normal distribution, though Student t-test was used, with statistical significance of $p<0.05$.

Results

MT immunoexpression was identified in epithelial cells in all cases, with cells showing high heterogeneity of staining, from negative to strongly positive. In RC (Figure 1B) and DC (Figure 1E) MT staining was observed in all epithelial layers. In KOT, MT staining was present in basal layer and in the inferior half of suprabasal layer, and absent or rare in the

superior half (Figure 1H). In OOC, MT staining was visualized in basal layer and absent or scarce in suprabasal layer (Figure 1K). No significant difference was observed in IMT in basal layer among the lesions. Besides, considering IMT in suprabasal layer, significant difference was found only between RC and OOC. In all lesions staining was predominantly found in nuclei and cytoplasm simultaneously (Table 2). KOT (Figure 1I) showed higher number of Ki-67 positive cells than RC (Figure 1C), DC (Figure 1F), and OOC (Figure 1L). Median of IMT and IK, and comparisons among lesions are in Table 3. A strong inverse correlation was observed between IMT and IK in KOT (Pearson correlation coefficient [r]= -0.5126), and in OOC (r= -0.6140). However, a strong positive correlation in RC (Spearman correlation coefficient [r_s]= 0.6178) and a moderate positive in DC were identified (r= 0.4763). No statistical significant differences in IMT or IK were observed between Group A and Group B in RC (IMT median 91% and 91% respectively; IK mean 3%±2 and 4%±2), DC (IMT mean 86%± 4 and 92%±6; IK mean 2%± and 3%±), and KOT (IMT mean 78%±2 and 78%±7; IK mean 13%±3 and 13%±4). Similar results were observed when they were grouped (IMT median 85% and 87%; IK median 3 % and 5%).

Discussion

MT plays a main role in the homeostasis of essential metals, and it is also correlated with: 1) protection against toxicity of heavy metals and free radicals[11] 2) inhibition of cellular apoptosis;[8] 3) carcinogenesis;[7,12,13] 4) regulation of cellular differentiation and proliferation.[9,23] MT is mainly a cytoplasmatic protein, however, in the present study a predominance of cytoplasmatic and nuclear staining simultaneously was observed, which was also demonstrated in normal oral mucosa.[14] High levels of MT in cell nucleus may be related to the increased requirement of zinc for several metallo-enzymes and transcription factors during rapid growth. Function of nuclear MT may be to inhibit the cellular apoptosis, to protect the cell from free radicals, and to regulate gene expression during cell cycle.[24]

MT regulates cell differentiation as it potentially affects the activity of some transcriptional factors by donating zinc. Also, MT immunoexpression decreases towards tissue development, so a well differentiated tissue shows lower MT immunoexpression.[23] KOT shows none or scarce mature keratinocytes, suggesting alteration in the differentiation process. Unlikely, OOC shows higher degree of squamous differentiation.[3,25] It was verified by the identification of cytokeratin (CK) 10 in suprabasal and superficial layers of the epithelium in the OOC, thus revealing that a constant process of keratinization is taking place. On the other hand, OKC expressed CK 10 only in the superficial layer, indicating the absence or the scarcity of mature keratinocytes.[3] Taken together, these observations are in accordance to the lower IMT found in this study in OOC, when compared with KOT. Also a higher IMT in suprabasal layer was observed in KOT (33%±12) than in OOC (16%±07), although this was not statistically significant. Unlikely, in DC and RC no gradual maturation of epithelial cells occurs through their migration to upper layers,[26] and it was verified by the CK 10 stain superficial or absent in these lesions.[27] It is in accordance to the present study that showed a highest IMT in all epithelial layers in these lesions. In mouse epithelium, in situ hybridization revealed abundant MT-I mRNA in basal layer and lower in the spinous one. Conversely, MT-IV mRNA was undetectable in basal cells and showed high levels in spinous layer, thus, there is a switch in MT isoform synthesis during differentiation of these epithelia.[10] In the present study, the basal layer showed the highest IMT (MTI+II) in all cases. Further studies may be developed to evaluate MT-IV immunoexpression in these lesions.

It is possible that MT may inhibit in apoptosis by: 1) regulation of cellular zinc concentration through zinc-dependent antiapoptotic transcription factor nuclear κB; 2) inducing antiapoptotic oncogenes as *bcl-2 and c-myc*; and 3) inhibiting proapoptotic tumour suppressor proteins as activation of caspase-1 and -3 or cytochrome c leakage.[28] Many studies using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay have showed an inverse relation between MT immunoexpression and apoptosis.[7,29,30] However, other studies demonstrated positive correlations,[31,32] and no correlation.[33] In the present study, the high MT immunoexpression observed in odontogenic

cysts and KOT suggests a low apoptotic index, which may influence the biological behavior of these lesions. It was reported that KOT shows a higher apoptotic index than DC assessed by TUNEL and methyl green-pyronin.[5,18] Also, analyzing the results of Kimi *et al.*,[34] Edamatsu *et al.*,[35] and Suzuki *et al.*[36], performed in the same department and using the same methodology, it is possible to observe a similar immunoexpression of single-stranded DNA, ssDNA - an antibody which recognizes DNA fragmentation in nuclei during apoptosis, between DC and RC, and higher in KOT than RC and DC. In accordance with these findings, no difference in IMT was observed between RC and DC in the present study. The higher KOT apoptotic index compared with RC and DC[5,18,34-36] may be correlated with lower IMT, which was observed in the present study. Superficial layer, the superior half of suprabasal layer, is responsible for the higher apoptotic cells in KOT compared with DC assessed by TUNEL and methyl green-pyronin,[5,18] and exactly in this layer that a absent or rare MT expression was observed in KOT and present in DC.

OOC and KOT show similar apoptotic index by TUNEL assay,[37] however the authors did not specify the criteria for differentiation between these two cysts. Apoptotic cellular process is determined by interactions among bcl-2 family members, as bcl-2 (antiapoptotic) and bax (pro-apoptotic bcl-2) proteins. It seems that bcl-2/bax ratio is the factor correlated with apoptosis. Rangiani and Motahhary[6] observed a higher bcl-2/bax ratio in KOT compared with OOC, which may leads to higher cell survival rate. This finding can be correlated with the higher IMT in KOT than OOC found in our study. Also, the presence of MT in the inferior half of the suprabasal layer observed in KOT and not identified in OOC may also be correlated with the higher bcl-2/bax ratio in the first. These findings reinforce the role of MT as inhibitor of cellular apoptosis in KOT. A higher MT immunoexpression in KOT may help explaining the higher cell survival rate found in this lesion and its more aggressive behavior than OOC.

The correlation of the MT and cellular proliferation varies in accordance with the tissue. A positive correlation was observed between MT and Ki-67 in malignant lesions.[9,31,32,38,39] In the present study, this positive correlation was strong in RC and

moderate in DC cases. It is supposed that in these lesions MT can be a role in the cellular proliferation. No correlation was described in malignant,[12,13,40] hyperplasic,[38] benign and borderline lesions.[39] Although, MT immunoexpression in normal endometrium was inversely correlated with Ki-67,[38] as found in the present study in KOT and OOC cases. It is possible that in KOT and OOC the MT is more related to apoptosis inhibition and to cellular differentiation than to cellular proliferation. Previous studies reported a higher IK in KOT than in RC, DC or OOC,[5,18,19,25] and this were also observed in the present study.

Synthesis of MT is induced by inflammation.[24] However, in the presented study the inflammation did not interfere in the IMT and IK in RC, DC and KOT. de Paula *et al*.[41] reported alteration in IK in the presence of inflammation, but this study design was based on number of positive cells per unit length of BM. Kaplan and Hirshberg[42] recalculated these data to labeling index, and verified that the IK remained unchanged in the inflamed cysts. Also, these authors[42] observed no effect of inflammation on the overall IK.

IMT was highest in RC and DC followed by KOT and OOC. The differences in MT immunoexpression may be possible related with cellulatr differentiation and inhibition of apoptosis. Also, MT immunoexpression did seem to be inversely correlated with the cellular proliferation in KOT and OOC, but directly in RC and DC. Besides, IMT and IK did not seem to be influenced by inflammation.

Conflict of interest statement

None declared.

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Figure 1

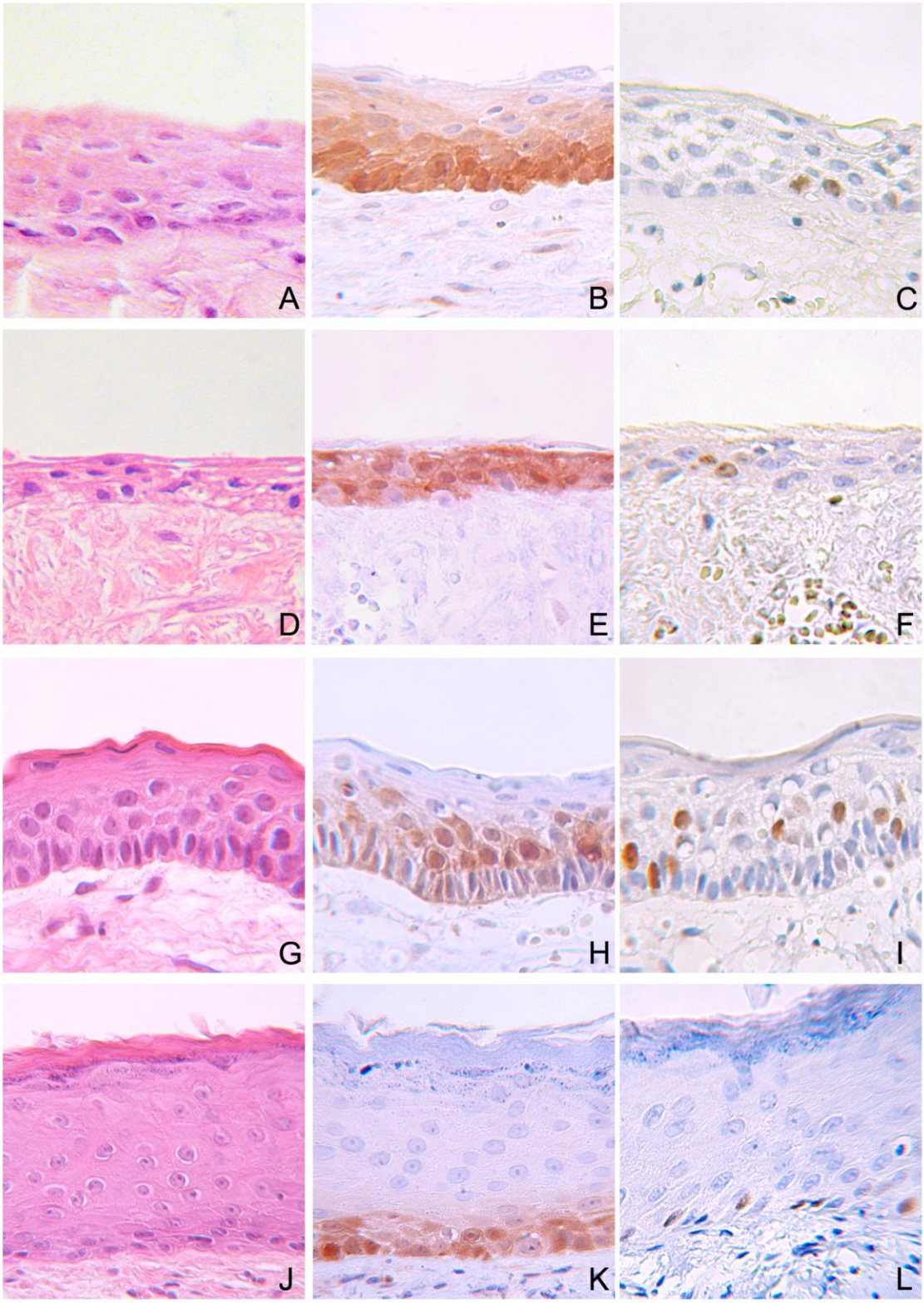

Figure Caption

Figure 1 – Radicular cyst: (A) cystic cavity lined by nonkeratinizing stratified squamous epithelium (Haematoxilin and eosin-HE, magnification X200); (B) It showed a high MT immunoexpression present in all layers (Streptavidin-biotin-SB, magnification X200). (C) It was observed few Ki-67 positive cells (SB, magnification X200). **Dentigerous cyst:** (D) cystic cavity lined by nonkeratinizing squamous epithelium of few layers (HE, magnification X200); (E) It exhibited a similar pattern of MT staining than RC (SB, magnification X200). (F) It was also observed few Ki-67 positive cells (SB, magnification X200). **Keratocystic odontogenic tumour:** (G) a cystic wall lined by stratified squamous epithelium with a well-defined basal layer of palisading columnar or cuboidal cells and with a corrugated surface of parakeratin (HE, magnification X200); (H) It was identified a decreased MT immunoexpression compared with RC and DC, and the staining was present in basal layer and in the inferior half of the suprabasal layer, and absent or rare in the superior half (SB, magnification X200) (I) It was identified a higher number of Ki-67 positive cells in KOT than RC and DC (SB, magnification X200). **Orthokeratinized odontogenic cysts:** (J) cystic wall lined by orthokeratinized squamous epithelium (HE, magnification X200); (K) It was observed a decreased MT immunoexpression compared with RC, DC and KOT, and the staining was visualized in basal layer and absent or scarce in suprabasal layer (SB, magnification X200) (L) It was observed a lower number of Ki-67 positive cells in OOC than KOT (SB, magnification X200).

Tables

Table 1

Antibodies, clones, dilutions, incubation and antigen retrieval details.

Antibody (Clone)	*Dilution*	*Incubation temperature/ time*	*Antigen retrieval/ pH*
MT-I+II (E9 / M0639)	1:100	4ºC / 18 hours	EDTA[a]/ 8.0
Ki-67 (MIB-1 / M7240)	1:100	Room / 18 hours	Citrate buffer[b] / 6.0

[a]1 mM ethylenediamine tetraacetic acid buffer (Vetec Química Fina, Rio de Janeiro, Brazil, 114); [b]10 mM citrate buffer (Lab Synth, Diadema, Brazil, A2270).

Table 2

Indexes of labeled cells for metallothionein stratified by cell compartment in radicular cyst, dentigerous cyst, keratocystic odontogenic tumour, and orthokeratinized odontogenic cyst, and comparisons among these lesions.

Lesion	**IMT% Median**		**IMT% Mean/SD**	***Comparisons***	***p value (Mann-Whitney U-test)***		***p value (Tukey Test)***
	N	**C**	**NC**		**N**[a]	**C**[b]	**NC**[c]
RC	01	03	83±11	RC compared with DC	NS	NS	NS
				RC compared with KOT	NS	NS	<0.05
				RC compared with OOC	NS	<0.008	<0.05
DC	01	03	84±06	DC compared with KOT	NS	NS	<0.05
				DC compared with OOC	NS	<0.008	<0.05
KOT	02	03	70±05	KOT compared with OOC	NS	<0.008	<0.05
OOC	06	00	54±03				

[a]Kruskal-Wallis, p>0.05; [b]Kruskal-Wallis, p<0.05; [c]ANOVA, p<0.05; SD= standard deviation values; N= nuclear only; NC= both nuclear and cytoplasmatic; C= cytoplasmatic only; NS= non-significative.

Table 3

Indexes of labeled cells for metallothionein and Ki-67 in radicular cyst, dentigerous cyst, keratocystic odontogenic tumour, and orthokeratinized odontogenic cyst, and comparisons among these lesions.

Lesion	***Number of cases***	***Median%***		***Comparisons***	***p value (Mann-Whitney U-test)***	
		IMT	***IK***		***IMT*[a]**	***IK*[a]**
RC	09	91	03	RC compared with DC	NS	NS
				RC compared with KOT	<0.008	<0.008
				RC compared with OOC	=0.009[b]	NS
DC	09	89	02	DC compared with KOT	<0.008	<0.008
				DC compared with OOC	<0.008	NS
KOT	08	78	12	KOT compared with OOC	<0.008	<0.008
OOC	04	63	04			

[a]Kruskal-Wallis, p<0.05; NS= non-significative; [b]In the limit of statistical significance.

ARTIGO 2 - Metallothionein immunoexpression in non-syndromic and in syndromic keratocystic odontogenic tumour

TITLE: Metallothionein immunoexpression in non-syndromic and in syndromic keratocystic odontogenic tumour

RUNNING TITLE: Metallothionein in keratocystic odontogenic tumour

SUMMARY

Odontogenic keratocyst has been re-classified by World Health Organization (2005) into a benign neoplasm and though it was re-named to keratocystic odontogenic tumour (KOT). KOT can occur sporadically (non-syndromic KOT) or in association with the Nevoid Basal Cell Carcinoma Syndrome (NBCCS- syndromic KOT). When associated with NBCCS, KOT exhibits a higher growth, destructive capacity and a tendency to develop more recurrences. Metallothionein (MT) is a protein which has been correlated with cellular apoptosis, differentiation and proliferation. It was hypothesized that these lesions show differences in MT expression. The goals of this study were to: 1) report and to compare the MT immunoexpression in non-syndromic and syndromic cases of KOT; 2) correlate MT immunoexpression with cellular proliferation in these lesions; and 3) evaluate the influence of inflammation in MT immunoexpression. Fourteen cases of KOT (8 non-syndromic and 6 syndromic KOT) were submitted to immunohistochemistry using anti-MT and anti-Ki-67. The lesions were grouped in accordance to grade of inflammation: mild-to-moderate (group A), and intense (group B). The indexes of MT (IMT) and Ki-67 (IK) were obtained. Statistical analysis was performed using BioEstat® 4.0 software. IMT was, with statistical significance, higher in non-syndromic KOT (78%± 05) than in the syndromic one (70%± 02), however no difference in IK (13%± 03 and 12%± 03 respectively) was observed. An inverse correlation observed between IMT and IK was strong in non-syndromic KOT (Pearson correlation coefficient [r]= -0.5126) and weak in syndromic KOT (r= -0.1678). No differences in IMT and in IK were observed between group A and group B. Syndromic KOT has different MT immunophenotype from non-syndromic one, which could be more contributory in cellular apoptosis than cellular proliferation. Besides, IMT and IK did not seem to be influenced by inflammation.

Keywords: odontogenic tumours, neoplasm, basal cell nevus syndrome, metallothionein, Ki-67 Antigen, MIB-1 antibody, immunohistochemistry.

Introduction

Odontogenic keratocyst has been re-classified by World Health Organization (2005)[1] as a benign odontogenic neoplasm derived from odontogenic epithelium and though it was re-named to keratocystic odontogenic tumour (KOT). This lesion shows a locally aggressive clinical behavior associated with a high rate of recurrence. KOT can occur sporadically (non-syndromic KOT) or can be multiple in association with the Nevoid Basal Cell Carcinoma Syndrome (NBCCS) or Gorlin syndrome (syndromic KOT), a rare autosomal-dominant condition characterized by multiple basal cell carcinomas, skeletal changes such as fused, bifid and splayed ribs, multiple KOTs and dyskeratosis of the palms and soles.[2]

There are differences between these lesions: 1) in the expression of bcl-1 and p53,[3] cytokeratin 17,[4] Fas, p16, DNA topoisomerase IIα,[5] GLI-1,[6] Bcl-2, $p27^{Kip1}$, c-erbB-2,[7] heparanase,[8] substance P;[9] 2) a higher frequency of intramural epithelial remnants and satellite cysts in syndromic KOT; 3) a greater number of cellular nuclei, epithelial height and basal nuclear density in non-syndromic KOT;[10,11] and 4) a higher frequency of multiple cysts in syndromic KOT[12]. These differences have been associated to a higher growth and destructive capacity and a tendency to develop more recurrences showed by syndromic KOT.[7,10,13] This supports the existence of a distinct immunophenotype and biological behavior of syndromic KOTs and non-syndromic KOTs.

Metallothionein (MT) is a low molecular weight protein that has a important role in homeostasis of essential metals, cellular differentiation and proliferation, and inhibition of cellular apoptosis. These functions are implicated in normal cellular process, and also may interfere in the biological behavior of many lesions. [14,15,16] This protein is detected under four isoforms (MT-I to IV). MT-I and MT-II isoforms are similar and observed in many tissues, including in oral epithelium. MT-III is found mainly in the central nervous system and MT-IV is only expressed in differentiated cells of stratified squamous epithelium.[17,18] In oral cavity, the immunoexpression of MT-I and MT-II evaluated simultaneously by MT monoclonal antibody E9 (MT-I+II) was performed in periodontitis;[19] amalgam tatoos,[20] premalignant,[21] and

malignant lesions.[14,22,23] It was hypothesized that syndromic and non-syndromic KOT show differences in MT expression, and this was not investigated yet.

Ki-67 is a nuclear protein correlated with cellular proliferation, present in all active phases of the cell cycle (G1, S, G2, M) but absent in G0.[24] The immunodetection of this protein has been widely used for evaluation of cellular proliferation in syndromic and non-syndromic KOTs.[7,25-28]

The goals of this study were to: 1) report and to compare the MT immunoexpression in syndromic and non-syndromic KOT; 2) correlate the MT immunoexpression with cellular proliferation in these lesions; and 3) evaluate the influence of the inflammation in MT immunoexpression.

Materials and methods

Specimens

Samples diagnosed as KOT: 8 cases non-syndromic (Figure 1A) and 6 syndromic (Figure 1C) were retrieved from files of Oral Pathology Service of Universidade Federal de Minas Gerais (Brazil). The histological slides stained with haematoxilin and eosin (HE) were reviewed, and the diagnosis were in accordance to the WHO classification (2005).[1] The diagnosis of NBCCS was made in the presence of two major criteria or one major and two minor criteria: 1) major criteria- multiple basal cell carcinomas or one in a patient under 20 years of age; KOTs of the jaws proven by histopathology; three or more palmar or plantar pits; bilamellar calcification of the falx cerebri; bifid, fused or splayed ribs; first-degree relative with BCNS; 2) minor criteria- macrocephalia determined after adjustment for height; congenital malformations as cleft lip or palate, frontal bossing, "coarse face", moderate of severe hypertelorism; other skeletal alterations as sprengel deformity, marked pectus deformity, marked syndactyly of the digits; radiological alterations as bridging of the sella turcica, vertebral anomalies such as hemivertebrae, fusion or elongation of the vertebral

bodies, modeling defects of the hands and feet, or flame-shaped lucencies of the hands or feet; ovarian fibroma; and medulloblastoma.[29] Cases of recurrent KOT and biopsies of lesions following decompression treatment were excluded. The study protocol was approved by the Committee of Ethics in Research of Universidade Federal de Minas Gerais (UFMG/COEP - number 15/08).

Inflammatory assessment

Using an optical microscope (Axiolab Zeiss, Germany) at 400x magnification, the inflammatory score was determined counting inflammatory cells adjacent to the epithelium in 20 consecutive fields, in one high power field (HPF)-depth from the basement membrane (BM). It was graded on: Grade 0 – no inflammation, Grade 1 – <15 cells/field, Grade 2 – 15–50 cells/field, and Grade 3 – >50 cells/field. The inflammatory score was calculated as the average of all HPFs examined. KOT cases were divided into two groups according to the inflammatory score: group A – grades 0–2 (mild-to-moderate), and group B – grade 3 (intense).[30] In non-syndromic KOT, 4 cases belonged to group A and 4 cases to group B. All cases of syndromic KOT belonged to group A.

Immunohistochemistry

Streptavidin-biotin (SB) standard protocol was performed. Sections of 4μm from paraffin-embedded blocks were deparaffinized and dehydrated and submitted to antigen retrieval buffer (Table 1) for 20 minutes at 98ºC. Blockage of endogenous peroxidase activity was made using 0.3% hydrogen peroxide in all cases, and the endogenous avidin block was made in accordance to Miller *et al*.[31] for MT. Sections were incubated with primary monoclonal antibodies (Dako, Carpinteria, CA, USA) listed in Table 1, and the detection was made using LSAB®+system, HRP Peroxidase Kit (Dako Corporation, Carpinteria, CA, USA, K0690) and 3.3'-diaminobenzidine tetrahydrochloride chromogen (DAB, Sigma Chemical, St.

Louis, USA, D5637). Mayer's Haematoxilin was used for counter staining. The positive control used was squamous cell carcinoma.

Immunohistochemical assessment

Twenty digital images were captured with a digital micro camera (JVC TK-1270/RGB, Tokyo, Japan) at 400x magnification, and analyzed using KS300 software coupled to a Carl Zeiss Image Analyzer (Oberkochen, Germany). To analyze MT and Ki-67 imunnoexpression stratified by layer, cells of the lining epithelium were divided into basal and suprabasal layers. The basal layer corresponded to columnar or cuboidal cells located from one to three rows at or near the basement membrane.[32] The suprabasal layer was composed of relatively large round cells above the basal layer. Also, the cell compartment with MT staining was registered: cytoplasmatic and nuclear, cytoplasmatic only or nuclear only, whereas the Ki-67 staining was only nuclear. The indexes of labeled cells for MT (IMT) and Ki-67 (IK) were obtained dividing the total positive cells per total epithelial cells and multiplying by 100.

Statistic analysis

BioEstat® 4.0 (BioEstat, Brazil) software was used in the statistical analysis. The Student t-test was employed in analysis of IK total, IK group A and B, IMT group A and B, IMT total, IMT citoplasmatic and nuclear, IMT basal, and IMT suprabasal as they had a normal distribution in Shapiro-Wilk tests. Mann-Whitney U-test was used in analysis of IMT nuclear and IMT cytoplasmatic as they did not show a normal distribution in Shapiro-Wilk tests. Statistical significance was accepted at $p<0.05$. Pearson's correlation was used to evaluate the correlation between IMT and IK in syndromic and in non-syndromic KOT as they did show a normal distribution. The correlation was graded on: weak – <0.30; moderate – 0.30-0.50; and Strong – >0.50.[33]

Results

MT immunoexpression was identified in epithelial cells in all cases, presenting the mosaic pattern, with cells showing high heterogeneity of staining, from negative to strongly positive. Non-syndromic KOT (Figure 1B) showed higher IMT than syndromic KOT (Figure 1E), and it was statistically significant. In both lesions the staining was predominantly found in nuclei and cytoplasm simultaneously (Table 2). Differences were not found in MT immunoexpression in basal or suprabasal layer between syndromic KOT and non-syndromic KOT. The mean of IMT and IK, and comparisons between the lesions are displayed in Table 3. Similar number of Ki-67 positive cells was observed between non-syndromic KOT (Figure 1C) and syndromic KOT (Figure 1F). An inverse correlation observed between IMT and IK was strong in non-syndromic KOT (Pearson correlation coefficient [r]= -0.5126), and weak in syndromic KOT (r= -0.1678). Neither statistical significant differences in IMT or in IK was observed comparing group A with group B in non-syndromic KOT (IMT mean 78%±02 and 78%±07, respectively; IK mean of 13%±03 and 13%±04), nor when syndromic KOT are grouped (IMT mean of 73%±04 and 78%±07; IK mean of 12 %± 03 and 13%±04).

Discussion

MT is an important protein related to homeostasis of essential metals, protection against toxicity of heavy metals and free radicals,[18] inhibition of cellular apoptosis,[15] carcinogenesis,[22] cellular differentiation, tissue development, and cellular proliferation.[34] In mammalian cells, MT is mainly a cytoplasmatic protein, but it can also be observed in nuclei.[35] However, in the present study MT was found mainly in cytoplasmatic and nuclear compartments simultaneously, similar to that reported in normal oral mucosa.[21] High levels of

MT in the nucleus have been related to an increased requirement of zinc for several metallo-enzymes and transcription factors during rapid growth.[36]

Apoptosis has diverse roles in tissue development and normal homeostasis, as well as in a variety of pathologic conditions.[15] MT may inhibit apoptosis by controlling cellular zinc levels through the zinc-dependent antiapoptotic transcription factor nuclear κB, by induction of antiapoptotic oncogenes, or by inhibition of proapoptotic proteins.[37] Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay and methyl green-pyronin demonstrated that the apoptotic index was slightly higher in syndromic than non-syndromic KOT, but the difference was not significant[26,38] Also, a higher apoptotic index was reported in syndromic KOT than non-syndromic evaluated by single-stranded DNA, ssDNA - an antibody that recognizes DNA fragmentation in nuclei during apoptosis.[5] This finding may be correlated with the present study, in which lower IMT was observed in syndromic KOT than in non-syndromic KOT, and this may signify a low inhibition of cellular apoptosis, with a consequent increase of apoptosis in syndromic KOT. Besides, syndromic KOT has a different MT immunophenotype from non-syndromic one. It is suggested that the more aggressive clinical behavior of syndromic KOT in relation to non-syndromic KOT may be not related to differences in the cell turnover, proliferation or apoptosis of the epithelial lining, but to the multiplicity of lesions and early development of syndromic KOTs.[26]

The correlation of the MT and cellular proliferation varies in accordance to the tissue. Normal endometrium MT immunoexpression was inversely correlated with Ki-67,[39] as found in the present study in syndromic and in non-syndromic KOT. It is suggest that MT is more related with apoptosis inhibition than cellular proliferation in these lesions. However, a positive correlation was observed between MT and Ki-67 in malignant lesions.[16,39,40] No correlation was also reported in malignant,[22,23] benign and borderline lesions.[39] The IK was similar in non-syndromic and syndromic KOT and it was also described by other authors.[26,27,38] However, other studies reported a higher Ki-67 expression in the syndromic KOT, but these studies design were based on calculations of the number of positive cells per unit length of BM (cells/μm BM).[25,28] Pan and Li[28] observed that the number of Ki-67 positive

cells/μm BM in KOTs with *PTCH1* mutation was significantly higher than in cases with no *PTCH1* mutation. As most mutations identified in patients with NBCCS or related non-syndromic KOTs resulted in premature truncation of the *PTCH1* protein. Pan and Li[28] also detected that KOTs harboring *PTCH1* truncation-causing mutations showed an even greater Ki-67 immunoexpression than those with non-truncation-causing mutations, and it was also observed when syndromic and non-syndromic KOTs were compared separately. These results suggest that *PTCH1* mutations, particularly those causing protein truncations, are associated with a subgroup of KOTs which shows increased proliferative activity and thus may be related to a higher recurrent tendency phenotype. Further studies correlating *PTCH1* mutation, MT immunoexpression and KOT recurrence should be performed to improve the knowledge about the aggressive behavior of syndromic or non-syndromic KOT. In basal layer, similar IK was observed between non-syndromic and syndromic KOT, and it was previously reported;[26] however a higher IK in suprabasal layer was observed in syndromic KOT than non-syndromic ones and it was different of the report of literature (acho que isto não está acrescentando o trabalho, o que vc acha?).[26]

Metals, hormones, cytokines, a variety of other chemicals, inflammation, and stress induce the synthesis of MT.[36] However, this was not observed in the presented study, in which the inflammation did not interfere in the IMT and IK immunoexpression. Kaplan and Hirshberg[41] also observed no significant effect of inflammation on the overall Ki-67 expression in KOT.

In conclusion, syndromic KOT has a different MT immunophenotype from the non-syndromic one, which can contribute more with cellular apoptosis than with cellular proliferation. Besides, MT immunoexpression does not seem to be related with the cellular proliferation in KOT. IMT and IK seems no influenced by inflammation.

Conflict of interest statement

None declared.

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Figure1

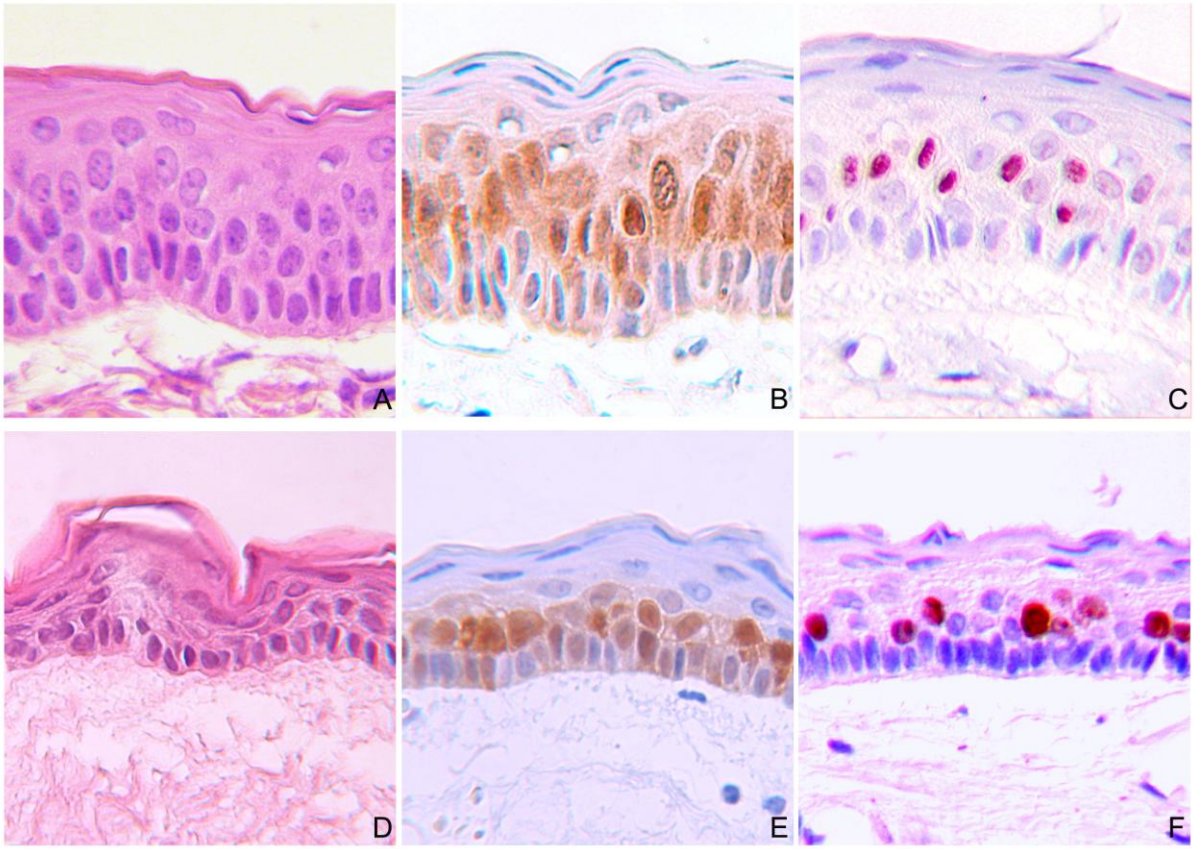

Figure caption

Figure 1- **Non-syndromic keratocystic odontogenic tumour:** (A) It was observed a thin connective tissue cystic wall lined by stratified squamous epithelium with a well-defined basal layer of palisading columnar or cuboidal cells and with a corrugated surface of parakeratin (Haematoxilin and eosin-HE, magnification X200); (B) MT staining was present in basal layer and in the inferior half of the suprabasal layer, and absent or rare in the superior half of the suprabasal layer (Streptavidin-biotin-SB, magnification X200); (C) Few positive cells to Ki-67 was identified (Streptavidin-biotin-SB, magnification X200). **Syndromic keratocystic odontogenic tumour** (D) the same histological characteristics of non-syndromic one (HE, magnification X200); (E) Compared with non-syndromic KOT, it was observed a lower MT staining (SB, magnification X200); (F) Also compared with non-syndromic KOT, it was observed a similar number of Ki-67 positive cells (Streptavidin-biotin-SB, magnification X200).

Tables

Table 1

Antibodies, clones, dilutions, incubation and antigen retrieval details.

Antibody (Clone)	*Dilution*	*Incubation temperature/ time*	*Antigen retrieval/ pH*
MT-I+II (E9 / M0639)	1:100	4ºC / 18 hours	EDTA[a]/ 8.0
Ki-67 (MIB-1 / M7240)	1:100	Room / 18 hours	Citrate buffer[b] / 6.0

[a]1 mM ethylenediamine tetraacetic acid buffer (Vetec Química Fina, Rio de Janeiro, Brazil, 114); [b]10 mM citrate buffer (Lab Synth, Diadema, Brazil, A2270).

Table 2

Indexes of labeled cells for metallothionein stratified by cell compartment in non-syndromic and syndromic keratocystic odontogenic tumour.

Lesion	***IMT %* Median**		***IMT% Mean/ SD***
	C	**N**	**NC**
Non-syndromic KOT	03[a]	02[b]	70[c] ± 05
Syndromic KOT	02[a]	09[b]	57[c] ± 05

[a]Mann-Whitney U-test: $p>0.05$; [b]Mann-Whitney U-test: $p<0.05$; [c]Student t-test: $p<0.05$; SD= standard deviation values; C= cytoplasmatic; N= nuclear; NC= both nuclear and cytoplasmatic.

Table 3

Indexes of labeled cells for metallothionein and Ki-67 in non-syndromic and syndromic keratocystic odontogenic tumour.

Lesion	*IMT% Mean/ SD*	*IK% Mean/DP*
Non-syndromic KOT	$78^{a} \pm 05$	$13^{b} \pm 03$
Syndromic KOT	$70^{a} \pm 02$	$12^{b} \pm 03$

Student t-test: $^{a}p<0.05$, $^{b}p>0.05$; SD= standard deviation values.

ARTIGO 3 - Metallothionein immunoexpression in benign odontogenic tumours

TITLE: Metallothionein immunoexpression in benign odontogenic tumours

RUNNING TITLE: Metallothionein in benign odontogenic tumours

Keywords: odontogenic tumour, ameloblastoma, metallothionein, Ki-67 Antigen, proliferating cell nuclear antigen.

ABSTRACT

BACKGROUND: Benign odontogenic tumours derive from tooth-forming tissues, and they present different biological behavior. Metallothionein (MT) is correlated with homeostasis of essential metals, cellular differentiation and proliferation, and inhibition of cellular apoptosis, and these processes may be associated with biological behavior of many lesions. The aims were: to compare MT immunoexpression among benign odontogenic tumours; and to correlate MT with cellular proliferation and inflammation.

MATERIAL AND METHODS: Solid ameloblastomas (SABs), squamous odontogenic tumours (SOTs), adenomatoid odontogenic tumours (AOTs), calcifying cystic odontogenic tumours (CCOTs), and calcifying epithelial odontogenic tumours (CEOTs) were submitted to immunohistochemistry for MT, Ki-67 and PCNA. Indexes of MT (IMT), Ki-67 (IK) and PCNA (IP) were obtained. In SAB, inflammatory cells were counted. Statistical analysis was performed using BioEstat® 4.0.

RESULTS: MT staining was variable among lesions. IMT was 93.1% in SAB, 52.9% in SOT, 38.4% in AOT, 76.8% in CCOT, and 0% in CEOT. Significant diferences were observed between SAB and SOT, AOT, or CCOT. A weak or strong positive correlation between IMT and IK or IP was observed in SAB, SOT and CCOT, but a strong inverse correlation was observed in AOT. In SAB, a strong positive correlation between inflammation and IMT was observed.

CONCLUSIONS: IMT variated among lesions and this may be due to its role in cellular differentiation and/or biological behaviour of tumours. Correlation of IMT and cellular proliferation was inverse in AOT, but direct in SAB, SOT and CCOT. Besides, in SAB, IMT seemed to be influenced by inflammation.

Introduction

Odontogenic tumours are benign, malignant and non-neoplastic lesions which derive from tooth-forming tissues. According to the types of tissues involved in their development, benign odontogenic tumours may be classified in following groups: 1) odontogenic epithelium without odontogenic ectomesenchyme; 2) odontogenic epithelium with odontogenic ectomesenchyme; 3) mesenchyme and/or odontogenic ectomesenchyme with or without odontogenic epithelium. Ameloblastoma (AB), squamous odontogenic tumour (SOT), calcifying epithelial odontogenic tumour (CEOT), and adenomatoid odontogenic tumour (AOT) are classified in the first group. The calcifying cystic odontogenic tumour (CCOT), formerly called calcifying odontogenic cyst, was classified in the second group. These tumours show diverse histopathologic features and clinical manifestations (1).

Metallothionein (MT) is a low molecular weight protein (6-7 kDa) characterized by high levels of cysteines, which are bound to metal ions, correlated with many important processes in the cell as homeostasis of essential metals, protection against toxicity of heavy metals and free radicals (2), regulation of cellular differentiation and proliferation (3, 4), and inhibition of cellular apoptosis (5). These functions are associated with normal cellular processes, and they also may be related with the biological behavior of many lesions (6, 7). It was hypothesized that there are differences in MT immunoexpression among benign odontogenic tumours, which should be correlated with degree of cellular differentiation and/ or biological behavior.

Proliferating cell nuclear antigen (PCNA) and Ki-67 proteins are involved in mechanisms of cellular proliferation and have been widely used for evaluation of cellular proliferation in odontogenic tumours (8-16). PCNA is a protein associated with the cell cycle, being an auxiliary protein of DNA polymerase-δ, and whose concentration increases through G1, peaks at the G1/S interphase and decreases through G2 phase (17). Ki-67 is present in all active phases of the cell cycle (G1, S, G2, M) and absent in G0 (18). Many studies have been correlating the MT expression with PCNA and KI-67 (3, 4, 19-22).

The present study approaches two aims: 1) to report and to compare the MT immunoexpression in benign odontogenic tumours, 2) to correlate the MT immunoexpression with cellular proliferation in Solid AB (SAB), SOT, AOT and CCOT, and with inflammation in SAB.

Material and methods

Study population

Samples diagnosed as SAB (5 cases plexiform type- Fig. 1a; 5 cases follicular type- some cases showing areas of cystic degeneration- Fig. 1c), SOT (4 cases- Fig. 1e), AOT (5 cases- Fig. 1g), CCOT (4 cases- Fig. 1i), and CEOT (3 cases- Fig. 1k) were selected from files of Oral Pathology Service of Universidade Federal de Minas Gerais (Brazil). Histological slides stained with haematoxilin and eosin (HE) were reviewed, and diagnosis were in accordance to the WHO classification (2005) (1). As plexiform and follicular SAB subtypes are the most common ones, they were evaluated. All CCOTs selected were of the cystic type, non-associated with odontoma, and showed a non-proliferative pattern (9). In one case of CEOT it was observed sheets of classical polyhedral epithelium with abundant eosinophilic cytoplasm alternated with zones of epithelium characterized by large cells with clear, foamy cytoplasm and distinct cell borders, and this case was classified as clear cell variant of CEOT. All lesions were intraosseous, except one case of extraosseous CCOT. Only one case of SAB was a recurrent lesion. All other cases were primary tumours.

The study protocol was approved by the Committee of Ethics in Research of Universidade Federal de Minas Gerais (UFMG/COEP-15/08).

Inflammatory assessment

Using an optical microscope (Axiolab Zeiss, Germany) at 400x magnification, inflammatory cells in the connective tissue adjacent to the epithelium were counted, in 10 consecutive fields. Inflammation was evaluated only in SAB due to the high number of cases.

Immunohistochemistry

Immunohistochemical reaction was performed using streptavidin-biotin standard protocol. Primary antibodies (Dako, Carpinteria, CA, USA), dilution, incubation time and temperature, and antigen retrieval buffer are listed in Table 1. Sections of 4μm from routinely processed paraffin-embedded blocks were deparaffinized and rehydrated. Specimens were immersed in antigen retrieval buffer and submitted for 20 minutes at 98ºC. Only for MT, the endogenous avidin was blocked in accordance to Miller *et al*. (23). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide. Sections were incubated with primary antibodies and the detection was performed using LSAB®+system, HRP Peroxidase Kit (Dako Corporation, Carpinteria, CA, USA, K0690) and 3.3'-diaminobenzidine tetrahydrochloride chromogen (DAB, Sigma Chemical, St. Louis, USA, D5637). Mayer's Haematoxilin was used for counter staining.

Immunohistochemical assessment

Slides were examined under an optical microscope (Carl Zeiss, model Axiostar 1122-100, Germany) at 400x magnification, and the number of positive tumoral epithelial cells among 500 cells was counted for each slide. To obtain the MT (IMT), PCNA (IP), and Ki-67 (IK) labelling indexes, the number of positive cells was divided to 500 and multiplied by 100. Peripheral and central cells were evaluated in SAB, SOT, AOT and CCOT. In AOT, the peripheral tumoral portion in contact with the connective tissue was composed by type II cells: enlongated cells and spindle-shaped cells forming strands in a cribiform pattern. These cells were considered peripheral cells. Adjacent to type II cells, type I cells were observed: columnar to cuboidal cells forming solid nodules, rosette-like and duct-like structures. These cells composed the central cells (24). In CCOT, the cuboidal basal cells were considered peripheral cells, and the central cells were composed by upper epithelial layers which resemble the stellate reticulum of the enamel organ. Also, the cell compartment with MT stain was registered: cytoplasmatic and nuclear, cytoplasmatic only or nuclear only; whereas the PCNA and Ki-67 staining were only nuclear.

Statistical analysis

BioEstat® 4.0 software (BioEstat, Brazil) was used for the statistical analysis. Since IMT, IP and IK did not have a normal distribution in Shapiro-Wilk tests; the Kruskal-Wallis and Mann-Whitney U-test were used. An adjustment for ten post hoc tests was done using Bonferroni procedure (5% level of statistical significance attained at $p < 0.005$) when all tumours were compared. On the other hand, the values for IMT in peripheral and central cells showed a normal distribution in Shapiro-Wilk tests, though the Paired t-test was used, with statistical significance of $p<0.05$. In plexiform and follicular SAB, since IMT, IP and IK did have a normal distribution, the Student t-test was applied to compare these subtypes, with statistical significance of $p<0.05$. In SAB, SOT, AOT and CCOT individualy, since the IMT, IK and IP had a normal distribution, Pearson correlation was used to correlate these indexes. In SAB, the Spearman's correlation was used to correlate the inflammation (it did not have a normal distribuition) with IMT, IK and IP. The correlation was graded in: weak – <0.30; moderate – 0.30-0.50; and strong – > 0.50 (25).

Results

MT immunoexpression was identified in a mosaic pattern, with epithelial cells showing high heterogeneity of staining from negative to strongly positive in all samples, excepted in cases of CEOT in which it was absent. Plexiform and follicular SAB showed a positive MT staining in columnar cells at the periphery and in central stellate cells (Fig. 1b). In metaplastic squamous cells and in some inner stellate cells, absence of MT reactivity was observed. In areas of cystic degeneration of follicular SAB, the basal and suprabasal layers showed positive MT stain, but absence of reactivity was observed in the flattened cells lining (Fig. 1d). In SOT, MT positive staining was observed in flat to cuboidal cells at the periphery. Most central cells showed negative staining to MT, but some cells showed positive stain (Fig. 1f). In AOT, MT labeling was positive in epithelial strands with a cribriform configuration; absent in columnar to cuboidal cells, with positive stain in some cells; and absent in the luminal pole of cells of ductlike spaces and in rosette-like structures (Fig. 1h). In CCOT, the staining was

positive in cuboidal basal cells. In upper epithelial layers most cells showed positive stain, but some cells surrounding ghost cells, the ghost cells, and flattened cells lining cystic surface showed negative stain (Fig. 1j). In CEOT, MT staining was absent in polyedrical cells (Fig. 1l), and only in one case the clear cells showed scarce stain to MT.

Except in CEOT cases, all lesions staining was predominantly found in nuclei and cytoplasm simultaneously. Considering the nuclei and cytoplasm compartments simultaneously, SAB showed the highest IMT, followed by CCOT, SOT, AOT, and CEOT. Significant diferences were observed between SAB and SOT, AOT, or CCOT (Table 2). This was also observed in IMT total (Table 3). IK was highest in SAB (plexiform type - Figure 2a, and follicular type- Figure 2c), followed by SOT (Figure 2e), AOT (Figure 2g), CEOT (Figure 2k) and CCOT (Figure 2i). IP was highest in SAB (plexiform type - Figure 2b, and follicular type- Figure 2d), followed by SOT (Figure 2f), CEOT (Figure 2l), CCOT (Figure 2j) and AOT (Figure 2h). For both IK and IP, significant diferences were observed between SAB and AOT, and SAB and CCOT (Table 3). In plexiform and follicular SAB, the IMT (92.3%±5.6 and 93.0%±4.1 respectively) and IP (91.2%±5.7 and 85.8%±13.2) were similar, but IK exihbited statistical significant differences (6.6%±2.9 and 3.2%±1.6). In SAB, SOT, AOT and CCOT, the peripheral cells showed higher IMT than the central ones (Table 4).

In SAB, the correlation between IMT and IK (Pearson correlation coefficient [r]= 0.0286) and IMT and IP (r= 0.2243) were weak and positive, but IP and IK showed a moderate positive correlation (r= 0.4093). Also, a strong correlation (Spearman correlation coefficient [rs]= 0.6000) was observed between inflammation and IMT. However, a weak correlation was observed between the inflammation and IK (rs= 0.1277) and IP (rs= 0.1879).

In SOT, the correlation between IMT and IK (r= 0.2656) was weak and positive; but IMT and IP (r= 0.7268), and IP and IK (r= 0.6557) showed a strong positive correlation. In CCOT, the correlation between IMT and IK was weak and positive (r= 0.2686); but IMT and IP (r= 0.5402), and IP and IK (r= 0.8697) showed a strong and positive correlation. In AOT, a strong inverse correlation was observed between IMT and IK (r= -0.5940), and in IMT and IP (r= - 0.7970); but a strong positive correlation was observed between IP and IK (r= 0.7921).

Discussion

MT is a cytoplasmatic protein which may cross the nuclear membrane by passive diffusion. MT may translocate to the nucleus during cell proliferation and differentiation. In nucleus, this protein may also inhibit cellular apoptosis (26). In normal oral mucosa, MT was identified predominantly in cytoplasmatic and nuclear compartments simultaneously (7). In the present study, the majority of positive cells showed nuclear and cytoplasmatic stain simultaneously.

MT affects the activity of some transcriptional factors by donating zinc, and regulating the cell differentiation. MT immunoexpression diminished with the tissue development, being correlated with the cellular morphological differentiation (27, 28). In the current study, IMT was high in peripheral cells of SAB, AOT and CCOT. This MT expression is coincidental with the CK14 immunoexpression in these lesions. CK14 is a cytokeratin found in basal layer of squamous stratified epithelium and is the main intermediate filament of odontogenic epithelium (29-31). The IMT in the peripheral cells of SOT was also high. In literature, the expression of CK14 was not studied in SOT yet. The high IMT in central stellate cells in SAB and CCOT was also coincidental with the CK14 expression in these lesions (29-31). In columnar to cuboidal epithelial cells of AOT, a low IMT was observed, and this was not coincidental with the high stain reported to CK14 in this lesion (29, 31). This may be due to a variable degree of cellular differentiation presented by AOT, what is reinforced by the variable positivity for CK 7 and 8, which are expressed in simple epithelia (31).

Differentiated cells of SAB (metaplastic squamous cells, in some inner stellate cells, and flattened cells lining cystic degeneration areas), SOT (squamous cells), and CCOT (squamous transitory cells surround the ghost cells, and flattened cells lining cystic surface), were negative to MT. These cells are positive to CK10, CK13 or KL1, cytokeratins which indicates squamous differentiation (29, 30, 32, 33). So, the absence of MT expression in these cells seems to be compatible with terminal squamous epithelial differentiation. In AOT, many columnar to cuboidal cells forming nests, rosette-like and duct-like structures showed negative MT stain. This was coincident with the positive stain to KL1 (34), but not coincidental with the absence of CK10 and CK13 described (29, 31). This variable

expression of cytokeratins may indicate variable degree of differentiation and may be reflected in MT immunoexpression. Besides, similarity in MT immunoexpression between plexiform and follicular types of SAB was observed in the present study, and this may be reflecting the similarity of cellular differentiation showed by the same cytokeratin distribution observed these tumours (33).

Absence of MT immunoexpression was observed in CEOT, except in one case in which scarce clear cells stained to MT. Positive stain for CK14 is described in all cells of CEOT. Lack of CK7 associated with vimentin expression, observed in some cases, seems to indicate tumoral indifferentiation. However, in others cases, the positive stain to CK 13, KL1, as well as the positive stain to CK7 associed with scarce vimentin, may be related with a well differentiated lesion (29, 35, 36). The absence of MT staining may be indicating a more differentiated lesion. The clear cell of CEOT showed positive stain to MT, and this is compatible with the CK 14 expression, and absence of CK13 exibited by these cells (37).

In addition to regulation of cellular differentiation, the role of MT in inhibition of cellular apoptosis has been evaluated (5, 27). In malignant lesions, studies evaluating apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay identified an inverse correlation between the immunoexpression of MT and apoptosis (38-40). However, positive correlation (41) as well as no correlation (22) were also described. The high IMT in SAB found in the present study suggests low apoptotic index, and this was verified in the literature. Few apoptotic cells were observed in this lesion evaluated by TUNEL assay and immunoexpression of single-stranded DNA, ssDNA- an antibody which recognizes DNA fragmentation in nuclei during apoptosis. Also, the central stellate cells of SAB showed a slightly lower IMT, which can suggest that in this region there is a lower inhibition of apoptosis, possibly permiting the presence of apoptotic cells in this region. In SAB, the report of apoptotic cells occurs in this region (16, 42, 43). No difference in IMT between the follicular and plexiform SAB was found in the present evaluation. Apoptotic index also did not reveal any difference between these subtypes (42). No study evaluated

the apoptotic index by TUNEL assay or ssDNA immunoexpression in SOT, AOT, CCOT and CEOT.

Beyond of regulation of differentiation cellular and apoptosis inhibition, MT has been also correlated with cellular proliferation. In the current study, the correlation of IMT and IK or IP was weak or strong and positive in SAB, SOT, and CCOT, which may indicate an influence of MT in cellular proliferation in these lesions. A positive correlation was also reported between IMT and IK in malignant lesions (3, 4, 21, 22), and between IMT and IP in malignant and benign lesions (19, 20). However, no correlation between IMT and IK was described in malignant (6, 44), benign, and borderline lesions (4); as well as between IMT and IP in malignant lesions (45). A moderate or strong inverse correlation was observed between IMT and IK or IP in AOT. It is possible that in this lesion MT does not have a role in cellular proliferation. This inverse correlation was also described in normal endometrium between IMT and IK or IP (21). A moderate or strong positive correlation of IP and IK was observed in all lesions, and this was previously reported in SAB (12). In spite of this positive correlation, values of IP were higher than IK and this may be due to the longer half-life time (20 h) of PCNA when compared to Ki-67 (60-90 min) (46), and to the association of PCNA with DNA repair processes. Though, Ki-67 is suggested to be more sensitive than PCNA in reflecting cellular proliferative activity (15).

In the current study, the plexiform SAB type showed a higher IK and similar IP when compared with follicular one. Reports of expression of these markers reveal inconsistent results, and the reason for this may be related to differences in the study methodology (12, 14). Rizzardi *et al*. (16) also reported a higher IK in plexiform type. However, the opposite (8, 14) as well as similar IK or IP (10, 13) were also verified between these lesions. A higher IP in SAB than in AOT (13), and a higher IK or IP in SAB than in CCOT were reported (9, 15), and it was also verified in the present study. However, when the proliferative variant of CCOT is considered, a similar value of IK was observed between these lesions (9). No study compared the IK or IP expression between SAB and SOT or CEOT. The CEOT IK reported in the present study is similar to a previous one (11). In the present study, SOT showed

lower indexes of IP and IK than SAB, although without statistical significance, and this may be related to a blander behavior. However, no previous studies evaluated IP and IK in SOT.

The synthesis of MT is induced by metals, hormones, cytokines, other chemicals, inflammation, and stress (2). In the presented study, inflammation was strongly correlated with the IMT in SAB, and it is possible that in this lesion MT is estimulated by inflammation. However, a weak correlation between inflammation and IK and IP was observed. No previous study evaluated the influence of inflammation in the immunoexpression of these proteins in SAB.

MT immunoexpression shows a variable pattern of expression depending on the lesion and it may be associated with cellular differentiation and/or biological behaviour.

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Conflict of interest statement

None declared.

Table 1 - Primary monoclonal antibodies.

Antibody (Clone)	*Dilution*	*Incubation temperature/ time*	*Antigen retrieval/ pH*
MT-I+II (E9 / M0639)	1:100	4ºC/ 18 hours	EDTA*/ 8.0
PCNA (PC10 / MO879)	1:1000	Room/ 18 hours	Citrate buffer** / 6.0
Ki-67 (MIB-1/ M7240)	1:100	Room/ 18 hours	Citrate buffer** / 6.0

*1 mM ethylenediamine tetraacetic acid buffer (Vetec Química Fina, Rio de Janeiro, Brazil, 114); **10 mM citrate buffer (Lab Synth, Diadema, Brazil, A2270).

Table 2. Indexes of labeled cells for metallothionein stratified by cellular compartment in solid ameloblastoma, squamous odontogenic tumour, adenomatoid odontogenic tumour, calcifying cystic odontogenic tumour, and calcifying epithelial odontogenic tumour.

Lesion	*Number of cases*	*IMT Median %*			*Comparison*	*p value (Mann-Whitney U-test)*		
		C	*N*	*NC*		*C*[a]	*N*[a]	*NC*[a]
SAB	10	07	3.5	79.6	SAB compared with SOT	NS	NS	<0.005
					SAB compared with AOT	<0.005	NS	<0.005
					SAB compared with CCOT	NS	NS	<0.005
					SAB compared with CEOT	NS	NS	NS
SOT	04	00	1.5	51.5	SOT compared with AOT	NS	NS	NS
					SOT compared with CCOT	NS	NS	NS
					SOT compared with CEOT	NS	NS	NS
AOT	05	0.6	0.6	36.0	AOT compared with CCOT	NS	NS	NS
					AOT compared with CEOT	NS	NS	NS
CCOT	04	6.7	4.1	67.9	CCOT compared with CEOT	NS	NS	NS
CEOT	03	00	00	00				

[a]Kruskal-Wallis, p<0.05; NS= non-significative; C= cytoplasm; N=nuclear; NC= nuclear and cytoplasm.

Table 3. Indexes of labeled cells for metallothionein, Ki-67 and PCNA of solid ameloblastoma, squamous odontogenic tumour, adenomatoid odontogenic tumour, calcifying cystic odontogenic tumour, and calcifying epithelial odontogenic tumour.

Lesion	*Number of cases*	*Median %*			*Comparison*	*p value (Mann-Whitney U-test)*		
		IMT	*IK*	*IP*		*IMT*[a]	*IK*[a]	*IP*[a]
SAB	10	93.1	4.3	90.4	SAB compared with SOT	<0.005	NS	NS
					SAB compared with AOT	<0.005	<0.005	<0.005
					SAB compared with CCOT	<0.005	<0.005	<0.005
					SAB compared with CEOT	NS	NS	NS
SOT	04	52.9	2.9	82.5	SOT compared with AOT	NS	NS	NS
					SOT compared with CCOT	NS	NS	NS
					SOT compared with CEOT	NS	NS	NS
AOT	05	38.4	02	60.2	AOT compared with CCOT	NS	NS	NS
					AOT compared with CEOT	NS	NS	NS
CCOT	04	76.8	1.7	65.5	CCOT compared with CEOT	NS	NS	NS
CEOT	03	00	1.8	79.8				

[a]Kruskal-Wallis, $p<0.05$; NS= non-significative.

Table 4. Indexes of labeled cells for metallothionein in peripheral and central cells of solid ameloblastoma, squamous odontogenic tumour, adenomatoid odontogenic tumour, and calcifying cystic odontogenic tumour.

Lesion	*Number of cases*	*IMT Mean %/SD*		*p value (Paired t-test)*
		P[a]	*C*[b]	*P*[a] *x C*[b]
SAB	10	93.6±4.0	91.3±6.1	<0.05
SOT	04	77.2±11.4	35.3±11.6	<0.05
AOT	05	88.8±7.7	22.4±13.0	<0.05
CCOT	04	96.4±5.0	72.5±8.7	<0.05

P= Peripheral cells; C= Central cells; NS= non-significative; [a]In AOT corresponds to epithelial strands, and in CCOT corresponds to cuboidal basal cells; [b]In AOT corresponds to columnar to cuboidal cells forming solid nodules, rosette-like and duct-like structures, and in CCOT corresponds to the upper epithelial layers.

FIGURE 1

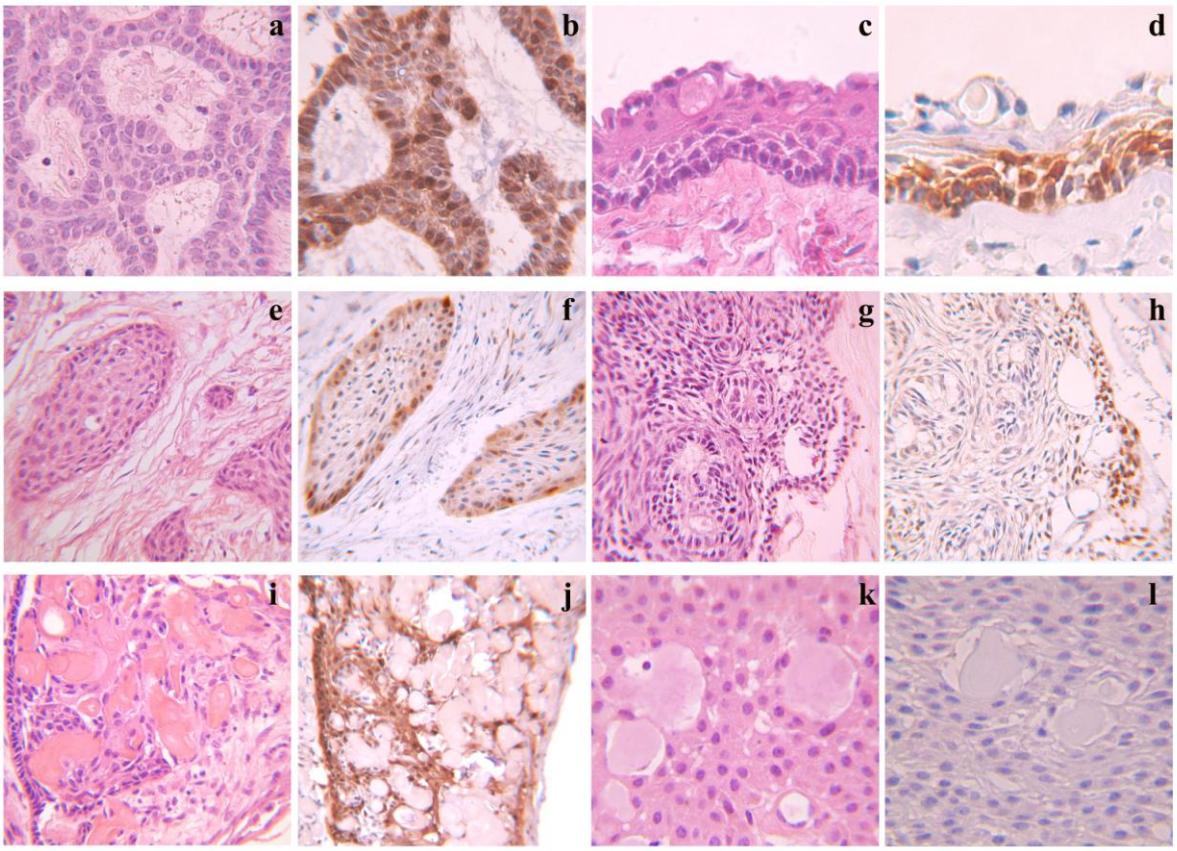

FIGURE 1 LEGEND

Figure 1-: **Plexiform type of solid ameloblastoma: (a)** peripherical cells of epithelial strands are columnar, hyperchromatic, and lined up in a palisaded fashion. The central cells are composed by loosely arranged stellate cells (Haematoxilin-eosin stain- HE; magnification X400); **(b)** positive MT staining in columnar cells at the periphery and in central stellate cells (Streptavidin-biotin- SB, magnification X400). **Cystic structure of follicular solid ameloblastoma (c):** cystic epithelial lining showing columnar or cubic cells at the basal layer and loosely arranged stellate cells in suprabasal layer (HE; magnification X400); **(d)** positive MT stain in the basal and suprabasal layers, but absent in the flattened cells lining (SB, magnification X400). **Squamous odontogenic tumour: (e)** epithelial islands showing flat to cuboidal cells at the periphery enclosing cells with squamous differentiation (HE; magnification X400); **(f)** positive MT stain in peripheral cells, and negative in the most of central cells, with positive stain in some cells (SB, magnification X400). **Adenomatoid odontogenic tumour: (g)** columnar to cuboidal cells forming solid nodules, rosette-like and duct-like structures, and strands of epithelium with a cribriform configuration (HE; magnification X400); **(h)** positive MT stain in cells of epithelial strands in a cribiform pattern, absent in columnar to cuboidal cells with positive stain in some cells, and absent in the luminal pole of cells of ductlike spaces and in rosette-like structures (SB, magnification X400). **Calcifying cystic odontogenic tumour: (i)** cystic lining showing cuboidal basal cells with hyperchromatic nuclei forming a palisade, and fusiform upper cells, sometimes resembling the stellate reticulum, with clusters of ghost cells (HE; magnification X400); **(j)** positive MT stain in basal cells and in the most of cells of upper epithelial layers, but negative in some cells surround the ghost cells, in the ghost cells, and in flattened cells lining cystic surface (SB, magnification X400). **Calcifying epithelial odontogenic tumour: (k)** sheets of polygonal cells with large eosinophilic cytoplasm, pleomorphic nuclei, prominents nucleoli, desmossomes, and presence of amyloid structure (HE; magnification X400); **(l)** absence of MT stain in polygonal cells (SB, magnification X400).

FIGURE 2

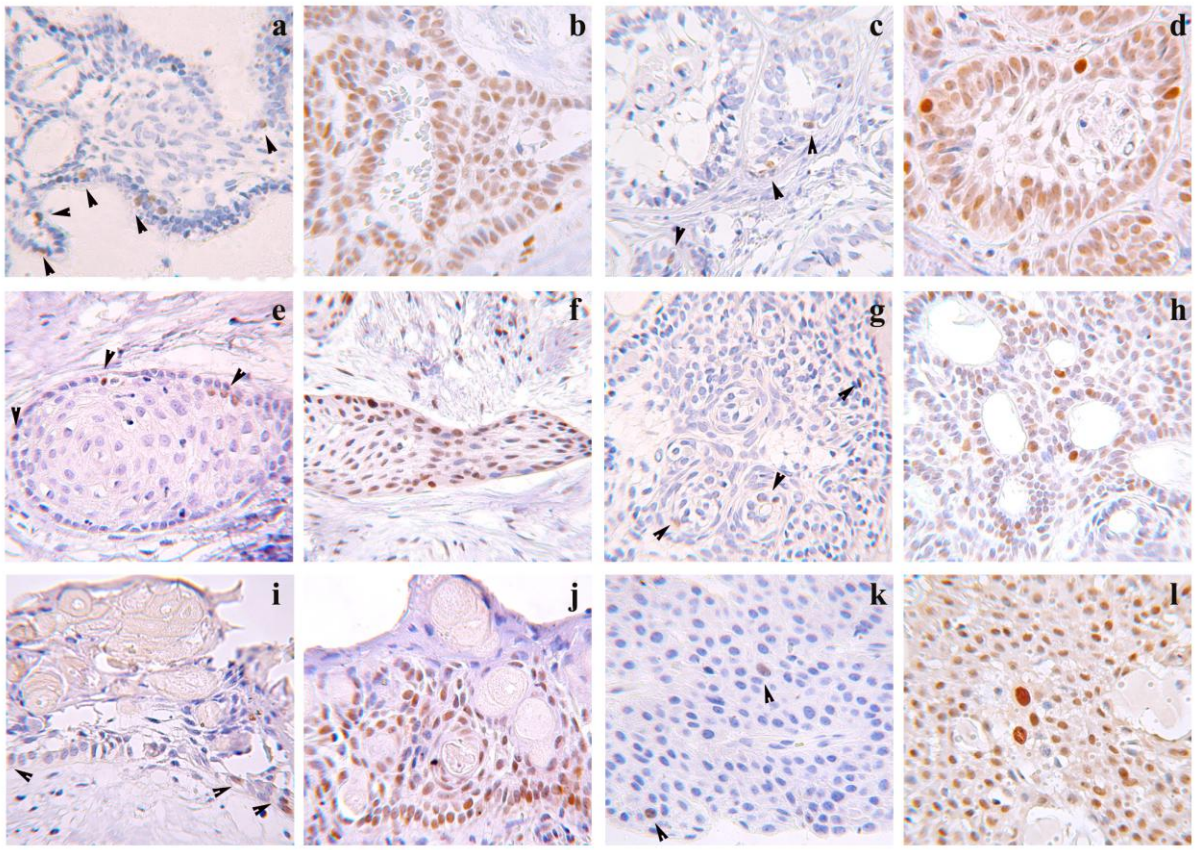

FIGURE 2 LEGEND

Figure 2-: Streptavidin-biotin- SB, magnification X400- **Plexiform type of solid ameloblastoma: (a)** Scarce Ki-67 positive cells were identified in peripherical cells of epithelial strands; **(b)** A large number of PCNA positive cells was visualized at the periphery and in central portion. **Follicular type of solid ameloblastoma (c):** It was observed a lower number of Ki-67 positive cells in these suptype than the plexiform ones; **(d)** It was identified similar numbers of PCNA positive cells than the plexiform subtype. **Squamous odontogenic tumour: (e)** It was identified Ki-67 positive cells in periphery of epithelial islands, and the number of positive cells was lower than in ameloblastoma; **(f)** Positive PCNA stain was visualized in peripheral and in central cells, and the number of positive cells was also lower than in ameloblastoma. **Adenomatoid odontogenic tumour: (g)** Positive stain of Ki-67 was present in rosette-like and duct-like structures, and in strands of epithelium with a cribriform configuration, and the number of positive cells was lower than in ameloblastoma; **(h)** Positive PCNA cells were observed in large portion of tumoral cells, and the number of positive cells was also lower than in ameloblastoma. **Calcifying cystic odontogenic tumour: (i)** It was noted Ki-67 positive cells mainly in basal layer, and the number of positive cells was lower than in ameloblastoma; **(j)** Positive PCNA cells were observed in large portion of tumoral cells, and the number of positive cells was also lower than in ameloblastoma. **Calcifying epithelial odontogenic tumour: (k)** Scarce Ki-67 positive cells was identified, and the number was lower than in ameloblastoma; **(l)** Positive PCNA cells were observed in the majory of tumoral cells, and the number was also lower than in ameloblastoma.

8 CONCLUSÕES

No presente estudo, a MT foi expressa de forma diferenciada em cistos odontogênicos e tumores odontogênicos benignos. Essas diferenças parecem ser determinadas pelo grau de diferenciação tecidual e pelo índice apoptótico.

O IMT foi mais alto no CR, seguido pelo CD, TOC e COO; e as diferenças entre eles foram estatisticamente significantes, exceto entre o CR e o CD. O IMT foi variável entre as lesões e isto possivelmente pode ser devido ao seu papel na diferenciação celular e na inibição da apoptose.

O IMT foi maior nos casos não associados à SCNB que nos casos associados à SCNB, o que pode significar uma diminuição na inibição da apoptose nos casos associados à SCNB, favorecendo um maior índice apoptótico.

O IMT foi maior no ABS, seguido pelo TOCC, TOE e pelo TOA, sendo ausente no TOEC e as diferenças foram estatisticamente significantes entre o ABS e as lesões: TOE, TOCC e TOA. Essas diferenças podem ser reflexo do papel da MT na diferenciação celular e/ou no comportamento biológico desses tumores.

A MT foi positivamente correlacionada com a proliferação nos cistos e tumores odontogênicos, com exceção do TOC (associados ou não à SBCN), COO e TOA.

A MT parece não ser influenciada pela inflamação no CR, CD e TOC, mas parece ser influenciada no ABS.

9 CONSIDERAÇÕES FINAIS

Dando continuidade aos trabalhos desenvolvidos durante o período do mestrado nesta instituição, surgiu o interesse pela proteína MT, quando desenvolvemos então a avaliação dessa proteína nas lesões potencialmente malignas de boca, resultando na publicação de um artigo. Foi quando também surgiu o interesse em avaliar a MT também nos cistos e tumores odontogênicos, tema da presente tese.

A MT tem sido relacionada a eventos de diferenciação e proliferação celular e inibição da apoptose. A imuno-expressão da MT diminui com o desenvolvimento tecidual, de forma que tecidos bem diferenciados apresentam uma menor expressão de MT. Na maioria dos estudos tem sido observada uma correlação inversa entre a MT e a apoptose, e direta entre a MT e o PCNA ou o Ki-67. Foi observado, no presente estudo, diferenças na expressão da MT nas lesões odontogênicas. Essas diferenças foram correlacionadas com características específicas do epitélio nas diferentes lesões, como índice apoptótico, proliferação e diferenciação celular, que podem interferir no comportamento biológico dessas lesões.

O TOC foi reclassificado pela OMS, em 2005, como um tumor devido ao seu comportamento clínico agressivo, a alta taxa de recorrência, o padrão destrutivo e a sua associação com a SCBN. Mesmo após a reclassificação, existe discussão acerca de sua natureza. Estudos recentes permanecem comparando a expressão de várias proteínas entre os cistos odontogênicos e o TOC, devido a essas lesões apresentarem diferenças no comportamento clínico e em determinadas características epiteliais, mas também similaridades como a formação da cavidade cística revestida por epitélio e aspectos radiográficos. No presente estudo, o IMT foi mais alto no CR, seguido pelo CD, TOC e COO; e as diferenças entre eles foram estatisticamente significantes, exceto entre o CR e o CD. O IMT foi variável entre as lesões e isto possivelmente pode ser devido ao seu papel na diferenciação celular e na inibição da apoptose. Isto foi postulado uma vez que o COO apresenta um maior grau de maturação epitelial comparado com o TOC, que por sua vez exibe maior grau de maturação que o CD e CR. Um maior índice apoptótico foi descrito para

o TOC quando comparado com o CR e o CD. Também foi descrita uma maior tendência a sobrevida celular no TOC comparado com o COO. No TOC e no COO, uma inversa correlação foi observada entre o IMT e o IK, o que possivelmente indica uma maior participação da MT na diferenciação celular e na inibição da apoptose que na proliferação celular nessas lesões. Já no CR e no CD uma correlação direta forte ou moderada foi observada, indicando que nessas lesões a MT pode estar influenciando a proliferação.

O TOC pode se apresentar na forma esporádica (não associada à SBCN) ou associada à SCBN. Quando associado à SCBN exibe maior crescimento, maior potencial destrutivo e maior tendência à recorrência. A expressão de várias proteínas tem sido estudada nessas lesões, revelando diferenças, o que suporta a existência de um imuno-fenótipo distinto. Além disso, um maior índice apoptótico tem sido reportado nos casos associados à SCBN quando comparada com os não associados. No presente estudo, o IMT foi maior nos casos não associados à SCNB que nos casos associados à SCNB, o que pode significar uma diminuição na inibição da apoptose nos casos associados à SCNB, favorecendo um maior índice apoptótico.

Os tumores odontogênicos apresentam variáveis comportamentos biológicos. O ABS apresenta um grande potencial destrutivo e uma alta taxa de recorrência, enquanto o TOE e o TOCC são lesões localmente destrutivas com rara recorrência. O TOA é um neoplasma não agressivo, não invasivo, com raros relatos de recorrência. O TOEC apresenta um comportamento localmente destrutivo, com uma taxa de recorrência de 14%. No presente estudo, o IMT foi variável entre as lesões, sendo maior no ABS, seguido pelo TOCC, TOE e TOA. O TOEC não revelou marcação para MT. O ABS exibiu o maior IMT que pode ser um fator contribuinte ao seu comportamento mais agressivo, com maior taxa de recorrência. Na literatura, observam-se variáveis padrões de diferenciação das células que compõem essas lesões. Esse variável grau de diferenciação foi compatível com a variável imuno-expressão da MT nessas lesões. No presente estudo, o alto IMT no ABS sugeriu um baixo índice apoptótico, e esse baixo índice apoptótico é relatado na literatura. Uma correlação positiva do IMT com marcadores de proliferação celular foi identificada no ABS, TOE e no TOCC,

podendo indicar uma influência da MT na proliferação celular dessas lesões. Entretanto, é possível que no TOA a MT não exerça um papel na proliferação celular, já que uma correlação inversa foi observada entre o IMT e os marcadores de proliferação celular.

A marcação para PCNA foi realizada para os tumores odontogênicos, pois nessas lesões a avaliação da proliferação celular nos estudos prévios é muito variada, sendo que algumas avaliam somente o Ki-67, outras somente o PCNA, e outras ambos Ki-67 e PCNA. Desta forma poderíamos comparar os nossos resultados com a literatura. Avaliação do PCNA nos cistos odontogênicos e no TOC também foi realizada, mas não foi incluída na tese, pois os resultados apresentaram divergências com os achados da literatura.

O presente estudo revela diferenças na expressão da MT nas diversas lesões odontogênicas, que podem estar associadas com diferenças na proliferação, diferenciação e apoptose celular. Esses achados fundamentam o desenvolvimento de posteriores estudos envolvendo avaliação conjunta de citoqueratinas (CK 14 e 10), marcador de apoptose (caspase 3 ativa) e MT, que poderão contribuir para a elucidação dos mecanismos pelos quais a MT influencia as células epiteliais dessas lesões.

A avaliação da MT na odontogênese é outro aspecto a ser avaliado, uma vez que pode auxiliar na elucidação da exata origem dos cistos e tumores odontogênicos. Outro ponto a ser explorado seria a expressão da MT-IV nessas lesões, já que esta é expressa nas células diferenciadas do epitélio estratificado escamoso, podento contribuir também na avaliação da diferenciação celular dessas lesões. Além disso, foi verificado que muitos estudos avaliam a apoptose nos ABS, no entanto, raros estudos o fazem no TOA, TOCC, TOE e no TOEC. Futuros estudos poderão ser desenvolvidos neste sentido, já que a apoptose é um processo que está intrinsicamente relacionado com o comportamento biológico dessas lesões.

No presente estudo também se verificou que a inflamação não influencia a imuno-expressão da MT nos cistos odontogênicos e no TOC. Entretanto, uma forte correlação foi verificada entre a inflamação e a expressão de MT no ABS. Estudos posteriores avaliando a

expressão de citocinas poderão elucidar a possível influência da inflamação na expressão da MT.

ANEXOS

ANEXO A- PARECER DO COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 15/08

Interessado(a): Prof. Ricardo Alves de Mesquita
DCPCO
Faculdade de Odontologia - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 2 de abril de 2008, o projeto de pesquisa intitulado **"Imuno-expressão da metalotioneína em cistos odontogênicos e tumores odontogênicos com componete epitelial"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

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ANEXO B- GUIA DE AUTORES DA REVISTA ORAL ONCOLOGY

Oral Oncology

A Journal Related to Head & Neck Oncology & Pathology

Guide for Authors

A Journal Related to Head & Neck Oncology & Pathology

Submission checklist

It is hoped that this list will be useful during the final checking of an article prior to sending it to the journal's Editor for review.

Ensure that the following items are present:

•One Author designated as corresponding Author:

E-mail address

Full postal address

Telephone(s) and fax numbers

• All necessary files have been uploaded

• Keywords (as comprehensive as possible)

• All figure captions

• All tables (including title, description, footnotes)

• The Author Form has been completed and uploaded to EES (or sent to the Editorial Office via fax)

Further considerations:

• Manuscript has been "spellchecked" and is written in good English• Title is clear and unambiguous

• References are in the correct format for this journal • All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Colour figures are clearly marked as being intended for colour reproduction on the Web (free of charge) and in print or to be reproduced in colour on the Web (free of charge) and in black-and-white in print

• If only colour on the Web is required, black and white versions of the figures are also supplied for printing purposes

• The manuscript conforms to the limits imposed on original research and review articles (2500 words for original research articles and 3000 words for review articles with a maximum of five tables and figures)

For any further information please contact the Author Support Department at authorsupport@elsevier.com

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Oral Oncology will consider manuscripts prepared according to the guidelines adopted by the International Committee of Medical Journal Editors ("Uniform requirements for manuscripts submitted to biomedical journals", available as a PDF from www.icmje.org). Authors are advised to read these guidelines.

Previous Publication

Submission of an article implies that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, without the written consent of the Publisher.

Online-only Publication

Due to the large number of high-quality papers submitted to *Oral Oncology* the time taken for accepted articles to be published in print has risen significantly. There may be a delay of many months before an accepted paper is selected for publication in a printed issue. *Oral Oncology* offers authors the opportunity to select online-only publication as their preferred option for publishing original research and review papers in the journal, rather than print publication. Letters to the Editor which are accepted for publication and errata and corrigenda will be published online-only and will not appear in print.

Any material which is published online-only will be published online on ScienceDirect as paginated and fully citable electronic article. It will be listed in the contents page of a printed issue and the full citation and abstract will be published in print. The citation and abstract of the paper will also still appear in the usual abstracting and indexing databases, including PubMed/Medline, Current Contents/Clinical Medicine and the Science Citation Index. Authors will be asked to select which publication option they would prefer when submitting their paper to the Editorial Office.

Randomised Controlled Trials

All randomised controlled trials submitted for publication in *Oral Oncology* should include a completed Consolidated Standards of Reporting Trials (CONSORT) flow chart. Please refer to the CONSORT statement website at http://www.consort-statement.org for more information. *Oral Oncology* has adopted the proposal from the International Committee of Medical Journal Editors (ICMJE) which require, as a condition of consideration for publication of clinical trials, registration in a public trials registry. Trials must register at or before the onset of patient enrolment. The clinical trial registration number should be included at the end of the abstract of the article. For this purpose, a clinical trial is defined as any research project that prospectively assigns human subjects to intervention or comparison groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Studies designed for other purposes, such as to study pharmacokinetics or major

toxicity (e.g. phase I trials) would be exempt. Further information can be found at www.icmje.org.

Ethics

Work on human beings that is submitted to *Oral Oncology* should comply with the principles laid down in the Declaration of Helsinki; Recommendations guiding physicians in biomedical research involving human subjects. Adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, amended by the 29th World Medical Assembly, Tokyo, Japan, October 1975, the 35th World Medical Assembly, Venice, Italy, October 1983, and the 41st World Medical Assembly, Hong Kong, September 1989. The manuscript should contain a statement that the work has been approved by the appropriate ethical committees related to the institution(s) in which it was performed and that subjects gave informed consent to the work. Studies involving experiments with animals must state that their care was in accordance with institution guidelines. Patients' and volunteers' names, initials, and hospital numbers should not be used.

Patient Consent Guidelines

Studies on patients or volunteers require ethics committee approval and informed consent which should be documented in your paper. Patients have a right to privacy. Therefore, identifying information, including patients' images, names, initials, or hospital numbers, should not be included in videos, recordings, written descriptions, photographs, and pedigrees unless the information is essential for scientific purposes and you have obtained written informed consent for publication in print and electronic form from the patient (or parent, guardian or next of kin where applicable). If such consent is made subject to any conditions, Elsevier must be made aware of all such conditions. Written consents must be provided to Elsevier on request.Even where consent has been given, identifying details should be omitted if they are not essential. If identifying characteristics are altered to protect

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If such consent has not been obtained, personal details of patients included in any part of the paper and in any supplementary materials (including all illustrations and videos) must be removed before submission.

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At the end of text, under a subheading "Conflict of interest statement" all authors must disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work. If there are no conflicts of interest, please state "None declared".

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All sources of funding should be declared as an acknowledgment at the end of the text.

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To avoid unnecessary errors you are strongly advised to use the "spellchecker" function of your wordprocessor.

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Original research articles submitted to the journal must be 2500 words in length or less, with a maximum of five figures and tables. Review articles submitted to the journal must be 3000 words or less in length, with a maximum of five tables or figures.

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Keywords: Immediately after the abstract provide a maximum of ten keywords, to be chosen from the Medical Subject Headings from Index Medicus. These keywords will be used for indexing purposes. It is usually necessary to include keywords such as *Oral Cancer*, or *Head and Neck cancer* .

Abbreviations: Define abbreviations or acronyms that are not standard in this field at their first occurrence in the article: in the abstract but also in the main text after it. Ensure consistency of abbreviations throughout the article.

Text: This should start on the third page and should be subdivided into the following sections: Introduction, Patients (or Materials) and Methods, Results, and Discussion.

References: Responsibility for the accuracy of bibliographic citations lies entirely with the authors.

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. "Unpublished data" and "Personal communications" are not allowed. As an alternative, say in the text, for example, '(data not shown)' or '(Dr F.G. Tomlin, Karolinska Institute)'. Citation of a reference as "in press" implies that the item has been accepted for publication and a copy of the title page of the relevant article must be submitted.

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Examples:

1. Llewellyn CD, Johnson NW, Warnakulasuriya KAAS. Risk factors for squamous cell carcinoma of the oral cavity in young people - comprehensive literature review. *Oral Oncol* 2001;**37**(5):401-418.

2. Gullick WJ, Venter DJ. The c-erbB2 and its expression in human tumors. In: Waxman J, Sikora K, editors. The molecular biology of cancer. Oxford: Blackwell Scientific, 1989. p. 38-53.

3. Scully C, Cawson RA. Medical Problems in Dentistry. 5th edition Oxford: Butterworth-Heinemann. 2004

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ANEXO C- GUIA DE AUTORES DA REVISTA JOURNAL OF ORAL PATHOLOGY & MEDICINE

Journal of Oral Pathology & Medicine

Official Publication of the International Association of Oral Pathologists, The American Academy of Oral & Maxillofacial Pathology, The British Society for Oral & Maxillofacial Pathology, the British Society for Oral Medicine and the Scandinavian Society of Oral Pathology & Oral Medicine

Edited by:

Erik Dabelsteen

Print ISSN: 0904-2512

Online ISSN: 1600-0714

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Current Volume: 38 / 2009

ISI Journal Citation Reports® Ranking: 2008: 24/55 Dentistry, Oral Surgery & Medicine;

40/68 Pathology

Impact Factor: 1.63

TopAuthor Guidelines

Content of Author Guidelines: 1. General, 2. Ethical Guidelines, 3. Manuscript Submission Procedure, 4. Manuscript Types Accepted, 5. Manuscript Format and Structure, 6. After Acceptance

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Useful Websites: Submission Site, Articles published in Journal of Oral Pathology & Medicine, Author Services, Blackwell Publishing's Ethical Guidelines, Guidelines for Figures

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Journal of Oral Pathology & Medicine publishes manuscripts of high scientific quality representing original clinical, diagnostic or experimental work in oral pathology and oral medicine. Papers advancing the science or practice of these disciplines will be welcomed, especially those which bring new knowledge and observations from the application of techniques within the spheres of light and electron microscopy, tissue and organ culture, immunology, histochemistry, immunocytochemistry and molecular biology. Review papers on topical and relevant subjects will receive a high priority and articles requiring rapid publication because of their significance and timeliness will be included as brief reports not exceeding three printed pages. All submitted manuscripts falling within the overall scope of the Journal will be assessed by suitably qualified reviewers, but manuscripts in an incorrect format will be returned to the author without review.

Please read the instructions below carefully for details on the submission of manuscripts, the journal's requirements and standards as well as information concerning the procedure after a manuscript has been accepted for publication in *Journal of Oral Pathology & Medicine*. Authors are encouraged to visit Wiley-Blackwell Publishing Author Services for further information on the preparation and submission of articles and figures.

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HEINIC GS, GREENSPAN D, MACPHAIL LA, *et al*. Oral Histoplasma capsulatum infection in association with HIV infection: a case report. J Oral Pathol Med 1992; 21: 5-9.

(2) Corporate author

European Collaborative Study. Risk factors for mother-to-child transmission of HIV-1. Lancet 1992; 339: 1007-12.

(3) No author given

Anonymous. `The importance of being early' [leader]. Br Dent J 1991; 170: 167.

(4) Journal supplement

MØLLER-PETERSEN J. Evaluation of diagnostic tests. Design and phases. Scand J Clin Lab Invest 1992; 52: suppl. (208): 35-50.
CROSS SS, SCHOLFIELD JH, KENNEDY A, COTTON DWK. Measuring the fractal dimension of tumour borders. J Pathol 1992; 168: 117A (abstr).

(5) Journal paginated by issue

HILLAM C. Dentistry in Europe in the 1790's. Dent Historian 1992; 22: (May): 31-4.

(6) Book

PINDBORG JJ. Atlas of diseases of the oral mucosa. Copenhagen: Munksgaard, 1992: 50-66.

(7) Chapter in a book

VAN DER WAAL I. Salivary gland neoplasms. In: PRABHU SR, WILSON DF, DAFTARY DK, JOHNSON NW, eds. Oral diseases in the tropics. Oxford: Oxford Medical, 1992; 478-86.

(8) Published proceedings paper

DRINNAN AJ. Review of the literature: educational aspects of oral medicine. In: MILLARD HD, MASON DK, eds. World workshop on oral medicine. Chicago: Year Book Medical, 1989; 5-11.

(9) Agency publication

MUIR C, WATERHOUSE J, MACK T, POWELL J, WHELAN S. Cancer incidence in five continents: Vol. 5. Lyon: International Agency for Research on Cancer, 1987; IARC Scientific Publications No. 88.

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CHUNGPANICH S. The diagnostic and prognostic potential of nucleolar organizer regions in oral epithelial dysplasia. MMedSci Thesis, University of Sheffield, 1989.

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