ANDRESA BORGES SOARES

AVALIAÇÃO DA ANGIOGÊNESE E DA LINFANGIOGÊNESE DURANTE A PROGRESSÃO TUMORAL DO CARCINOMA EX-ADENOMA PLEOMÓRFICO

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Tese de Doutorado apresentada à Pós Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção de título de Doutor em Ciências Médicas, área de concentração Ciências Biomédicas

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A Deus...

"O Senhor é meu pastor e nada me faltará!

Deita-me em verdes pastos e guia-me mansamente em águas tranqüilas.

Refrigera a minha alma, guia-se pelas veredas da justiça, por amor do seu nome.

Ainda que eu ande pelo vale da sombra da morte, não temerei mal algum, porque Tu estás comigo, a Tua vara e o Teu cajado me consolam.

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Salmo 22

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“A vida é a arte do encontro,
embora haja tanto desencontro pela vida,
é preciso encontrar as coisas certas da vida,
para que ela tenha o sentido que se deseja.
Assim,
a escolha de uma profissão
também é a arte de um encontro.
Porque uma vida só adquire vida
quando a gente empresta nossa vida,
para o resto da vida.”

(Vinícius de Moraes)

AP	Adenoma Pleomórfico
ATV	Área total vascular
CXAP	Carcinoma Ex-Adenoma Pleomórfico
DVL	Densidade Vascular Linfática
MDV	Microdensidade Vascular
VEGF	Fator de Crescimento Endotelial Vascular
VEGFR	Receptor do Fator de Crescimento Endotelial Vascular

Pág.

RESUMO

Carcinoma ex-adenoma pleomórfico (CXAP) é uma neoplasia geralmente de alto grau, com risco moderado para metástase cervical. O objetivo do trabalho foi analisar a vascularização tumoral sanguínea e linfática numa série de CXAP, que representam as diferentes fases da seqüência adenoma-carcinoma. Em oito CXAP precoces (intracapsulares ou minimamente invasores), oito avançados (francamente invasores) e 10 adenomas pleomórficos (AP) sem transformação maligna usamos os seguintes anticorpos: D2-40, Ki-67, CD34, CD105, α-SMA, CK7 e 14. A vascularização sanguínea foi estudada através da microdensidade vascular intratumoral (MDV) e área vascular total (AVT). A vascularização linfática, através da microdensidade vascular linfática (MVL) intratumoral e peritumoral. Em relação à vascularização sanguínea, a MDV pelo CD 105 mostrou forte correlação positiva com a progressão tumoral, enquanto que a MDV pelo CD 34 e a ATV não apresentaram nenhuma correlação. Carcinomas com diferenciação mioepitelial apresentavam MDV pelo CD 105 mais baixa, mas os mais altos valores de ATV. Quanto à vascularização linfática, em AP e CXAP precoces, foram encontrados raros, se algum, linfático intratumoral, enquanto que em CXAP avançados eles eram mais numerosos, porém Ki 67 negativos. Êmbolos peri e intratumorais foram vistos apenas nos CXAP sem diferenciação mioepitelial. Em conclusão, com o CD 105 demostrou-se forte correlação positiva entre angiogênese e progressão tumoral. A vascularização linfática é formada predominantemente por vasos pré-existentes, encontrados principalmente nos CXAP avançados. Tanto linfáticos intratumorais como peritumorais são condutores de êmbolos neoplásicos. CXAP com diferenciação mioepitelial mostram atividade angiogênica mais baixa associada a valores mais altos de área vascular e capacidade mais baixa para invadir vasos linfáticos.

ABSTRACT

Carcinoma ex pleomorphic adenoma (CXPA) usually is a high-grade carcinoma with a moderate risk for neck metastasis. In a series of CXPAs, which represent the different phases of the adenoma–carcinoma sequence, we investigated lymphatic vascular density (LVD), lymph vessel endothelial proliferation and whether an angiogenic switch would take place during the malignant transformation of PA into carcinoma and during tumor progression. The CXPAs were classified into two main groups: early tumors - 8 cases (4 intracapsular and 4 minimally invasive carcinomas), and advanced tumors – 8 cases (8 widely invasive carcinomas). In addition, 10 cases of pleomorphic adenoma (PA) without malignant transformation were also analyzed. The following antibodies were used: CD34, CD 105, D2-40, Ki-67, α-smooth-muscle actin, vimentin, cytokeratins 7 and 14. Microvascular density (MVD) for CD 105 increased significantly during tumor progression whereas this was not the case for CD 34 MVD. Comparing widely invasive CXPA with and without myoepithelial differentiation, CXPA with myoepithelial differentiation showed a significantly lower number of CD 105 positive vessels but revealed higher total vascular area (TVA) values. In terms of lymph vessels, PA without malignant transformation and early CXPA contained rare, if any, lymph vessels whereas in widely invasive carcinomas they were more numerous but Ki-67 negative. Carcinomatous emboli were found in peritumoral as well as in intratumoral lymphatics only in advanced CXPA without myoepithelial differentiation. In conclusion, the antibody CD 105 shows an angiogenic switch during the progression from adenoma to carcinoma in salivary glands. In terms of lymphatic vessels, in CXPA, the lymphatic network is mainly composed of pre-existing lymphatics which are mainly found in advanced CXPA. In the widely invasive CXPA, intratumoral as well as peritumoral lymphatics are a conduit for carcinoma cells. Carcinomas with myoepithelial differentiation show low angiogenesis associated with high TVA value and the neoplastic cells seem to have a lower vascular invasion capacity.

1- INTRODUÇÃO GERAL

1.1- Adenoma Pleomórfico

Tumores das glândulas salivares são raros, representando apenas 2 a 6,5% de todas as neoplasias da região de cabeça e pescoço (Auclair *et al.* 1992; Toida *et al.* 2005). O Adenoma Pleomórfico (AP) é a neoplasia mais comum das glândulas salivares, correspondendo a: na parótida 59 a 66% dos tumores (Sungur *et al* 2002; Vargas *et al*. 2002; Ito *el al.* 2005), na submandibular 26 a 67% (Yasumoto *et al*. 1992; Nagler & Laufer 1997; Pinkston & Cole 1999; Rapidis *et al*. 2004; Ito *et al*. 2005) e, nas glândulas salivares menores, de 33 a 57% (Loyola *et al*. 1995, Rivera-Bastidas *et al*. 1996, Kusama *et al*. 1997; Toida *et al.* 2005; Ito *et al*. 2005).

Conhecido antigamente como tumor misto, o AP é uma neoplasia benigna de origem epitelial composta por células que demonstram diferenciação epitelial e mesenquimatosa (Ellis & Auclair 1996). O termo tumor misto se referia à mistura dos elementos epiteliais e mesenquimatosos, enquanto que o termo AP enfatiza a grande diversidade morfológica deste tumor.

O AP pode afetar indivíduos de qualquer idade. No entanto, é mais freqüente em pacientes na quinta e sexta década de vida (Ellis & Auclair 1996; Rivera-Bastidas *et al.* 1996; Alves *et al*. 2002; Toida *et al.* 2005). Quanto ao gênero, as mulheres são geralmente mais afetadas do que os homens (Ellis & Auclair 1996; Alves *et al.* 2002; Toida *et al*. 2005; Ito *et al*. 2005).

Clinicamente, apresenta-se como um aumento de volume assintomático, de consistência firme, crescimento lento e que pode se tornar grande quando não tratado (Ellis & Auclair 1996). Na glândula parótida, a maioria dos AP acomete a região inferior do lóbulo superficial, causando abaulamento sobre o ângulo da mandíbula, à frente da orelha. Inicialmente, o tumor é móvel, mas quando não tratado perde a sua mobilidade e se torna nodular e fixo. AP recidivantes de parótida são geralmente multinodulares e aparecem, clinicamente, como múltiplos e pequenos nódulos fixos à palpação (Ellis & Auclair 1996).

O AP de glândula submandibular provoca poucos sinais ou sintomas clínicos, sendo, na maioria das vezes, o aumento de volume a única manifestação clínica da lesão. Entretanto, em alguns casos têm sido relatadas dores e dificuldade na fala e deglutição (Alves *et al*. 2002).

Nas glândulas salivares menores, o AP apresenta-se como massa de crescimento lento, assintomática e recoberta por mucosa normal. Raramente são ulcerados, exceto quando são traumatizados secundariamente. O palato é o local mais freqüente, seguido pela mucosa jugal, lábio superior e região retromolar (Wal *et al*. 1992; Rivera-Bastidas *et al.* 1996; Toida *et al*. 2005). Quando presentes no palato duro, os tumores são geralmente fixos, devido à estreita relação da mucosa com o osso adjacente. Nas demais localidades, o tumor é móvel, na maioria dos casos (Ellis *et al*, 1991).

Histologicamente, o AP apresenta uma grande diversidade morfológica. O componente epitelial do AP é composto por células epiteliais e mioepiteliais, as quais podem formar ninhos, lençóis, cordões e estruturas ductiformes com duas camadas celulares, uma mais internamente do tipo epitelial e outra externamente do tipo mioepitelial (Ellis & Auclair 1996). As células mioepiteliais podem apresentar forma cúbica, fusiforme ou plasmocitóide. Vários autores têm relatado o potencial das células mioepiteliais em se transformar em células epidermóides, mesenquimatosas e condrócitos, além de secretarem produtos como elastina, mucinas mesenquimatoso e membrana basal. Desta maneira, as células mioepiteliais são diretamente responsáveis pela formação e variedade estromal do AP (Skalova *et al*. 1992). Este estroma pode ter aspecto mixóide, hialino, condróide, ósseo e, na maioria dos AP, encontra-se uma mistura destes componentes (Ellis & Auclair 1996).

O AP é, geralmente, um tumor encapsulado, bem circunscrito. No entanto, a cápsula pode ser incompleta ou estar infiltrada por células neoplásicas. Cápsula incompleta é mais comum nos tumores de glândulas salivares menores (Ellis & Auclair 1996).

O tratamento do AP consiste na sua completa remoção cirúrgica. Na parótida, nos casos onde o tumor se localiza no lóbulo superficial, é realizada uma parotidectomia superficial com pequena margem de tecido normal e preservação do nervo facial. Nos casos em que a neoplasia está localizada no lóbulo profundo da parótida, uma parotidectomia total é recomendada (Hardingham 1993; Yamashita *et al.* 1993; Wasylik *et al*. 2001). Os tumores de glândulas submandibulares devem ser tratados com remoção da lesão e da glândula envolvida (Alves *et al*. 2002). Já aqueles de glândulas salivares menores devem ser tratados através de remoção cirúrgica com margem de segurança (Ellis & Auclair 1996).

Raramente o AP pode sofrer transformação maligna, podendo dar origem a três subtipos de tumor misto maligno: (1) Adenoma Pleomórfico Metastatizante, no qual tanto o tumor primário quanto a metástase apresentam características histológicas de um tumor benigno, (2) Carcinoma Ex-Adenoma Pleomórfico (CXAP), caracterizado pela transformação maligna do componente epitelial do AP, (3) Adenoma Pleomórfico Maligno Verdadeiro ou também chamado de Carcinossarcoma, na qual tanto o tumor primário como as metástases consistem de tumor de origem epitelial e mesenquimatosa (Olsen & Lewis 2001). O mais comum dentre esses é o CXAP, que representa cerca de 3,6% de todos os tumores de glândulas salivares e 11,7% dentre os malignos (Gneep *et al*. 2000; Olsen & Lewis 2001).

1.2- Carcinoma Ex-Adenoma Pleomórfico

O CXAP é definido pela Organização Mundial de Saúde como uma neoplasia epitelial maligna que se origina no AP. Para o diagnóstico histológico de tal entidade é necessário o achado do AP residual junto com o carcinoma (Barnes *et al.* 2005).

A exata patogenia do CXAP é ainda bastante controversa. A maioria dos autores acredita que este tumor ocorra da transformação maligna do componente epitelial de um AP. Beahrs *et al.* (1957) relataram que a média de idade dos pacientes com AP era 10 anos menor do que a dos pacientes com CXAP. Além disso, os pacientes relatavam a presença de uma massa por muitos anos que, de repente, crescia, com o aparecimento de outros sintomas, concomitantemente.

Eneroth & Zetterberg (1974) estudaram a composição do DNA do AP com menos de um ano de história clínica, e observaram que estes apresentavam uma população celular diplóide. Ao contrario, os AP com mais de cinco anos de história mostravam população celular tetraplóide, muito similar ao encontrado no CXAP, sugerindo então que nos AP mais velhos as células podem sofrer uma transformação que pode induzir ao aparecimento de um componente carcinomatoso.

Alguns achados clínico-patológicos têm sido descritos como fatores de risco para a malignização. Os principais fatores clínicos são: idade avançada do paciente, tumores recidivados, de longa duração e com origem na glândula submandibular. Necrose, atipia nuclear, extensa hialinização, invasão para tecidos adjacentes e aumento da atividade mitótica têm sido relatados como os achados microscópicos da transformação maligna. (Ellis & Auclair 1996).

Clinicamente, o CXAP apresenta-se como uma massa assintomática. No entanto, dor, ulceração da pele, disfagia, parestesia ou paralisia do nervo facial podem estar presentes. O tempo de duração dos sintomas pode variar de um mês a 52 anos (Olsen & Lewis 2001). Acima de 80% dos casos de CXAP têm sido descritos nas glândulas salivares maiores, principalmente na parótida (86%) e submandibular (14%). Aproximadamente dois terços dos casos de CXAP das glândulas salivares menores ocorrem no palato (Tortoledo *et al*. 1984; Olsen& Lewis 2001).

Histologicamente pode haver dificuldade para se identificar o componente do AP residual, pois este pode ser extremamente pequeno ou estar muito hialino ou hipocelular (Olsen & Lewis 2001). Muitos tipos histológicos de carcinoma podem ser vistos no CXAP, inclusive numa mesma lesão pode ser encontrado mais de um subtipo (Costa Rosa *et al*. 1996). Carcinoma do ducto salivar, carcinoma indiferenciado, adenocarcinoma polimorfo de baixo grau, carcinoma mioepitelial e adenocarcinoma SOE são os subtipos mais freqüentes (Tortoledo *et al*. 1984; Lewis *et al.* 2001). Alguns tipos histológicos são de diagnóstico mais fácil devido à presença das atipias celulares e mitoses. Outros, no entanto, exibem pouca ou nenhuma atipia, de tal modo que o diagnóstico de malignidade só é estabelecido pelo caráter infiltrativo da neoplasia para estruturas adjacentes (Gneep *et al*., 2000).

O CXAP pode ser classificado em: intracapsular, minimamente invasor e francamente invasor; de acordo com a extensão da invasão tumoral além da cápsula do AP. Esta extensão além da cápsula é considerada, por alguns autores, como o principal critério de prognóstico para este tipo de tumor, independente, inclusive, do tipo histológico (Tortoledo *et al.* 1984; Brandwein *et al*. 1996; Lewis *et al*. 2001). CXAPs francamente invasores são usualmente relatados como tumores agressivos causando, freqüentemente, metástase e óbito, enquanto que carcinomas intracapsulares e minimamente invasores têm sido considerados como tumores de baixo grau de agressividade (Gnepp *et al.* 2000; Lewis *et al.* 2001; Felix *et al*. 2002). Tortoledo *et al*. (1984) observaram que tumores com menos de 8mm de invasão não apresentaram metástase. Para Lewis *et al.* (2001) este valor foi de 5mm e para Brandwein *et al.* (1996) foi de apenas 1,5mm. No entanto, Felix *et al*. (2002) relataram o primeiro caso de um CXAP completamente restrito aos limites do AP que apresentou metástase linfonodal na evolução, mostrando que mesmo os CXAP intracapsulares podem apresentar um grau mais elevado de agressividade.

O tratamento é baseado na localização do tumor, acometimento dos tecidos adjacentes, subtipo histológico e grau de agressividade. Remoção cirúrgica é o tratamento de escolha para a maioria dos CXAPs,

sendo que radioterapia pode ser administrada nos casos de tumores de alto grau (Olsen & Lewis 2001; Kariya *et al*., 2006). O prognóstico depende do tamanho do tumor, grau e subtipo histológico e extensão da invasão carcinomatosa além da cápsula tumoral (Olsen & Lewis 2001; Tortoledo *et al*. 1984).

1.3- Angiogênese

Angiogênese é a formação de novos vasos sanguíneos, ocorrendo em diversas situações fisiológicas e patológicas, tais como tecido de cicatrização, menstruação, desenvolvimento embrionário, crescimento tumoral, psoríase e artrite reumatóide (Folkman 1995). O desenvolvimento destes novos vasos sanguíneos é um fenômeno complexo resultante de uma seqüência de eventos envolvendo a dissolução da membrana basal, migração de células endoteliais, proliferação, formação luminal e anastomoses com outros vasos (Shima *et al.* 1995).

A angiogênese é um processo fundamental para o crescimento tumoral, pois nenhum tumor pode ultrapassar o tamanho de $1mm^3$ sem vascularização. Portanto, a angiogênese vai fornecer nutriente e oxigênio para o metabolismo do tumor e remoção dos produtos tumorais residuais (Vaupel *et al*. 1989; Denckamp 1993). A angiogênese não constitui apenas um pré-requisito para o crescimento tumoral, mas, também, para a formação de metástase, pois sem acesso à vascularização, as células tumorais são incapazes de causar metástase (Sharma *et al*. 2005).

Srivastava *et al* (1988) realizaram o primeiro estudo demonstrando uma correlação entre o aumento da angiogênese e a probabilidade de metástase em melanoma. Posteriormente, diversos estudos também evidenciaram uma correlação entre a angiogênese tumoral e o risco de metástase em câncer de mama, próstata, intestino, pulmão e cérebro (Fox *et al.* 1996). No entanto, alguns autores não encontraram nenhuma correlação entre angiogênese tumoral e metástase (Page & Jesen, 1995), e Lindmark *et al*. (1996) descreveram, inclusive,

melhor prognóstico associado com o aumento da angiogênese em câncer retal. Esta discrepância entre os autores, provavelmente, pode ser devida aos anticorpos utilizados, métodos de contagem e diferenças no tratamento dos tecidos, o que afetaria a preservação antigênica e, conseqüentemente, o resultado do exame imunoistoquímico.

Recentemente, em algumas neoplasias, também foi demonstrado papel fundamental da angiogênese na progressão tumoral, ou seja, na evolução do estágio pré-neoplásico para o francamente carcinomatoso; e do carcinoma *in situ* para a neoplasia invasora (Choi *et al*. 2005, Swelam *et al*. 2005).

Em 1971, Folkman propôs que a terapia anti-angiogênica poderia ser coadjuvante no tratamento do câncer e, desde então, diversos estudos têm sido realizados para tentar identificar marcadores vasculares. Entretanto não há, ainda, um marcador específico para vascularização tumoral, sendo que os anticorpos disponíveis detectam células endoteliais em proliferação, células endoteliais ativadas, marcadores de hipóxia, receptores de fatores de crescimento, antígenos de células endoteliais indefinidos e de proteínas expressas na membrana basal recém-formada ou remodelada (Duff *et al*. 2003).

Alguns marcadores imunoistoquímicos têm sido utilizados para a evidenciação dos vasos sangüíneos; dentre eles os mais utilizados são CD31, CD34, fator de von Willebrand e CD105 (Uzzan *et al*. 2004; Choi *et al*. 2005).

O fator de von Willebrand ou também chamado de fator VIII é demonstrado em todos os vasos de grande calibre e, focalmente, em capilares (Akagi *et al*. 2002). Este marcador não é específico para vasos sangüíneos, pois pode também marcar vasos linfáticos (Guidi *et al.* 1994). A glucoproteína transmembrana CD31 (molécula de adesão de células endoteliais-plaquetárias) é encontrada em células endoteliais e algumas células hematopoéticas (Akagi *et al*. 2002; Mietinnen *et al.* 1994). Contudo, este é um bom marcador de células endoteliais corando, com mesma intensidade, vasos de grande e pequeno calibre, tanto em tecido normal quanto em tumores, podendo, ocasionalmente,

corar células carcinomatosas (Mietinnen *et al.* 1994). O CD 34 é outra proteína de transmembrana encontrada na superfície de células endoteliais, especialmente as células em atividade angiogênica (Kuzu *et al*. 1992), corando vasos tanto em tecido normal quanto tumoral. Este marcador também pode ser evidenciado em outras células neurais mesenquimatosas (Lindenmayer & Miettinen 1995). Devido à robustez e à facilidade do uso, o CD34 tem sido considerado um ótimo marcador de endotélio vascular, que sobrepuja o CD31 e o fator VIII (Vermeulen *et al*. 2002).

CD 105 é uma glicoproteína de membrana homodimérica de 180kD que é expressa em células endoteliais humanas em proliferação e regulada por hipóxia (Kumar *et al*. 1996; Brekken *et al.* 2002; Sanchez-Elsner *et al.* 2002). Tais propriedades têm feito do CD 105 um importante marcador de vasos neoformados, o que é mostrado na literatura em inúmeros trabalhos. Este anticorpo é intensamente imunomarcado somente em vasos neoformados, não sendo expresso em vasos pré-existentes e tecido normal (Kumar *et al*. 1996; Bodey *et al.* 1998; Kumar *et al.* 1999; Minhajat *et al*. 2006).

Recentemente, alguns autores têm sugerido a correlação entre a menor sobrevida em câncer retal e o alto índice de densidade vascular de vasos neoformados, sendo, portanto o CD 105 também utilizado como um importante marcador de prognóstico (Romani *et al.* 2006). Paralelamente, Li *et al* (2000) relataram um aumento do nível de CD105 em amostra de plasma de pacientes com câncer de mama quando comparado com um grupo controle, sugerindo que o CD105 também pode ser utilizado para identificar pacientes com alto risco de metástase e de recidivas.

A angiogênese pode ser avaliada através da microdensidade vascular (MDV) e área total vascular (ATV). A MDV é o valor médio da contagem do número de vasos em uma determinada área, sendo selecionadas as áreas mais vascularizadas (“hotspots”). A ATV é o valor médio da área total ocupada pelos vasos em uma determinada área do tumor, sendo também selecionados os “hotspots” (Sharma *et al*. 2005).

Vários estudos têm mostrado que alguns tumores contêm fatores capazes de efetuar o processo de formação de novos capilares, sendo estes fatores denominados de fatores angiogênicos (D'Amore & Thompson 1991; Vaupel *et al*. 1989; Klagsbrun & D'Amore 1991; Denckamp 1993; Zagzag 1995). O fator de crescimento endotelial vascular (VEGF) é um dos mais importantes fatores angiogênicos, sendo um agente indutor da diferenciação endotelial, estando presente tanto em células endoteliais como em células epiteliais, neoplásicas ou não (Swelan *et al*. 2005). A expressão gênica do VEGF pode ser regulada por hipóxia, citocinas e mutações no gene p53 (Ferrara & Davis-Smyth 1997; Swelan *et al*. 2005). O VEGF tem sido relacionado à patogênese, potencial metastático e, portanto, prognóstico de diversos tumores, devido à sua associação com a angiogênese (Lin *et al*. 2003).

1.4- Linfangiogênese

Os vasos linfáticos são a principal via de metástase para a maioria dos tipos de carcinoma (Xuan *et al.* 2005). No entanto, pouco se sabe a respeito do mecanismo de linfangiogênese (formação de novos vasos linfáticos) e os meios através do qual ocorre a metástase. Acredita-se que, durante a embriogênese, a linfangiogênese possa ocorrer através de dois mecanismos: 1) uma nova diferenciação do endotélio linfático através de linfoblastos e/ou 2) um brotamento através de veias preexistentes (Oliver G *et al*. 2002; Agarwal *et al.* 2005).

Um dos motivos da dificuldade de se estudar os vasos linfáticos era a falta de um anticorpo específico. Alguns anticorpos têm sido utilizados na tentativa de resolver este problema, tais como VEGF-C, podoplanina, LYVE-1 e Prox-1 (Kaipainen *et al.* 1995; Breiteneder-Geleff *et al.* 1999; Banerji *et al.* 1999; Wigle & Oliver 1999; Stacker *et al.* 2001). No entanto, nenhum destes anticorpos apresentou uma marcação específica para vasos linfáticos, podendo também marcar vasos sanguíneos e outros tipos de células (Breiteneder-Geleff *et al* 1997; Valtola *et al*. 1999; Partanen *et al*. 2000; Jussila & Alitalo 2002; Mouta Carreira *et al.* 2001).

O VEGF-C se liga ao VEGFR-3, que é um receptor demonstrado em células vasculares embrionárias, principalmente no endotélio linfático na sua fase tardia do desenvolvimento e pós-embrionária. Por esta razão, o VEGFC foi o primeiro marcador utilizado na tentativa de distinguir vasos sanguíneos de vasos linfáticos (Agarwal *et al* 2005). Posteriormente, um estudo realizado por Jussila *et al* (1998) demostrou que VEGFC também pode ser expresso em vasos sanguíneos embrionários e posteriormente ser re-expresso em vasos sanguíneos tumorais. Por está razão, atualmente este anticorpo não tem sido utilizado como um marcador específico para vasos linfáticos (Kaipanen *et al.* 1995; Schoppmann *et al* 2002).

O LYVE-1 (lymphatic vascular endothelium-specific marker -1) é um receptor para hyalurona, que por sua vez é expressa durante a fase inicial do desenvolvimento do endotélio linfático, marcando, portanto vasos que apresentam características ultraestrutural de linfáticos. No entanto, a expressão de LYVE – 1 não é restrita a vasos linfáticos, que pode, também, ser observado em células endoteliais sinusoidais do fígado e baço, sincicitrofoblasto de placenta e em macrófagos (Cursiefen *et al*. 2003; Mouta Carreira *et al*. 2001; Stacker *et al*. 2002).

Prox-1 é um "homeobox" que está associado ao crescimento e prologamento dos brotos vasculares linfáticos durante o desenvolvimento e amadurecimento do endotélio linfático em adultos. Entretanto, este marcador não é específico para vasos linfáticos, podendo ser expresso em células não endoteliais de fígado, coração, pâncreas e sistema nervoso (Stacker *et al.* 2002).

A podoplanina, o Prox -1 e o LYVE – 1 são os marcadores existentes mais específicos para o endotélio linfático. Dentre estes, a podoplanina tem sido considerada o mais específico e o mais utilizado na pesquisa de vasos linfáticos tumorais (tabela 1) (Schoppmann *et al*. 2002; Breiteneder-Geleff *et al* 1997; Breiteneder-Geleff *et al.* 1999; Breiteneder-Geleff *et al.* 1999; Wigle & Oliver 1999; Banerji *et al.* 1999; Jackson *et al* 2001). Podoplanina é uma glicoproteína de membrana de 38-KDa que é intensamente e seletivamente expressa em vasos linfáticos, não apresentando marcação em vasos sanguíneos (Kanh *et al*. 2002;

Choi *et al*. 2005). Entretanto, também pode ser imunomarcada em alguns tecidos e células normais, tais como osteócitos, condrócitos, podócitos de rim, leptomeninges, células alveolares tipo I do pulmão, células de Purkinje, ceratinócitos basais da pele, cérvix uterino e de esôfago, células ependimárias, miofibroblastos, e células mioepiteliais na mama, glândulas salivares e brônquio (Ordoñez, 2006). Na literatura não há um consenso sobre podoplonina e D2-40, pois alguns autores acreditam se tratar de dois marcadores distintos (Agarwal *et al* 2005), enquanto outros relatam ser o mesmo marcador, considerando o D2-40 como uma podoplonina disponível comercialmente (Ordoñez, 2006).

Tabela 1- Principais marcadores utilizados para a evidenciação de vasos linfáticos

Marcador	***Expressão em Vasos Linfáticos***	***Expressão em Vasos Sanguíneos***	***Referências***
VEGF - C	++	+	Kaipainen *et al*. 1995; Ji & Kato 2003
Prox-1	+++	+	Wigle & Oliver 1999; Wilting *et al*. 2002
LYVE-1	+++	+	Banerij *et al*. 1999; Prevo *et al*. 2001
Podoplanina (D2-40)	+++	-	Breiteneder-Geleff *et al*. 1999; Kriehuber *et al*. 2001

Fonte: Ji RC. Histol Histopathol 2005.

Através da utilização destes marcadores, vasos linfáticos peritumorais e intratumorais têm sido evidenciados (Stacker *et al.* 2001). Entretanto, há uma grande controvérsia em relação aos vasos intratumorais. Alguns autores acreditam que os vasos linfáticos pré-existentes são suficientes para provocar metástase, e que os vasos intratumorais podem estar ausentes ou colapsados (Leu *et al*. 2000). Além disso, experimento realizado em modelo animal mostrou que os vasos linfáticos presentes na periferia dos tumores são dilatados e

funcionantes, enquanto que os vasos intratumorais são comprimidos e não funcionais, confirmando a hipótese de que vasos pré-existentes peritumorais são suficientes para provocar metástase (Padera *et al*. 2002). Entretanto, Beasley *et al* (2002) relataram a presença de vasos intratumorais em carcinomas de cabeça e pescoço, e através de um estudo com dupla marcação imunoistoquímica (LYVE-1XKi67), mostraram que estes vasos apresentavam as células endoteliais com alta atividade proliferativa, evidenciando portanto, que estes eram neoformados e não pré-existentes. Resultados semelhantes foram observados em melanomas cutâneos (Skobe *et al*. 2001; Dadras *et al*. 2003; Straume *et al*. 2003).

Os achados na literatura sobre vasos linfáticos são ainda bastante conflitantes, mostrando que é necessário mais estudo para tentar esclarecer o verdadeiro mecanismo da relação dos vasos linfáticos com os tumores.

2- JUSTIFICATIVA GERAL

No estroma tumoral, os vasos sangüíneos e linfáticos desempenham papéis cruciais, pois os primeiros são particularmente importantes para o crescimento, manutenção e progressão das neoplasias, enquanto que os linfáticos são a principal via de metástase para a maioria dos tipos de carcinoma. Entretanto, até recentemente, não havia anticorpos específicos para o endotélio linfático e nem anticorpos que distinguissem vasos sanguíneos pré-existentes de vasos sanguíneos tumorais neoformados, ou seja, que permitissem medir a verdadeira atividade angiogênica da neoplasia.

Na literatura, há escassos trabalhos sobre vascularização tumoral em neoplasias de glândula salivar, sendo que, até o momento, não há estudos sobre a vascularização sanguínea e linfática no carcinoma que se origina no adenoma pleomórfico. O adenoma pleomórfico é o tumor mais comum da glândula salivar e o carcinoma-ex-adenoma pleomórfico (CXAP) representa cerca de 4,5% a 15% de todos os cânceres destas glândulas. É considerado um carcinoma agressivo, de alto grau e com risco moderado para metástase cervical.

Conhecimento sobre vascularização tumoral é considerado essencial para a terapia antiangiogênica, que tem emergido como uma alternativa adjuvante para a terapia anti-câncer tradicional, além de aumentar o entendimento da relação tumor / estroma e da carcinogênese.

3- OBJETIVOS

3.1- Objetivos gerais

Estudar a vascularização sanguínea e linfática durante a progressão tumoral do carcinoma-ex-adenoma pleomórfico.

3.2- Objetivos específicos

Em adenomas pleomórficos sem transformação maligna e em CXAP subdivididos em precoces (não invasor além da cápsula do adenoma pleomórfico ou minimamente invasor) e avançados (francamente invasores além da cápsula do adenoma pleomórfico) serão analisadas:

a) Com relação a vascularização sanguínea:

- a microdensidade vascular intratumoral com os anticorpos CD34 (marcador pan-endotelial) e CD105 (marcador de neoangiogênese).
- a área vascular total intratumoral.

b) Com relação à vascularização linfática

- a densidade linfática intratumoral, peritumoral e dos tecidos normais adjacentes ao tumor.
- a freqüência de êmbolos neoplásicos em linfáticos intra e peritumorais.
- a freqüência de células endoteliais proliferantes (Ki 67 positivas) em linfáticos intratumorais e peritumorais

4- ARTIGOS

Artigo 1- Angiogênese

Artigo 2- Linfangiogênese

Virchows Archiv

Angiogenic switch during tumor progression of carcinoma ex-pleomorphic adenoma.

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Angiogenic switch during tumor progression of carcinoma ex-pleomorphic adenoma

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ABSTRACT

We analyzed the tumor vascularisation in carcinomas ex-pleomorphic adenoma (CXPA) in order to investigate the angiogenic switch during the malignant transformation of pleomorphic adenoma (PA) to carcinoma and during tumor progression. In 8 cases of early CXPA (intracapsular and minimally invasive tumors), 8 of advanced CXPA (widely invasive tumors) and 10 of PA without malignant transformation, tumor vascularisation was assessed in histological samples by measuring total microvascular area (TVA) and microvessel density (MVD) using CD 34 and CD 105 antibodies. MVD for CD 105 increased significantly during tumor progression whereas this was not the case for CD 34 MVD. Comparing widely invasive CXPA with and without myoepithelial differentiation, CXPA with myoepithelial differentiation showed a significantly lower number of CD 105 positive vessels but revealed higher TVA values. In these tumors, the neoplastic cells usually formed larger hypovascularized aggregates that were often surrounded by large-sized vessels. In conclusion, the antibody CD 105 reveals an angiogenic switch during the progression from adenoma to carcinoma in salivary glands. The degree of angiogenesis and the total vascular area have distinctive patterns in CXPA with and without myoepithelial differentiation. Low angiogenesis associated with high TVA value is more characteristic of CXPA with myoepithelial differentiation.

Key words: carcinoma, pleomorphic adenoma, angiogenesis, malignant transformation

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INTRODUCTION

Angiogenesis, the formation of new blood vessels from pre-existing ones, has been considered a critical process for tumor growth, invasion and metastasis [20, 24]. The ability to induce and sustain angiogenesis occurs during tumor development, via an angiogenic switch from vascular quiescence [12]. Besides that, blood can be supplied by a non-angiogenic process, the so-called co-option mechanism, well-documented in some human cancers, where the neoplastic cells use the existing blood vessels without eliciting an angiogenic response [9, 22, 28].

Tumor vascularisation has rarely been studied in salivary gland tumors [5, 30].

In humans, the most common salivary gland tumor, the pleomorphic adenoma (PA) usually shows a poorly vascularized myxoid and chondroid stroma [26]. Carcinoma ex-pleomorphic adenoma (CXPA) is the principal form of malignancy arising in PA and accounts for 4.5% -15% of all cancers of the salivary glands [7, 11, 18]. They are usually high-grade carcinomas, with frequent metastases and a poor clinical outcome [7, 10, 11, 17, 18].

In salivary carcinoma spontaneously arising in HER-2/neu transgenic mice it has recently been suggested that the richly vascularized pre-existing salivary tissue is able to support tumor onset and progression with no need for an angiogenic switch [4].

Taking these results into consideration we investigated the question whether an angiogenic switch would take place during the malignant transformation of PA into carcinoma and during tumor progression.

In a series of CXPAs, which represent the different phases of the adenoma–carcinoma sequence, ie. *in situ* (intracapsular), minimally invasive, and invasive carcinoma, tumor vascularisation was assessed in histological samples by measuring the total microvascular area (TVA) and microvessel density (MVD) using CD 34 and CD 105 antibodies. PA without malignant transformation was used as control. CD34 has been reported as an optimal pan-endothelial marker [9] that stains not only "newly forming" vessels but also normal ones trapped within tumor tissues. In contrast, CD 105 (endoglin), a proliferation-associated and hypoxia-inducible protein expressed in angiogenic endothelial cells, has been considered as a marker of neoangiogenesis [9, 24, 27, 29].

MATERIAL AND METHODS

The study was performed in 16 cases of carcinoma ex-pleomorphic adenoma which were retrieved from the files of the Department of Pathology of the University of Campinas. All diagnoses were reviewed by two pathologists (AA and VCA) using 5μm sections obtained from formalin-fixed paraffin embedded samples and routinely stained with hematoxylin-eosin. CXPA

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was defined as a malignant epithelial neoplasm arising in association with a primary or recurrent PA. The tumors were classified according to extension of invasion beyond the capsule of the previous PA as: intracapsular (without invasion) – 4 cases, minimally invasive (≤ 1.5 mm of invasion) – 4 cases and widely invasive – 8 cases. Demographic and clinical information was obtained from the patients' medical records.

Immunohistohemistry

One paraffin block from each case was chosen for the immunohistochemical study [23] and the following antibodies were used (table 1): CD34 and CD 105 for detection of blood vessels and α-smooth-muscle actin (α-SMA), vimentin, cytokeratins 7 (CK 7) and 14 (CK14) - for the detection of epithelial and myoepithelial cells. The 5µm sections were deparaffinized, hydrated and endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide. For all antibodies, except for alpha-SMA and anti-CD 105, antigen retrieval (AR) was achieved by boiling in a steamer immersed in citrate buffer (pH: 6.0, for 30 minutes). For CD 105 AR was performed using 0.4% pepsin for 30 minutes (Table 1). Only the sections for CD 105 were incubated at 37° C with protein block serum free (code x0909, Dako, SA, Denmark) for 30 minutes. Subsequently, for all antibodies the sections were incubated overnight at 4°C with the primary antibody and afterwards with the EnVision polymer HRP and Envision+ (code K1491, DAKO, SA, Denmark) for 1h at 37°C. Sections were stained for 5min at 37°C with 3.3'-diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin. Negative controls were run by omitting primary antibodies.

Table 1: Details of the antibodies used for immunohistochemistry.

Specificity	Clone	Isotype	Dilution	Source	Buffer (AR)
CD34	QBEnd 10	IgG_1	1:50	Dako*	Citrate
CK7	OV-TL 12/30	IgG_1	1:100	Dako*	Citrate
CK 14	LL002	IgG_3	1:1000	Neomarkers†	Citrate
Vimentin	V9	IgG_1	1:100	Dako*	Citrate
α-SMA	1A4	IgG_{2a}	1:200	Dako*	None
CD 105	SNG	IgG_1	1:10	Dako*	Pepsin

* Dako Corporation, Glostrup, Denmark.

† LabVision Neomarkers, Fremont, CA, USA.

Microvessel Density and Microvessel Area Assessments

Immunohistochemical reactions for CD34 and CD 105 were interpreted by two authors (AA and AS) using a double-headed microscope and the most vascularized areas at low power magnification (hotspots) within each tumor were chosen for further analysis. For quantitative study 10 digital images per case were obtained using a CCD camera adapted to an Olympus CX30 microscope (X40 objective, 0.44 mm field diameter)and analyzed with Imagelab analysis software (version 2.4), which allows manual segmentation of target areas (vessels). Microvascular density for CD34 as well as for CD 105 was considered the mean number of microvessel count and total vascular area the area occupied by microvessels per unit area of the tumor. Vessels with muscular walls were excluded.

Statistical analysis

For comparison of the variables between the different tumor types we used the Kruskal-Wallis test. In order to assess changes during the tumor progression we codificated adenomas as 0, minimally invasive carcinomas as 1 and widely invasive carcinomas as 2 and followed the changes of the variables with a Sperman rank order correlation. Results with $p < 0.05$ were considered significant

RESULTS

The CXPA group included 11 women and 5 men and the tumors were located in the parotid glands in 9 cases, in the submandibular glands in 5, and in the minor salivary glands in 2. The average age of the intracapsular CXPA group was 47, in the minimally invasive 67 and in frankly invasive 65. The CXPAs were classified into two main groups according to the extent of invasion beyond the capsule of the previous PA: early tumors - 8 cases (4 intracapsular and 4 minimally invasive carcinomas), and advanced tumors – 8 cases (8 widely invasive carcinomas).

The PA group without malignant transformation included 5 women and 5 men, the average age was 41 and the tumors were located in the parotid glands in 8 cases and in the submandibular glands in 2.

Microvascular density (MVD) for CD 34 and CD 105

Figures 1, 2, 3 and 4 illustrate positive vessels and MVD for CD105 and CD34 in all groups. The mean vessel density of CD 105 stained vessels was significantly different between the groups ($p < 0.0001$; Kruskal Wallsi test). During the adenoma carcinoma sequence the MVD of CD 105 gradually increased (Figure 3) with a highly significant strong positive correlation ($R = 0.793$; $p <$

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0.0001). In PA without malignant transformation (Figure 1 and 3) a low number of small CD 105 positive vessels were detected (mean 3.33). However, in the early CXPA (intracapsular and minimally invasive carcinomas) these vessels were more numerous (mean 9.0) (Figure 1 and 3). The greatest increment in CD 105 MVD was seen in the widely invasive CXPA (mean 33.6), with carcinomas without myoepithelial differentiation revealing higher values (mean 46.5) than those with such cellular differentiation (mean 16.33) (Figure 2 and 3).

In relation to MVD for CD 34, no significant differences (p=0.15) were observed in the subgroups that represent the adenoma- carcinoma sequence (Figure 4, adenoma – mean 10.49, early CXPA – 7.66, advanced CXPA – 8.71, CXPA without myoepithelial differentiation 9.35, and CXPA with myoepithelial differentiation 7.87).

Total vascular area (TVA)

Total vascular area was significantly different among the tumors (p=0.005); Kruskal-Wallis test.

Figure 5 illustrates TVA in all groups of adenoma-carcinoma sequence. In widely invasive CXPAs, the TVA value was higher (mean 179.4) than in intracapsular and minimally invasive CXPAs (mean 63.8). In widely invasive CXPA, comparing tumors with and without myoepithelial differentiation, TVA value was higher in the former (mean 260.2 and 118.9 respectively). In widely invasive tumors with myoepithelial differentiation the carcinoma cells often formed large hypovascularized cellular aggregates that were surrounded by large vessels (Figure 2c) whereas in those without such cellular differentiation the carcinomatous aggregates usually contained a smaller quantity of cells and the vessels around them were thinner (Figure 2d).

DISCUSSION

Studies over the last decade have assumed that quantification of microvessel density may reveal the degree of angiogenic activity in a tumor [13]. However, a crucial question in angiogenesis is what proportion of the tumor vascular network is due to pre-existing normal host vessels or newly formed tumor vessels, which truly represent tumor angiogenesis [24]. Therefore, it has been suggested that markers that are preferentially expressed in angiogenic vessels, such as CD 105, would be more appropriate to quantify angiogenesis [6, 8]. In this context, in recent works a distinctive pattern of expression of pan-endothelial markers (CD34 and CD 31) and CD 105 was found in benign and malignant lesions, where MVD for CD 105 was more indicative of tumor angiogenesis [3, 19].

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In the current study, a series of CXPAs that represent the lesion in the different carcinogenesis phases was analyzed in relation to MVD for CD 34 and CD 105 as well as total vascular area, thus allowing the characterization of tumor vascularisation and the assessment of angiogenic activity during tumor progression.

In our series when the different subgroups that represent the adenoma- carcinoma sequence (adenoma – intracapsular / minimally invasive CXPA – widely invasive CXPA) were compared, MVD for CD 105 showed a strongly significant positive correlation with tumor progression whereas MVD for CD 34 revealed no difference. These results suggest that an angiogenic switch was required during the process of malignant transformation of PA into carcinoma and also reinforce the idea that MVD for CD 105 may give a more accurate measure of the tumor angiogenic activity than a pan-endothelial marker [2]. Furthermore, our findings in salivary adenoma-carcinoma sequence were similar to those reported in the process of human colorectal cancer development where CD 105, but not CD 34, gradually increased in adenoma-carcinoma sequence [2]. Nevertheless, CXPA differs markedly from salivary carcinoma spontaneously arising in HER-2/neu transgenic mice since in this animal model an angiogenic switch was not detected in any phase of carcinogenesis. However, it should be noted that CXPAs are usually high-grade carcinomas with frequent metastases whereas the salivary carcinoma in the murine model is a slow-growing tumor with low metastatic capability and, therefore there are probably differences in terms of oxygen and nutrient consumption rates of the tumor cells.

The onset of angiogenesis has been considered a hallmark of neoplastic transformation [13]. In benign lesions the expression of CD 105 in the microvessels has been described as barely visible, contrasting with that in carcinoma where it is marked [3, 19]. In our series, in PA without malignant transformation, CD 105 positive vessels were rare and usually small. Nevertheless, in PA harboring intracapsular or minimally invasive carcinoma they were more numerous suggesting that angiogenesis was activated in these early malignant lesions. These findings are in agreement with those reported in multistep tumorigenesis models. In these models, angiogenesis was found to be activated in midstage lesions before the appearance of full-blown tumors suggesting that neovascularization is a prerequisite for the rapid clonal expansion associated with the formation of macroscopic tumors [12]. However, in macroscopic tumors the major factor contributing to vessel density is metabolic demand which frequently increases during tumor progression to accommodate an increased metabolic need of cancer cells [13, 24]. In this sense in our salivary adenoma-carcinoma sequence the most significant increment regarding MVD for CD 105 was observed in the widely invasive CXPA group suggesting that a marked modification in metabolic burden occurred when the carcinoma cells infiltrate the tissues outside the confines of the original PA.

However, it is known that MVD varies widely with tumor type. Carcinomas arising in PA encompass a wide spectrum of histological tumor types [1]. In our series, comparing widely invasive CXPA with and without myoepithelial differentiation, a marked difference in terms of MVD for CD 105 (neoangiogenesis) was detected. CXPA with myoepithelial differentiation showed a lower number of CD 105 positive vessels. Angiogenesis is influenced by the net balance between angiogenic factors that stimulate and those that inhibit vessel growth [12] and it has been suggested that myoepithelial cells may inhibit angiogenesis [25]. Therefore, this non-angiogenic property of the myoepithelial cell could be an explanation for the lower MVD for 105 found in CXPA with such cellular differentiation.

Total vascular area (TVA) has been less studied than MVD but recent studies have emphasized its prognostic value [14, 15, 16, 24]. Our series showed significant findings in relation to TVA. Although widely invasive CXPA with or without myoepithelial component presented a MVD for CD 34 without significant difference, the total vascular area assessed by this antibody revealed a marked difference among them. Widely invasive CXPA with myoepithelial component showed the highest TVA mean value. In these tumors, the neoplastic cells usually formed larger hypovascularized aggregates that were often surrounded by large-sized vessels. In contrast, in CXPA without myoepithelial differentiation, the carcinomatous aggregates usually contained a smaller quantity of cells with several small-sized vessels among them. These differences in vessel shape, size and distribution between the two types of carcinomas could also be explained by some properties of myoepithelial cells. These may produce an abundant extracellular matrix devoid of vessels and containing bound angiogenic inhibitors [21]. It is likely that large-sized blood vessels are needed for cellular nutrition in carcinomas with myoepithelial component to compensate for the low angiogenesis inside the large cellular aggregates. Regarding benefit from antiangiogenic therapies and measuring the response to such a therapy it has been suggested that localization of CD 105 to angiogenic tissue could have potential when selecting patients [6]. Whether these differences appointed in our study could be relevant in terms of antiangiogenic treatment is only a topic of speculation at the present moment.

In conclusion, the antibody CD 105 reflects more clearly the angiogenic changes during the progression from adenoma to carcinoma in salivary glands than CD 34, revealing an angiogenic switch during this process. The degree of angiogenesis and the total vascular area have distinctive patterns in CXPA with and without myoepithelial differentiation. Low angiogenesis associated with high TVA value is more characteristic of CXPA with myoepithelial differentiation.

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FIGURE LEGENDS

Figure 1 - CD34 (at left) and CD 105 (at right) positive vessels: in pleomorphic adenoma without malignant transformation (a, b) and in intracapsular CXPA (c, d).

Figure 2 - CD34 (at left) and CD 105 (at right) positive vessels: in widely invasive CXPA with myoepithelial component (c, d) – large hipovascularized carcinomatous aggregates are surrounded by larger vessels, than seen in widely invasive CXPA without myoepithelial differentiation (a, b)– carcinomatous aggregates contain fewer cells and the vessels around them are thinner.

Figure 3 – Box and whisker plot of the microvascular density (MVD) for CD 105: 1) pleomorphic adenoma without malignant transformation, 2) intracapsular and minimally invasive CXPA, 3) widely invasive CXPA, 4) CXPA without myoepithelial differentiation, 5) CXPA with myoepithelial differentiation. The limits of the box represent the 25 per cent and 75 per cent percentils and the centre bar the median. The whiskers are equivalent to the 5 and 95 per cent percentils.

Figure 4 - Box and whisker plot of the microvascular density (MVD) for CD 34: 1) pleomorphic adenoma without malignant transformation, 2) intracapsular and minimally invasive CXPA, 3) widely invasive CXPA, 4) CXPA without myoepithelial differentiation, 5) CXPA with myoepithelial differentiation. The limits of the box represent the 25 per cent and 75 per cent percentils and the centre bar the median. The whiskers are equivalent to the 5 and 95 per cent percentils.

Figure 5 - Box and whisker plot of the total microvascular area (TVA) for CD 34: 1) pleomorphic adenoma without malignant transformation, 2) intracapsular and minimally invasive CXPA, 3) widely invasive CXPA, 4) CXPA without myoepithelial differentiation, 5) CXPA with myoepithelial differentiation. The limits of the box represent the 25 per cent and 75 per cent percentils and the centre bar the median. The whiskers are equivalent to the 5 and 95 per cent percentils.

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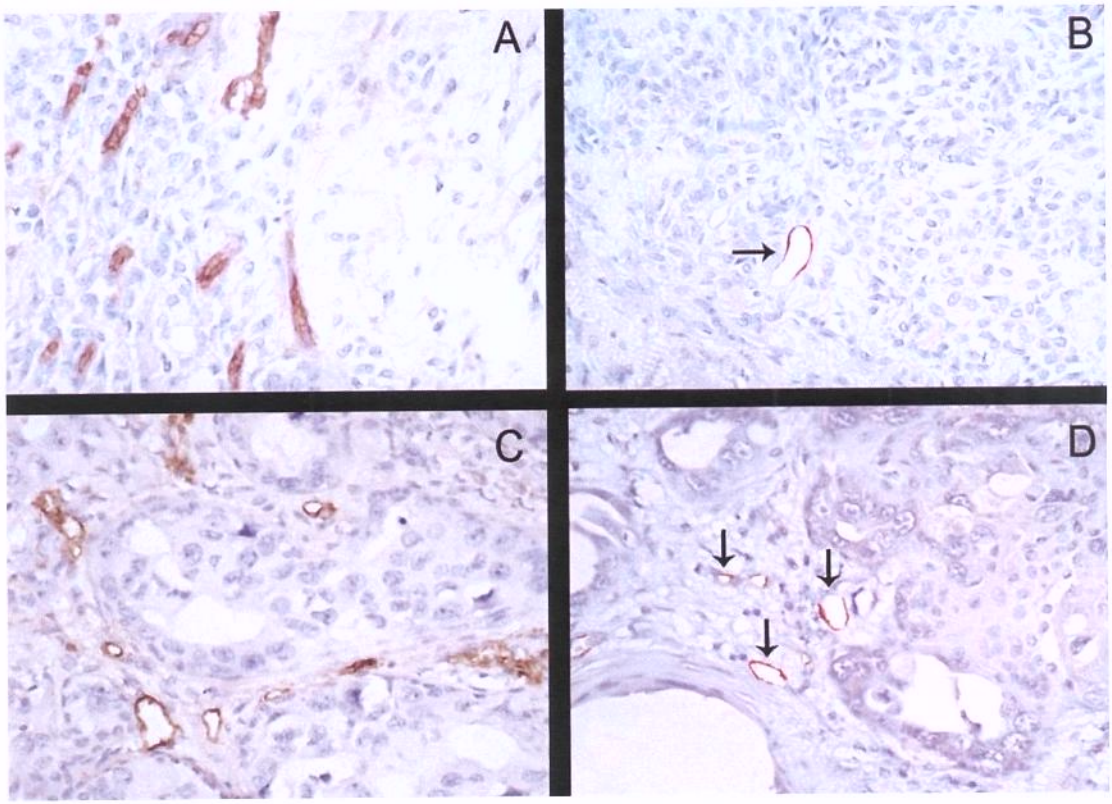

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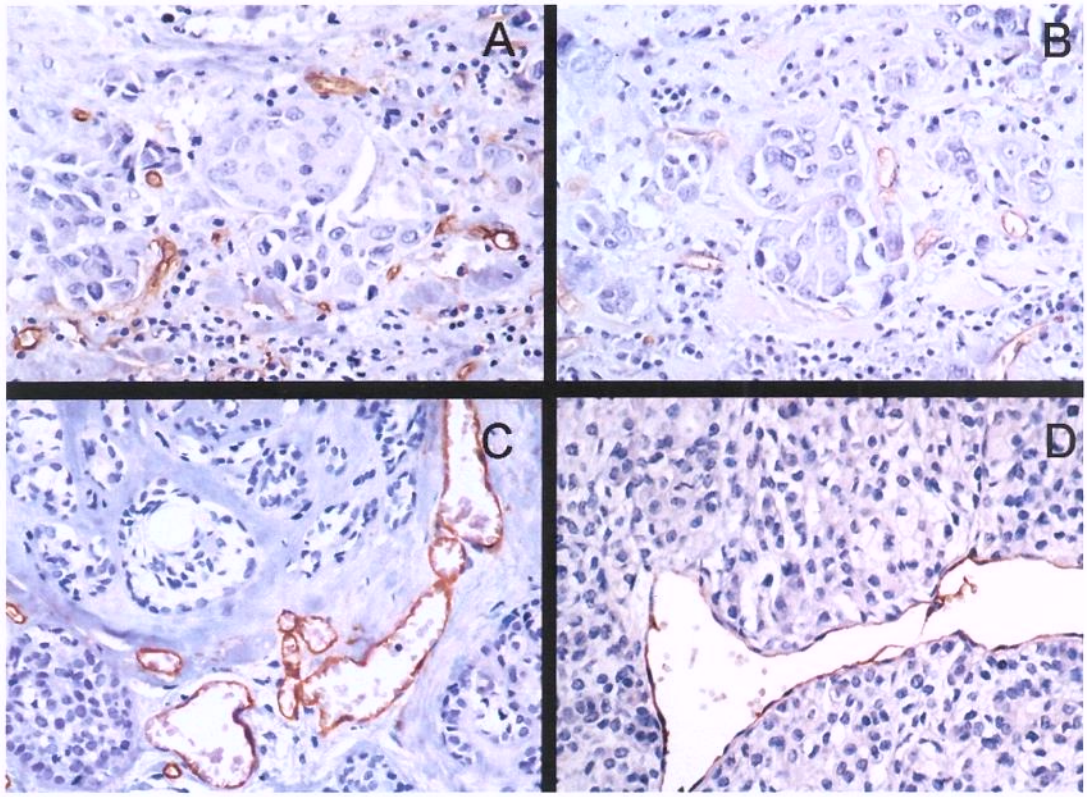

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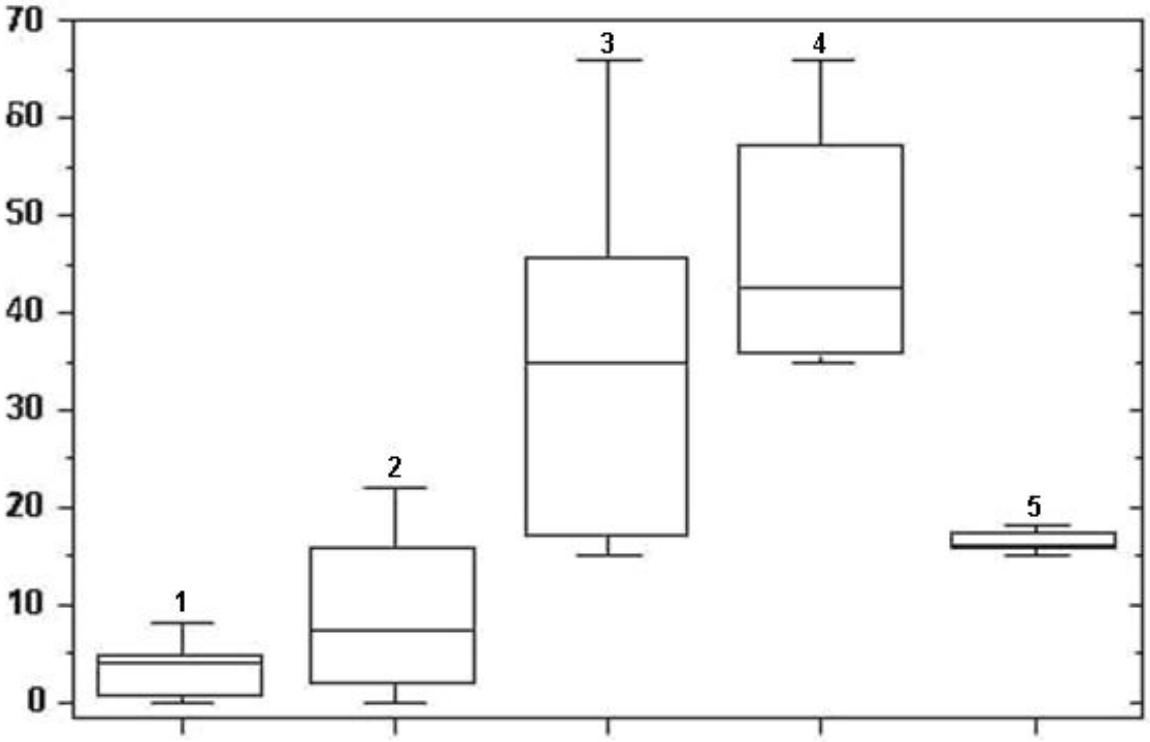

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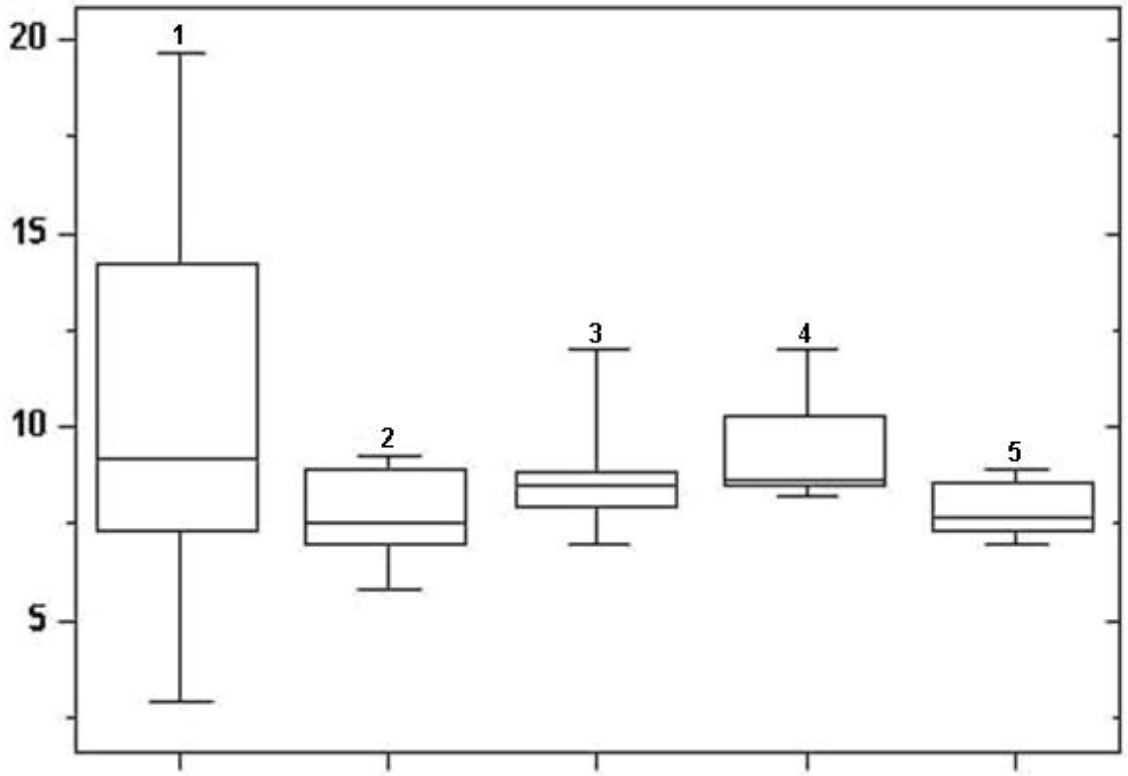

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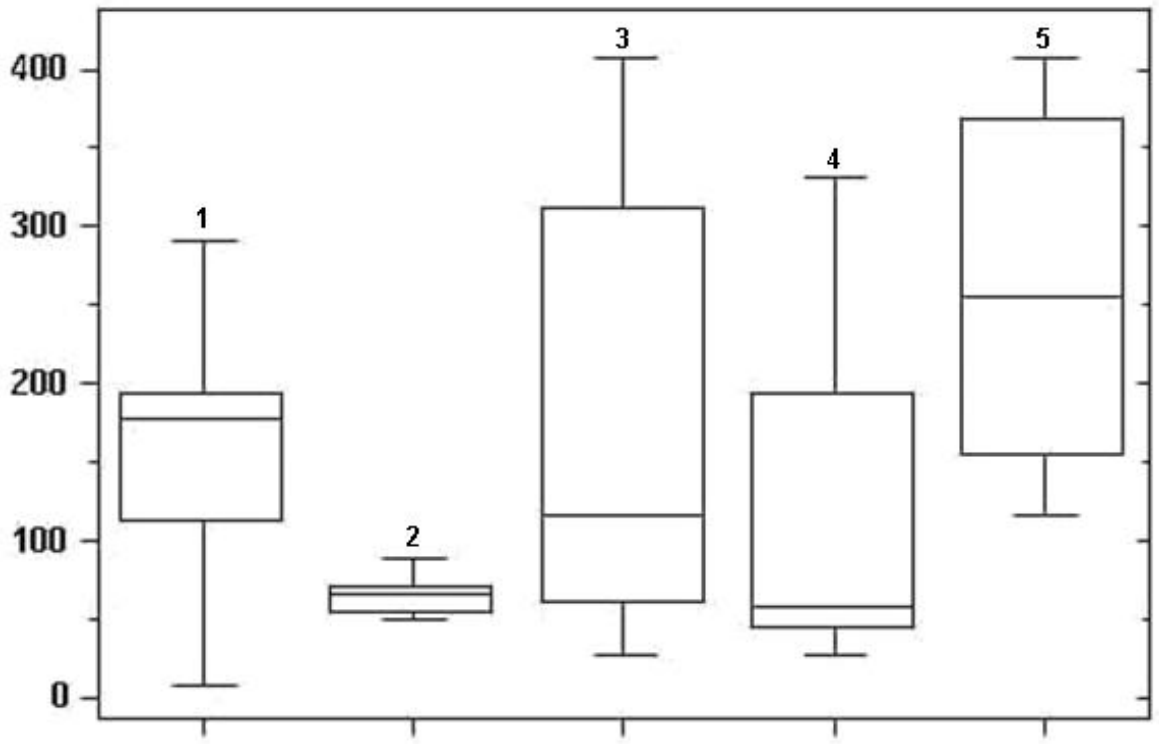

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Histopathology

Lymphatic vascular density and lymphangiogenesis during tumor progression of carcinoma ex-pleomorphic adenoma

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Abstract

Aims: To assess lymphatic vascular density (LVD) and lymph vessel endothelial proliferation in a series of carcinoma ex pleomorphic adenoma (CXPA) that represents the tumor in the different carcinogenesis phases and tumor progression.

Methods: In 8 cases of early CXPA (intracapsular and minimally invasive tumors), 8 of advanced CXPA (widely invasive tumors) and 10 of pleomorphic adenoma (PA) without malignant transformation, lymphatic vessels and proliferating cells were detected using the antibodies D2-40 and Ki 67 respectively.

Results: Comparing early tumors with advanced ones, LVD was not significantly different at the tumor margin. In contrast, regarding intratumoral lymphatics, PA without malignant transformation and early CXPA contained rare, if any, lymph vessels whereas in widely invasive carcinomas they were more numerous. However, neither intratumoral nor peritumoral LVD were increased in comparison to adjacent normal salivary gland tissue. In no case did dual immunohistochemistry using D2-40 and the cell proliferation marker Ki-67 reveal the existence of proliferating lymphatics. Carcinomatous emboli were found in peritumoral as well as in intratumoral lymphatics only in advanced CXPA without myoepithelial differentiation.

Conclusion: In CXPA, the lymphatic network is mainly composed of pre-existing lymphatics which are rare in tumors that have not infiltrated outside the confines of the original PA. In the widely invasive CXPA, intratumoral as well as peritumoral lymphatics are a conduit for carcinoma cells, but in carcinomas with myoepithelial differentiation, the neoplastic cells seem to have a lower invasion capacity.

Key Words: carcinoma ex-pleomorphic adenoma, lymph vessel, lymphangiogenesis;, lymphatic vascular density, pleomorphic adenoma

Lymphatic vascular density and lymphangiogenesis during tumor progression of carcinoma ex-pleomorphic adenoma.

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Keywords: lymphangiogenesis; carcinoma ex-pleomorphic adenoma; pleomorphic adenoma; lymph vessel; lymphatic vascular density.

Word count: 4746

ABSTRACT

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TAKE-HOME MESSAGES

•Intracapsular (in situ) and minimally invasive CXPAs as well as PA without malignant transformation showed rare, if any, intratumoral lymph vessels.

•In widely invasive CXPA, carcinomatous emboli were found in peritumoral as well as in intratumoral lymphatics

•In no case did dual immunohistochemistry using D2-40 and the cell proliferation marker Ki-67 reveal the existence of proliferating lymphatics suggesting that the lymphatic network is mainly composed of pre-existing vessels

•In CXPA with myoepithelial differentiation, the inherent properties of the myoepithelial cells are likely to contribute to its low tendency to invade lymph vessels.

INTRODUCTION

Pleomorphic adenoma (PA) is the most common benign tumor arising in salivary glands.[1] Carcinoma ex pleomorphic adenoma (CXPA), an epithelial malignancy that arises in or from a pleomorphic adenoma (PA),[1] has been considered to belong to the salivary carcinoma group which has moderate risk for neck metastasis.[2] This tumor accounts for between 4.5% and 15% of all cancers of these glands.[3-5]

In human cancers, lymphatic vascular density (LVD) has been analyzed within the main neoplastic mass (intratumoral lymphatics) as well as at the tumor margin (peritumoral lymphatics). Pre-existing peritumoral lymphatics have been considered functional, accessible and sufficient for lymphatic metastasis.[6-8] Intratumoral lymphatics, in spite of being proposed as nonfunctional in tumor models, are associated with an adverse clinical outcome and nodal metastasis in certain types of human tumors, such as cutaneous melanoma[9] and squamous cell carcinoma of head and neck, and uterine cervix.[10 11] However, in other neoplasms, such as breast, ovarian, endometrial and lung cancers, [7 12-14] no intratumoral lymphatic network has been found.

Furthermore, there is great debate whether tumors promote newly formed vessels (lymphangiogenesis) or whether pre-existing lymphatics provide the main avenue for nodal metastasis.[15 16] Evidence for lymphangiogenesis has been found in melanoma and head neck squamous carcinoma [10 17 18] but not in other malignancies such as breast cancer.[7 16 19- 21] Consequently, it has been hypothesized that the reported discrepancies may reflect genuine differences between the malignant behavior of various human neoplasms.[10]

To our knowledge, this is the first study in which lymphatic vascular density (LVD) and lymph vessel endothelial proliferation have been assessed in carcinoma-ex-pleomorphic adenoma. Lymphatic vessels were detected using the monoclonal antibody D2-40 that is considered an excellent lymphatic endothelium marker.[22 23] However, as D2-40 reactivity has also been described in non-endothelial normal cells and up-regulated in certain kinds of neoplastic cells,[23 24] we also aimed to verify its immunoreactivity in the cellular components of CXPA.

MATERIAL AND METHODS

The present study was approved by the Committee of Ethics of the University of Campinas, Brazil and was performed in 16 cases of CXPA and 10 cases of PA without malignant transformation which were retrieved from the files of the Department of Pathology of the University of Campinas. CXPA was defined as a malignant epithelial neoplasm arising in association with a primary or recurrent PA. These tumors were classified according to extent of invasion beyond the capsule of the previous PA[1] as: intracapsular (without invasion) – 4 cases, minimally invasive (≤ 1.5 mm of invasion) – 4 cases and widely invasive – 8 cases. Demographic and clinical information was obtained from the patients' medical records.

Immunohistohemistry

The following antibodies were used (table 1): D2-40 - for detection of lymphatic vessels, CD34 - for blood vessels, Ki-67 - for proliferating endothelial and epithelial cells, and α-smooth-muscle actin (α-SMA), vimentin, cytokeratins 7 (CK 7) and 14 (CK14) - for classifying the carcinomas according to the presence of epithelial and / or myoepithelial cells.

Single-staining for D2-40, α-SMA, vimentin, CK 7 and CK14 were carried out using the EnVision System (DAKO, Denmark). [25] Briefly, 5μm sections from each paraffin block were deparaffinized, hydrated and endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide. The antigen retrieval (AR) was achieved by boiling them in a steamer immersed in citrate buffer (pH: 6.0) except for alpha-SMA, which did not undergo any AR (Table 1). After cooling, the sections were incubated at 4°C, with the primary antibody, overnight and then with the EnVision polymer HRP (code K1491, DAKO, SA, Denmark) for 1h at 37°C. Subsequently, sections were stained for 5min at 37°C with 3.3'- diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin.

Double-labelling immunohistochemical staining (EnVision doublestain, code K1395, Dakopatts S/A, Denmark) was performed for D2-40/ CK14, D2-40/ CD34 and Ki67/ D2-40. Briefly, a monoclonal antibody anti-D2-40 or anti-Ki67 (Table 1) was applied after antigen retrieval using Tris-EDTA buffer (pH: 8,9) and incubated overnight at 4°C; detection was achieved using the EnVision polymer HRP and DAB to

visualize the binding of the first antibody. The sections were then incubated with a second antibody against CK-14, CD34 or D2-40 (Table 1) at 4°C overnight. EnVision polymer linked to alkaline phosphatase and fast red as a substrate chromogen system were used to complete the second immunostaining.

The isotype-matched negative controls did not show coloured-precipitate on the tissue, which indicates that artifactual staining was minimized.

Table 1: Details of the antibodies used for immunohistochemistry.

Specificity	Clone	Isotype	Dilution	Source	Buffer (AR)
CD34	QBEnd 10	IgG_1	1:50	Dako*	Citrate
CK7	OV-TL 12/30	IgG_1	1:100	Dako*	Citrate
CK 14	LL002	IgG_3	1:1000	Neomarkers†	Citrate
Vimentin	V9	IgG_1	1:100	Dako*	Citrate
α-SMA	1A4	IgG_{2a}	1:200	Dako*	None
D2-40	D2-40	IgG_1	1:200§	Dako*	Tris-EDTA

* Dako Corporation, Glostrup, Denmark.

† LabVision Neomarkers, Fremont, CA, USA.

§ Except for double-staining when the dilution was 1:100

Evaluation of staining

Immunohistochemical reactions for D2-40, D2-40/CK14, D2-40/CD34, and D2-40/Ki-67 were interpreted by two authors (AA and AS) using a double-headed microscope. The initial evaluation was performed to verify the expression pattern of the antibodies in both single and double-stained sections.

The quantitative analysis of the intratumoral and peritumoral lymphatic vessel density was carried out in sections that were single-stained for D2-40, as previously described.[26] Because this antibody is also expressed by myoepithelial cells, double stains for D2-40/CK14, and D2-40/CD34 on serial sections were used to confirm the identity of lymphatic vessels. Three distinct sets of measurements were performed in each tumor section, in which single lymphatic vessels were manually counted at 200X (0.7386 mm^2 field): a) 3 fields with the most vascularized areas (hotspots) were identified within the tumor mass (intratumoral lymphatic density), b) within an area 500μm from the tumor border (peritumoral lymphatic density) and c) within the normal

tissue adjacent to the lesion. We also evaluated: a) whether there was invasion of the D2-40 intratumoral or peritumoral lymphatic vessels by carcinoma cells (tumor emboli), and b) whether benign and malignant myoepithelial as well as epithelial cells expressed D2-40. Finally, lymphatic endothelial proliferation was assessed by counting the number of Ki-67 positive lymphatic endothelial cells per lymphatic vessel.

Statistical analysis

For comparison between different tumor groups we used the Kruskall-Wallis test. Comparisons between intratumoral, peritumoral and normal tissue in the same tumor group were done with the help of the Wilcoxon test. All calculations were performed with the help of the SAS 8.0 and SPSS 10.0 programs. Significance level was always 0.05.

RESULTS

The CXPA group included 11 women and 5 men and the tumors were located in the parotid glands in 9 cases, in the submandibular glands in 5, and in the minor salivary glands in 2. The average age of the intracapsular CXPA group was 47, in the minimally invasive 67 and in frankly invasive 65. The CXPAs were classified into two main groups according to the extent of invasion beyond the capsule of the previous PA: early tumors - 8 cases (4 intracapsular and 4 minimally invasive carcinomas), and advanced tumors – 8 cases (8 widely invasive carcinomas). In early tumors, nodal metastasis at the time of primary surgery was not present in any cases whereas in advanced tumors it was detected in 2 out of 7 cases.

The PA group without malignant transformation included 5 women and 5 men, the average age was 41 and the tumors were located in the parotid glands in 8 cases and in the submandibular glands in 2.

Lymphatic Vessel Density and Endothelial Proliferation

Normal salivary gland and pleomorphic adenoma without malignant transformation

In the normal salivary glands, LVD was 8.75 ± 1.16. Lymphatic vessels were dispersed in adipose tissue and adjacent to the interlobular salivary ducts and blood vessels but were not seen within the intralobular stroma. In pleomorphic adenoma without malignant transformation, rare lymph vessels were detected within the tumor in one out of ten cases (fig 1A).

Early tumors (intracapsular and minimally invasive CXPA) – 8 cases

In this group, according to cellular composition, all cases were composed of carcinoma without myoepithelial differentiation and in only two of them, rare and small intratumoral lymph vessels were observed (fig 1B). In contrast, at the tumor margin, LVD was 6.63 ±3.89 and the distribution as well as the morphological appearance of the lymphatic vessels was similar to that seen in normal salivary glands (fig 1C,D). Comparing the density of lymphatics in normal salivary glands with that in the peritumoral as well as intratumoral region (fig 2A), only the latter was significantly lower ($p= 0.34$, $p= 0.04$ respectively).

Advanced tumors (widely invasive CXPA) – 8 cases

In widely invasive CXPA, intratumoral LVD was 6.25 ±7.09 and peritumoral LVD 7.14± 4.53 (fig 2B). Comparing normal salivary glands with the intratumoral as well as the peritumoral region, no significant difference was found in terms of LVD ($p=0.59$ and $p=0.32$ respectively). According to cellular composition, these tumors were classified as: carcinomas with myoepithelial differentiation, four cases, and without, four cases. In the former group, intratumoral lymphatics were detected in two cases and they were morphologically similar to those in normal tissues (fig 3C). In CXPA without myoepithelial differentiation, intratumoral lymphatic vessels were observed in three cases and in two of them, dilated lymphatics containing carcinoma emboli were seen within the tumor mass as well as at the tumor margin (fig 3A,B).

Comparing early with advanced CXPA groups, the peritumoral region of advanced tumors showed a slight increase in LVD but the difference was not significant ($p= 0.79$). In contrast, intratumoral LVD was significantly lower in early tumors than in advanced ones ($p=0.04$).

All lymphatic endothelial cell nuclei were negative for proliferation marker Ki 67, whereas strong positivity was seen in adjacent tumor cells and blood endothelial cells, which served as a positive internal control (fig 3D).

D2-40 expression in tumor cells

In PA without malignant transformation and in CXPA, variable proportions of benign as well as malignant myoepithelial cells showed D2-40 expression in the cytoplasm. Among the myoepithelial cells, D2-40 positivity was found in those from tubulo-ductal structures (fig 4A), and in solid cellular masses. Regarding epithelial cells, D2-40 expression was detected in only two cases (one intracapsular and one widely invasive CXPA) and in these, only in malignant cells. In the widely invasive CXPA, the carcinomatous cells showed squamous differentiation and D2-40 was expressed predominantly within the basal tumor cell layer, with enhanced membrane-staining pattern (fig 4B).

In normal salivary glands, D2-40 stained myoepithelial cells were located in the acinar and ductal structures.

DISCUSSION

Based on the extent of carcinomatous invasion beyond the PA capsule, CXPA may be classified as intracapsular, minimally invasive and frankly invasive.[27] Intracapsular and minimally invasive carcinomas have been considered low-grade neoplasias, in which metastasis rarely occurs.[28-30] In contrast, widely invasive CXPA are typically high-grade carcinomas with frequent metastases and disease-related deaths .[27 28]

In the current study, a series of CXPA that represent the tumor in the different carcinogenesis phases was analyzed, thus allowing the characterization of tumor lymphatics and the assessment of lymphangiogenesis during tumor progression.

In our series, peritumoral LVD was not significantly different in intracapsular and minimally invasive CXPA (early tumors) compared with widely invasive ones (advanced tumors). In contrast, regarding intratumoral lymphatics, early CXPA contained rare, if any, lymph vessels whereas in widely invasive carcinomas they were more numerous. However, neither intratumoral nor peritumoral LVD increased in comparison to adjacent normal salivary gland tissue. In addition, in no case did dual immunohistochemistry using D2-40 and the cell proliferation marker Ki-67 reveal the existence of proliferating lymphatics, contrasting with the strong expression of Ki-67 in adjacent tumor cells and blood endothelial cells. These results suggest that lymphangiogenesis did not occur, or was minimal, during the process of malignant transformation of PA into carcinoma and, thus, peritumoral as well as intratumoral lymphatic vessels were pre-existing. Therefore, carcinoma arising in PA is similar to breast cancer,[7 19 31] where lymphangiogenesis was not detected and intratumoral lymphatics were considered "entrapped vessels" adjacent to pre-existing normal lobules and ducts.[19] Nevertheless, CXPA differs markedly from head and neck squamous cell carcinoma [10 17] and malignant melanoma ,[18] where proliferating intratumoral lymphatics have been seen. These findings lend support to the hypothesis that tissue specific tumor features might be responsible for the presence or absence of a "lymphangiogenic switch".[31] However, the mechanisms involved in the promotion of newly formed lymph vessels and the role of this phenomenon in tumor progression still need to be clarified. In breast carcinogenesis, as lack of lymphangiogenesis cannot be explained by the absence of proteins associated to lymphangiogenic growth (VEGF-C and VEGF-D) or

of their receptors (VEGFR-3)[32-33] Vleugel *et al* [31] suggested that factors that contribute to lymphangiogenic growth inhibition should be investigated.

Regarding neoplastic lesions representing the early phases of tumor progression, rare studies have assessed LVD and lymphangiogenesis. In these, comparing premalignant with pre-invasive intraductal proliferations of the breast and nevi with malignant melanoma in situ, no significant increase was found in terms of LVD.[19 31 34] In our series, intracapsular (in situ) and minimally invasive CXPAs as well as PA without malignant transformation showed rare, if any, intratumoral lymph vessels. However, it should be noted that intracapsular CXPA shows significant differences in relation to other pre-invasive epithelial proliferations. These refer to neoplasias restricted to the epithelium without stromal invasion whereas intracapsular CXPA defines a tumor that has not infiltrated outside the confines of the original PA ,[1] which is composed of an intimate admixture of ductal epithelium, myoepithelium and stroma.[1] The stroma is produced by adenoma cells and consists of an admixture of mucoid, myxoid and chondroid tissues. Non-neoplastic stroma (pre-existing stroma), in which the lymph vessels are situated, was only found beyond the PA capsule which could explain the presence of intratumoral lymphatics only in widely invasive CXPA. This idea is in agreement with the findings reported by Vleugel *et al* [31] in breast ductal carcinoma in situ. These authors suggested that the inverse correlation between lymph vessel density and lesion size could be due to replacement of stroma by tumor epithelium, rather than the collapse of lymph vessels.

Dilated peritumoral lymph vessels, often containing tumor emboli, have been reported in different types of human cancer suggesting that these lymphatics are sufficient for metastatic dissemination.[7 19] In contrast, intratumoral lymphatics have been considered non-functional due to the results of fluorescent dye uptake measurements.[20] However, in head neck squamous cell carcinomas it has been shown that intratumoral lymphatics are involved in nodal metastasis. [10 17 35]In the current study, in widely invasive CXPA, carcinomatous emboli were found in peritumoral as well as in intratumoral lymphatics. These findings suggest that in the advanced phase of tumor progression, intratumoral lymphatics should be regarded as an additional pathway that might increase the propensity of the CXPA to metastasize. Nevertheless, at the early phase, where the malignant cells are still contained within the parent PA, the scarcity of intratumoral lymph vessels probably contribute to the very low metastatic risk of these tumors.

Besides the presence of peritumoral as well as intratumoral lymph vessels, other factors related to tumor biology may contribute to increase the risk of metastases.[8 19] Carcinomas arising in PA encompass a wide spectrum of histological patterns in which malignancies with epithelial and / or myoepithelial differentiation[36] are included. In the present investigation, lymphatic emboli and cervical metastases were present only in widely invasive carcinomas without myoepithelial differentiation suggesting that the frequency of these events may also be influenced by tumor cellular composition. In CXPA with myoepithelial differentiation, the inherent properties of the myoepithelial cells (including secretion of low levels of matrix-degrading proteinases and of high levels of various anti-invasive proteinase inhibitors)[37] are likely to contribute to its low tendency to invade lymph vessels and, thus, to metastasize.

In relation to D2-40 reactivity with other non-endothelial cells, this antibody recognizes an epitope on the podoplanin molecule that is expressed in myoepithelial cells of the normal salivary glands.[23] Podoplanin seems to play an important role in mediating cellular contractile properties and cytoskeletal reorganization .[23] In our series, benign and malignant myoepithelial cells were D2-40 positive suggesting that this antibody might be used as an additional tool to identify neoplastic myoepithelial cells in salivary gland tumors.

Regarding carcinomatous cells, Schacht *et al*,[23] Dumoff *et al* [24] and Ordóñez [38] reported expression of D2-40 in skin, uterine cervix and lung squamous cell carcinomas respectively. Antibody D2-40 was generated against an oncofetal M2A antigen (40-kd O-linked sialoglycoprotein), which is expressed in the fetal testis and in germ cell neoplasia .[39] It has been suggested that this antigen could have a role in epithelial tumor progression by enhancing tumor cell spread via lymphatic vessels. However, in our series only a few D2-40 positive carcinoma cells were observed in two cases (intracapsular and widely invasive CXPA respectively) but in both nodal metastases were not detected. Interestingly, in one of them (a widely invasive CXPA), the carcinoma cells showed squamous differentiation and D2-40 was expressed predominantly within the basal tumor cell layer. Based on these findings we speculate whether D2-40 expression is implicated in squamous cell differentiation since podoplanin expression has been found in normal basal keratinocytes and predominantly in poorly differentiated squamous cell carcinoma.[23]

In conclusion, in CXPA, the lymphatic network is mainly composed of pre-existing lymphatics which are rare in tumors that have not infiltrated outside the

confines of the original PA. In the widely invasive CXPA, intratumoral as well as peritumoral lymphatics are a conduit for carcinoma cells, but in carcinomas with myoepithelial differentiation, the neoplastic cells seem to have a lower invasion capacity.

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LEGENDS

Figure 1 - Immunostaining of lymphatic vessels in pleomorphic adenoma (PA) without malignant transformation and in intracapsular and minimally carcinoma ex-pleomorphic adenoma (CXPA). (A) In PA without malignant transformation and (B) in intracapsular CXPA, rare and small intratumoral D2-40-positive lymph vessels (arrows) are seen. (C) In minimally invasive CXPA, the carcinoma cells invade the tissues beyond the PA capsule and (D) at the tumor margin, regularly-shaped D2-40-positive lymph vessels (brown, arrows) are seen close to a small salivary duct with CK 14-positive basal cells (red) and at some distance from neoplastic cells (arrows) (double immunostaining with D2-40 and CK 14 antibodies).

Figure 2 – Box and whisker plot of the lymphatic vascular density (LVD). (A) in intracapsular and minimally invasive CXPA (early CXPA) 1) intratumoral LVD; 2) peritumoral LVD; 3) LVD in normal tissue adjacent to the lesion. (B) in widely invasive CXPA (advanced CXPA): 1) intratumoral LVD; 2) peritumoral LVD; 3) LVD in normal tissue adjacent to the lesion. The limits of the box represent the 25 per cent and 75 per cent percentils and the centre bar the median. The whiskers are equivalent to the 5 and 95 per cent percentils.

Figure 3 - Immunostaining of lymph vessels in widely invasive CXPA. (A) In CXPA without myoepithelial differentiation (i.e., CXPA composed of epithelial cells only), peritumoral D2-40-positive lymph vessels containing tumor emboli (arrow) are seen. The inset shows the carcinoma emboli. (B) Within the tumor mass, dilated intratumoral D2-40-positive (brown) lymph vessels containing malignant epithelial cells within the lumen and CD34-positive (red) blood capillaries are seen (double immunostaining with D2-40 and CD 34 antibodies). (C) CXPA composed of myoepithelial cells only; note the regularly-shaped intratumoral D2-40 positive lymph vessel without tumor embolus. The inset shows CXPA composed of malignant myoepithelial cells of hyaline type. (D) At the tumor margin, regularly-shaped, non-proliferating lymphatic vessels (red) are found in CXPA with myoepithelial differentiation. Note the D2-40-negative blood vessel (arrow) and the tumor cells with positive nuclear Ki-67 staining (double immunostaining with D2-40 and Ki-67 antibodies).

Figure 4 - D2-40 expression in non-endothelial cells. (A) In adenoma areas of CXPA, benign myoepithelial cells of tubulo-ductal structures are stained by D2-40 (brown) and CK14 (red) (double immunostaining with D2-40 and CK14 antibodies). (B) In the widely invasive CXPA with squamous differentiation, D2-40 (brown) is expressed in the basal tumor cell layer and in lymph vessels (arrows). Note the CD34 (red) positive blood vessels (double immunostaining with D2-40 and CD 34 antibodies).

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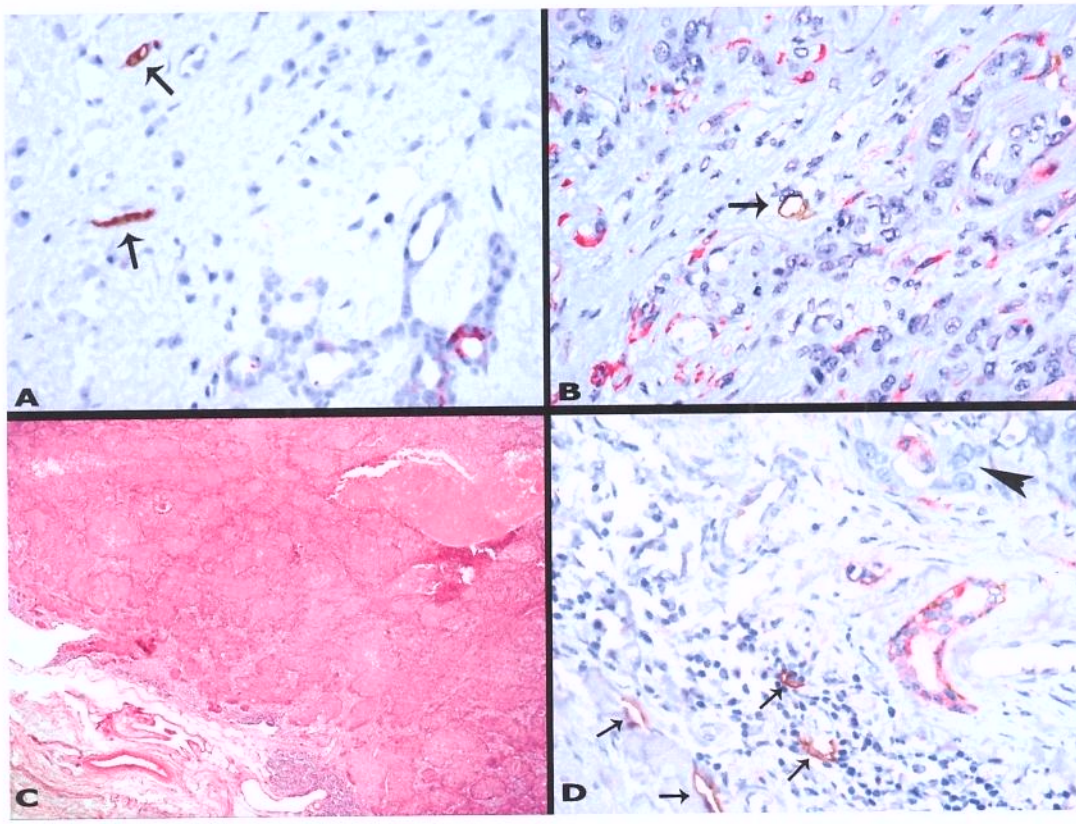

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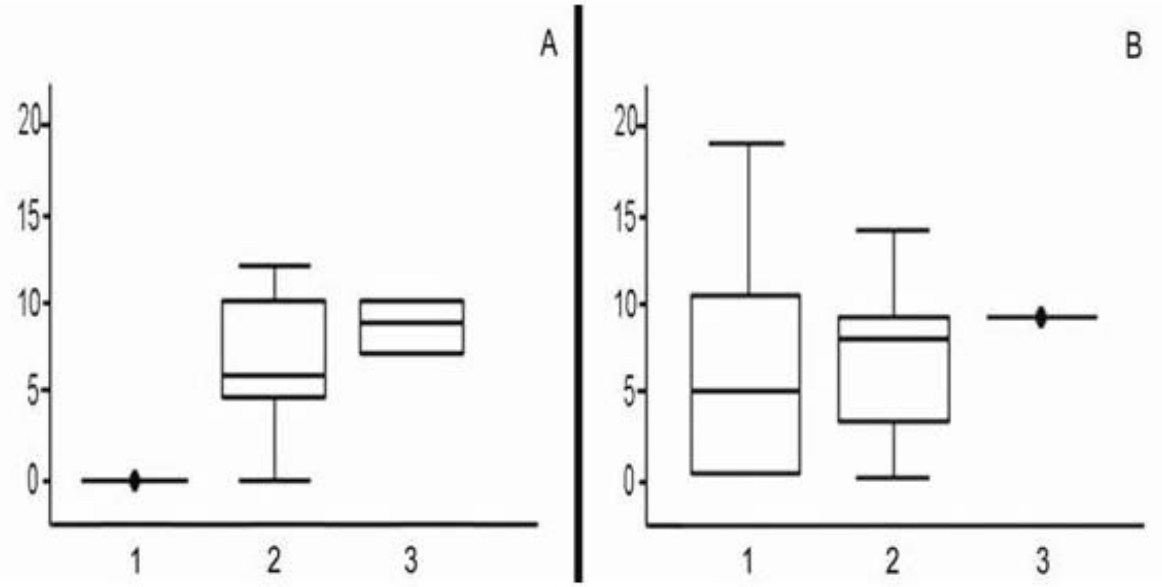

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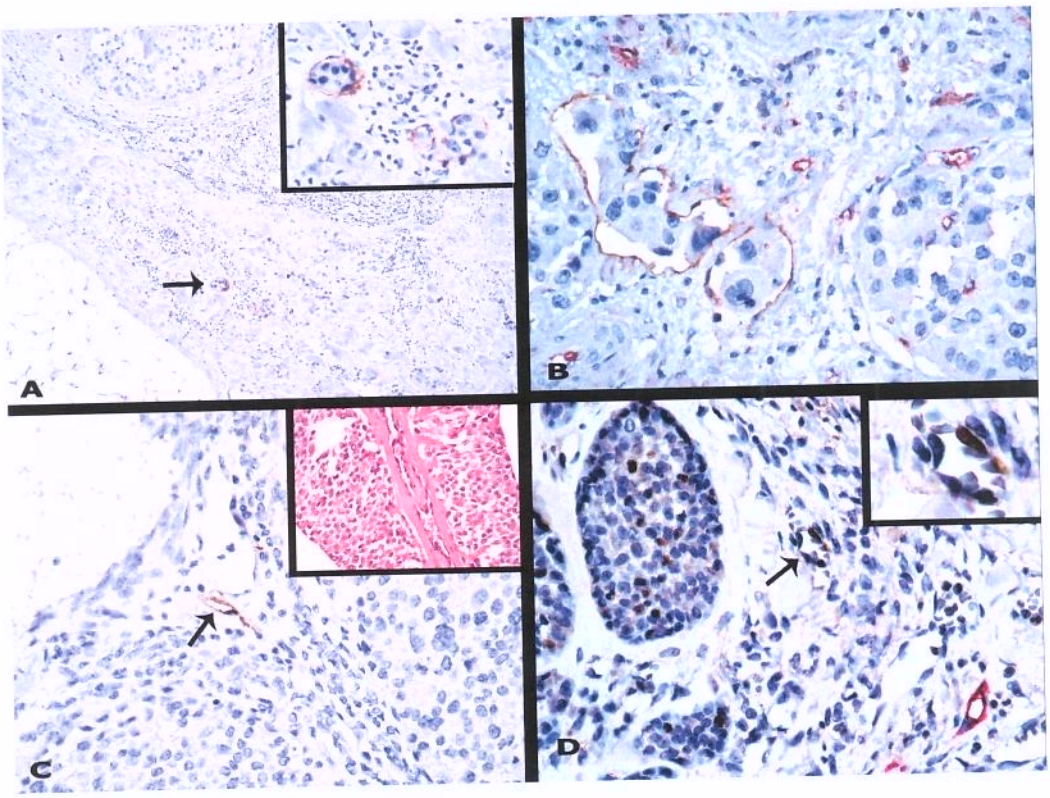

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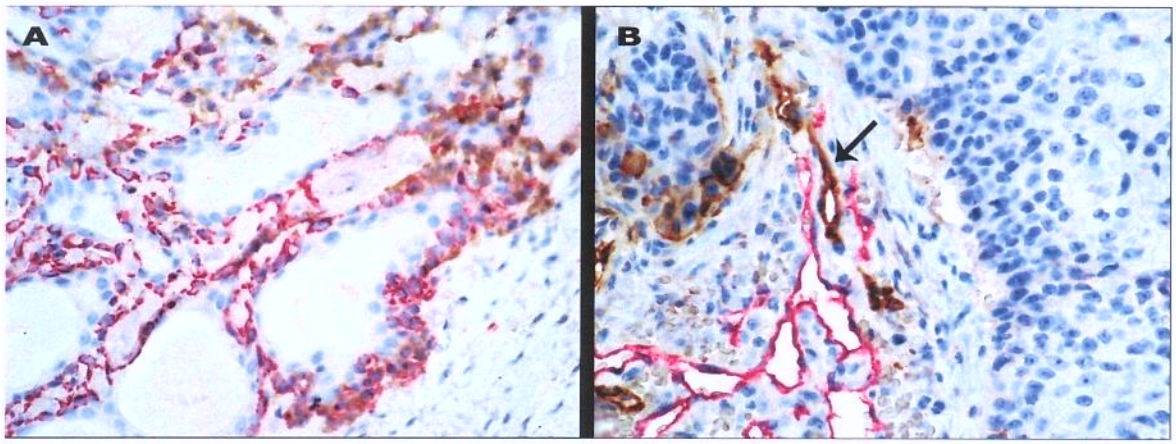

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5- DISCUSSÃO GERAL

O CXAP é uma neoplasia epitelial maligna de glândulas salivares, na qual evidências de um AP ainda podem ser encontradas (Barnes *et al.* 2005). Pela possibilidade de se observar, na mesma lesão, áreas benignas e malignas lado a lado, o CXAP torna-se um modelo interessante para se estudar a seqüência adenoma-carcinoma.

O CXAP pode ser classificado em intracapsular, minimamente invasor ou francamente invasor, de acordo com a extensão carcinomatosa além da cápsula (Gnepp *et al*. 2000). Os intracapsulares e minimamente invasores são considerados neoplasias de baixo grau de malignidade, cujo comportamento biológico é semelhante ao do próprio AP (Brandwein *et al*. 1996; Lewis *et al* 2001), havendo apenas um caso relatado na literatura de um CXAP intracapsular com metástase linfonodal (Felix *et al*. 2002). Em contraste, os francamente invasores são considerados como tumores agressivos que, freqüentemente provocam metástase levando o paciente a óbito (Gnepp *et al*. 2000; Lewis *et al* 2001).

A metástase pode ser classificada em local, regional (por via linfática) e distante (por via sanguínea). As duas últimas são mais comuns, o que tem levado a estudos amplos dos vasos sanguíneos e linfáticos ao longo dos anos (Pepper 2001). Atualmente, com o surgimento de novos anticorpos mais específicos para proliferação endotelial vascular sanguínea e para o endotélio linfático, tanto a angiogênese como a linfangiogênese tem sido pesquisadas em diversos tumores, mostrando ter um papel fundamental no crescimento tumoral, invasão e metástase (Uhr *et al*. 1997; Gastl *et al.* 1997; Choi *et al*. 2005). O nosso estudo avaliou a angiogênese e a linfangiogênese no AP e nas diferentes fases da progressão tumoral do CXAP, sendo analisados 10 casos de AP sem tranformação maligna, 8 de CXAP na fase precoce (4 intracapsulares e 4 minimamente invasores) e 8 francamente invasores.

5.1- Vascularização Sanguínea no Carcinoma-Ex-Adenoma Pleomórfico

Angiogênese é a formação de novos vasos sanguíneos através da migração e proliferação de células endoteliais dos vasos pré-existentes (Beck & D'Amore 1997). É um processo fundamental para que ocorram diversos

eventos fisiológicos e patológicos, além de ser considerado um pré-requisito para o crescimento tumoral e metástase (Uhr *et al*. 1997; Gastl *et al.* 1997). A progressão da angiogênese resulta do balanço entre os fatores que estimulam e dos que inibem o crescimento vascular, além de fatores não angiogênicos, tais como o oxigênio e a taxa do consumo de nutrientes pelas células tumorais (Hlatky *et al*. 2002).

A técnica mais utilizada para se avaliar a angiogênese é pela contagem dos microvasos (microdensidade vascular – MDV) vistos por meio de marcadores imunoistoquímicos de células endoteliais (Choi *et al*. 2005). Esta técnica permite estudar, não somente a angiogênese, como também a neoangiogênese. Em nosso estudo, nós não encontramos nenhuma diferença entre os diferentes grupos estudados em relação à MDV quando analisada com o anticorpo CD34, que é um marcador pan-endotelial. Em contraste, quando avaliado a MDV com o anticorpo CD105, que é um marcador de neoangiogênese, foi observada uma intensa correlação entre a neoangiogênese, identificada com o emprego deste marcador e a progressão tumoral, sugerindo que, durante a transformação maligna do AP para CXAP, é necessário que ocorra neoangiogênese.

Nossos resultados, nas glândulas salivares, são similares àqueles observados na seqüência adenoma-carcinoma colon-retal, na qual também foi encontrado aumento da MDV pelo CD105 e nenhuma diferença significante para o CD34 (Akagi *et al*. 2002). Estes achados enfatizam que a MDV, analisada com o CD105 possa dar uma medida mais precisa da atividade angiogênica no tumor, permitindo ainda determinar quando ocorre a mudança angiogênica na seqüência adenoma-carcinoma (Akagi *et al*. 2002). Além disso, recentemente, alguns estudos, comparando a MDV pelo CD105 com aquela pelo CD34, em carcinoma gástrico e carcinoma não “oat cell” do pulmão, demonstraram que o CD105 é superior para se avaliar, além da angiogênese, o prognóstico (Tanaka *et al*. 2001; Ding *et al*. 2006). Alguns autores têm levantado a hipótese de que a identificação seletiva dos vasos neoformados possa ter importância fundamental para a terapia anti-angiogênica, que é uma alternativa co-adjuvante para a terapia anti-câncer tradicional (Huang *et al*. 1997).

O inicio da angiogênese tem sido considerado como um marco na transformação neoplásica (Hlatky *et al*. 2002). Em lesões gástricas benignas, a expressão do CD105 na rede microvascular foi descrita como sendo pouco visível, contrastando com aquela observada no carcinoma gástrico, no qual foi encontrada uma alta expressão deste anticorpo (Ding *et al.* 2006). Em nossos resultados, APs sem transformação maligna apresentaram raros e pequenos vasos marcados para o CD105. Ao contrário, nos AP com carcinoma intracapsular ou minimamente invasor foi observado maior número de vasos corados por esse anticorpo, sugerindo que a angiogênese já é ativada na fase precoce da transformação maligna. Estes achados corroboram com aqueles relatados em modelos experimentais da carcinogênese, em que a angiogênese tem sido encontrada ativada antes do aparecimento do tumor macroscópico, sugerindo que a neovascularização é um pré-requisito para que ocorra expansão clonal e formação da neoplasia (Hanahan & Weinberg 2000).

Em tumores já formados, o principal fator que contribui para a densidade vascular é a demanda metabólica, que freqüentemente está aumentada durante a progressão tumoral, devido à maior necessidade metabólica das células cancerosas (Hlatky *et al*. 2002, Sharma *et al.* 2005). Em nossa seqüência adenoma-carcinoma, o principal aumento da MDV pelo CD105 foi observado no grupo dos carcinomas francamente invasores, sugerindo que há um significante aumento da neoangiogênese, nessa fase de infiltração carcinomatosa além da cápsula do adenoma, provavelmente refletindo importante alteração metabólica durante a progressão tumoral.

Na literatura, é descrito que a MDV pode variar muito de acordo com o tipo do tumor. Carcinomas surgindo de AP podem apresentar um amplo espectro de padrões histológicos e, dentre eles, há aqueles com e sem diferenciação mioepitelial (Altemani *et al*. 2005). Em nossos resultados, quando comparada a MDV pelo CD105 nos carcinomas francamente invasores com e sem diferenciação mioepitelial, foi observada uma diferença significante entre esses dois grupos. O CXAP com diferenciação mioepitelial apresentou baixo número de

vasos positivos para o CD105, o que poderia ser explicado pela propriedade das células mioepiteliais de inibirem a angiogênese (Sternlicht & Barsky, 1997).

A área total vascular (ATV) tem sido menos estudada que a MDV. Entretanto, estudos recentes têm enfatizado a importância da ATV para avaliar prognóstico (Korkolopoulou *et al*. 2001; Korkolopoulou *et al*. 2002; Laitakari *et al.* 2004; Sharma *et al.* 2005). Nosso estudo mostrou resultados significantes em relação à ATV. Apesar da MDV pelo CD34 no CXAP francamente invasor não mostrar nenhuma diferença em relação aos tumores com e sem diferenciação mioepitelial, a ATV por esse anticorpo mostrou diferença significante nestes dois tipos de carcinomas. CXAPs francamente invasores com componente mioepitelial apresentaram maior média da ATV. Nestes tumores, as células neoplásicas formavam, freqüentemente, agrupamentos grandes hipovascularizados, que eram circundados por vasos sanguíneos dilatados. Em contraste, nos CXAP sem diferenciação mioepitelial, os agrupamentos carcinomatosos geralmente eram menores, continham pouca quantidade de células e havia freqüentes pequenos vasos entre eles. Esta diferença em relação ao número, forma, tamanho e distribuição dos vasos entre os dois tipos de carcinoma, pode ser explicada por algumas propriedades das células mioepitelais. Estas células podem produzir matriz extracelular abundante livre de vasos e conter fatores que inibem a angiogênese (Nguyen *et al*. 2000). É provável que vasos sanguíneos mais dilatados sejam necessários para a nutrição celular em carcinomas com componentes mioepitelial, para compensar a baixa atividade angiogênica dentro dos agrupamentos celulares. Entretanto, ainda é uma questão aberta se esta diferença em relação à área vascular e angiogênese entre carcinomas com e sem diferenciação mioepitelial possa ser relevante para um futuro tratamento antiangiogênico.

5.2- Vascularização Linfática no Carcinoma-Ex-Adenoma Pleomórfico

O sistema linfático compreende uma rede de capilares, vasos maiores, gânglios linfáticos e ductos, que têm por principal função transportar a linfa para todo o corpo (Jackson *et al.* 2001; Pepper 2001; Reis Filho & Schmitt 2003).

Além disso, observações clínicas e patológicas mostram que, na grande maioria dos carcinomas, as células neoplásicas são transportadas através do sistema linfático (Pepper 2001). As principais razões para este acontecimento são: os capilares linfáticos serem maiores que os sanguíneos, não possuem uma membrana basal contínua, apresenta uma velocidade interior de fluxo bem mais baixa do que a observada no sistema sanguíneo e pela composição da linfa ser muito similar a do fluido intersticial, favorecendo, desta maneira, a sobrevivência das células neoplásicas (Pepper 2001; Sleeman *et al*., 2001; Swartz 2001; Swartz & Skobe 2001).

A linfangiogênese é definida como a formação de novos vasos linfáticos (Jackson *et al.* 2001; Karkkainen et al. 2001; Pepper 2001; Sleeman *et al*., 2001; Reis Filho & Schmitt 2003). Apesar da grande quantidade de informações sobre o papel da angiogênese em neoplasias humanas, pouco se sabe sobre a linfangiogênese em tumores (Carmeliet e Jain, 2000). Uma das principais razões para esta limitação de pesquisas sobre linfangiogênese era a ausência de marcadores específicos para as células endoteliais linfáticas, até recentemente. Com o surgimento dos anticorpos VEGR3, LYVE-1, Prox1 e D2-40, o conhecimento aumentou (Pepper 2001; Dadras *et al.* 2003; Maula *et al.* 2003; William *et al.* 2003; Agarwal *et al.* 2005), porém, as informações sobre o mecanismo molecular da linfangiogênese e o caminho percorrido pelas células neoplásicas até a invasão linfática ainda são limitadas.

Atualmente, na literatura, há controvérsia sobre como as células tumorais se disseminam através dos vasos linfáticos. Alguns autores acreditam que a metástase ocorra apenas através de vasos pré-existentes localizados na margem do tumor (vasos peritumorais) (Carmeliet & Jain 2000; William *et al.* 2003; Agarwal *et al.* 2005), enquanto outros autores relatam que há neoformação vascular linfática no interior do tumor (vasos intratumorais) e que esta seria importante para causar metástase (Pepper 2001; Dadras *et al.* 2003; Maula *et al.* 2003). Estes últimos acreditam que as células tumorais podem produzir fatores linfangiogênicos que, além de induzirem à proliferação e dilatação

dos linfáticos peritumorais, levam a proliferação dos intratumorais, o que explicaria a existência destes vasos no interior dos tumores (Franchi *et al*. 2004)

Os autores que defendem a ausência de vasos linfáticos intratumorais a explicam pelo fato das células neoplásicas crescerem em espaço confinado, o que geraria stress mecânico ou hipertensão intersticial, que causaria, por conseqüência, compressão dos vasos neoformados (Helmlinger *et al*., 1997). Além disso, devido ao aumento da pressão intersticial no interior do tumor, os vasos linfáticos dos tecidos adjacentes não conseguiriam penetrar no estroma tumoral. Os linfáticos peritumorais, por sua vez, por sofrerem pouco stress mecânico, geralmente estão dilatados devido ao maior fluxo linfático, aumentando as chances de entrada das células neoplásicas (Swartz & Skobe, 2001). Por esta razão, os vasos linfáticos na periferia dos tumores têm sido considerados funcionantes e suficientes para causar metástase linfática (Padera *et al*. 2002).

Em nosso estudo, a densidade vascular linfática (DVL) peritumoral não foi significantemente diferente, quando comparados o CXAP precoce (intracapsular/minimamente invasor) com o avançado (francamente invasor). Entretanto, em relação aos vasos linfáticos intratumorais houve uma diferença entre os diversos grupos estudados. No AP sem transformação maligna e no CXAP precoce foram observados raros ou ausência de vasos linfáticos intratumorais, enquanto que no CXAP avançado estes vasos estavam presentes em maior número. Entretanto, quando realizada a reação de dupla marcação imunoistoquímica com os anticorpos D2-40 X Ki67, não foram observadas células endoteliais linfáticas em proliferação, sugerindo que não houve linfangiogênese durante o processo de transformação maligna do AP para carcinoma, ou seja, que tanto os vasos intratumorais quanto os periféricos eram pré-existentes e não neoformados. Além disso, quando comparada a DVL dos vasos intratumorais e periféricos na fase tardia do CXAP com o tecido glandular normal adjacente, não foi notada diferença estatisticamente significante.

Os nossos achados são semelhantes àqueles observados em câncer de mama, no qual também não houve linfangiogênese. Os vasos linfáticos intratumorais foram considerados pré-existentes e, portanto, pertencentes ao

estroma do hospedeiro (Williams *et al*. 2003; Straume *et al*. 2003; Vleugel *et al*. 2004; Agarwal *et al*. 2005). Contudo, nossos achados diferem dos encontrados em carcinomas de cabeça e pescoço e melanoma, no qual foram detectados linfáticos intratumorais neoformados (Beasley *et al*. 2002; Dadras *et al.* 2003; Straume *et al*. 2003; Kyzas *et al*. 2005). Vleugel *et al* (2004) sugerem que características específicas do tecido no qual se origina o tumor possam ser responsáveis por estas diferenças, ou seja, pela presença ou ausência de linfáticos intratumorais.

O mecanismo de como ocorre a formação de novos vasos linfáticos e o seu papel na progressão tumoral ainda não está totalmente esclarecido. Em câncer de mama, a ausência de linfangiogênese não pode ser explicada apenas pela falta de proteínas associadas a fatores de crescimento linfático (VEGF-C e VEGF-D) ou de seus receptores (VEGFR-3), de tal modo que Vleugel *et al* (2004) sugeriram que outros fatores, por exemplo, que contribuem para a inibição do crescimento de vasos linfáticos devam ser investigados com mais detalhe.

Na literatura, raros estudos têm sido realizados a respeito da DVL e linfangiogênese em lesões pré-neoplásicas ou na fase inicial da progressão tumoral. Giorgarize *et al* (2004) estudaram a DVL comparando nevo melanocítico com melanoma *in situ*, não observando nenhuma diferença entre as lesões. Resultados semelhantes foram relatados na mama, quando a proliferação intraductal pré-neoplásica foi comparada com a pré-invasora (Vleugel *et al*. 2004; Agarwal *et al*. 2005). Em nosso estudo, também não foi observada nenhuma diferença entre o AP sem transformação maligna e o CXAP intracapsular (*in situ*) e minimamente invasor.

Quando comparada a DVL intratumoral na fase precoce com aquela na fase avançada do CXAP, nossos resultados mostraram diferença estatística, sendo que ocorreu aumento da DVL na fase avançada. Uma possível explicação é que o estroma do AP, por ser produzido pelas próprias células do tumor, não contém vasos linfáticos. Na fase precoce, o CXAP está contido dentro do adenoma e, portanto, nestes tumores, os linfáticos são raros ou inexistentes. Entretanto, na fase avançada, como o carcinoma invade os tecidos normais do

hospedeiro localizados além da cápsula do adenoma, é possível observar vasos linfáticos pré-existentes, o que explicaria, então, os vasos intratumorais no CXAP francamente invasor, mas não no CXAP intracapsular ou minimamente invasor. Esta hipótese está de acordo com os achados de Vleugel *et al.* (2004) em carcinoma ductal *in situ* da mama. Estes autores mostraram que há uma correlação inversa entre densidade vascular linfática e o tamanho da lesão, devido à substituição progressiva do estroma do hospedeiro, que contém os vasos linfáticos, pelo epitélio tumoral.

Em vários tipos de câncer tem sido relatada a presença de vasos linfáticos peritumorais dilatados contendo êmbolos neoplásicos, sugerindo que estes vasos, pré-formados, sejam suficientes para causar metástase (Williams *et al*. 2003; Agarwal *et al*. 2005). Em nossos resultados, no CXAP na fase avançada foi observada a presença de êmbolos neoplásicos tanto nos vasos peritumorais como nos intratumorais. Estes achados sugerem que na fase avançada da progressão tumoral, os linfáticos intratumorais podem representar um caminho adicional para as células neoplásicas se disseminarem. Além disso, a ausência de vasos intratumorais na fase inicial do CXAP provavelmente contribui para o baixo risco de metástase nestes tumores.

Além da presença de vasos linfáticos peritumorais e intratumorais, outros fatores relacionados à biologia tumoral podem contribuir para o aumento do risco de metástase e, dentre estes, a composição histológica do tumor (Agarwal *et al.* 2005; Ji 2005). Em nosso estudo, êmbolos neoplásicos linfáticos e metástase cervical foram observados somente nos carcinoma sem diferenciação mioepitelial, sugerindo que a composição das células tumorais pode influenciar no prognóstico do tumor. No CXAP com diferenciação mioepitelial, as propriedades inerentes às células mioepiteliais, entre elas a secreção em baixo nível de proteínas degradadoras de matriz e o alto nível de vários inibidores de proteínas anti-invasoras, parecem contribuir para a baixa tendência de invasão vascular linfática e, conseqüentemente, de metástase (Sternlich & Barsky 1997).

O D2-40 é um anticorpo novo no mercado, havendo ainda poucos estudos sobre a sua expressão nos tecidos; portanto, paralelamente ao estudo da linfangiogênese, nós também analisamos a expressão do anticorpo D2-40 nas células neoplásicas. O D2-40 é um anticorpo que reconhece um epítopo na molécula de podoplanina, que está presente em células não endoteliais, dentre elas, em células mioepiteliais de glândulas salivares normais (Schacht *et al*. 2005). Em nossos resultados, D2-40 foi imunoexpresso em células mioepiteliais benignas e malignas, sugerindo que este anticorpo pode ser utilizado como um marcador adicional para se identificar células mioepiteliais em tumores de glândulas salivares.

Schacht *et al.* (2005); Dumoff *et al.* (2005) e Ordóñez (2006) relataram expressão de D2-40 em células escamosas carcinomatosas da pele, colo uterino e pulmão. D2-40 foi criado contra um antígeno oncofetal M2A (uma sialoglicoproteína) que é expresso em testículo fetal e em neoplasias de células germinativas (Marks *et al.* 1990). Além disso, tem sido sugerido que este antígeno pode ter papel importante na progressão tumoral, por aumentar a invasão linfática e, conseqüentemente, metástase. Entretanto, em nossa série, em apenas dois casos (um CXAP intracapsular e um francamente invasor) foi observada imunoexpressão de D2-40 em células carcinomatosas e, em nenhum havia metástase linfonodal no momento da cirurgia. Curiosamente, em um dos casos (francamente invasor) as células carcinomatosas apresentavam diferenciação escamosa e o D2-40 estava expresso predominantemente na camada basal tumoral. Baseados nestes resultados e no fato da podoplanina estar expressa em queratinócitos basais normais e em carcinomas de células escamosas pouco diferenciado (Schacht *et al*. 2005), especulamos se a expressão de D2-40 poderia estar relacionada com a diferenciação escamosa celular.

6- CONCLUSÃO GERAL

A) Quanto à vascularização sanguínea

- O anticorpo CD 105 permite medir mais apuradamente a atividade angiogênica tumoral do que o CD 34.

- Com o anticorpo CD 105 foi detectada atividade angiogênica em CXAP, sendo que esta apresenta forte correlação com progressão tumoral.

- Embora CXAP francamente invasores apresentem área vascular total maior do que aquela do CXAP precoce, a diferença não é estatisticamente significante.

- CXAP com e sem diferenciação mioepitelial apresentam padrões distintos quanto ao grau de atividade angiogênica e de área vascular. Atividade angiogênica baixa e valor de área vascular alto são mais característicos de CXAP com diferenciação mioepitelial.

B) Quanto à vascularização linfática

- Linfáticos intratumorais são raros, se existentes, em adenomas pleomórficos e CXAP não invasores (intracapsulares) e minimamente invasores.

- Em carcinomas francamente invasores, a rede linfática é composta predominantemente por vasos pré-existentes, visto que não são detectadas células proliferantes (Ki 67 positivas) em linfáticos.

- Os vasos linfáticos tanto intratumorais como peritumorais são condutores de êmbolos neoplásicos. Entretanto, em CXAP com diferenciação mioepitelial, provavelmente devido às propriedades inerentes das células mioepiteliais, há baixa tendência para invasão destas estruturas e, conseqüentemente, de metástases.

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*Segundo as normas de Vancouver.