Ana Flávia de Mattos Costa

"Identification of genetic alterations in adenoid cystic carcinoma with high-grade transformation"

"Identificação das alterações genéticas do carcinoma adenóide cístico com transformação para alto grau"

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Ana Flávia de Mattos Costa

"Identification of genetic alterations in adenoid cystic carcinoma with high-grade transformation"

Orientadora: Profa. Dra. Albina Messias de Almeida Milani Altemani
Coorientador: Dr. Marinus Antonius Jacobus Hermsen

"Identificação das alterações genéticas do carcinoma adenóide cístico com transformação para alto grau"

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Epígrafe

"Podemos escolher recuar em direção à segurança ou avançar em direção ao crescimento. A opção pelo crescimento tem que ser feita repetidas vezes, e o medo tem que ser superado a cada momento."

Resumo

O carcinoma adenóide cístico pode raramente sofrer desdiferenciação, um fenômeno também referido como transformação para alto grau. Contudo, alguns casos de carcinoma adenóide adenóide cístico foram descritos mostrando transformação para adenocarcinomas que não são pobremente diferenciados, indicando que a transformação para alto grau pode não refletir necessariamente um estágio mais avançado da progressão tumoral, mas sim uma transformação em uma outra forma histológica, que pode abranger um amplo espectro de carcinomas em termos de agressividade. Inicialmente, investigamos a expressão das proteínas reguladas pela hipóxia (HIF-1α, VEGF, GLUT-1 e CD105), dado que a hipóxia contribui para a agressividade tumoral e, pode, também, promover um fenótipo desdiferenciado em certos tipos de câncer. Em seguida, analisamos um importante ponto de interesse em relação ao carcinoma adenóide cístico com transformação para alto grau, o seu pior prognóstico, que é sugerido ser comparável ou até pior do que o subtipo sólido. Para isso, comparamos as alterações genéticas do carcinoma adenóide cístico com transformação para alto grau com o subtipo sólido e, com os aspectos clínicos e patológicos de ambos os tumores. Além disso, em outro trabalho, usamos a hibridização genômica comparativa em microarranjo para comparamos o perfil genético de ambos os componentes histológicos do carcinoma adenóide cístico com transformação para alto grau. Atenção especial foi dada à expressão da proteína e à translocação cromossomal do gene MYB, que está sendo considerado o maior evento precoce e oncogênico do carcinoma adenóide cístico clássico. Nossos resultados mostraram que o carcinoma adenóide cístico com transformação para alto grau pode apresentar uma complexidade genética similar ao subtipo sólido e, também, que o processo de transformação não é sempre acompanhado pelo acúmulo de alterações genéticas, o que indica uma progressão paralela de ambos os componentes do carcinoma adenóide cístico transformado. Em contrapartida à expressão da proteína MYB, a translocação entre MYB/NFIB não é necessariamente um evento precoce e, bem como a hipóxia, não são fundamentais para o desenvolvimento destes tumores. Finalmente, o estudo advindo do carcinoma adenóide cístico com transformação para alto grau, também nos permitiu fazer uma revisão sobre o assunto. Neste outro estudo fizemos um panorama sobre os recentes conceitos na classificação histopatológica dos tumores de glândula salivar com desdiferenciação/transformação para alto grau descritos na literatura. Destaque também foi dado aos achados imuno-histoquímicos e genéticos que podem ajudar no diagnóstico de cada um destes tumores.

Abstract

Adenoid cystic carcinomas can occasionally undergo dedifferentiation, a phenomenon also referred to as high-grade transformation. However, cases of adenoid cystic carcinomas have been described showing transformation to adenocarcinomas that are not poorly differentiated, indicating that high-grade transformation may not necessarily reflect a more advanced stage of tumor progression, but rather a transformation to another histological form, which may encompass a wide spectrum of carcinomas in terms of aggressiveness. The aim of this study was to gain more insight in the biology of this pathological phenomenon. Firstly, we investigated expression of proteins regulated by hypoxia (HIF-1α, VEGF, GLUT-1 and CD105), given that hypoxia contributes to aggressive tumor behavior and can also promote a dedifferentiated phenotype in certain types of cancer. Hereafter, we analyzed an important point of interest of adenoid cystic carcinoma with high-grade transformation that is its proposed poor prognosis to be comparable to or even worse than solid subtype. Therefore, we compared the genetic changes of transformed and solid subtype adenoid cystic carcinomas and correlated the results to their clinico-pathological features. In addition, in another work, we used microarray comparative genomic hybridization to compare the genetic profiles of both histological components of adenoid cystic carcinomas with high-grade transformation. Special attention was given to chromosomal translocation and protein expression of MYB, recently being considered to be an early and major oncogenic event in adenoid cystic carcinomas. Our data showed that transformed adenoid cystic carcinoma with high-grade transformation may present a genetic complexity similar to the solid subtype and, also that the process of high-grade transformation is not always be accompanied by an accumulation of genetic alterations; which indicate a parallel progression of the two histological components of transformed adenoid cystic carcinoma. In contrast to MYB protein expression, MYB/NFIB translocation is not necessarily an early event and, as hypoxia, not fundamental for the development of these tumors. Finally, the study that comes from of adenoid cystic carcinoma with high-grade transformation also allowed us to do a review about it. In this study we made an overview of the latest concepts in histopathological classification of salivary gland tumors with dedifferentiation / high-grade transformation described in the literature. Highlight was also given to immunohistochemical and genetic findings that can help in the diagnosis of each of these tumors.

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1. Introdução

O carcinoma adenóide cístico com transformação para alto grau foi primeiramente descrito como "desdiferenciado" por Cheuk e colaboradores em 1999 (Cheuk et al., 1999). Posteriormente, a desdiferenciação no carcinoma adenóide cístico foi reconhecida por diversos autores (Moles et al., 1999; Chau et al., 2001; Nagao et al., 2003; Ide et al., 2003; Brackrock et al., 2005; Hayashido et al., 2005; Sato et al., 2006). Em 2007, Seethala e colaboradores sugeriram o termo "transformação para alto grau", que desde então, tem sido adotado por muitos autores, inclusive para outros tumores de glândula salivar, tais como o carcinoma de células acinares e o carcinoma epitelial-mioepitelial (Seethala et al., 2007; Skálová et al., 2009; Roy et al., 2010; Costa AF et al., 2011). Este termo foi considerado mais apropriado pelo fato de que a linha original de diferenciação epitelial ainda pode ser reconhecida dentro do componente de alto grau (Seethala et al., 2007). Seja qual for a terminologia correta, é evidente que o carcinoma adenóide cístico com transformação para alto grau é uma entidade clínica e patológica distinta do carcinoma adenóide cístico clássico, porém esta controvérsia também demonstra a incerteza sobre este fenômeno.

1.1 Carcinoma adenóide cístico

O carcinoma adenóide cístico (CAC) é um tumor maligno de origem epitelial, descrito pela primeira vez por Billroth em 1856 (Billroth, 1856), que o denominou inicialmente de "cilindroma" por sua aparência histológica cribriforme, formada por células tumorais com falso lúmen cilíndrico. Representam aproximadamente 10% de todas as neoplasias salivares epiteliais e 30% dos tumores das glândulas salivares menores, sendo o carcinoma mais frequente naquelas localizadas no palato. O restante, ou seja, 70% estão divididos entre as glândulas parótida e submandibular. Aparentemente, não existe predileção por sexo, exceto por uma alta incidência em mulheres, quando ocorre na glândula submandibular. Todas as idades são atingidas, porém pacientes idosos são os mais acometidos. A taxa de sobrevida em cinco anos é de aproximadamente 35%, entretanto, 80% a 90% dos pacientes morrem em 10 ou 15 anos (Kokemueller et al., 2004; El-Naggar e Huvos, 2005).

O CAC é constituído por dois tipos de células, as células ductais (luminais) e as mioepiteliais (abluminais), as quais são separadas por material extracelular constituído por lâmina basal, fibras colágenas, elastina e glicosaminoglicanas (Jaeger et al., 2008). Este tumor apresenta três padrões de crescimento: cribriforme, tubular e sólido (El-Naggar e Huvos, 2005). O subtipo histológico tubular é caracterizado por condutos bem formados, com luz central, delimitados por uma dupla população celular. O subtipo mais frequente, cribriforme, se caracteriza por ninhos de células com espaços microcísticos cilindromatosos contendo material hialino ou mucóide basófilo. Quando o CAC perde a característica tubular ou cribriforme, é denominado de sólido, sendo formado por ninhos de células basalóides, com moderado pleomorfismo, mitoses e, por vezes, apresentando necrose. Este subtipo é o menos frequente (Figura 1).

Acredita-se que exista uma correlação entre o subtipo histológico e o prognóstico. O padrão sólido é considerado o de pior prognóstico global, principalmente em relação ao desenvolvimento de metástases à distância e elevada taxa de mortalidade (da Cruz Perez et al., 2006; Bradley, 2004). Szanto e, estabeleceram a classificação em graus: I - quando os padrões de crescimento cribriforme e/ou tubular são predominantes, II - quando há menos de 30% de padrão sólido (grau II) e III - mais que 30% de padrão sólido, figura 1 (Szanto et al., 1984).

O CAC é caracterizado por um curso clínico indolente, porém prolongado, e normalmente com um resultado fatal devido as recidivas locais ou metástases (van der Wal et al., 2002). Muitos outros parâmetros foram correlacionados com fatores prognósticos em pacientes com CAC, incluindo o estado da margem cirúrgica, estágio clínico, localização e invasão perineural (da Cruz Perez et al., 2006). As metástases ganglionares não são frequentes neste tumor e variam de 5 a 25% (El-Naggar e Huvos, 2005), enquanto as metástases à distância ocorrem em 33 a 40% dos casos e podem aparecer décadas depois do tratamento do tumor primário, principalmente no pulmão e ossos (Fordice et al., 1999).

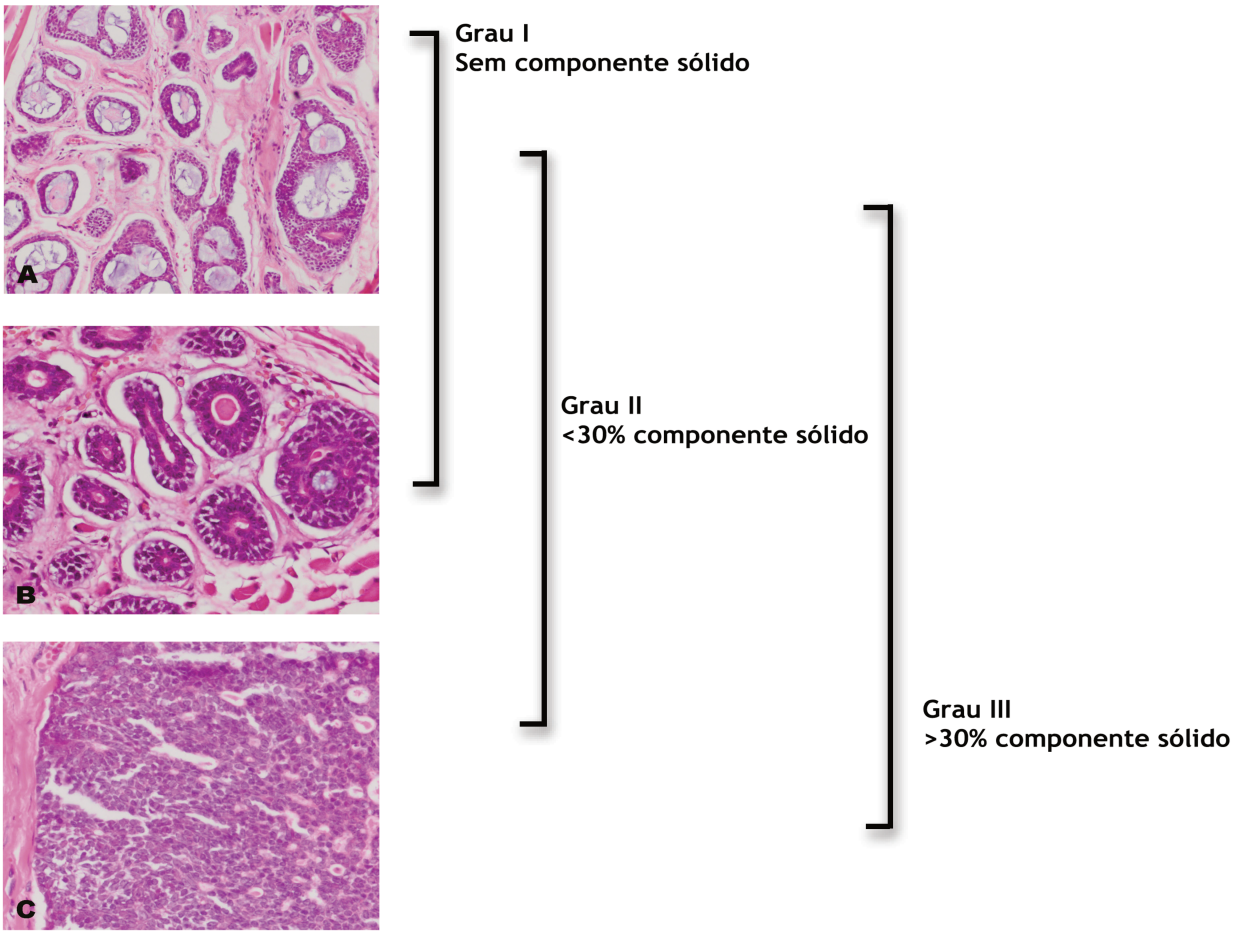

Figura 1. Padrões e graus histológicos do CAC. A, cribriforme; B, tubular; C, sólido. Grau I - quando os padrões de crescimento cribriforme e/ou tubular são predominantes; Grau II - quando há menos de 30% de padrão sólido e Grau III - mais que 30% de padrão sólido.

1.2 Carcinoma adenóide cístico com transformação para alto grau

O CAC pode raramente sofrer transformação para alto grau (CAC-TAG). Seethala e colaboradores, foram os primeiros a estabelecerem critérios morfológicos para diferenciar o CAC clássico daquele com transformação (Seethala et al., 2007). Foram propostos **critérios maiores** (tamanho nuclear no mínimo 2 a 3 vezes maiores do que o CAC graus I e II, ninhos celulares sólidos e confluentes ocupando um campo de maior aumento, desmoplasia fibrocelular, áreas escamóides, micropapilas e no mínimo, a perda focal da camada mioepitelial) e **menores** (cromatina vesicular, membrana nuclear espessada e irregular, nucléolo proeminente e central, citoplasma escasso a moderado, comedonecrose, microcalcificações, mais de 10 mitoses por campo de maior aumento e mais de 50% das células carcinomatosas Ki-67 positivas). Para o diagnóstico de CAC-TAG é necessário no mínimo três critérios maiores, sendo que as áreas escamóides, as micropapilas e a perda da diferenciação mioepitelial são considerados achados morfológicos exclusivos da área transformada. Na literatura não é sugerida uma porcentagem mínima de componente transformado para que o tumor seja diagnosticado como CAC-TAG (Seethala et al., 2007). Figura 2.

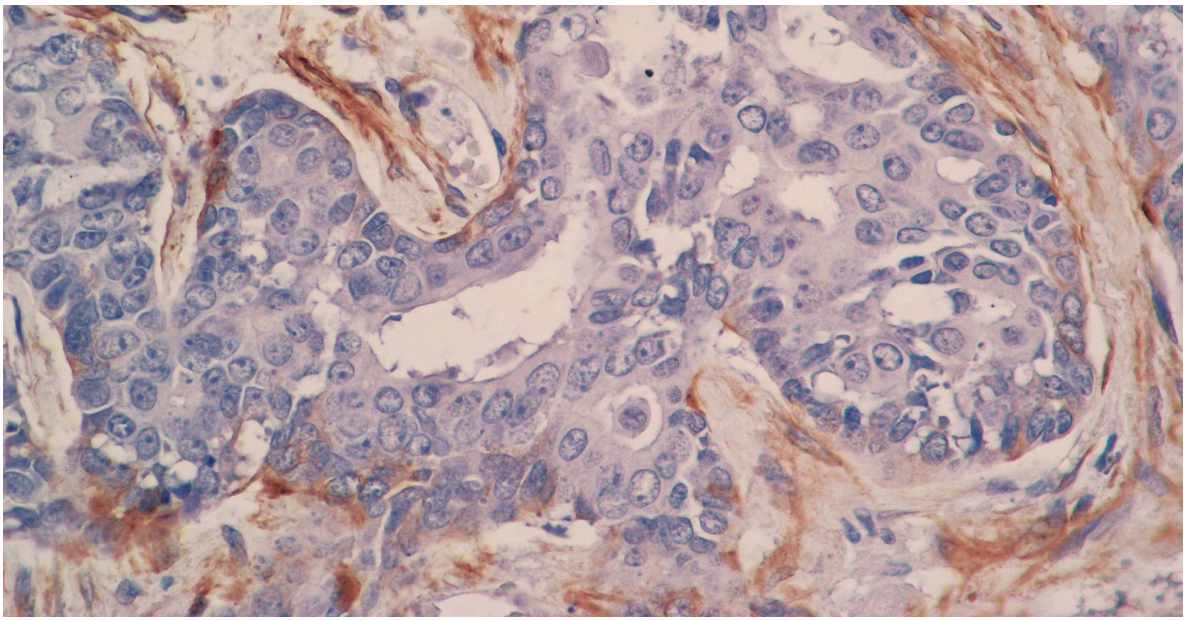

Figura 2. Imuno-histoquímica do CAC-TAG. Marcação do anticorpo actina de músculo liso na área transformada do CAC mostrando a perda focal da diferenciação mioepitelial.

A transformação para alto grau pode ocorrer no tumor primário ou na recidiva de um CAC clássico e, mais comumente, afeta as glândulas sinonasais mucoserosas, glândulas submandibulares e palato (salivares menores). Existe uma leve predileção para os homens (1,4: 1), são geralmente tumores agressivos, com envolvimento extraglandular e de tecidos ósseos. Diferentemente do CAC clássico, o envolvimento de linfonodos regionais é de quase 60%, sugerindo que o esvaziamento cervical poderia ser uma conduta terapêutica para este tumor (El-Naggar e Huvos, 2005; Seethala et al., 2007).

1.3 Alterações genéticas e moleculares no CAC clássico

O CAC clássico não parece precisar de um grande número de alterações moleculares para seu desenvolvimento, cerca de 22 mutações somáticas foram encontradas por tumor (Ho et al., 2013). Esta taxa é bastante baixa quando comparada a outros tumores sólidos, como por exemplo ao carcinoma de células escamosas (Agrawal et al., 2011). A baixa prevalência de mutações em oncogenes e genes supressores de tumor pode indicar que a condução do processo neoplásico no CAC seja direcionada primeiramente por alterações em genes reguladores da transcrição e, agravado por alterações na estrutura da cromatina (Frierson e Moskaluk, 2013). Stephens e colaboradores identificaram através do sequenciamento do exoma do CAC, que oncogenes e genes supressores de tumor mais comumente alterados como: *TP53, RB1, Her2/Neu, BRAF, EGFR, KRAS, PTEN* e *KIT* foram praticamente ausentes e as alterações em *PIK3CA* e *CDKN2A* ocorreram em apenas um caso (Stephens et al., 2013). Uma mutação sem sentido (nonsense) foi encontrada no éxon 8 de um CAC (Gomes et al., 2012), corroborando com a ideia de que mutações em TP53 não parece ser um evento frequente no CAC. Ho e colaboradores, também através do sequenciamento do exoma destes tumores, encontraram mutações em genes que codificam reguladores do estado da cromatina, como *SMARCA2, CREBBP* e *KDM6A*, sugerindo que há regulação epigenética na oncogênese do CAC (Ho et al., 2013).

Persson e colaboradores demonstraram que a fusão do fator de transcrição *MYB* com o gene *NFIB* gerada pela translocação t(6;9) (q22-23;p23-24), provavelmente seja o maior evento oncogênico no CAC. O resultado é o aumento na expressão de *MYB*, que leva à alteração na expressão de genes alvos envolvidos no controle do ciclo celular, apoptose, angiogênese e adesão celular (Persson et al., 2009; Mitani et al., 2010; Brill et al., 2011). No entanto, a expressão de

MYB também pode ser desregulada por outro mecanismo não relacionado a fusão de *MYB/NFIB* (Persson et al., 2012). Tanto que a sensibilidade e a especificidade de sua expressão é de 82 e 86%, respectivamente (Brill et al., 2011), mas a fusão entre *MYB/NFIB* é observada somente em 57% dos casos (Ho et al., 2013). A real participação deste evento na carcinogênese do CAC ainda precisa ser esclarecida.

Além das alterações em *MYB*, as alterações genéticas mais frequentes parecem ocorrer também no cromossomo 6, e estas parecem estar mais correlacionadas com um curso clínico agressivo (Nordkwist et al., 1994; Queimado et al., 1998; El-Rifai et al., 2001 Stallmach et al., 2002; Kishi et al., 2003; Rutherford, 2006). Outros exemplos de alterações no CAC é o ganho nos cromossomos 7, 8 e 19 (Mark et al., 1996; Martins et al., 1996; Nordkwist et al., 1994; El-Rifai et al., 2001; Vékony et al., 2007). Em relação ao padrão de crescimento, o padrão sólido normalmente apresenta mais deleções quando comparado com o padrão tubular e/ou cribriforme (El-Rifai et al., 2001; Vékony et al., 2007). Outros estudos moleculares no CAC clássico estão descritos na tabela 1.

Apesar da alta expressão do receptor para o fator de crescimento epidérmico (EGFR) e de c-Kit no CAC, o tratamento contra estes alvos terapêuticos não mostraram resposta clínica satisfatória (Clauditz et al., 2012; Mino et al., 2003; Laurie et al., 2011). Entretanto, foram identificadas mutações recorrentes na via *FGF-IGF-PI3K*, que podem representar novos caminhos para uma terapia alvo mais eficiente no CAC (Ho et al., 2013).

Tabela 1. Revisão da literatura das alterações genéticas e epigenéticas no CAC clássico.

Gene candidato	Banda cromossomal	Metilação	LOH	Duplicação	Deleção	Referência
CDKN2C (p18)	1p32	x			x	Rao et al., 2008; Daa et al., 2008
D3S1029	3p21.2-21.3		x			Yamamoto et al., 1996
MLH1	3p22.3	x				Zhang et al., 2007
RASSF1A	3p21.3	x				Li et al., 2005;Lee et al., 2008
CTNNB1	03p21.3	x		x		Daa et al., 2004; Kasamatsu et al., 2005
PIK3CA	3q26			x		Serqueiros-Santiago et al., 2009
FRG1	4q35			x		Kasamatsu et al., 2005
APC	5q22			x		Daa et al., 2004
CDKN1A (p21)	6p21	x				Daa et al., 2008
PLAGL1	6q23				x	El-Rifai et al., 2001, Rao et al., 2008
ESR1	6q25.1		x			Rutherford et al., 2006
TSP-2	6q25-27		x			Kishi et al.,2003
PARK2	6q26		x			Stallmach et al., 2002
ERBB1	7p11.2			x		Serqueiros-Santiago et al., 2009
CDK6	7q21			x		Kasamatsu et al., 2005
FGFRI	8p11			x		Rao et al., 2008
D8S602	8p22		x			Yamamoto et al., 1996
CDKN2A (p14/p16)	9p21	x	x			Cerilli et al., 1999; Kishi et al., 2005; Maruya et al., 2003
CDKN2B (p15)	9p21	x				Daa et al., 2008
Interf. A	9p21-22		x			Yamamoto et al., 1996; Kishi et al., 2005
PTEN	10q23.3	x				Kishi et al., 2005
CD44	11p12			x		Kasamatsu et al., 2005
CCND1	11q13			x		Serqueiros-Santiago et al., 2009; Frierson et al., 2002; Kasamatsu et al., 2005
ATM	11q23			x		Kasamatsu et al., 2005
CDKN1B (p27)	12p13.2	x		x	x	Kishi et al., 2005; Kasamatsu et al., 2005
D12S1301	12q12				x	El-Rifai et al., 2001, Rao et al., 2008; Rutherford et al., 2005
CHFR	12q24		x	x		Rutherford et al., 2005; Kasamatsu et al., 2005
RB	13q14	x	x			Yamamoto et al., 1996; Kishi et al., 2005
BRCA3	13q21				x	El-Rifai et al., 2001
TSC2	16p13.3			x		Vécony et al., 2007
CDH1	16q22	x				Maruya et al., 2004; Zhang et al., 2007
p53	17p13.1		x			Yamamoto et al., 1996
HIC1	17p13.3			x		Vécony et al., 2007
CDKN2D (p19)	19p13	x				Daa et al., 2008
KLK3	19q13.33			x		Vécony et al., 2007
TIMP3	22q12.3			x		Toida et al., 2001
PDGFB	22q13			x		Freier et al., 2005

LOH, loss of heterozygosity (perda de heterozigosidade).

1.4 Alterações genéticas e moleculares no CAC com transformação para alto grau

As alterações moleculares no CAC-TAG não são muito conhecidas, principalmente devido à raridade e aos poucos estudos dedicados a este tumor. Exemplos incluem a mutação e/ou LOH em *TP53* (Chau et al., 2001; Cheuk et al., 1999). Além disso, estudos imuno-histoquímicos mostraram alta expressão da proteína p53 e elevado índex de Ki-67 (Seethala et al., 2007). Outros marcadores imuno-histoquímicos avaliados foram c-Kit, Her2/Neu e a Ciclina D1, porém a participação destes é controversa a respeito da sua participação na transformação do CAC (Cheuk et al., 1999; Moles et al., 1999; Chau et al., 2001; Ide et al., 2003, Seethala et al., 2007). Seethala e colaboradores, através da hibridização genômica comparativa em microarranjo, observaram uma maior quantidade de deleções na área convencional do tumor, enquanto que na área transformada foram encontrados mais ganhos, principalmente em 8q24, onde está localizado o gene *MYC* (Seethala et al., 2011). Portanto, os mecanismos genéticos e moleculares responsáveis pela transformação para alto grau no CAC ainda precisam ser elucidados.

1.5 Outros fatores relacionados à desdiferenciação/transformação para alto grau

Nosso conhecimento sobre a biologia tumoral foi amplamente beneficiado pelos enormes progressos na genética (oncogenes, genes supressores de tumores, anormalidades cromossômicas, etc.) e, na epigenética, que regula a expressão dos genes. Porém, outros fatores também são importantes na carcinogênese, principalmente pelo potencial terapêutico, como o metabolismo energético e as células-tronco cancerosas (Hanahan e Weinberg, 2011).

A hipóxia tem surgido como um dos principais fatores relacionados à agressividade tumoral, pois contribui para a diversidade celular intratumoral (Harris, 2002). É conhecido que a hipóxia pode promover um fenótipo desdiferenciado em certos tipos de câncer, como no carcinoma ductal de mama e nas células de neuroblastoma (Jögi et al., 2003 e Axelson et al., 2005). Nestes tumores, as células tumorais se ajustam ao ambiente hipóxico regulando negativamente seus padrões de expressão gênica diferenciados e desenvolvendo características de células tronco.

Uma das formas pelo qual as células respondem à hipóxia é através do fator indutor de hipóxia 1 alfa (HIF-1α). O HIF-1 consiste das subunidades alfa e beta e sua estabilidade depende principalmente da subunidade alfa (Semenza, 2000). Sob normóxia, o HIF-1α é degradado, enquanto que durante a hipóxia, a degradação é inibida levando à estabilização da proteína. Após a estabilização, HIF-1α é transportado para dentro do núcleo onde é heterodimerizado com o HIF-1β, para transativar a expressão de numerosos genes de resposta à hipóxia, incluindo um fator pró-angiogênico, o fator de crescimento endotelial vascular (VEGF) e os transportadores de glicose (Harris, 2002; Gatenby e Gillies, 2004).

Os transportadores de glicose (GLUT) participam da captação de glicose pelas células e, entre as várias isoformas de GLUT, o GLUT-1 é expresso ubiquamente. Sob hipóxia, HIF-1 regula positivamente a expressão e a função de GLUT-1 para manter a produção celular de energia através da via glicolítica (Behrooz et al., 1999; Chen et al., 2001). No entanto, nas células cancerosas, o metabolismo da glicose também é regulado positivamente em condições de normóxia (Gatenby e Gillies, 2004). Por esta razão, a expressão de GLUT-1 nas células tumorais tem sido considerada um marcador de aumento na absorção de glicose e, consequente metabolismo glicolítico, bem como de hipóxia (Gillies e Gatenby, 2007). O padrão de expressão de proteínas nos ninhos tumorais está sendo considerado como um reflexo do mecanismo de indução do GLUT-1. Nos ninhos tumorais, a expressão de GLUT-1 pode ser observada no centro (denominada de padrão antiestromal) ou nas camadas mais periféricas (proestromal) (Haber et al., 2002; Kalir et al., 2002; Bursten et al., 2006). O padrão antiestromal tem sido explicado pela indução de uma via sensível à hipóxia (Kalir et al., 2002; Bursten et al., 2006), e sua frequente associação com zonas necrosadas suporta esta hipótese. Inversamente, a expressão de GLUT-1 nas células próximas ao estroma e, portanto, próximas às fontes de oxigênio capilar (expressão de GLUT-1 proestromal) acredita-se que seja induzida por outros fatores não hipóxicos (Bursten et al., 2006).

Dentro da grande família de reguladores angiogênicos, VEGF é a molécula mais importante no controle do crescimento dos vasos sanguíneos e, também, o mais bem estudado fator de crescimento ativado pelo HIF-1α. O VEGF participa na angiogênese fisiológica e patológica, inclusive naquela associada ao crescimento tumoral (Ferrara et al., 2003). O VEGF estimula a

proliferação de células endoteliais, vasculogênese e a remodelação angiogênica (Adams e Alitalo, 2007). Nos tumores, as células cancerosas são a maior fonte de VEGF (Harris, 2002).

A hipóxia não somente regula positivamente o principal fator angiogênico, o VEGF, mas também a expressão da endoglina (CD105) nas células endoteliais (Li et al., 2003 e Duff et al., 2003). A regulação positiva deste componente acessório do receptor para o fator de crescimento transformante beta (TGF-β) parece exercer um efeito antiapoptótico, melhorando a sobrevivência das células endoteliais sob estresse hipóxico (Li et al., 2003). O CD105 é altamente expresso em células endoteliais ativadas/mitóticas e foi descrito como um marcador poderoso da neoangiogênese tumoral (Li et al., 2001).

Cheuk e colaboradores sugeriram que a radiação pode ser um fator contribuinte para que a desdiferenciação ocorra no CAC (Cheuk et al., 1999). Eles descreveram três casos com desdiferenciação e, dois deles haviam sido submetidos à radioterapia. Somente mais um caso de CAC foi descrito na literatura sofrendo desdiferenciação após radiação (Brackrock et al., 2005). Porém, a desdiferenciação pós-radioterapia já foi descrita em um carcinoma próstata e, em todos os casos isso significou um pior prognóstico para os pacientes (Wheeler et al., 1993).

2. Objetivos

2.1 Objetivo geral

O presente trabalho teve como objetivo geral identificar e comparar as alterações genéticas presentes na área convencional e transformada do CAC-TAG, através da hibridização genômica comparativa em microarranjo.

2.2 Objetivos específicos de cada artigo publicado

Capítulo 1. Levels and patterns of expression of hypoxia-inducible factor-1α, vascular endothelial growth factor, glucose transporter-1 and CD105 in adenoid cystic carcinomas with high-grade transformation.
Objetivo: investigar a expressão imuno-histoquímica de proteínas reguladas pela hipóxia (HIF-1α, VEGF, GLUT-1 e CD105) no CAC-TAG e no CAC convencional e correlacioná-la com aspectos clínicos e patológicos.

Capítulo 2. Genetic profile of adenoid cystic carcinomas (ACC) with high-grade transformation versus solid type.
Objetivo: comparar as alterações genéticas presentes na área transformada do CAC-TAG com o subtipo sólido do carcinoma adenóide cístico clássico, através da hibridização genômica comparativa em microarranjo. Correlacioná-las com os aspectos clínicos dos pacientes.

Capítulo 3. Analysis of *MYB* oncogene in transformed adenoid cystic carcinomas reveals distinct ways of tumor progression.
Objetivo: Comparar as alterações genéticas presentes na área convencional e transformada do CAC-TAG através da hibridização genômica comparativa em microarranjo. Analisar a expressão do gene *MYB* através da imuno-histoquímica e validar a translocação entre *MYB/NFIB* pela hibridização fluorescente in situ.

Capítulo 4. Current concepts on dedifferentiation/high-grade transformation in salivary gland tumors.
Objetivo: Revisar os recentes conceitos histopatológicos, imuno-histoquímicos e genéticos dos tumores de glândula salivar com transformação para alto grau.

Capítulo 1

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Levels and patterns of expression of HIF-1α, VEGF, GLUT-1 and CD105 in adenoid cystic carcinomas with high-grade transformation

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Abbreviations: ACC, adenoid cystic carcinoma; ACC-HGT, adenoid cystic carcinoma with high-grade transformation, AR, antigen retrieval, EC, endothelial cell, GLUT, glucose transporter; HIF-1, hypoxia-inducible factor-1; MVD, microvascular density; SD, standard deviation, VEGF, vascular endothelial growth factor.

Abstract

Aims: To compare the expression of proteins regulated by hypoxia between adenoid cystic carcinoma (ACC) with high-grade transformation (ACC-HGT) and ordinary ACC.

Methods and Results: In 8 ACC-HGT and 18 ordinary ACC, expression of HIF-1α, VEGF, GLUT-1 and microvascular density (MVD) by CD105 (a hypoxia-inducible protein expressed in angiogenic endothelial cells) was determined. Expression levels of HIF-1α and VEGF as well as CD105-MVD did not differ significantly between: a) transformed and conventional areas (TA and CA, respectively) of ACC-HGT, b) CA and ordinary ACC. HIF-1α was detected in 100% of cases and presented a diffuse staining pattern. No significant association was found between levels of HIF-1α expression and tumor size, metastasis and recurrence. GLUT-1 showed a prostromal staining pattern and was observed exclusively in TA (3/6 cases) and in only 3 out of 14 ACC.

Conclusions: Both the absence of significant alterations in levels of expression of HIF-1α, VEGF and CD105 and the patterns of expression of HIF-1α and GLUT-1 suggest that hypoxia may not play a key role in the process of high-grade transformation of ACC. Although HIF-1α expression is a common finding in ACC, it cannot be used as a marker of tumor aggressiveness.

Introduction

Adenoid cystic carcinoma (ACC) is among the most frequent malignant tumours of the salivary glands. It is characterized by prolonged clinical course, with frequent local recurrences, late onset of metastases, and fatal outcome.[1] High-grade transformation is an uncommon phenomenon in salivary carcinomas and has been associated with increased tumour

aggressiveness.[2-8] In ACC with high-grade transformation (ACC-HGT), the clinical course tends to be accelerated and with a high propensity for lymph node metastases. [4, 9]

In cancer biology it has been proposed that hypoxia turns tumours more aggressive by altering the expression of specific genes and is an important contributor to cell diversity within the lesion.[10] Hypoxia has been reported to promote a dedifferentiated phenotype in certain types of cancer such as ductal breast carcinoma in situ and in neuroblastoma cells. [11-12] In these tumours, cancer cells adjust to the hypoxic environment by down-regulating their differentiated gene expression patterns and developing stem cell-like characteristics.

One way that cells respond to hypoxia is through hypoxia-inducible factor-1 (HIF-1). HIF-1 consists of α and β subunits and its stability mostly depends on the α subunit. [13] Under normoxia, HIF-1α is degraded whereas during hypoxia this process is inhibited leading to protein stabilization. Stabilized HIF-1α is transported into the nucleus where it heterodimerizes with HIF-1β to transactivate the expression of numerous hypoxia-response genes, including the pro-angiogenic vascular endothelial growth factor (VEGF) and glucose transporters.[10, 14]

The glucose transporters (GLUT) mediate cellular glucose uptake and among the several isoforms, GLUT-1 is ubiquitously expressed. Under hypoxia, HIF-1 up-regulates expression and function of GLUT-1 to maintain cellular production of energy through the glycolytic pathway. [15-16] However, in cancer cells glucose metabolism is also upregulated in normoxic conditions.[14] For this reason, in tumour cells GLUT-1 expression has been considered a marker of increased glucose uptake and glycolytic metabolism as well as of hypoxia. [17] The pattern of protein expression in tumour nests has been considered to reflect the mechanisms of GLUT-1 induction. In the tumour nests, GLUT-1 staining can be observed in the center (called antistromal pattern) or at the most peripheral layers (prostromal). [18-20] The former pattern has been explained by induction of a hypoxia-sensing pathway [19-20], and its frequent association with necrotic zones has lent support to this hypothesis. On the other hand, GLUT-1 expression in cells closest to stroma and, thus, to stromal capillary oxygen sources (prostromal GLUT-1 staining) is believed to be induced by factors other than hypoxia.[20]

In the large family of angiogenic regulators, VEGF is the most important molecule that controls blood vessel growth and the best studied HIF-1α-activated growth factor. VEGF participates in physiological and pathological angiogenesis, including that associated with tumour

growth.[21] VEGF stimulates endothelial cell (EC) proliferation, vasculogenesis and angiogenic remodeling. [22] In tumours, cancer cells are the major source of VEGF.[10]

Hypoxia upregulates not only the major angiogenic factors, such as VEGF, but also the endothelial expression of CD105 (endoglin). [23-24] This accessory component of the transforming growth factor β (TGF-β), when upregulated appears to exert an antiapoptotic effect, enhancing EC survival under hypoxic stress[23]. CD105 is highly expressed in activated/ mitotic EC and has also been reported as a powerful marker of tumour neoangiogenesis.[25]

Given that hypoxia contributes to aggressive tumour behavior and can also promote a dedifferentiated phenotype in certain types of cancer, we investigated expression of proteins regulated by hypoxia (HIF-1α, VEGF, GLUT-1 and CD105) in a series of ACC-HGT and ordinary ACC. The findings were correlated to clinical-pathological characteristics and follow up data of the patients.

Material and Methods

The present study was approved by the Committee of Ethics of the University of Campinas, Brazil and was performed in 26 ACC samples (7 ACC-HGT and 18 ordinary tumours) which were retrieved from the files of the Department of Pathology of the University of Campinas. One ACC-HGT case was retrieved of the Department of Pathology of the Hospital Universitario Central de Asturias, Spain. Hematoxylin–eosin-stained slides from each tissue block were reviewed to confirm the pathological diagnosis and to select a representative section for immunohistochemical study. Clinical information was obtained from the patients' medical records. The transformed areas were identified according to the recommendations of Seethala et al (2007)[4], and in all cases at least three of the major criteria were present: proliferation of tumour cells with at least a focal loss of myoepithelial cells surrounding tumour nests, nuclear size at least 2-3 times the size of ordinary ACC nuclei, thickened irregular nuclear membranes and prominent nucleoli in a majority of cells[4] (Figure 1).

Immunohistochemistry

The antibodies used in this study were HIF-1α, VEGF-A, GLUT-1 and CD105 (Table 1). For immunohistochemical staining, 5 μm sections from each para*ffi*n block were depara*ffi*nized, hydrated and endogenous peroxidase activity was quenched by immersion of the slides in 3%

hydrogen peroxide. The antigen retrieval (AR) was achieved by boiling in a steamer immersed in citrate buffer (pH 6.0), except for HIF-1α and CD105. AR was performed using TrisEDTA (pH 9.0) for HIF-1α and 0.4% pepsin for 30 min for CD105. Only the sections for CD105 were incubated at 37°C with protein block serum free (code X0909, Dako, SA, Denmark) for 30 min. Subsequently, for all antibodies, the sections were incubated overnight at 4°C with the primary antibody and afterwards with the EnVision peroxidase system, dual link (K4061, DAKO, Carpenteria, CA, USA) for 1 h at 37°C. After washing, sections were stained for 5 min at 37°C with 3.3'- diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin. Appropriate positive and negative controls were used throughout.

Evaluation of staining

Microvessel density (MVD) by CD105 and HIF-1α positive cell counting

Three hotspot areas were chosen in each tumour stained by CD105 and HIF-1α for counting of vessels and HIF-1α positive cells, respectively. However, the chosen areas were not necessarily the same for the two markers (CD105 and HIF-1α). MVD was the mean number of vessels evaluated in three 40x fields, corresponding to 0.456 mm^2. HIF-1α index was scored as the percentage of positive cells in relation to all tumour cells in three 40x fields in each sample. The pattern of HIF-1α was noted as: a) purely diffuse (throughout the tumour without emphasis on areas with necrosis), b) perinecrotic (only positive staining around a necrotic area), or c) a combination of these two (mixed). [26]

Quantification of GLUT-1 and VEGF expressions in carcinoma cells

The expression of GLUT-1 and VEGF was performed in the same areas of the tumours (in parallel sections). For GLUT-1 and VEGF, when the number of positive cells was more than 10%, the case was judged positive. For VEGF, in each stained section, the relative numbers of neoplastic positive cells were considered: (a) in ordinary ACC in relation to all neoplastic cells; and (b) in ACC-HGT in relation to all neoplastic cells of conventional and transformed areas, respectively. The quantity of VEGF positive cells was assessed using a three-tiered scale: 10-25%, 25-50% and >50% of positive cells. For GLUT-1, only the pattern of staining was noted since its quantification was performed in a previous study of our group. [27] The pattern of staining was classified as antistromal (with GLUT-1 negative cells at the tumour-stromal interface and

positive cells in the central zone of tumour nests) or prostromal (with GLUT-1 positive cells at the most peripheral layers). [20]

Statistical analysis

A Fisher exact $\chi 2$ test was used to assess the associations among categorical data. Kruskal-Wallis test and Mann-Whitney U test were used for comparison of the numeric variables between the groups as appropriate. For comparison of numeric variables between conventional and HGT areas of ACC-HGT, the Wilcoxon signed rank test was used. Data were presented as mean ± SD (standard deviation), and the results with $p < 0.05$ were considered significant. All the statistical procedures were performed using SPSS software for Windows, version 12.0 (SPSS® Inc. Illinois, USA).

Results

Clinical follow-up

The clinical and pathological data of all cases are summarized in Table 2. Median follow-up time was 86 months (6 to 184 months) for ordinary ACC and 50 months (7 to 154 months) for ACC-HGT.

Expressions of HIF-1α*, GLUT-1 and VEGF in carcinoma cells*

In the normal salivary gland surrounding the tumour, expression of HIF-1α (nuclear staining) and VEGF (cytoplasmic staining) was occasionally observed, whereas GLUT-1 (membrane staining) was detected only in erythrocytes.

HIF-1α was seen as purely diffuse pattern of expression in 100% of cases (except for 6 ordinary ACC and 2 ACC-HGT in which HIF-1α staining was not available). In ACC-HGT this pattern of expression was observed in conventional as well as transformed areas, except for one case where HIF-1α expression was noticed around necrotic areas in the transformed component. Necrosis was found only in three ACC-HGT and all were located in the transformed area. The percentage of positive cells ranged from 22.7% to 79.6% (mean 54.3%) in ordinary ACC, 41.8% to 53.3% (mean 47.4%) in ACC-HGT conventional areas and 12.5% to 64.6% (mean 41.3%) in the transformed areas. The differences between the groups were not significant (Table 3, Figure 2) No significant association was found between mean level of nuclear staining for HIF-1α and tumour size (T1/T2 versus T3/T4 $p=0.118$), metastasis ($p=0.385$) and recurrence ($p=0.509$).

Regarding GLUT-1, only prostromal pattern of expression was observed (Figure 3). GLUT-1 positive cells were found in 3 out of 14 cases (21.4%) in ordinary ACC group and in 3 out of 6 (50%) in ACC-HGT one. In the latter, only the transformed component presented GLUT-1 positivity, and this aspect was already detailed in our previous study. [27]

VEGF expression in tumour cells was detected in 100% of cases (Figure 4). Strong VEGF expression (>50% of cells) was seen in 16/18 ordinary ACC, whereas in ACC-HGT it was observed in 6/8 conventional and 3/8 transformed areas. The differences between the groups were not significant (Table 4).

Microvessel density (MVD) by CD105

CD105-MVD ranged from 1.54 - 43.30 vessels/mm^2 in ordinary ACC, 3.72 - 24.12 vessels/mm^2 in ACC-HGT conventional areas and 0 - 12.50 vessels/mm^2 in ACC-HGT transformed ones (Figure 3). Comparing ordinary ACC (mean 11.51, SD 10.33) with conventional area of ACC-HGT (mean 9.85, SD 7.87), no significant difference was found, and neither did CD105-MVD differ significantly between conventional and transformed areas of ACC-HGT (Table 3).

Discussion

The main point of interest in ACC-HGT lies in their proposed poor prognosis as compared to the ordinary ACC. [4] The pathogenesis of high-grade transformation in ACC, however, still remains unclear and so far there are only few studies on genetic or metabolic changes associated with the tumour phenotypic modification. [7, 27] Regarding cellular metabolism, we have recently shown that ACC-HGT demonstrates increased expression of GLUT-1 and mitochondrial antigen, suggesting that transformation of ACC into ACC-HGT changes the metabolic state of cancer cells imposing an increased utilization of energy.[27] In the current series, as hypoxia is believed to be one of the mechanisms behind increased tumour aggressiveness, we explored the expression of proteins which are regulated by this event.

HIF-1α plays an essential role in O_2 homeostasis[28] and HIF-1α has been found to be expressed in the majority of the common human cancers. [29] However, the frequency of HIF-1α positivity is highly variable among tumours and in some types, such as endometrioid endometrial carcinoma[26] and poorly differentiated invasive breast carcinoma [30], protein expression was

detected in all cases. To our knowledge, this is the first time that expression of HIF-1α has been investigated in ACC. We found that 100% of ordinary as well transformed ACC exhibited HIF-1α expression, whereas this was only occasionally observed in normal salivary gland tissue adjacent to the tumour. Furthermore, the HGT areas of ACC did not show significant differences in terms of degree of HIF-1α expression when compared to: a) conventional areas in the same tumour and b) ordinary ACC. These findings suggest that there is no significant change in HIF-1α induction during the process of high-grade transformation in ACC. Moreover, in our series, the absence of association between HIF-1α expression level and metastasis and recurrence suggests that HIF-1α protein expression could not be used as a marker of tumour aggressiveness. In the literature, the correlation between levels of HIF-1α expression with tumour histological grade and biological behavior is variable and seems to depend on the tumour type.[31] In breast carcinoma, levels of HIF-1α have been reported to increase as the degree of malignancy increases indicating its potential association with poor clinical outcome.[30] In contrast, in endometrial carcinoma, high HIF-1α was significantly associated with low-grade tumours.[32]

Recently it has been proposed that different HIF-1α expression patterns in cancer cells have different prognostic implications and could have different induction mechanisms as well. [26, 33] HIF-1α expression can be observed throughout the tumour (diffuse pattern), around necrotic foci (perinecrotic) or a combination of these two patterns. Perinecrotic HIF-1α expression is believed to be related to hypoxia because of its strong association with expression of hypoxia-associated genes (GLUT-1 and carbonic anhydrase IX). [26, 33-34] This pattern of expression has been associated with more aggressive tumours as well. [26, 33] In contrast, with diffuse HIF-1α expression, major downstream effects have been reported to be lacking, leading to the hypothesis that this expression pattern might be regulated by oxygen-independent mechanisms .[26, 33] In fact, it is known that a range of factors, including activation of oncogenic signaling pathways and inactivation of tumour suppressor genes can lead to HIF-1α overexpression under aerobiosis[14, 35] In the current series, regardless of their histological classification as ordinary ACC or ACC-HGT, all cases presented a diffuse pattern of HIF-1α expression and in the majority no GLUT-1 positivity was found. These findings reinforce the idea that diffuse HIF-1α expression may be induced by oxygen-independent mechanisms and therefore, hypoxia probably would not be the main factor inducing stabilization of HIF-1α in ACC.

In our previous study on GLUT-1 in ACC-HGT, we showed that the transformed areas exhibited more frequent protein expression.[27] In the current series, in same samples, we analysed the pattern of GLUT-1 expression, since this may reflect different mechanisms of induction, similar to what has been reported regarding HIF-1α expression. [18-20] In agreement with our findings of diffuse (non-hypoxic) expression of HIF-1α, we only found a prostromal pattern of GLUT-1 staining, which is believed to be induced by non-hypoxic mechanisms as well. [20] Increased glycolysis is a normal response to proliferation since the glycolytic pathway provides a series of precursors which are essential for cell growth. [10, 36] In this sense, the characteristically higher proliferation index of the transformed component of ACC-HGT [4,27] could be an explanation not only for its higher GLUT-1 expression but also for its prostromal (non-hypoxic) pattern.

Finally, when comparing VEGF expression and CD105-MVD between transformed and conventional areas in ACC-HGT and the latter with ordinary ACC, we did not detect significant differences. As hypoxia regulates VEGF secretion by tumour cells as well as expression of CD105 on the endothelium surface [10, 37], our findings suggest that tissue oxygen tension in the transformed component probably did not differ significantly from that in the conventional area. Although hypoxia is an important inductor of VEGF and CD105 expression, there are other mechanisms that can upregulate VEGF production, such as growth factors, cytokines, enzymes and inactivation of tumour suppressor genes. [38] Given that HIF-1 has a central role in the transcriptional activation of VEGF regardless of the mechanisms of gene induction[39], we cannot discard oxygen-independent inductors of VEGF owing to the diffuse pattern of HIF-1α expression. CD105 expression is induced by hypoxia via the HIF-1 complex as well, which binds a functional consensus hypoxia-responsive element in the endoglin promoter.[40] Therefore, stabilization of HIF-1α by oxygen-independent mechanisms could also induce CD105 expression. Interestingly, CD105 and VEGF gene promoters share similarities regarding the structure of the hypoxia-responsive elements in the so-called HIF-1 ancillary sequences. [40] Thus, further studies are needed to clarify the up-regulating pathways of VEGF and CD105 in ACC.

In conclusion, our findings suggest that hypoxia may not play a key role in the process of high-grade transformation in ACC. This is supported by a) the absence of significant alterations in levels of expression of HIF-1α, VEGF and CD105 and b) the patterns of expression of HIF-1α

and GLUT-1. Although HIF-1α expression is a common finding in ACC, it cannot be used as a marker of tumour aggressiveness.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Table 1. Antibodies used for immunohistochemistry

Specificity	Clone	Dilution	Source	AR
HIF 1 α	H1α67	1:50	Santa Cruz *	TrisEDTA
VEGF-A	A-20	1:500	Santa Cruz *	Citrate
CD105	SN6h	1:10	Dako †	Pepsin
GLUT 1	Polyclonal	1:400	Abcam ††	Citrate

* Santa Cruz Biotechnology, Santa Cruz, USA

† Dako, Carpinteria, USA

†† Abcam, Cambridge UK

Table 2. Clinical follow-up

Case	Age	Sex	Site	T	Treatment	Local recurrence	Distant metastasis	Follow-up (mo)	Outcome
Ordinary ACC									
1	48	F	Submandibular	T1	S+RT	Yes	Yes	72	NA
2	60	M	Palate	T4	S+RT	Yes	No	127	Dead
3	52	F	Buccal mucosa	T4	S+RT	Yes	No	184	Alive
4	29	F	Palate	T1	S+RT	Yes	Yes	125	Alive
5	21	F	Submandibular	T2	S+RT	No	No	93	NED
6	49	F	Palate	T4	S+RT	No	Yes	63	DOD
7	30	M	Parotid	T1	S+RT	No	No	84	NED
8	44	F	Submandibular	NA	S	No	Yes	132	NA
9	44	M	Parotid	T1	S+RT	No	No	82	NED
10	64	F	NA	T4	S+RT+CT	No	Yes	55	DOD
11	27	F	Submandibular	T4	S+RT	Yes	No	75	Alive
12	NA	NA	NA	NA	S	NA	NA	NA	NA
13	55	F	Rhinopharynx	T1	S	NA	NA	NA	NA
14	58	F	Larynx	NA	S+RT	No	Yes	74	Alive
15	19	F	Lacrimal gland	NA	S	NA	NA	6	NA
16	66	F	Buccal mucosa	T2	S+RT	No	No	39	NED
17	42	M	Minor salivary gland	T4	S	NA	NA	NA	NA
18	56	F	Minor salivary gland	NA	S	NA	NA	NA	NA
ACC-HGT									
1	44	F	Submandibular	T2	S+RT	No	No	18	NA
2	55	F	Palate	T4	S+RT	No	No	154	NED
3	65	M	Paranasal sinus	T4	S+RT	No	No	8	Dead
4	49	F	Parotid	T3	S+RT	No	Yes	44	Alive
5	61	F	Paranasal sinus	T2	S+RT	Yes	NA	144	NED
6	64	F	Submandibular	T2	S+RT	No	Yes	7	DOD
7	58	F	Lip	T2	S	No	No	18	NED
8	47	M	Palate	T4	S	No	Yes	12	Alive

ACC- adenoid cystic carcinoma; CT- chemotherapy ; DOD- died of disease; F- female; HGT- high-grade transformation; M-male ; Mo - months; NA - not available; NED - no evidence of disease;

RT - radiotherapy ; S - Surgery; T - TNM classification.

Table 3. HIF-1α expression in carcinoma cells and CD105-MVD

Test	Antibody	Ordinary ACC (mean ± SD)	ACC-HGT (mean ± SD)		*P*
			CA	TA	
Mann Whitney	CD105	11.51 ± 10.33*	9.85± 7.87*		0.72
	HIF-1α	54.3 ± 17.8 †	47.4 ± 4.1 †		0.371
Wilcoxon	CD105		9.85 ± 7.87*	4.38 ± 3.72*	0.128
	HIF-1α		47.4 ± 4.1 †	41.3 ± 19.0 †	0.463

ACC - adenoid cystic carcinoma, CA - conventional area, HGT, high-grade transformation; MVD - microvascular density, SD - standard deviation; TA -transformed area.

*** Vessels/mm^2**

† Percentage of positive cells

Table 4. Vascular endothelial growth factor (VEGF) expression in carcinoma cells

Amount of positive cells (%)	N	Ordinary ACC	N	ACC-HGT CA	*P* X^2
<50	2	2.00 ± 0.00	2	2.00 ± 0.00	0.563
>50	16	3.00 ± 0.00	6	3.00 ± 0.00	

Amount of positive cells (%)	N	ACC-HGT CA	N	ACC-HGT TA	*P McNemar*
<50	2	2.00 ± 0.00	5	1.80 ± 0.45	1.000
>50	6	3.00 ± 0.00	3	3.00 ± 0.00	

ACC, adenoid cystic carcinoma; CA, conventional area; HGT, high-grade transformation; TA, transformed area

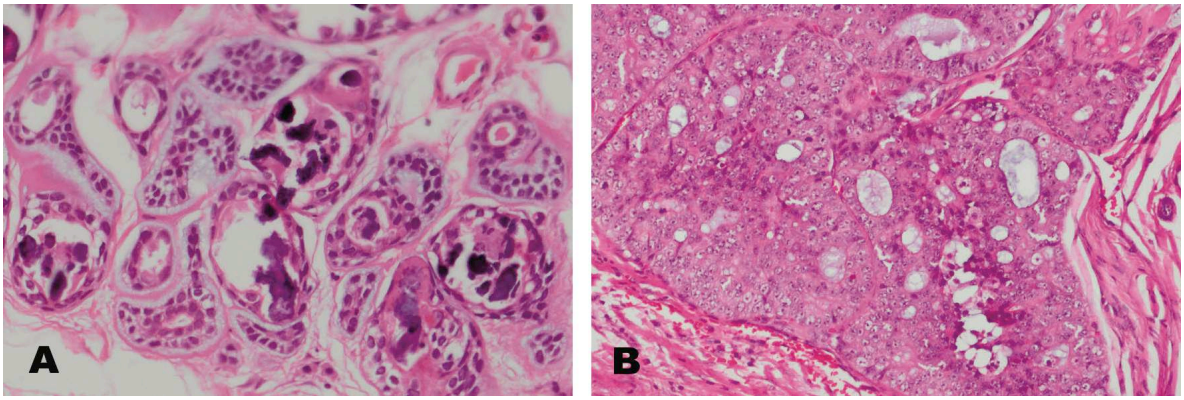

Figure 1. Adenoid cystic carcinoma with high-grade transformation. **A** – conventional area; **B** – high-grade area: poorly differentiated adenocarcinoma showing large nuclei (2-3 times the size of those in the conventional area) (HE X200).

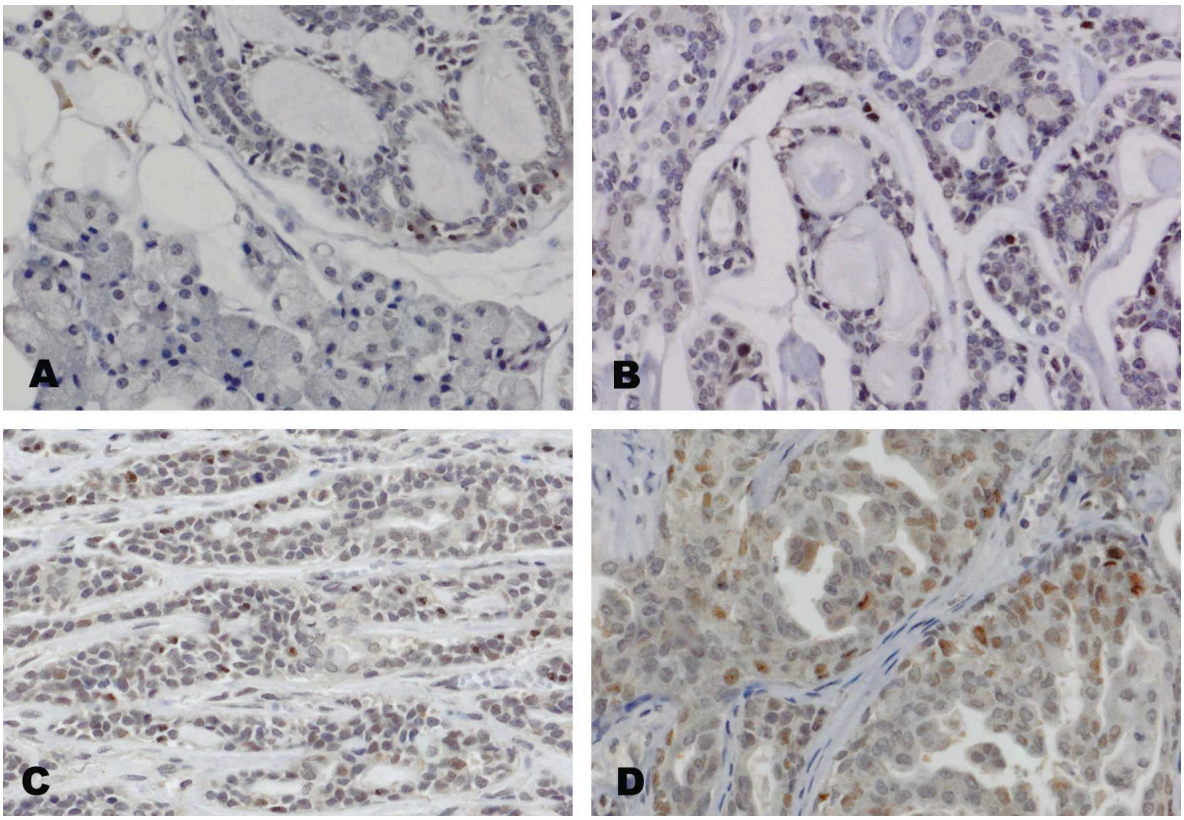

Figure 2. Expression of hypoxia-inducible factor 1α (HIF-1α) (dark nuclei). **A, B**: ordinary adenoid cystic carcinoma (ACC) – note the absence of expression in the normal salivary gland adjacent to the tumor **(A)** which contains positive cells. **C, D**: conventional and transformed areas (respectively) in ACC- high-grade transformation (HGT). Ordinary and ACC-HGT show a diffuse pattern of expression of HIF-1α. (x400).

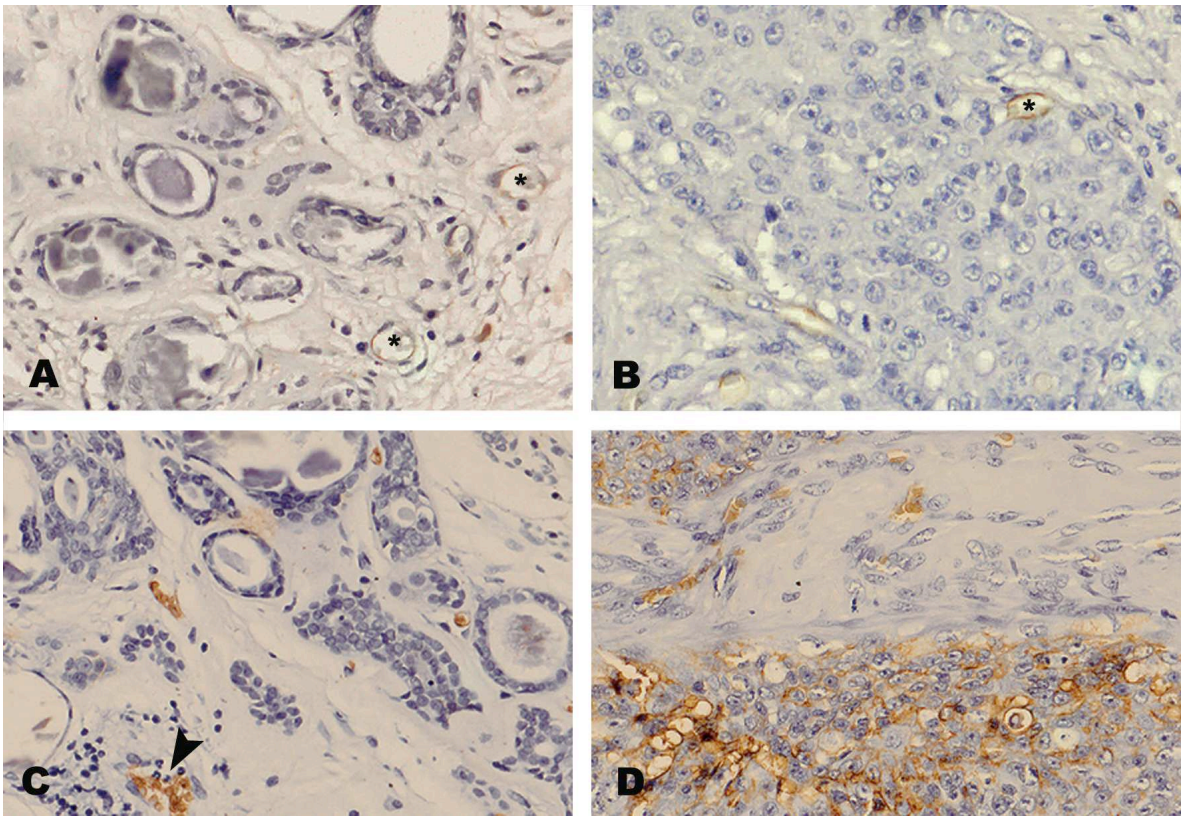

Figure 3. A, B – CD105 positive vessels (*) in conventional **(A)** and transformed **(B)** areas of adenoid cystic carcinoma with high-grade transformation (ACC-HGT). **C** - absence of glucose transporter 1 (GLUT1) expression in conventional area of ACC-HGT (only erythrocytes are GLUT1 positive - arrowhead). **D** – prostromal GLUT1 expression in the transformed component. (x400).

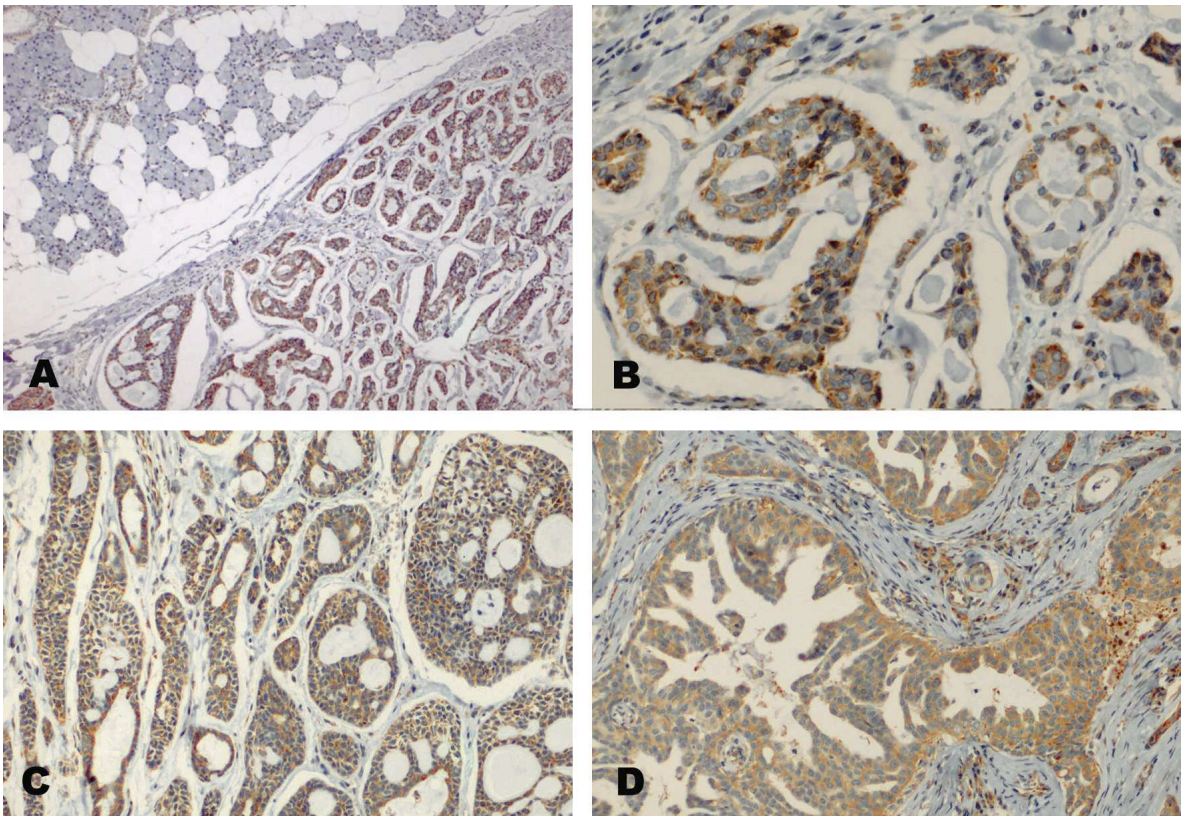

Figure 4. VEGF expression in carcinoma cells. **A, B** ordinary adenoid cystic carcinoma (ACC) (in **A** note the absence of expression in the normal salivary gland surrounding the tumor). **C, D** ACC high-grade transformation (HGT) – conventional **(C)** and transformed **(D)** areas. Ordinary and ACC-HGT show similar levels of VEGF expression. (**A** – x100; **B, C** and **D** - x400).

Capítulo 2

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Reprinted from Cellular Oncology, 2011, Aug;34(4):369-379, Genetic profile of adenoid cystic carcinomas (ACC) with high-grade transformation versus solid type,

Genetic profile of adenoid cystic carcinomas (ACC) with high-grade transformation versus solid type.

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Abstract

Background: ACC can occasionally undergo dedifferentiation also referred to as high-grade transformation (ACC-HGT). However, ACC-HGT can also undergo transformation to adenocarcinomas which are not poorly differentiated. ACC-HGT is generally considered to be an aggressive variant of ACC, even more than solid ACC. This study was aimed to describe the genetic changes of ACC-HGT in relation to clinico-pathological features, and to compare results to solid ACC. **Methods:** Genome wide DNA copy number changes were analyzed by microarray CGH in ACC-HGT, 4 with transformation into moderately differentiated adenocarcinoma (MDA) and two into poorly differentiated carcinoma (PDC), and 5 solid ACC. In addition, Ki67 index and p53 immunopositivity was assessed. **Results**: ACC-HGT carried fewer copy number changes compared to solid ACC. Two ACC-HGT cases harboured a breakpoint at 6q23, near the cMYB oncogene. The complexity of the genomic profile concurred with the clinical course of the patient. Among the ACC-HGT, p53 positivity significantly increased from the conventional to

the transformed (both MDA and PDC) component. **Conclusion:** ACC-HGT may not necessarily reflect a more advanced stage of tumor progression, but rather a transformation to another histological form in which the poorly differentiated forms (PDC) presents a genetic complexity similar to the solid ACC.
Keywords: Adenoid cystic carcinoma; high-grade transformation; dedifferentiation; microarray CGH.

1. Introduction

Adenoid cystic carcinoma is a slow-growing tumor presenting a dual cellular composition, i.e., ductal (luminal) and myoepithelial cell differentiation and three major growth patterns: tubular, cribriform and solid [1]. The solid growth pattern has been considered to be an adverse prognosticator [2-4] and in a three-tired system for grading ACC, tumors having more than 30% of the solid component are classified as grade III or poorly differentiated. Grade I tumors are those with tubular and cribriform areas but without solid components whereas Grade II are pure or mixed cribriform with less than 30% of solid areas [3]. Grade III ACC has been associated with increased disease mortality and greater frequency of aneuploidy than grade I or II tumors [5] Furthermore, comparing low-grade foci with high-grade ones within the same tumor revealed a greater number of mutations at either the p53 or Rb genes in the latter [6].

ACC can occasionally undergo transformation into poorly differentiated adenocarcinoma or undifferentiated carcinoma. This phenomenon has been referred to as dedifferentiation or high-grade transformation (ACC-HGT), and there have been only 33 reported cases so far [7-16]. This process was first believed to occur in low-grade ACCs without morphological recognizable changes, as an abrupt transition, but recently cases have been described showing a gradual transformation of solid ACC into high-grade adenocarcinoma [12, 13].

ACC-HGT is generally considered to be an aggressive variant of ACC, even more than solid ACC [13]. However, recently it has been claimed that high-grade transformation of ACC may, in addition to poorly differentiated carcinomas, also result in adenocarcinomas with a moderate differentiation [16].

The pathogenesis of high-grade transformation of ACC is poorly understood, partly because few studies have been dedicated to this tumor type. Some molecular studies reported TP53 mutations, loss of heterozygosity at the TP53 locus [7, 9], and strong overexpression of p53

protein in high-grade components [7, 9, 11, 13], suggesting that p53 alterations may play a significant role in the pathogenesis of high-grade transformation of ACC [9].

Genetic characterization of these lesions may give more insight in this complex matter. This study was aimed to compare the ACC-HGT and solid type ACC using genome-wide high-resolution microarray CGH analysis. In addition, the genetic changes were correlated with clinical outcome.

2. Material and Methods

2.1. Material

The present study included 11 paraffin embedded carcinoma samples of patients with ACC diagnosed between 1996 and 2007: 6 cases ACC-HGT and 5 cases solid ACC. Five cases of ACC-HGT were obtained from the archives of the Department of Pathology of the University of Campinas, Brazil, 1 case of ACC-HGT of the Department of Pathology of the Hospital Universitario Central de Asturias, Spain and 5 cases of solid ACC of the Department of Pathology, VU University Medical Center, The Netherlands. The solid ACC have previously been described as part of a larger series [17]. Hematoxylin-Eosin (H&E) stained slides from each tissue block were reviewed (Figure 1) to confirm the pathological diagnosis. The transformed component was identified according to the criteria described by Seethala et al. [13] and all cases showed the following features: proliferation of tumor cells with at least a focal loss of myoepithelial cells surrounding tumor nests, nuclear size at least 2 -3 times the size of tubular /cribriform ACC nuclei, thickened irregular nuclear membranes and prominent nucleoli in a majority of cells. In addition (Table 1), based on the degree of gland formation (differentiation), cellular pleomorphism and mitotic activity, the transformed components were classified into: moderately differentiated adenocarcinomas (MDA) when at least 2/3 exhibited gland differentiation and poorly differentiated carcinomas (PDC) those with scarce or absent gland differentiation [18]. The conventional component was classified in a three-tired system proposed by Szanto et al [3], which is widely used in the literature.

The ACC-HGT group consisted of 1 male and 5 female patients ranging from 44 to 65 years of age, with a median of 56 years. Two tumors occurred in the submandibular gland, 2 in paranasal sinus and 1 in palate and 1 in parotid gland. Four cases underwent transformation into MDA and two into PDC. The solid ACC group comprised of 2 male and 3 female patients

ranging from 33 to 66 years of age, with a median of 52 years. Three tumors occurred in the parotid gland and 1 in the submandibular gland. The exact origin of case 2 could not be determined; it concerned a large mass located in the oropharynx and nasopharynx. The follow-up time was 7-144 months (mean 55 months) for the ACC-HGT and 7-81 months (mean 43 months) for the solid ACC. The clinical and pathologic data of all cases are summarized in Table 1.

2.2. Microdissection and DNA extraction

Tumor tissue of 5 solid ACC and 6 ACC-HGT was obtained from 10 paraffin sections of 10 µm. Regions of interest (conventional and transformed areas) of the tumors were carefully dissected manually, on the basis of H&E-stained slides. Tumor DNA was extracted using Qiagen extraction kits (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. Special care was taken to obtain high quality DNA from the formaldehyde-fixed, paraffin-embedded tissues. DNA extracted from archival material can be partly degraded and cross-linked, the extent of which depends on the pH of the formaldehyde and the time of the fixation before paraffin embedding. To improve the quality of the isolated DNA, we have applied an elaborate extraction protocol especially for paraffin tissues, which includes thorough deparaffination with xylene, methanol washings to remove all traces of the xylene, and 24-hour incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet is dried and digested for 3 days in lysis buffer with high doses of proteinase K (final concentration 2 ug/uL, freshly added twice a day). Finally, the DNA was purified with Qiagen columns (QIAamp DNA mini-kit Qiagen GmbH, Hilden, Germany). With this protocol, most formaldehyde-fixed, paraffin-embedded tissue samples yielded DNA of relatively good quality, with A260/A280 values between 1,7 and 2.0 measured by Nanodrop (Thermo Scientific, Wilmington DE, USA) and lengths between 2000 and 20,000 bp. Before performing microarray CGH, we performed an additional quality test using the ENZO Bioscore Screening and Amplification kit (Enzo Life Sciences, Lörrach, Germany). The assay consists of an isothermal whole genome amplification reaction using 100 ng of DNA, followed by purification by QIAquick PCR Purification columns (Qiagen GmbH, Hilden, Germany) and measurement of the DNA concentration by Nanodrop (Thermo Scientific, Wilmington DE, USA). Only those samples that gave a total yield of 3.0 or more were used for microarray CGH analysis.

2.3. Microarray CGH

Microarray CGH analysis was performed as described previously by Buffart et al [19]. Briefly, sample DNA and reference DNA (extracted and pooled from blood of 18 different healthy female donors) were differently labeled by using the Enzo Genomic DNA Labeling kit according to the manufacturer's instructions (Enzo Life Sciences, Lörrach, Germany). Five hundred nanograms test and 500 ng pooled reference DNA were hybridized to a 180k oligonucleotide array (SurePrint G3 Human CGH Microarray Kit 4 x 180K, Agilent Technologies, Palo Alto CA, USA). Hybridization and washing took place in a specialized hybridization chamber (Agilent Technologies). Images were acquired using a Microarray scanner G2505B (Agilent Technologies, Amstelveen, Netherlands). Analysis and data extraction were quantified using feature extraction software (version 9.1, Agilent Technologies). Normalization of the calculated ratios was done against the mode of the ratios of all autosomes. Graphics were plotted using a moving average of log2 ratios of 5 neighboring clones. Gains and losses were defined as deviations of 0.2 or more from log2 ratio = 0.0. High-level amplification was considered when at least 2 neighboring clones reached a log2 ratio of 1.0 or higher. The locations of possible copy number variations (rather than copy number alterations) were verified with the database of genomic variants and mapped according the human genome build NCBI 36 (http://projects.tcag.ca/variation/). The microarray CGH analyses of the 5 solid ACC have been performed as part of a previous study, using a homemade microarray consisting of approximately 4.500 BAC-PAC clones [17].

2.4. Immunohistochemistry

One paraffin block from each case was chosen for the immunohistochemical study and the following antibodies (DAKO, Carpenteria, California) were used: Ki67 (MIB1- dilution 1:150), p53 (DO-7, dilution: 1:100) and alpha smooth muscle actin (α-SMA, 1A4 - dilution 1:200). The 5µm sections were deparaffinized, hydrated and endogenous peroxidase activity was quenched by immersion of the slides in 3% hydrogen peroxide. Antigen retrieval (AR) was performed for Ki-67 and p53 by using Tris-EDTA (pH 9.0), heating 5 minutes in a pressure cooker. For α-SMA, AR was not done. Staining was done at room temperature on an automatic staining workstation (Autostainer; DAKO, Carpenteria, California). Subsequently, for all antibodies the sections were incubated with the primary antibody, and afterwards with the Envision peroxidase system (Envision Plus; DAKO, Carpenteria, California) and with 3.3'-

diaminobenzidine tetrahydrochloride (DAB) chromogen used as the substrate (DAKO). Counterstaining with hematoxylin for 1 minute was the final step. After staining, the slides were dehydrated through graded alcohols and mounted with a coverslip. Negative controls were run by omitting primary antibodies.

Immunostaining of alpha smooth muscle actin (α-SMA) and p63, the latter as part of a previous study [16], was done in all cases of ACC-HGT for detection of myoepithelial cells contributing the selection of the transformed area for DNA extraction. In Ki-67 and p53 stained sections, three hotspot areas were chosen for counting of positive cells at 40X magnification. To quantify positive and negative cells, images were obtained from three areas and analyzed with Imagelab analysis software (version 2.4, Softium informática LTDA-ME, São Paulo, Brasil). Ki-67 and p53 indexes were calculated as the percentage of positive cells in relation to all tumor cells in these three areas in each sample.

2.5. Statistical analysis

Possible correlations between genetic and clinico-pathological parameters were statistically analyzed by SPSS 12.0 software for Windows (SPSS® Inc. Illinois, USA), using the Fisher exact Chi2 test and Student's t-test. Kaplan-Meier analysis was performed for estimation of survival, comparing distributions of survival through the logarithmic range test (log-rank test). P values below 0.05 were considered significant.

3. Results

3.1. Clinical follow up

All patients except the case 1 of solid ACC underwent radiotherapy after resection. Chemotherapy was not used in any case. During the follow-up period, 3 of 6 ACC-HGT and 4 of 5 solid ACC developed either a recurrence or a metastasis. The overall survival (Figure 3a) of the 6 ACC-HGT was more favorable than the 5 solid ACC (mean 58 versus 42 months) and this was the same with regard to the disease-free survival (mean 52 versus 33 months), although this did not reach statistical significance (Figure 3b).

3.2. Microarray CGH

Two of 6 ACC-HGT yielded a bad quality of DNA and had to be excluded from microarray CGH analysis (Table 2). Two ACC-HGT showed only one aberration; gain of whole chromosome 16 in case B and loss of 4q13.2-q22.3 in case D. Cases E and F harbored 6 and 11 changes,

respectively. A detailed description of all copy number changes is given in table 3 and figure 4. Two aberrations were recurrent, loss of 6q23.3-qter and gain of chromosome 8, both in cases E and F. These two cases also shared two transition points (where a change in the copy number begins or ends), which may indicate a translocation breakpoint. One lies in chromosomal band 6q23.3 at point 135.7 Mbp, and the second recurrent breakpoint in band 9p22.3 at point 14.1 Mbp (Figure 5).

The genome wide profiles of the ACC-HGT differed much from the solid ACC, both in number of alterations (Table 2) and in the specific chromosomes involved in alterations (Figure 4). The average number of alterations in the 4 ACC-HGT was 4.7 (3 gains and 1.7 loss) whereas the solid ACC demonstrated on average 21.8 events (19 gains and 2.8 losses). The 5 solid ACC showed many recurrent events, of which the most striking were gains at 9q33-q34, 11q13, 11q25, 12q13, 16p13, 16q24, 19 and 22 and loss at 14q. Only few of the recurrent aberrations in solid ACC were also seen in the ACC-HGT (Figure 4).

3.3 Immunohistochemistry

All ACC-HGT showed at least focal loss or absence of α-SMA and p63 [16] immunoreactivity for myoepithelial cells at the periphery of tumor nests in the transformed component, demonstrating the loss of biphasic ductal-myoepithelial differentiation in this area (Figure 2).

The Ki-67 index showed a trend for higher expression in the transformed component (both MDA and PDC) compared to the conventional areas (mean 19.6 versus 34.2, p=0,093) (Figure 2 and Table 2). Among the ACC-HGT group, a correlation was found between the degree of differentiation (Table 1) of the transformed component and the Ki-67 index. The proliferation index was significantly lower in the MDA than in the PDC group (mean 24.7 versus 53.3, p=0,028). A comparison of the transformed component of ACC-HGT with solid ACC, showed no significant difference in Ki-67 index (mean 34.2 versus 33.0, respectively; p=0,917). When comparing the solid ACC to MDA and PDC group separately, no significant differences were found in Ki-67 index (mean solid 33.0 versus MDA 24.7 and PDC 53.3; p=0,502 and p=0,270). PDC did show a trend for a higher index but unfortunately there were only two cases in the series.

Neither did we find a significant difference between the solid conventional area of ACC-HGT (cases B-F, regardless of the degree of differentiation MDA or PDC) and the solid ACC, (mean 20.7 versus 33.0; p=0,276).

In all cases, both components of ACC-HGT showed positive p53 staining (Figure 2 and Table 2). The p53 expression was significantly higher in the transformed component (both MDA and PDC) than in the conventional area (mean 62.9 versus 38.9, p=0,000), for group. No significant difference was found between the groups MDA and PDC (mean 63.4 versus 62.0; p=0,713). Similar expression of p53 without significant differences was observed between ACC-HGT and solid ACC (mean 62.9 versus 64.0; p= 0,929) and also between MDA or PDC group and solid ACC (mean 63.4 (MDA) and 62.0 (PDC) versus 64.0; p=0,968 and p= 0,929, respectively). The solid conventional areas (cases B-F) of the ACC-HGT group showed a trend towards lower p53 expression compared to the solid ACC group (mean 38.2 versus 64.0; p=0,085).

3.4. Clinicopathological-genetic correlations

The 2 cases of ACC-HGT with transformation into MDA (cases B and D) showed the lowest number of copy number abnormalities and one of the patients was a long-term survivor, who did not develop recurrence or metastasis. Conversely, the solid ACC group and ACC-HGT with transformation into PDC carried the highest number of abnormalities and had the worst clinical course; 3 out of 5 patients with solid type of ACC and one out of two with PDC died of disease (Table 1 and 2).

4. Discussion

An important point of interest with ACC-HGT lies in their proposed poor prognosis, which is suggested to be comparable to or even worse than solid ACC. The median survival of the largest reported series of ACC-HGT, in which all cases were poorly differentiated carcinomas, was estimated at 12 months [13], while in solid ACC this is approximately 36-48 months [3]. In addition to recurrence and distant metastasis, a high propensity for lymph node metastasis has been observed, which would indicate a role for neck dissection in these patients [13]. However, ACC-HGT may encompass a wide spectrum of tumors in morphological appearance and probably in biological behavior as well [16].

To date, genetic studies on ACC-HGT have almost exclusively been restricted to protein expression studies with immunohistochemistry [7-9; 11-13; 15, 16]. Here, we applied a high resolution microarray CGH analysis in an attempt to uncover genes involved in high-grade transformation of ACC, supplemented by immunohistochemical analysis of Ki67 and p53 and clinico-pathological data. In addition, we contrasted our data to an existing set of microarray CGH data on solid ACC [17].

An interesting finding in our study was the correlation between the number of chromosomal aberrations and the degree of gland differentiation of the transformed component in the ACC-HGT group. The two MDA had relatively simple genomic profiles carrying one single abnormality, whereas the two PDC showed a higher number of alterations. Solid ACC exhibited even higher numbers of chromosomal aberrations.

ACC with many chromosomal aberrations have been reported to be more aggressive and associated with less favorable outcome than those with few alterations [17]. Also in our series of ACC-HGT the complexity of the genomic profile grossly concurred with the clinical course of the patient: case F with transformation into PDC showed the worst clinical course and the highest number of chromosomal abnormalities. This patient developed lymph node and distant metastasis, and died of disease 7 months after diagnosis. In contrast, case B (a MDA with a single chromosomal aberration) was a long-term survivor and did not present metastasis or local recurrence. However, owing to our small number of cases, this association between degree of gland differentiation of the transformed component, amount of chromosomal aberrations and clinical outcome needs further confirmation in a larger series.

Two recurrent chromosomal changes were found: deletion at 6q23.3-qter and gain of whole chromosome 8, both in the two cases of PDC. Both aberrations have been found previously by cytogenetic and LOH analyses in salivary gland tumors [20-24] and in ACC [25-28]. Rao and collaborators [26] reported that gain of chromosome 8 was significantly associated with ACC solid type. In our series, in the ACC-HGT with PDC, the conventional component was of the solid type. Therefore, we believe that this chromosomal aberration in PDC areas is residual of the parent ACC. In our solid ACC group four cases out of 5 also showed gains of chromosome 8 reinforcing its association with this subtype of ACC.

In the two PDC cases, especially interesting were breaks found at the 6q and 9p regions, because they both occurred at exactly the same localization, which could indicate unbalanced

chromosomal translocations. Both 6q23 and 9p22 have previously been found involved in translocations in salivary gland tumors [20-24; 29], and in ACC [25-28]. Interestingly, recurrent translocations between 6q23 and 9p13-23 have been identified before in ACC [20, 21], and very recently, Persson et al [30] identified the genes MYB at 6q23 and NFIB at 9p22 being the fusion partners of this translocation, leading to chimeric transcripts predominantly consisting of MYB and overexpression of MYB protein [30]. In our two cases, the two breaks at 9p22 were located within NFIB in one, and just 100 kb centromeric to NFIB in the other case. At 6q23.3-qter the two breakpoints were identical, lying within the gene AHI1, which is a close neighbour telomeric of cMYB. Hence it remains unclear if our results confirm the findings of Persson et al.[30]. Overexpression of the oncogene AHI-1 has been implicated in the tumorigenesis of cutaneous T-cell lymphoma [31] and chronic myeloid leukemia [32]. However, we found no studies on a role for AHI-1 in solid tumors.

Although TP53 mutations and/or loss of heterozygosity at the TP53 locus have been suggested to play a role in the pathogenesis of high grade transformation of ACC [9; 11], in the current series none of the cases of ACC with transformation showed chromosomal aberration at 17p13, the locus of TP53. However, positive immunostaining is indicative of mutations in TP53 [33] and all cases in the current series showed positive p53 protein immunoexpression, increasing in the transformed component, suggesting a pivotal role of TP53 in the transformation of ACC.

Finally, our findings do not lend support to the hypothesis that ACC-HGT as a single group or separately (MDA and PDC) is more aggressive than solid ACC [13]. Due to the low number of cases, we cannot conclude whether PDC are more aggressive than MDA. Our data do suggest that the clinical course in ACC-HGT is dependent of the amount of chromosomal abnormalities, in which the poorly differentiated forms (PDC) presents a genetic complexity similar to the solid ACC. Although our series of cases is too small for strong conclusions, we do believe our data are valid, because we found agreement between the genetic, morphology, proliferation index and clinical data. Perhaps ACC-HGT does not necessarily reflect a more advanced stage of tumor progression, but rather a transformation to another histological form, which encompasses a wide spectrum of carcinomas in terms of aggressiveness [16]. Therefore, the term 'high grade transformation' may not be adequate for cases of ACC with transformation into MDA. In addition, we propose that for prognostication of ACC-HGT, histopathological classification may

be supplemented by genomic profiling by microarray CGH copy number analysis or other genome-wide analysis techniques.

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Table 1. Clinicopathological parameters

Case	Age	Sex	Site	T	Treatment	Local Recurrence	Distant Metastasis	Follow-up (mo)	Time disease free (mo)	Outcome	Histological Features		Glandular Differentiation
											CA	TA	
ACC with transformation													
A	44	F	Submandibular	T2	SE+RT	No	No	18	18	NA	T/C	MDA	frequent
B	55	F	Palate	T4	SE+RT	No	No	140	132	NED	S	MDA	frequent
C	65	M	Paranasal sinus	T4	SE+RT	No	No	8	8	Dead	S	MDA	frequent
D	49	F	Parotid	T3	SE+RT	No	Liver	33	19	Alive	S	MDA	frequent
E	61	F	Paranasal sinus	T2	SE+RT	Yes	No	144	132	Alive	S	PDC	scarce
F	64	F	Submandibular	T2	SE+RT	No	Liver	7	5	DOD	S	PDC	scarce
Solid ACC													
1	46	M	Submandibular	T1	SE	No	Liver, lung, bone	7	2	DOD	S	-	-
2	58	F	Oropharynx/Nasopharynx	NA	SE+RT	Yes	No	64	46	DOD	S	-	-
3	57	M	Parotid	NA	SE+RT	Yes	Liver	10	10	Alive	S	-	-
4	66	F	Parotid	T2	SE+RT	Yes	No	81	56	DOD	S	-	-
5	33	F	Parotid	T2	SE+RT	No	No	51	51	Alive	S	-	-

DOD, died of disease; F, female; M, male; MDA, moderately differentiated adenocarcinoma; Mo, months; NA, not available; NED, no evidence of disease; PDC, poorly differentiated carcinomas; RT, radiotherapy; S, solid;SE, surgical excision; T, TNM classification, TA, transformed area; T/C, tubular/cribriform.

Table 2. Summary of microarray CGH and Ki-67 index

Cases	CNA			Ki-67		p53	
	Total	Gains	Losses	CA	TA	CA	TA
ACC with transformation							
A	-	-	-	13,7	12,5	42,5	63,1
B	1	1	0	8,5	20,8	34,0	63,5
C	-	-	-	29,0	32,0	39,6	62,5
D	1	0	1	27,9	33,6	34,3	64,5
MDA mean	**1,0**	**1,0**	**1,0**	**19,8**	**24,7**	**37,6**	**63,4**
E	6	4	2	25,5	59,9	50,2	67,7
F	11	7	4	13,0	46,7	33,1	56,3
PDC mean	**8,5**	**5,5**	**3,0**	**19,3**	**53,3**	**41,7**	**62,0**
Total mean	**4,7**	**3,1**	**1,7**	**19,6**	**34,2**	**38,9**	**62,9**
Solid ACC							
1	34	31	3	45,0	-	75,0	-
2	25	23	2	5,0	-	65,0	-
3	27	24	3	35,0	-	80,0	-
4	16	11	5	60,0	-	15,0	-
5	7	6	1	20,0	-	85,0	-
Total mean	**21,8**	**19**	**2,8**	**33,0**	**-**	**64,0**	**-**

CNA - copy number alterations detected by microarray CGH. CA, conventional area; TA, transformed area

Table 3. Detailed description of all gains and losses in 4 transformed ACC.

Case	Alteration	Chromosomal band	Begin (kbp)	End (kbp)	Size (kbp)	Candidate genes*
B	Gain	16pter_qter	0	88669	88669	many
D	Deletion	04q13.2_q22.3	67364	98431	31067	many
E	Deletion	06q23.3_qter	135698	170753	35055	AHI1
		12q13.11	47175	52417	5242	many
	Gain	08pter_qter	0	146272	146272	many
		09pter_p22.3	0	14402	14402	NFIB
		10q26.13	123228	123254	26	FGFR2
		11q21	95558	95715	157	MAML2
F	Deletion	06q23.3_qter	135698	170753	35055	AHI1
		09pter_p22.3	0	14119	14119	NFIB
		09p22.3_p22.2	14119	18476	4357	many
		09p21.3_p13.1	21574	38514	16940	many
	Gain	07pter_qter	0	158821	158821	many
		08pter_qter	0	146272	146272	many
		06pter_q23.3	0	135698	135698	AHI1
		10q11.21_q11.22	45492	47944	2452	PTPN20A
		18pter_qter	0	76116	76116	many
		19pter_qter	0	63789	63789	many
		20pter_qter	0	62432	62432	many

*Candidate genes were only given when the gain or loss, or the breakpoint of a gain or loss, concerned one unique gene.

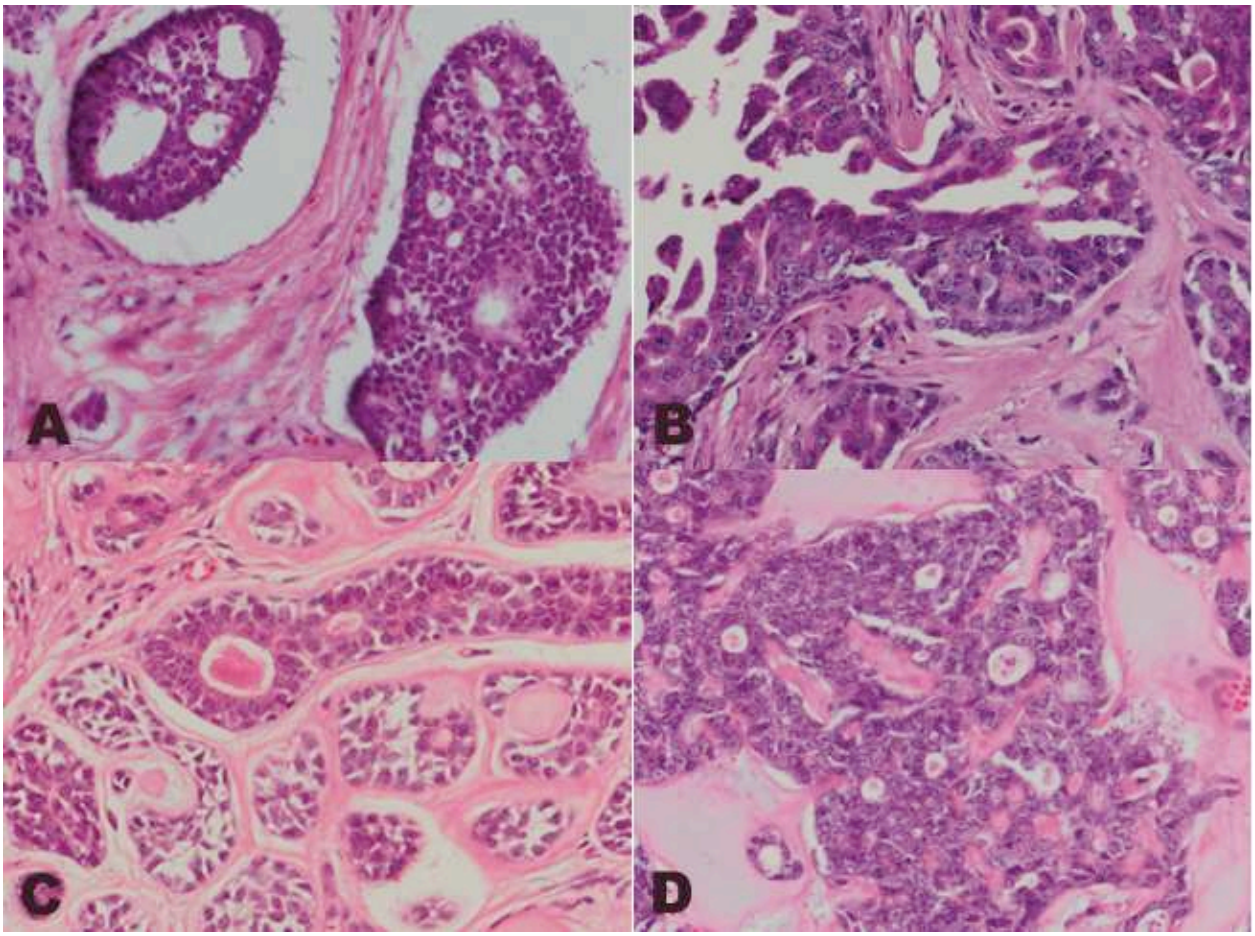

Figure 1. ACC-HGT to a moderately differentiated adenocarcinoma, case C (A, B). ACC-HGT to poorly differentiated carcinoma, case F (C, D). H&E original magnification 200x (A, B, C and D).

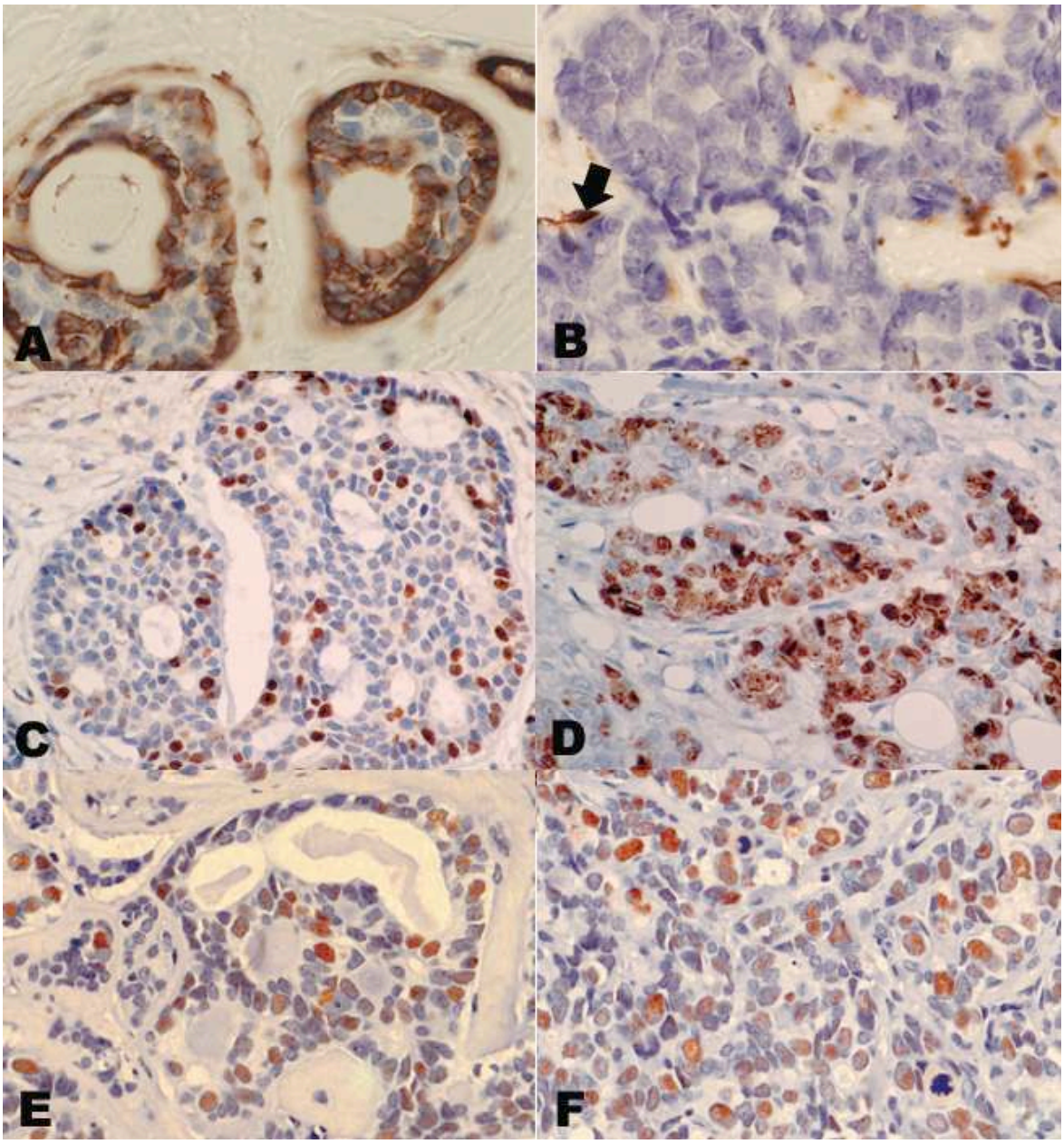

Figure 2. Expression of α-SMA in myoepithelial cells of conventional area (A) and transformed area (B) of ACC-HGT (Case F). The transformed component shows few positive myoepithelial cells (arrows) for α-SMA, demonstrating the loss of biphasic ductal-myoepithelial differentiation in this area. Ki-67 (Case C) and p53 (Case A) expression in conventional areas (C, E) and in transformed component (D, F). Original magnification 400x (A, B, C and D).

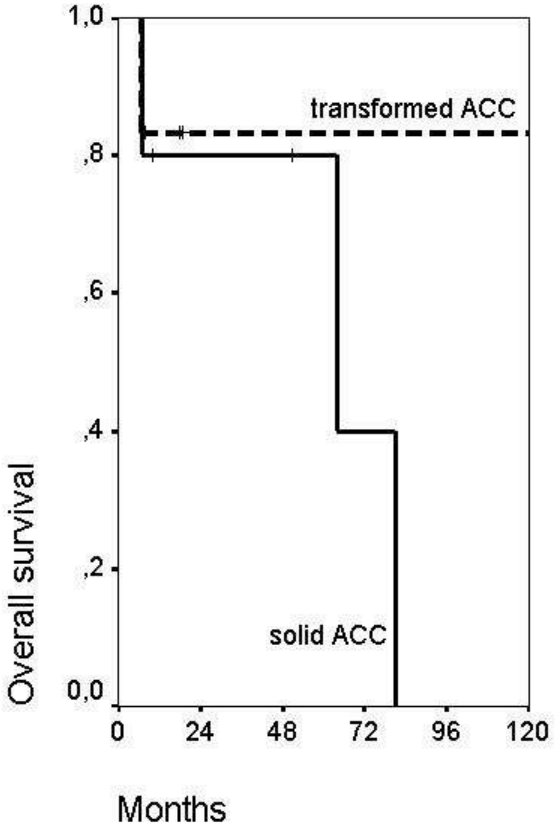

Figure 3a. Kaplan Meier curve showing the overall survival of 6 cases ACC-HGT versus 5 cases solid ACC.

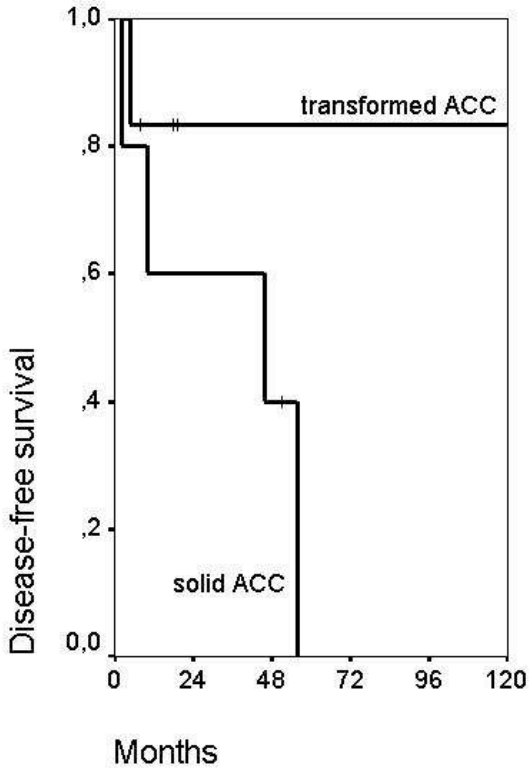

Figure 3b. Kaplan Meier curve showing the disease-free survival of 6 cases ACC-HGT versus 5 cases solid ACC.

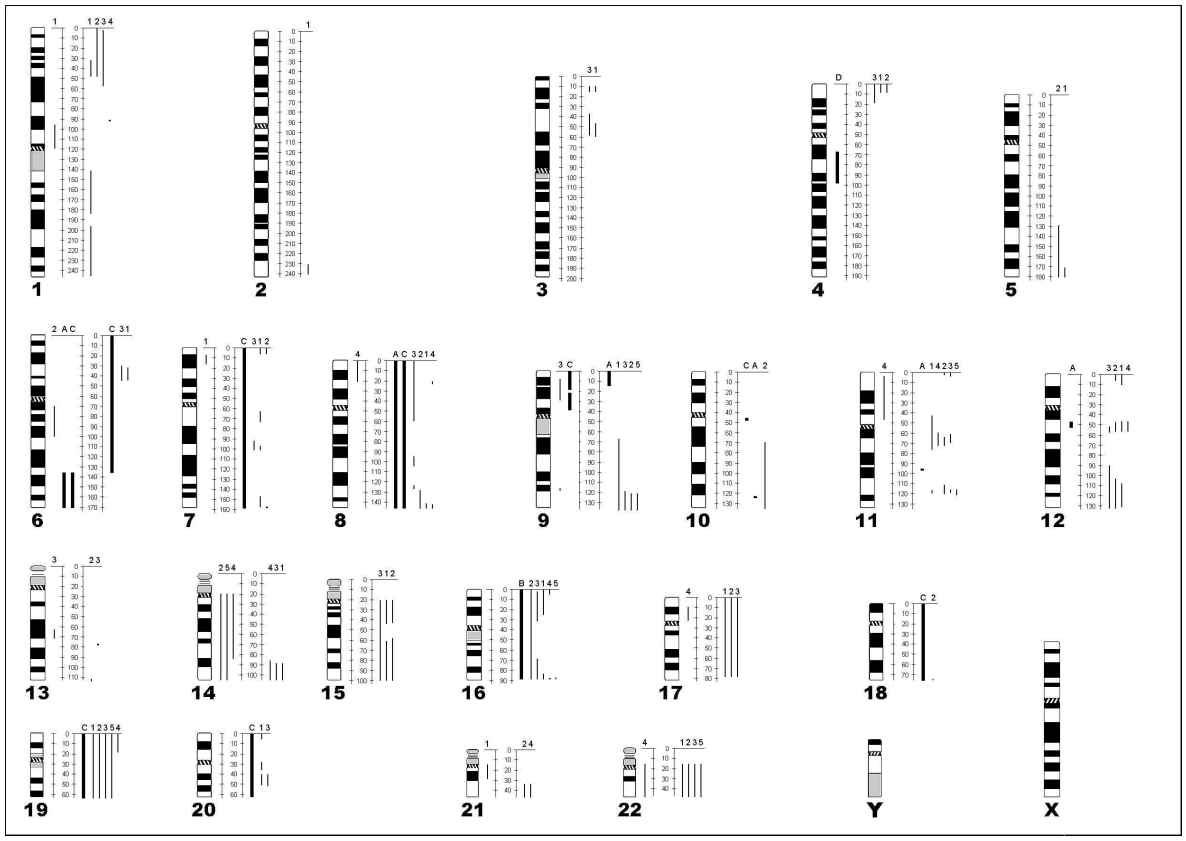

Figure 4. Overview of all copy number alterations of 4 ACC-HGT (cases B, D, E and F) and 5 solid ACC (cases 1-5). To the right of the pictogram of each chromosome, a scale is placed expressing the number of megabasepairs (Mpb) counting from pter to qter. Copy number losses are presented as bars left to the Mbp-scale and copy number gains to the right.

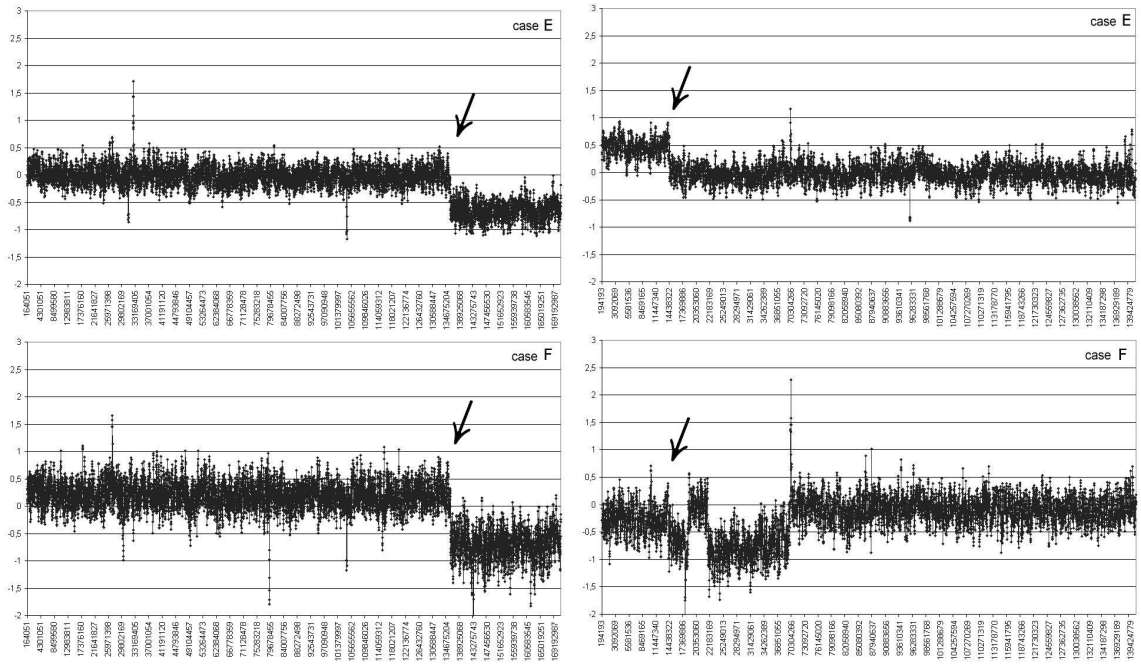

Figure 5. The left panel shows the microarray CGH profile of chromosome 6 of cases E and F, both carrying a telomeric deletion that begins at the same 6q23.3 breakpoint, marked by an arrow. The right panel shows the microarray CGH profile of chromosome 9 of cases E, carrying a gain, and of case F carrying three distinct regions with copy number loss. The arrows mark the common 9p22.3 breakpoint.

Capítulo 3

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Published in Laboratory Investigation, 2014, April 14, in press, Analysis of MYB oncogene in transformed adenoid cystic carcinomas reveals distinct pathways of tumor progression. For figures and tables please visit the original article in http://www.nature.com/labinvest/index.html.

Análise do oncogene MYB nos carcinomas adenóides císticos transformados revela diferentes vias de progressão tumoral

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Palavras-chave: carcinoma adenóide cístico; transformação para alto grau; *MYB*; hibridização genômica comparativa em microarranjo; hibridização fluorescente in situ.

Resumo

Os carcinomas adenóides císticos podem ocasionalmente sofrer desdiferenciação, um fenômeno também referido como transformação para alto grau. Contudo, casos de carcinomas adenóides císticos foram descritos sofrendo transformação para adenocarcinomas que não são pobremente diferenciados, indicando que a transformação para alto grau pode necessariamente não refletir um estágio mais avançado da progressão tumoral, mas uma transformação para outra forma histológica, que pode englobar um grande espectro de carcinomas em termos de agressividade. O objetivo deste estudo foi ganhar mais conhecimento sobre este fenômeno patológico através do perfil genético de ambos os componentes histológicos. Utilizando-se a hibridização genômica comparativa, nós comparamos as alterações no número de cópias do DNA do genoma das áreas convencionais e transformadas de oito casos de carcinomas adenóides císticos com transformação para alto grau, compreendendo quatro casos com transformação para adenocarcinomas moderadamente diferenciados e outros quatro casos para carcinomas pobremente diferenciados. No geral, os casos de carcinomas pobremente diferenciados mostraram maior número total de alterações no número de cópias do que os casos de adenocarcinomas moderadamente diferenciados, e isso foi correlacionado com um pior curso clinico. Atenção especial foi dada a translocação cromossomal e a expressão da proteína do gene *MYB,* que recentemente foi considerado ser o maior evento oncogênico e precoce nos carcinomas adenóides císticos. Nossos dados também mostraram que o processo de transformação para alto grau não é sempre acompanhado pelo acúmulo de alterações genéticas; pois tanto o componente convencional quanto o transformado abrigaram alterações genéticas únicas, que indicam uma

progressão paralela. Nossos dados demonstraram ainda que a translocação entre *MYB/NFIB* não é necessariamente um evento precoce ou fundamental para a progressão do carcinoma adenóide cístico com transformação para alto grau.

Introdução

O carcinoma adenóide cístico com transformação para alto grau (CAC-TAG) foi primeiro descrito como desdiferenciado por Cheuk e colaboradores em 1999 (Cheuk et al, 1999). Posteriormente, a desdiferenciação no CAC foi reconhecida por diversos autores (Moles et al, 1999; Chau et al, 2001; Nagao et al, 2003, Ide et al, 2003, Brackrock et al, 2005, Hayashido et al, 2005; Sato et al, 2006). Em 2007, Seethala e colaboradores sugeriram o termo "transformação para alto grau", que desde então, tem sido adotado por muitos outros autores, inclusive para outros tumores de glândula salivar, tais como o carcinoma de células acinares e o carcinoma epitelial-mioepitelial (Seethala et al, 2007; Skálová et al, 2009; Roy et al, 2010, Costa et al, 2011a, Nagao, 2013). Este termo foi considerado mais apropriado pelo fato de que a linha original de diferenciação epitelial ainda pode ser reconhecida dentro do componente de alto grau (Seethala et al., 2007). Contudo, nosso grupo recentemente descreveu casos mostrando transformação para adenocarcinomas que não são pobremente diferenciados, ou seja, não de "alto grau" (Bonfitto et al, 2010). Independente da exata terminologia, é claro que o carcinoma adenóide cístico com transformação para alto grau é uma entidade clínica e patológica distinta do carcinoma adenóide cístico clássico, apresentando evidentes aspectos morfológicos como a perda focal ou total da diferenciação mioepitelial, aumento nuclear, micropapilas e áreas escamóides no componente transformado (Seethala et al, 2007). O CAC-TAG apresenta maior índice Ki67 e

expressão mais forte de p53 no componente transformado quando comparado a área convencional (Costa et al, 2011b). Além disso, em contraste com o CAC clássico, o CAC-TAG parece ter um curso clínico acelerado e alta propensão para metástases linfonodais (Seethala et al, 2007; El-Naggar e Huvos, 2005). Até o presente momento, a combinação dos critérios morfológicos e a expressão do Ki67 é ainda a ferramenta mais útil na identificação do componente transformado no CAC-TAG (Costa et al, 2011a).

Poucos estudos foram publicados sobre as aberrações genéticas e moleculares no CAC-TAG. Dois estudos com hibridização genômica comparativa em microarranjo (um deles inclusive de nosso grupo) produziram resultados divergentes, provavelmente devido ao pequeno número de casos (Costa et al, 2011b; Seethala et al, 2011). Seethala et al (2011), relataram deleções na áreas convencionais, enquanto que os ganhos foram encontrados principalmente nas áreas transformadas, principalmente em 8q24, a localização do gene *MYC*. Nosso estudo anterior revelou tanto ganhos quanto perdas na área transformada.

As aberrações mais freqüentes foram o ganho de todo o cromossomo 8 e, também, simultâneo ganhos em 6q23/ perda em 9p22, possivelmente indicando a translocação entre *MYB/NFIB*. Além disso, nós encontramos que o alto número de aberrações cromossomais foi inversamente correlacionado com o nível de diferenciação glandular, sugerindo que a transformação para alto grau pode não necessariamente refletir um estágio mais avançado da progressão tumoral (Costa et al, 2011b). Semelhante ao CAC clássico, o CAC-TAG contém poucas alterações no número de cópias ou mutações (Seethala et al., 2011; Costa et al., 2011b; Ho et al., 2013; Stephens et al., 2013). Frierson e Moskaluk sugeriram que a transformação

neoplásica pode ser dirigida por um limitado número de alterações em genes reguladores da transcrição e, agravada pelas mudanças na estrutura da cromatina (Frierson e Moskaluk, 2013). Infelizmente, até o momento, não há estudos feitos sobre a reprogramação transcripcional e epigenética no CAC-TAG.

Os mecanismos moleculares que engatilham a transformação morfológica nos tumores salivares continuam obscuros. Em um estudo anterior realizado pelo nosso grupo, nos analisamos somente as áreas transformadas de um pequeno número de casos de CAC-TAG (quatro casos), utilizamos a hibridização genômica comparativa em microarranjo (Costa et al, 2011b). Com a finalidade de melhorar ou compreender o fenômeno da transformação para alto grau no CAC, neste estudo, acrescentamos mais quatro casos e ambos os componentes, convencional e transformado, foram analisados, através da hibridização genômica comparativa em microarranjo, hibridização fluorescente in situ e imuno-histoquímica. Atenção especial foi dada a translocação e a expressão da proteína *MYB,* que atualmente estão sendo consideradas o maior evento oncogênico e precoce no CAC clássico (Persson et al, 2009; Mitani et al, 2010; West et al, 2011; Fehr et al, 2011; Brill et al, 2011) e, o qual, até agora, não foi estudado no CAC-TAG. Nossos achados revelaram diferenças genéticas entre os dois componentes de cada tumor, sugerindo distintas vias de progressão tumoral.

Material e Método

Amostras

Oito blocos de parafina de CAC-TAG foram recuperados dos arquivos do Departamento de Anatomia Patológica da Universidade de Campinas, Brasil e do Hospital Universitário central de Astúrias, Espanha. Lâminas coradas com hematoxilina e eosina de cada caso foram revisadas para confirmar o diagnostico patológico. A área convencional de cada tumor foi classificada de acordo com o padrão histológico predominante como proposto por Szanto et al (1984): grau I (padrões cribriforme e/ou tubular predominantes); grau II (menos de 30% de padrão solido) e grau III (mais do que 30% de padrão solido). A área transformada foi identificada de acordo com os critérios propostos por Seethala et al (2007) e, em todos os casos pelo menos três dos maiores critérios propostos por ele estavam presentes: células tumorais com alto índice proliferativo, perda focal ou total da diferenciação mioepitelial ao redor dos ninhos tumorais, tamanho nuclear de 2 a 3 vezes maior do que no CAC clássico, membranas nucleares grossas e irregulares e nucléolos proeminentes na maioria das células. Este estudo foi aprovado pelo comitê de ética.

Além disso, baseado no nível de diferenciação celular, pleomorfismo celular e atividade mitótica, a área transformada foi classificada em: adenocarcinomas moderadamente diferenciados (AMD) quando pelo menos 2/3 exibiram diferenciação glandular (**Figura 1A**) e carcinomas pobremente diferenciados (CPD) quando a diferenciação glandular foi escassa ou ausente, **Figura 1B** (Wenning, 2008)**.**

Imuno-histoquímica

Cortes de 5 μm de blocos de parafina foram desparafinizados em xilol, reidratados através de uma serie de etanol descendente e submetido a recuperação antigênica por calor em banho-maria com a solução de Tris-EDTA pH 9.0 por 30 minutos. Posteriormente, os cortes foram imersos em 0.3% de peróxido de hidrogênio e em metanol e então, encubados com o anticorpo primário *MYB* (ab45150, Abcam, Cambridge, UK), e depois, com o sistema EnVision peroxidase (K4061, DAKO, Carpenteria, USA) por 1 h a 37°C. A concentração do anticorpo foi 1:300 e carcinomas de colón como tecido controle. Após a lavagem, os cortes foram corados por 5 minutos a 37°C com tetracloretodiaminobenzidina (DAB) e contra-corados com hematoxilina. Os controles negativos foram executados com a omissão do anticorpo primário. Para todos os tumores, três áreas com maior expressão foram escolhidas tanto no componente convencional quanto no transformado por contagem das células positivas no aumento de 40x utilizando o software de analise Imagelab (versão 2.4, Softium informática LTDA-ME, São Paulo, Brasil). A expressão de MYB foi analisada pela porcentagem de células positivas em relação a todas as células tumorais nessas três áreas de cada caso.

Extração do DNA

Dois cilindros de 5 mm de tecido parafinado foram coletados; um da área convencional e outro da área transformada de cada caso utilizando-se um punch descartável para biopsia (Kolplast LTDA, São Paulo, Brazil). A área do punch foi selecionada com base nos cortes de

hematoxilina e eosina (Prince et al, 2007). Cuidado especial foi tomado para obter uma alta qualidade do DNA do tecido parafinado. O DNA extraído de tecido parafinado pode ser parcialmente degradado e conter muitas ligações cruzadas, a medida depende do pH do formaldeído e o tempo de fixação antes de o material ter sido parafinado. Nós aplicamos um elaborado protocolo de extração especialmente desenvolvido para tecidos parafinados (Costa et al, 2011b), que inclui desparafinização em xilol e lavagens em metanol para remover todos os vestígios do xilol e, 24hs de incubação em 1 mol/L em tiocianato de sódio para reduzir as ligações cruzadas. Posteriormente, o pellet de tecido foi digerido por três dias em proteinase K com concentração final de μg/μL. Finalmente, a extração do DNA foi feita usando o kit QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Microarray CGH

A análise da hibridização genômica comparativa em microarranjo foi anteriormente descrita (Buffart et al, 2008). O DNA referência (extraído e agrupado de sangue de 18 doadoras saudáveis) e os casos de DNA foram marcados diferentemente utilizando-se o kit Enzo Genomic DNA Labeling de acordo com as instruções do fabricante (Enzo Life Sciences, Lörrach, Germany). Quinhentos nanogramas de cada foram hibridizados com 180k oligonucleotide array (SurePrint G3 Human CGH Microarray Kit 4 × 180K, Agilent Technologies, Palo Alto, CA, USA). A hibridização e a lavagem foram feitas em uma câmara especial (Agilent Technologies). As imagens foram adquiridas utilizando-se um scanner G2505B (Agilent Technologies, Amstelveen, Netherlands). A analise e os dados foram quantificados utilizando-se o software de

extração (version 9.1, Agilent Technologies). A normalização das relações calculadas foi feita contra o modo e as proporções de todos os autossomas. Gráficos foram plotados usando uma média móvel de log 2 proporções de 5 clones vizinhos. Os ganhos e perdas foram definidos como desvios de 0,2 ou mais de log relação 2 = 0.0. Uma amplificação de alto nível foi considerada quando pelo menos dois clones vizinhos atingiram um índice de log 2 de 1.0 ou superior. As localizações das possíveis alterações no numero de copias foram verificadas com o banco de dados de variantes genômicas e mapeadas de acordo com o genoma humano construído por NCBI 36 (http://projects.tcag.ca/variation/).

Hibridização fluorescente in situ

Para identificar a translocação entre MYB/NFIB, foi realizada a hibridização fluorescente in situ (FISH) em cortes de parafina de 4 μm de CAC-TAG em ambos os componentes utilizando-se sondas disponíveis comercialmente (BlueGnome, Cambridge, UK). Baseado nos pontos de quebras observados no microarray CGH, nós escolhemos três marcadores diferentes de sonda para FISH, a sonda de cor verde para 6q23 próximo ao gene *MYB* e, a de cor laranja para 9p22 próximo ao gene NFIB. Detalhes das sondas e respectivos pontos de quebra detectados pelo microarray CGH são observados na **Figura 2.** Os cortes foram desparafinizados em xilol, reidratados em uma série de etanol e pré-tratados em banho-maria a 99^{o}C, utilizando-se o kit acessório para FISH em parafina (DAKO histology FISH accessory kit, DakoCytomation, Carpenteria, USA). Após a lavagem, os cortes foram digeridos com pepsina por 10 minutos a 37^{o}C e, desidratados em uma série de etanol. Simultaneamente, tanto as sondas quanto os cortes

sofreram desnaturação em uma placa quente por 5 minutos a 82ºC e hibridização a 45ºC por toda noite em câmera úmida. Em seguida, os cortes foram lavados em uma solução rigorosa por 10 minutos a 65ºC e desidratados, novamente, em uma serie de etanol e montados em um meio antidesbotamento (Vectashield - Vector laboratories, Burlingame, USA) contendo 4,6-dia-mino-2-phenylindole (DAPI) para contra-coloração. As imagens foram capturadas e processadas utilizando-se o microscópio de fluorescência Olympus BX61 e as imagens foram processadas pelo software Image J 1.43u(Wayne Rasband National Institutes of Health, USA). Foi definido como fusão positiva quando os sinais verde e laranja das sondas do FISH, 6q23 e 9p22, respectivamente, se interceptaram em mais de 20% do total do número de células.

Análise estatística

Todos os procedimentos estatísticos foram realizados utilizando-se o software SPSS para Windows, versão 12.0 (SPSS® Inc., IL, USA). O teste Mann–Whitney foi utilizado para comparações entre variáveis numéricas entre os grupos. Para a comparação entre as variáveis numéricas entre as áreas convencionais e transformadas do CAC-TAG, foi utilizado o teste de Wilcoxon. Todos os valores p foram considerados two-tailed e foi adotado 95% de intervalo de confiança, somente valores $p < 0.05$ foram considerados significantes.

Resultados

Aspectos clínicos

Os dados clínicos e patológicos deste estudo está representado na **Tabela 1.** Quatro casos sofreram transformação para AMD (Casos cac1, cac2, cac3 e cac4) e quatro em CPD (casos cac5, cac6, cac7 e cac8). A media de meses referente ao seguimento clinico dos pacientes foi de 52 meses (7 a 154 meses). A média de idade foi de 55 anos (variou de 44 a 65 anos) e seis pacientes foram mulheres e dois homens. Dois tumores ocorreram na glândula submandibular, dois no seio paranasal, dois no palato, um na glândula parótida e um no lábio. Durante o seguimento, três pacientes desenvolveram metástases e um paciente desenvolveu recidiva local. Seis pacientes receberam radioterapia e quimioterapia após a remoção cirúrgica.

Análise das alterações no número de cópias

O microarray CGH revelou alterações no número de cópias em todos os oito casos, do qual quatro deles na área transformada e os outros quatro em ambas as áreas. O ganho mais recorrente foi de todo cromossomo 8. A região genômica mais frequentemente amplificada foi 9pter-p22/p23, englobando o gene *NFIB*. Outros eventos freqüentes foram o ganho de todo cromossomo 19 e da região 11q21. A região genômica 6q23.3-qter, englobando *MYB*, foi a perda mais frequente. Alterações simultâneas em 6q23.3 e 9pter-p22p23, possivelmente indicando a translocação entre *MYB/NFIB*, ocorreram em cinco dos 8 casos (2 AMD e 3 CPD): duas vezes em ambos os componentes, duas vezes somente na área transformada e uma vez na área convencional somente. Em geral, o grupo CPD mostraram significantemente maior número de alterações no número de cópias do que o grupo de AMD (76 versus 11, respectivamente; $p = 0.017$, teste Mann-Whitney), tanto na área convencional quanto transformada. Das 76 alterações

do grupo CPD, 54 foram na área transformada e 22 na área convencional. O grupo de AMD mostrou um total de 3 alterações na área convencional e de 8 na área transformada. Em ambos os grupos, os ganhos foram mais freqüentes que perdas.

A comparação entre os perfis genomicos da área convencional e transformada revelou diferenças nas alterações no número de cópias, embora não significantes (p=0.061, teste de Wilcoxon). Do total de 47 ganhos em oito casos, 37 foram na área transformada e 10 na área convencional. As áreas transformadas contiveram mais perdas do que as áreas convencionais (25 versus 15, respectivamente) total de 40 perdas. Um caso de CPD (cac5) mostrou um perfil genético idêntico em ambos os componentes do tumor. Em quatro casos (3 AMD e 1 CPD), não houve alterações genéticas na área convencional, mas múltiplas anormalidades no componente transformado correspondente. Em dois casos (ambos CPD), a área transformada apresentou as mesmas alterações genéticas do que a sua área convencional correspondente, mais alterações adicionais. Finalmente, um caso (AMD) mostrou uma deleção compartilhada entre ambos os componentes, enquanto que área convencional apresentou aberrações adicionais. A descrição detalhada de todas as alterações no número de cópias são apresentadas nas **Figuras 1 A e B.**

Translocação e expressão da proteína MYB

Em cinco casos (cac3, cac4, cac5, cac6 e cac8), as alterações no número de cópias envolvendo pontos de quebra (a posição aonde o ganho e a perda começa e termina) próximos a 6q23 e 9p22 foram observadas simultaneamente. Em quatro casos houve a perda de 6q23-qter e o ganho de 9pter-9p22 e, em um caso (cac6) apresentou uma pequena deleção em 6q23 em

combinação com a deleção em 9p22 (**Figuras 1A e B).** O FISH foi realizado em esses cinco casos para investigar se isso indicava a translocação entre *MYB/NFIB*. Em dois casos (cac3 e cac6) os pontos de quebra foram observados somente na área transformada: cac3 foi fusão positiva e cac6 foi fusão negativa. Os casos cac5 e cac8 mostraram pontos de quebra em ambas as áreas: somente cac8 foi fusão positiva tanto nas áreas convencional quanto na área transformada. No cac4, o ponto de quebra foi visto somente na área convencional e foi fusão positiva (**Figura 3).**

A proteína MYB foi expressa em todos os oito casos de CAC-TAG, exceto na área transformada do cac3. Nas áreas convencionais, a marcação foi completamente nuclear com forte expressão nas células mioepiteliais e falta de expressão nas células ductais (**Figura 3**). As áreas transformadas mostraram menor expressão de MYB do que a área convencional em cinco casos. A alta expressão de MYB nas áreas transformadas foi observada nas células ductais e, nas células mioepiteliais MYB mostrou um padrão de expressão focal (**Figura 3**). Contudo, a diferença na expressão de MYB entre as áreas convencionais e transformadas do CAC-TAG não foram estatisticamente significante (p = 0.123, teste Wilcoxon). Os resultados da expressão imuno-histoquímica e a analise da fusão de MYB estão resumidas na **Tabela 2.**

Correlações clinicas e patológicas

A complexidade do perfil genético do tumor concorreu com o curso clínico do paciente. Os quatro casos com transformação em AMD mostraram um menor número de alterações no número de cópias. Entre elas, somente o cac4 mostrou metástase para fígado e pulmão após 2 anos. O

caso cac2 foi o com maior tempo de sobrevida sem recidivas ou metástases. Por outro lado, o grupo de CPD mostrou maior número total de alterações no número de cópias do que o grupo de AMD. Somente o cac7 mostrou curso clínico sem recidivas ou metástases embora somente 25 meses de seguimento estivesse disponível. Os casos cac6 e cac8 mostraram um maior número de alterações no número de cópias e pior curso clínico. Nenhuma diferença no aspecto clínicos ou patológico foi encontrado entre os casos com translocação positiva ou negativa de *MYB/NFIB* ou expressão de MYB. A expressão de MYB foi mais forte em ambas as áreas convencionais e transformadas do grupo de CPD comparado aos casos de AMD. Porém, somente as diferenças entre as áreas transformadas dos grupos CPD e AMD foram estatisticamente significantes (área convencional, $p = 0.083$ e área transformada, $p = 0.043$, teste Mann-Whitney), **Tabela 2**.

Discussão

A controvérsia a respeito da terminologia, desdiferenciação ou transformação para alto grau no CAC, reflete a incerteza sobre esse fenômeno. Independentemente do CAC, um número crescente de tipos de tumores de glândula salivar foram descritos sofrendo transformação para alto grau ao invés de desdiferenciação (Skálová et al, 2009; Roy et al, 2010; Costa et al, 2011a; Nagao et al, 2013). Contudo, outros tumores exibindo esse fenômeno, incluindo liposarcomas, neuroblastomas e carcinomas de mama, ainda são referidos como desdiferenciados (Helczynska et al, 2003; Coindre et al, 2010; Jögi et al, 2003). Assumindo que a desdiferenciação implica na regressão de uma célula terminalmente diferenciada para um estagio menos diferenciado dentro da sua própria linhagem (Jopling et al, 2011), então, a desdiferenciação de um tumor

convencional para um de alto grau poderia significar o próximo passo na progressão linear tumoral. Portanto, nós esperávamos encontrar alterações genéticas comuns na área convencional e transformada e alterações adicionais na área transformada. Porém, nossos resultados sugeriram que o componente de alto grau nem sempre progride diretamente da sua contraparte convencional e que o processo de transformação para alto grau pode ser resultado tanto de uma progressão linear ou paralela. Nossa análise do perfil genômico do CAC-TAG indicou que cinco casos podem ter progredido da área convencional para a transformada pela aquisição mais anormalidades genéticas na área transformada (cac1, cac2, cac3, cac6, cac7). Contudo, três casos (cac4, cac5 e cac8) mostraram perfis genéticos que não se enquadram em esse modelo de progressão. Eles aparentam ter uma progressão clonal até certo ponto, substanciado pela observação de alterações no número de cópias idênticas em ambos os componentes do tumor. Após este ponto, a progressão linear, não mais explica os eventos genéticos. O cac5 mostrou alterações genéticas idênticas em ambos os componentes, achados similares foram reportados em liposarcomas e carcinosarcomas (Vékony et al, 2009; Horvai et al, 2009). No cac8, a área convencional e transformada demonstrou tanto alterações no número de cópias comuns e únicas, sugerindo uma progressão paralela. O cac4 mostrou uma alteração em comum em ambos os componentes, e duas alterações adicionais somente na área convencional. Em esse último caso, as anormalidades genéticas que determinam a transformação para alto grau parecem terem sido adquiridas precocemente na tumorigênese ou pelo menos antes do início da alteração fenotípica (**Figura 4)**.

Uma relevante questão é qual as alterações genéticas no CAC-TAG são precoces, são eventos iniciadores do tumor e, quais alterações poderiam ser relacionadas com a progressão do tumor e comportamento clínico. Foi sugerido que alterações de número de cópias em todo cromossomo poderiam representar eventos precoces (Ricke et al, 2008). Nós encontramos seis dos oito casos com ganho em todo cromossomo, como no 8, 19, 6, 7, 16, 18, 20, 21 e 22 (**Figura 1 A e B),** em ambos os componentes. Contudo, um número de casos mostrou alterações em todo o cromossomo somente no componente transformado (cac1, cac2 e cac3), o que parece sugerir ser um evento tardio na progressão tumoral.

A translocação t(6:9)(q22-23; p23-24) resultando na fusão do oncogene *MYB com* o fator de transcrição NFIB foi recentemente considerado ser o maior e precoce evento no CAC clássico (Person et al, 2009; Mitani et al, 2010; Fehr et al, 2011; Brill et al, 2011; Mitani et al, 2011). Contudo, em nossos casos de CAC-TAG, a translocação *MYB/NFIB* não é, provavelmente, um evento precoce ou tardio. Primeiro, porque isso foi observado somente na área convencional ou somente na transformada e, segundo, porque o componente transformado pode ter surgido em um estágio precoce do desenvolvimento do tumor e ter seguido um caminho paralelo, adquirindo alterações genéticas independentemente do componente convencional. Isso implica que o CAC-TAG talvez não seja necessariamente um estágio mais avançado na progressão tumoral do CAC clássico.

Nossa análise do FISH confirmou a translocação *MYB/NFIB* em três do cinco casos onde a análise do microarray CGH indicou alterações no número de cópias simultâneas em 6q22-23/9p22-23 (**Tabela 2)**. Interessantemente, nestes três casos os pontos de quebra estavam

localizados proximal a *MYB*, enquanto que os dois casos que não apresentaram translocação apresentaram pontos de quebra distal a *MYB,* dentro do gene *AHI1*. Dentre os casos que apresentaram a translocação, os casos cac3 e cac4 apresentaram deleções terminais 6q23-qter, enquanto que o cac8 mostrou várias alterações no número de cópias em vários níveis ao longo do cromossomo 6, incluindo uma pequena deleção localizada dentro da banda 6q23.3, proximal a *MYB* e, englobando também os genes *ALDH8A1* e *HBS1L* (**Figura 1 A e B**). A maioria das translocações *MYB/NFIB* descritas na literatura mostram o ponto de quebra dentro do gene *MYB* (Persson et al, 2009; Mitani et al, 2010), embora mostrem também pontos de quebra próximos a *MYB*, ou seja, fora de *MYB* (Mitani et al, 2011). Os desequilíbrios genômico envolvendo 9p foram observados em cinco casos. Três casos (cac3, cac4 e cac5) mostraram ganho na região 9pter-p22.3 e, o cac8 mostrou um pequeno ganho em 9p23 (**Figura 1 A e B**). No cac6, a região 9pter-p22.3 foi deletada, bem como uma pequena região em 9p21.3-p13.1. Todos os cinco casos abrigaram números de cópias normais ou ganhos em 9pter. Essa variabilidade entre números de cópias envolvendo 9p não é incomum no CAC clássico e, indicam que para os rearranjos em 9p a preservação da parte telomérica de NFIB é crucial (Persson et al, 2012).

A expressão de MYB no CAC clássico foi observada previamente ocorrer tanto em tumores com fusão positiva ou negativa entre *MYB/NFIB* (West et al 2011). Entre nossos casos de CAC-TAG, somente o cac3 mostrou ausência na expressão de MYB na área transformada, mesmo este caso sendo a translocação positiva confirmada pelo FISH. A alta expressão de MYB no CAC clássico foi descrita em tumores com pontos de quebra da translocação centroméricos a *MYB,* semelhante ao cac4 e cac8 nesse estudo (West et al, 2011; Mukai et al, 2006). Em contraste, o

cac5 e o cac6 mostraram expressão positiva de MYB e não contém a translocação. Nas áreas convencionais a marcação foi completamente nuclear, semelhante ao descrito para o CAC clássico, ou seja, forte expressão nas células mioepiteliais e ausência de expressão nas células ductais (Mitani et al, 2010; West et al, 2011; Brill et al, 2011). Na maioria de nossos casos de CAC-TAG, o componente transformado mostrou menor expressão de MYB do que no componente convencional, exceto nos casos cac2 e cac8 **(Tabela 2)**, possivelmente porque existe uma perda parcial ou total da diferenciação mioepitelial nas áreas transformadas (West et al, 2011). Isso sugere que no CAC-TAG existem outros mecanismos de ativação de MYB, como já foi demonstrado em tumores de colón e mama (Ramsay et al, 2008). A expressão de MYB pode ser considerada um evento precoce, uma vez que está presente em ambos os componentes do tumor.

A correlação entre o número de aberrações cromossomais e o nível de diferenciação glandular do componente transformado no CAC-TAG (Costa et al, 2011b) foi confirmado nesse estudo maior. Os casos CPD contém mais genótipos complexos e ainda mostram um comportamento clinico mais agressivo. Esse achado suporta a ideia de que o CAC pode sofrer transformação em adenocarcinomas com varáveis níveis de diferenciação, ou até para outros tipos histológicos (Altemani et al, 2013), que não são sempre de alto grau, contribuindo para a controvérsia relacionada a este fenômeno.

Em conclusão, esse estudo forneceu novas compreensões sobre as vias de progressão tumoral do CAC-TAG, através da comparação do perfil genético entre os componentes convencional e transformado. Ênfase foi dado na translocação *MYB/NFIB*. Nós mostramos que o processo de

transformação para alto grau não é sempre acompanhado pelo acúmulo de alterações genéticas; pois ambos os componentes, convencional e transformado, abrigaram alterações genéticas únicas, o que indica uma progressão tumoral paralela. Nossos dados também demonstraram que a translocação *MYB/NFIB* não é necessariamente um evento precoce ou fundamental para a progressão do CAC-TAG, como sugerido previamente na literatura para o CAC clássico (Persson et al, 2009; Mitani et al, 2010).

Conflitos de interesse

Os autores declaram não ter conflitos de interesse.

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Capítulo 4

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Current concepts on dedifferentiation/high-grade transformation in salivary gland tumors

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Abstract

The concept of dedifferentiation had previously been used in salivary gland carcinomas. Recently, the term “high-grade transformation” was introduced for adenoid cystic carcinoma, acinic cell carcinoma, epithelial-myoepithelial carcinoma and polymorphous low-grade adenocarcinoma and may better reflect this phenomenon, although transformation into moderately differentiated adenocarcinoma (i.e. not 'high grade') has also been described. Among the immunohistochemical markers, Ki-67 seems to be the only one that can help distinguish between the conventional and transformed components; however, the combination of morphological criteria is still sovereign. The overexpression of p53 was observed in the

transformed component in all tumors types studied, despite few cases having been demonstrated to carry mutations or deletions in TP53 gene. Genetic studies in salivary gland tumors with dedifferentiation/high-grade transformation are rare and deserve further investigation. This review aims at providing an overview on the recent concepts in histopathological classification of salivary gland tumors, complemented by immunohistochemical and genetic findings.

1. Introduction

The concept of dedifferentiation was first proposed by Dahlin and Beabout in 1971 [1], when they described dedifferentiated chondrosarcoma as a distinct clinicopathologic entity characterized by a low-grade chondrosarcoma juxtaposed to a histologically different high-grade sarcoma [1]. Lately, dedifferentiation has been recognized in a variety of salivary gland carcinomas, including adenoid cystic carcinoma [2], mucoepidermoid carcinoma [3], myoepithelial carcinoma [4], epithelial-myoepithelial carcinoma [5] and acinic cell carcinoma [6].

Dedifferentiation is the progression of cells towards a less differentiated state in which the original line of differentiation is no longer evident [7]. The term dedifferentiation might not be properly used in epithelial tumors, especially when the dedifferentiated component is still recognizable as carcinoma or adenocarcinoma [8]. Recently, Seethala et al. introduced the term 'high-grade transformation' for adenoid cystic carcinomas. This term better reflects the fact that the dedifferentiated component often maintains some features of the original tumor, such as glandular differentiation [8-10]. In recent studies, our group and others have demonstrated that adenoid cystic carcinomas can also undergo transformation to adenocarcinomas which are not poorly differentiated, suggesting that also the term ‘high-grade transformation’ may not be adequate, at least in the case of adenoid cystic carcinoma [11,12].

Although considerable progress has been made in elucidating the genetic events that underlie the progression of many malignancies, those involved in salivary gland tumors are still poorly understood and the relationship between histological progression and genetic events is not well defined. The general theory of monoclonal evolution assumes that the mutational complexity of a tumor increases with time and, therefore, tumor genomes with the fewest chromosome aberrations contained the earliest mutations in tumor progression [13]. In contrast, high-resolution comparative genomic hybridization (CGH) microarrays have been used to study the genome structure of heterogeneous breast tumors and shown that they progress by different

genomic rearrangement patterns [14]. Thus, the genomic heterogeneity can be ascribed to genetically distinct subpopulations, which contain a set of common mutations (early events) that are inherited and persistent throughout their evolution, while events unique to the profiles are late [15]. However, genetic alterations cannot solely explain the histological heterogeneity in tumors. Epigenetic alterations, such as DNA hypomethylation in tumor cells causes chromatin decondensation and chromosomal rearrangements that may result in chromosomal instability. Moreover, DNA hypermethylation of CpG islands near the promoter regions silences specific genes including tumor suppressor genes in cooperation with histone modification [16]. Therefore, the histological heterogeneity could also involve modifications of epigenetic switch.

This paper will give an overview on the recent concepts in histopathological classification of salivary gland tumors in which dedifferentiation/transformation has been described: adenoid cystic carcinoma, acinic cell carcinoma, epithelial-myoepithelial carcinoma, polymorphous low-grade adenocarcinoma, mucoepidermoid carcinoma and myoepithelial carcinoma. Below follows a one-by-one description of these tumors and emphasis will be placed on immunohistochemical and genetic findings.

2. Clinical and morphological features

Since dedifferentiation/transformation is extremely rare, there are few data to establish how the prognosis compares to that of their conventional counterparts. Several clinical features have proven to be relevant, such as lymph node metastasis in adenoid cystic carcinoma and acinic cell carcinoma [8, 9], recurrence in polymorphous low-grade adenocarcinoma and mucoepidermoid carcinoma [3, 17], and metastasis in epithelial-myoepithelial carcinoma [10].

The majority of **adenoid cystic carcinoma (AdCC)** with transformation occurs during the sixth decade or later and most commonly involves the sinonasal mucoserous glands, palate and submandibular glands. This tumor shows a slight male predominance; unlike conventional AdCC and is often detected at an advanced stage due to extra-glandular or bone involvement. One the most important clinical features is the high propensity for lymph node metastasis (57% versus 5-25% in conventional AdCC), suggesting that this tumor should be also placed in the high-risk category for neck dissection [8, 18]. Until the time of writing, the literature revealed a total of 36 cases [2, 11, 12, 19-27]. The median survival of the largest reported series of AdCC with transformation, in which all cases were poorly differentiated carcinomas, was estimated at 12 months [8]. However, AdCC with transformation into moderately differentiated adenocarcinoma

seems to present a slower course (in some cases comparable to conventional AdCC), in contrast to AdCC with transformation into poorly differentiated carcinoma which usually shows a more aggressive clinical course [11, 12]. Seethala et al. were the first to establish morphological criteria for differentiating AdCC with high-grade transformation [8]. At least three major criteria are required, proliferation of tumor cells with at least a focal loss of myoepithelial cells surrounding tumor nests, nuclear size at least 2 -3 times the size of tubular /cribriform AdCC nuclei, thickened irregular nuclear membranes and prominent nucleoli in a majority of cells. The squamous areas, micropapillary and the loss of myoepithelial differentiation are considered unique morphological findings in the area transformed [8]. Based on the degree of gland formation (differentiation), cellular pleomorphism and mitotic activity, Bonfitto et al. and Costa et al. classified the transformed components into: moderately differentiated adenocarcinomas, (when at least 2/3 of the transformed component presented gland formation) and poorly differentiated carcinomas (gland formation was scarce or absent) [11, 12]. These morphological features are observed in Figure 1. The literature does not suggest a minimum percentage of the transformed component [8].

The first **acinic cell carcinoma (AcCC)** with dedifferentiation/high-grade transformation of salivary gland was reported by Stanley, in 1988 [6]. Thirty-five cases have been described in the literature [6, 9, 28-40] and most of them showed poor clinical outcome, but in 3 cases described it remained unclear [28; 41, 42]. All cases reported to date were of parotid gland origin with involvement of both the superficial or deep lobes. Especially those AcCC in the deep parotid lobe have been associated with a poor clinical outcome [34]. The median age of, 58 years, is higher that reported for conventional AcCC, 44 years [33]. In contrast with its conventional counterpart, AcCC dedifferentiation/high-grade transformation shows a slight male predominance, high recurrence rate and high propensity for cervical lymph node metastasis, suggesting a role for neck dissection in management of patients [9, 34].

Dedifferentiated AcCC (Figure 2) generally show conventional low-grade AcCC juxtaposed with high-grade carcinoma, which may be either poorly differentiated adenocarcinoma or undifferentiated carcinoma. Both solid and microcystic patterns of AcCC have been described in the low-grade component. The high-grade component generally shows a population of anaplastic cells with abundant cytoplasm, large polymorphic nuclei and loss of

acinar differentiation. Furthermore, comedonecrosis, vascular and perineural invasion are typically observed in AcCC [6, 9, 34, 35, 37].

Epithelial-myoepithelial carcinoma (**EMC**) is a biphasic tubular neoplasm of clear myoepithelial cells surrounding small lumina lined by ductal epithelial cells. Typically, this is a low-grade malignancy that mainly occurs in the parotid gland and exhibits distinctive subtypes including tubular, papillary, cystic, and solid patterns [43]. In EMC, myoepithelial as well as ductal epithelial cells can transform into a high-grade carcinomatous component [10] which has gone under a variety of terminologies such as high-grade carcinoma [44, 45], dedifferentiated [5, 46-50], myoepithelial anaplasia [48] and myoepithelial carcinoma arising in EMC [2]. Recently the term 'EMC with high-grade transformation' has been proposed for the all lesions where a more aggressive carcinoma is observed regardless whether it originated as a gradual transition or an abrupt transformation of the ductal or myoepithelial component [10]. The reasons for the adoption of this term were: the difficulties in defining criteria for the cellular classification of the high-grade component, the possibility that some tumors may have features of both cell types and the fact that these lesions uniformly show worse prognosis than typical EMC.

A common feature in many of transformed EMC also is a history of indolent growth prior to the development of transformation [49]. Patients with EMC containing high-grade transformation were older than conventional EMC patients (mean 75.9 years) and most commonly involved the parotid glands, with frequent extraglandular extension. Of all 17 cases reported in the literature, 61.5% were female versus 38.5% males. They appeared more aggressive than conventional EMC, mainly due to high propensity for lymph node and distant metastasis, prominent infiltrative growth pattern and higher proliferative activity. However, little follow up information is available, with a mean of 27.6 months (range: 3 to 72 months). The worse prognosis suggests the need for wider excision, neck dissection and adjuvant radiotherapy [5, 10, 44, 45, 47-50].

Five cases of **polymorphous low-grade adenocarcinoma (PLGA)** with transformation to high-grade carcinoma described in the literature showed significant similarities in the morphology and in the origin, minor salivary gland of palate. These cases underwent transformation to poorly differentiated adenocarcinomas characterized by a predominantly solid and cystic growth pattern, nuclear atypia with prominent nucleoli and foci of necrosis [17, 51, 52] Lloreta et al. described one more case originating in the nasal cavity and adjacent sinuses with

extensive areas of undifferentiated carcinoma consisting of compact epithelial cell nests with central necrosis [53]. The histological transformation seems to have occurred after a protracted clinical course with multiple recurrences, a late phenomenon in tumor progression [17, 52]. The possible role of radiation therapy as an initiator of this transformation may have been important in the three of the five published cases of transformation in PLGA [51, 52]. Despite the fact that high-grade transformation is recognized as an event with a more aggressive clinical course, only one case of PLGA in the literature died in consequence of the disease [53] and none showed metastasis thereafter [17, 51, 52].

The only two cases reported of dedifferentiated **mucoepidermoid carcinoma (MEC)** in the literature, both presented a biphasic histology comprising a high grade component and a low grade component separated by a transition zone [3, 54]. The high grade component exhibited solid nests and sheetlike growth patterns, without glandular or cystic structures. Sheets of undifferentiated anaplastic or sarcomatoid growth with marked pleomorphism, frequent mitoses (>50%) and extensive necrosis were also observed, Figure 3 [3, 54].

The first case described by Nagao et al, concerned a 55 year old man with a parotid gland tumor. Despite two recurrences within a short period after surgery, the patient remained alive during the next 10 years [3]. The second case located in trachea, conversely, rapidly metastasized to pleura, mediastinal lymph nodes, abdominal wall and vertebral bones leading to death an 11 year old girl in < 3 months from diagnosis [54]. With few cases in the literature, it is difficult to establish clinical correlations in relation to the conventional MEC, but dedifferentiated MEC seems to reach the same broad age range than the conventional counterpart.

One case of dedifferentiated **myoepithelial carcinoma (MCa)** was described by Ogawa et al in 2003 [4]. Two histologically distinct neoplastic cell populations were observed in the multinodular tumor of parotid gland. The first population was diagnosed as low-grade MCa and occupied more than 80% of the tumor. The second population consisted of polygonal or short spindle cells with pleomorphism as well as infiltration and high mitotic rate suggesting undifferentiated carcinoma. Moreover, these tumor cells lost the immunohistochemical characteristics of myoepithelial differentiation. A 59-year-old man presented first recurrence in the primary site after 5 months and a second was observed 4 months later, although radiation therapy was used. The patient is alive and metastasis was not recognized [4].

3. Immunohistochemical profile

3a. Myoepithelial markers

Myoepithelial cells exhibit dual epithelial and smooth muscle characteristics and traditionally are stained with antibodies against myoid proteins, such as α-smooth muscle actin (α-SMA), muscle-specific actin (HHF35), vimentin or calponin. Recently, p63 has become a popular marker for abluminal cells (basal cells and myoepithelial cells) [55-57]. In tumors with myoepithelial component, such as AdCC and EMC, the participation of myoepithelial cells in the dedifferentiated/ high-grade transformed areas seems to differ markedly. In AdCC, the loss of myoepithelial component has been used as one of the major criteria to identify the transformed areas [8]. Thus the expression of p63, α-SMA and calponin should be absent or at least focal in the high-grade component [8]. In contrast, in EMC the myoepithelial participation appears to be important in the high-grade component [10], although the cells show a heterogeneous expression of the myoepithelial markers (Table 1). S-100 protein and p63 have been found to be diffusely or focally positive in the transformed component in many dedifferentiated/high-grade transformed EMC whereas α-SMA was rarely detected and calponin negative [5, 10, 44, 45, 49]. It should emphasized that neoplastic myoepithelial cells present a great plasticity in terms of immunoprofile and in EMC, even the conventional tumors show differences regarding the expression of myoepithelial markers [48]. In these, p63 expression is more frequently encountered than SMA.

3b. Other markers

The immunoprofile of the dedifferentiation/high-grade transformation in salivary gland tumors has been evaluated in many studies (Table 1), however, with variable and therefore inconclusive results, possibly due to the small number of cases. The marker that best distinguished between the transformed and the conventional components was Ki-67, since in all salivary gland tumor studied, an increased proliferation index was detected in the transformed component when compared to the conventional counterpart [2, 3, 5, 8, 9, 11, 12, 17, 20, 21, 24, 26, 27, 35, 44, 48, 50, 54]. However, a distinct cut-off for the proliferation index that could identify the transformed component has yet to be established and probably it is variable among the different types of tumors. The expression of p53 in the transformed areas showed, in most of the cases and in most of the salivary tumor types, higher levels than in the conventional areas. Nevertheless, Di Palma et al (1999) and Henley et al (1997) showed negative expression of this

protein in the dedifferentiated/high-grade component of AcCC [34, 35]. Fonseca et al and Sarode et al also showed lack of specificity to p53 in both components of their cases of EMC [49, 50]. These inconsistent results suggest that TP53 alteration is not the only mechanism for transformation in salivary gland tumors, but may indicate a poor prognosis, similar to what is known for conventional AdCC [58]. Cyclin D1 is an important regulator of the G1 phase of cell cycle [59]. Positive expression was observed in dedifferentiated/transformed and conventional area of AdCC, AcCC and EMC [5, 8, 9, 20, 24, 27]. However, in the MEC, Subraniam et al. did not find positive expression in the dedifferentiated area [54]. The precise mechanisms responsible for the observed cyclin D1 overexpression in dedifferentiated/high-grade transformed salivary gland tumors (Table 1) are not fully established; a role in dedifferentiation of AdCC has been suggested in early studies [2, 20]. Gene amplification of cyclin D1 might contribute, as has been described in conventional AdCC [60].

Many other markers such as b-catenin [9], E-cadherin [26], pRB [27], BCL2 [17] and glucose transporters (GLUT) have been studied in individual tumors and deserve to be analyzed in other salivary gland tumors with dedifferentiation/high-grade transformation. GLUT1 has been considered a key molecule regulating the transport and metabolism of glucose. Overexpression of GLUT1 has been correlated with poor prognosis, tumor aggressiveness and lymph node metastases [61]. Bonfitto et al. showed increased expression of GLUT1 in the transformed area when compared to conventional area of AdCC, suggesting a change in metabolic state of cancer cells imposing an increased utilization of energy. However, the authors did not find any correlation between GLUT1 expression and clinical outcome [11]. In summary, the immunohistochemical differences between the conventional and transformed areas require further studies.

4. Molecular profile

Studies on the genetic changes in salivary gland tumors with dedifferentiation/high-grade transformation are rare. Only few cases have been demonstrated to carry mutations or deletions in TP53 gene by loss of heterozygosity (LOH), polymerase chain reaction (PCR), and microarray comparative genomic hybridization (CGH) [9, 12, 20, 27, 34, 35]. However, p53 positive immunostaining is often indicative of mutations in TP53 and was observed in the transformed component in all tumors types studied [2-5, 8, 9, 11, 12, 17, 20, 21, 27, 24, 26, 54]. Therefore, the evidence suggests that p53 abnormalities may be implicated in the process of

dedifferentiation, although its real importance in this process should be clarified by further molecularstudies.

Fluorescence in situ hybridization (FISH) analysis did not demonstrate gene amplification in the transformed area with mild overexpression of HER-2/neu protein in acinic cell carcinoma [9]. DNA content has been studied only in dedifferentiated/high-grade transformed AcCC and EMC. Aneuploid AcCC were associated with poor clinical outcome whereas no aneuploid tumor was found in the EMC group [30, 34, 35].

Using a high-resolution microarray CGH analysis in AdCC with high-grade transformation, a correlation between the number of chromosomal aberrations and the degree of gland differentiation of the transformed component was found. The AdCC with transformation in moderately differentiated carcinomas carrying one single abnormality, whereas the AdCC with transformation in poorly differentiated carcinomas showed a higher number of alterations. These findings suggest that the AdCC with high-grade transformation may not necessarily reflect a more advanced stage of tumor progression, but rather a transformation to another histological form, which encompasses a wide spectrum of carcinomas in terms of aggressiveness [12]. A comparison of the microarray CGH results of the transformed and the conventional component in two cases of AdCC with high-grade transformation (unpublished data on cases in reference 12), showed identical genetic profiles. A search in the literature shows that this finding is not unusual. Among others, examples are dedifferentiated liposarcomas and biphasic carcinosarcomas [62, 63]. This indicates that the genetic abnormalities have been acquired early in tumorigenesis, or at least before the start of the phenotypic change. It may be speculated that the genetic changes not detectable by microarray CGH analysis such as mutation or epigenetic silencing underlies the phenotypic change.

In conclusion, the dedifferentiation/high grade transformation in salivary gland tumors seems to be a more complex process than simple progression through histological grades.
Currently, the most useful tool in identifying the transformed component is still a combination of morphological criteria aided by Ki67 expression analysis.

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Table 1. Immunoprofile of dedifferentiated/high-grade transformed areas in salivary gland tumors

Antibodies	Immunoprofile of high-grade transformed areas					
	AdCC	AcCC	EMC	PLGA	MEC	MCa
Proliferative antigen						
Ki-67	HI	HI	HI	HI	HI	HI
Cytokeratins						
AE1-AE3	+	+/F	+	+	+/-	F
CAM 5.2	+	NA	+/F	+	-	NA
34bE12	+	NA	F	NA	NA	NA
CK 5/6	NA	-	+	NA	NA	NA
CK 7	NA	-	+/-	+	NA	NA
CK 14	-/F	-	-/F	NA	NA	NA
CK 20	NA	-	NA	-	NA	NA
Myoepithelial cell						
S-100	+/F/-	+/-	+/F/-	+/-	-	-
α-SMA (alfa smooth muscle actin)	-/F	-	-/F	-	-	-
p63	-/F	NA	+/F/-	+/-	NA	NA
Vimentin	NA	+	+/-	+/-	NA	+
Calponin	F	-	-	-/F	NA	NA
HHF35 (muscle-specific actin)	-/F	NA	-	NA	NA	NA
Desmin	NA	NA	-	-	NA	NA
Cell Cycle Control						
p53	HI/+	HI/-	+/-	HI	HI/F	+
Cyclin D1	+/F	HI	+	NA	NA	+
Membrane Receptors						
C-kit (CD117)	+/-	-	NA	NA	-	NA
HER2/neu (c-erbB2)	+/-	+	-	+	-	NA
EMA (epithelial membrane antigen)	+	NA	+	+	+	NA
Structural Proteins						
GFAP (glial fibrillary acidic protein)	-/F	NA	-	+/-	-	NA
Cell Adhesion Proteins						
CEA (carcino embryonic antigen)	-/F	NA	+	+/-	-	NA
Steroid receptor						
Androgen receptor	-	-	NA	+/F	NA	NA

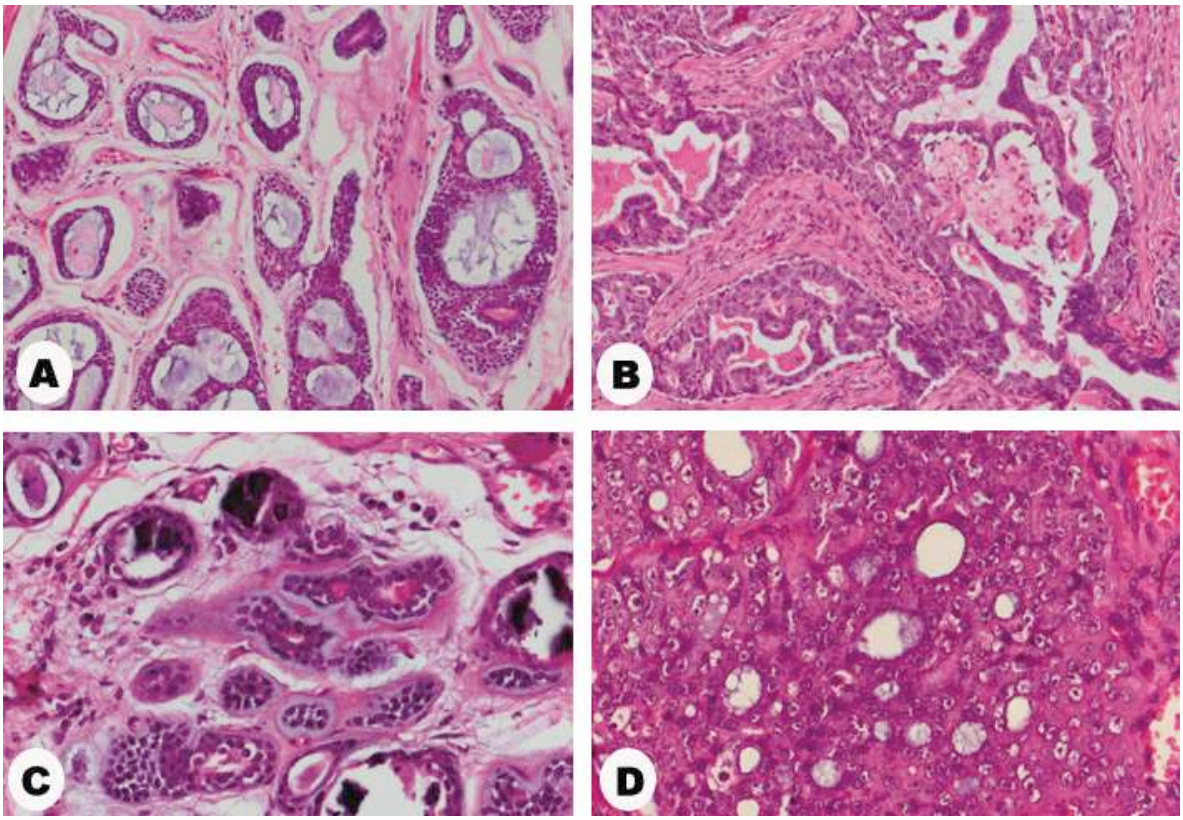

Figure 1. Adenoid cystic carcinoma with transformation to a moderately differentiated adenocarcinoma (A, B) and to poorly differentiated carcinoma (C, D). A and B, H&E original magnification 200x and C and D, 400x.

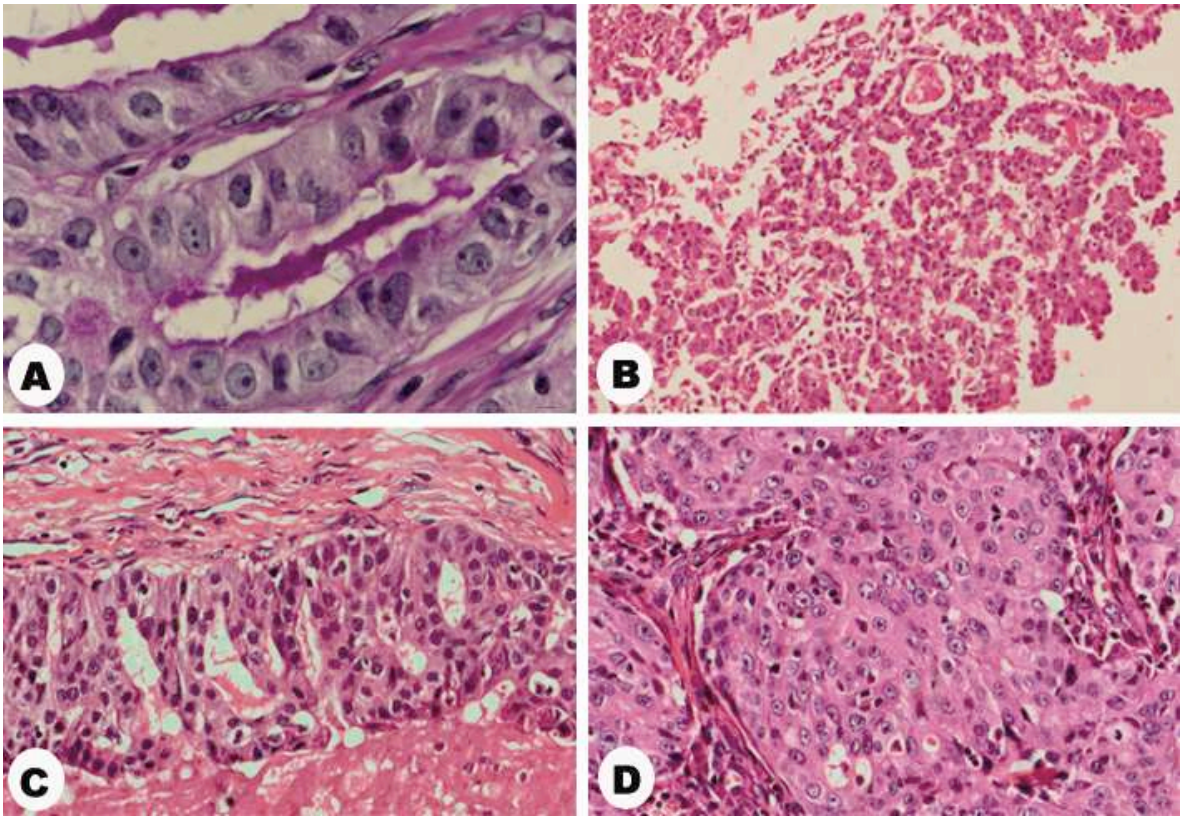

Figure 2. Acinic cell carcinoma with high-grade transformation to undifferentiated carcinoma. A, B and C, conventional component; D, high-grade transformed component. A, PAS original magnification 1000x; B, H&E original magnification 100x; C and D, H&E original magnification 400x.

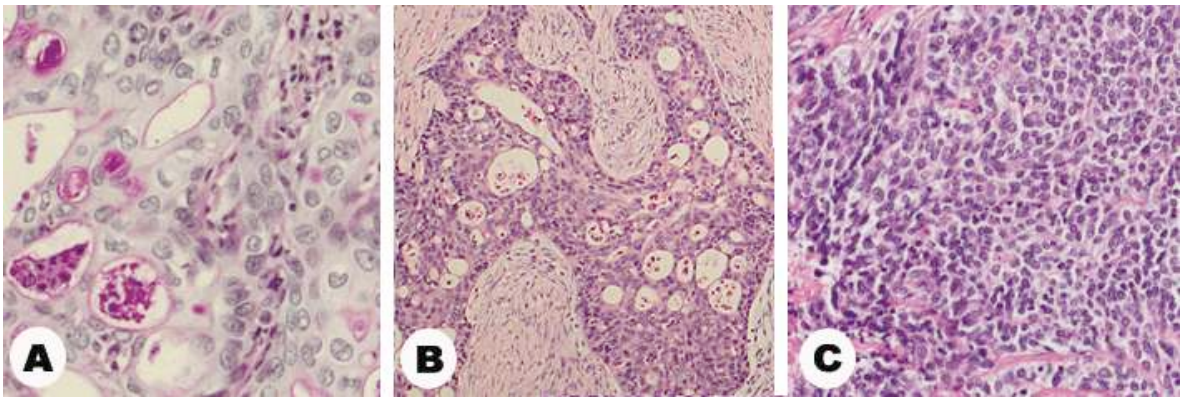

Figure 3. Mucoepidermoid carcinoma with dedifferentiation to undifferentiated carcinoma. A and B, conventional component; C, dedifferentiated component. A, PAS original magnification 400x; B, H&E original magnification 100x; C, H&E original magnification 200x.

Discussão

Os achados mais importantes de cada artigo publicado são discutidos a seguir.

Capítulo 1. Levels and patterns of expression of hypoxia-inducible factor-1α, vascular endothelial growth factor, glucose transporter-1 and CD105 in adenoid cystic carcinomas with high-grade transformation.

O HIF-1α, além de desempenhar um papel essencial na homeostase (Iyer et al., 1998), também é expresso na maioria dos cânceres humanos mais comuns (Zhong et al., 1999). No entanto, a frequência de positividade HIF-1α é altamente variável entre os tumores e, em alguns tipos, tais como o carcinoma endometrióide do endométrio (Seeber et al., 2006) e do carcinoma da mama invasivo pobremente diferenciado (Bos et al., 2001), a expressão da proteína foi detectada em todos os casos. Em nosso estudo observamos que em 100% dos CAC clássicos e dos transformados exibiram expressão HIF-1α, enquanto que no tecido da glândula salivar normal adjacente ao tumor, a expressão foi apenas ocasionalmente observada. Observamos também, ausência de alteração significante nos níveis de expressão do HIF-1α, VEGF e CD105 entre: (i) a área transformada e convencional do CAC-TAG e (ii) entre a área convencional do CAC-TAG e o CAC clássico. Portanto, esses achados sugerem que estes fatores não são importantes no processo de transformação do CAC. Além disso, na nossa série, a ausência de correlação entre o grau de expressão do HIF-1α e metástases e, também, recorrências, sugere que a expressão da proteína HIF-1α não pode ser utilizada como um marcador de agressividade tumoral. Quanto aos padrões de expressão de HIF-1α e GLUT-1, notamos que independentemente de sua classificação histológica, CAC clássico ou CAC-TAG, todos os casos apresentaram um padrão difuso de expressão HIF-1α e, na maioria dos casos nenhuma expressão positiva de GLUT-1 foi encontrada. Na literatura, o padrão difuso de expressão de HIF-1α tem sido associado à indução do fator por mecanismos independentes dos níveis de oxigenação. Portanto, possivelmente, a hipóxia não é o fator indutor principal da estabilização de HIF-1α no CAC. Em relação a GLUT-1, somente encontramos um padrão proestromal, que acreditamos ser também induzida por mecanismos não-hipóxicos.

Concluindo, considerados em conjunto, nossos achados sugerem que a hipóxia não desempenha um papel fundamental no processo de transformação para alto grau do CAC. Embora a expressão de HIF-1α seja usual no CAC, ela não pode ser usada como marcador de agressividade.

Capítulo 2. Genetic profile of adenoid cystic carcinomas (ACC) with high-grade transformation versus solid type.

O CAC-TAG é considerado uma variante agressiva do CAC clássico, tão ou mais agressivo que o CAC com padrão de crescimento sólido. A média de sobrevida destes tumores é estimada em 12 meses (Seeethala et al., 2007), enquanto que no subtipo sólido é de aproximadamente 36-48 meses (Szanto et al., 1984). Além disso, o CAC-TAG apresenta uma alta propensão à metástases ganglionares, diferentemente do CAC clássico, onde a taxa de envolvimento de linfonodos varia de 5% a 25% (El-Naggar e Huvos, 2005). A alta incidência de metástases para linfonodos no CAC-TAG sugere que estes pacientes deveriam ser submetidos ao esvaziamento cervical (Seethala et al., 2007). Contudo, tem-se defendido que o CAC-TAG pode sofrer transformação para adenocarcinomas que não são de alto grau, o que poderia influenciar seu comportamento biológico (Bonfitto et al., 2010).

O presente estudo teve como objetivo comparar as alterações genéticas das áreas transformadas de seis casos de CAC-TAG com cinco casos de CAC sólido, através da técnica de hibridização genômica comparativa em microarranjo e, correlacioná-las com o prognóstico de ambos os tumores.

Nossos resultados não deram suporte à hipótese de que o CAC-TAG, seja mais agressivo do que o subtipo sólido do CAC clássico (Seethala et al., 2007), mesmo quando a comparação foi feita entre o grupo de CAC-TAG com transformação para carcinomas pobremente diferenciados e o CAC sólido. Nossos dados sugeriram que o curso clínico no CAC-TAG é dependente da quantidade de anormalidades cromossômicas acumuladas e, os tumores pobremente diferenciados apresentaram uma complexidade genética semelhante ao CAC sólido. Portanto, nossos resultados corroboram a hipótese que o CAC-TAG não necessariamente deve refletir um estágio mais avançado na progressão tumoral, mas sim uma transformação em outra forma histológica, que engloba um amplo espectro de carcinomas em termos de agressividade, como sugeriu Bonfitto e colaboradores (Bonfitto et al., 2010). Com base nisto, o termo transformação para alto grau pode não ser o mais adequado para estes tumores, principalmente quando o CAC sofre transformação para adenocarcinoma moderadamente diferenciado.

Capítulo 3. Analysis of MYB oncogene in transformed adenoid cystic carcinomas reveals distinct ways of tumor progression.

Os mecanismos moleculares que engatilham a transformação morfológica nos tumores salivares ainda continuam obscuros. Este estudo inclui quatro casos que fizeram parte do artigo anterior (Capítulo 2), onde nós somente estudamos a área transformada dos tumores. Para este trabalho, acrescentamos mais dois casos (total de 8 casos) e, ambas as áreas, convencional e transformada, foram estudadas (total de 16 áreas). Utilizamos as técnicas de hibridização genômica comparativa em microarranjo, hibridização fluorescente in situ (FISH) e imuno-histoquímica. Atenção especial foi dada a expressão da proteína *MYB* e a translocação cromossomal entre *MYB/NFIB* t(6q23; 9p22), que atualmente está sendo considerada ser o maior e mais precoce evento no CAC clássico (Persson et al., 2009; Mitani et al., 2010; West et al., 2011; Fehr et al., 2011; Brill et al., 2011).

Nossos achados revelaram que o processo de transformação para alto grau não é sempre acompanhado pelo acúmulo de alterações genéticas, ambas as áreas, convencional e transformada abrigam alterações genéticas únicas, que indicam uma progressão tumoral paralela. Nossos resultados também demonstraram que a translocação entre *MYB/NFIB* não é necessariamente um evento precoce ou fundamental para a progressão do CAC-TAG, como é sugerido na literatura para o CAC clássico (Persson et al., 2009; Mitani et al., 2010).

Capítulo 4. Current concepts on dedifferentiation/high-grade transformation in salivary gland tumors.

O estudo advindo dos CAC-TAG também nos permitiu fazer uma revisão sobre o assunto. Neste estudo fizemos um panorama sobre os recentes conceitos na classificação histopatológica dos tumores de glândula salivar com desdiferenciação/transformação para alto grau descritos na literatura. Destaque também foi dado aos achados imuno-histoquímicos e genéticos que podem ajudar no diagnóstico de cada um destes tumores. Nossa conclusão foi que a ferramenta mais poderosa na identificação do componente transformado, ainda é a associação entre os critérios morfológicos, variável para cada tipo de tumor salivar e, a diferença na expressão do marcador imuno-histoquímico para proliferação celular Ki-67, entre a área convencional e transformada do tumor. Em relação aos aspectos genéticos destes tumores, mais estudos são necessários para elucidar este fenômeno, que parece ser um processo muito mais complexo do que uma simples progressão através de graus histológicos.

3

Conclusões

1. A ausência de uma alteração significante nos níveis de expressão do HIF-1α, VEGF e CD105 e os padrões de expressão de HIF-1α e GLUT-1 sugerem que a hipóxia pode não desempenhar um papel fundamental no processo de transformação para alto grau no CAC.

2. O CAC-TAG não reflete necessariamente um estágio mais avançado na progressão tumoral, mas uma transformação em uma outra forma histológica, na qual os tumores pobremente diferenciados apresentam uma complexidade genética semelhante ao CAC sólido.

3. O processo de transformação para alto grau não é sempre acompanhado pelo acúmulo de alterações genéticas; ambos os componentes, convencional e transformado, abrigam alterações genéticas únicas, que indicam uma progressão paralela. A translocação entre *MYB/NFIB* não é necessariamente um evento precoce ou fundamental para a progressão do CAC-TAG.

4. A ferramenta mais útil na identificação da área transformada dos tumores de glândula salivar com desdiferenciação/transformação para alto grau continua sendo a associação dos aspectos morfológicos (variável para cada tipo de tumor) e a maior expressão do Ki-67 na área transformada.

Artigos relacionados

Além dos artigos anteriormente mencionados, colaborei com o desenvolvimento de outros artigos com enfoque no CAC-TAG, nos quais sou coautora. Os artigos são:

1. Bonfitto VL, Demasi AP, **Costa AF**, Bonfitto JF, Araujo VC, Altemani A. High-grade transformation of adenoid cystic carcinomas: a study of the expression of GLUT1 glucose transporter and of mitochondrial antigen. J Clin Pathol. 2010 Jul; 63(7): 615-9.

2. Altemani A, **Costa AF**, Montalli VA, Mosqueda-Taylor A, Paes de Almeida O, León JE, Hermsen M. Signet-ring cell change in adenoid cystic carcinoma: a clinicopathological and immunohistochemical study of four cases. Histopathology. 2013 Mar; 62(4): 531-42.

Artigo relacionado 1

Artigo publicado na revista Journal of Clinical Pathology 2010 Jul;63(7):615-619

Reprinted from Journal of Clinical Pathology, 2010, Jul; 63(7): 615-9, High-grade transformation of adenoid cystic carcinomas: a study of the expression of GLUT1 glucose transporter and of mitochondrial antigen, Copyright 2010, with kind permission from BMJ group.

High-grade transformation of adenoid cystic carcinomas may result in adenocarcinomas with wide spectrum of differentiation. A study of the expression of GLUT1 glucose transporter and of mitochondrial antigen in the transformed component.

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Abstract

Aims: To broaden our understanding of phenomena involved in progression from classical adenoid cystic carcinomas (ACC) to tumors with high-grade transformation (ACC-HGT) we analyzed expression of proteins linked to cellular metabolism as well as the microvascular density (MVD) in conventional and transformed areas. We also compared the findings with ordinary ACCs.

Methods: In seven cases of ACC-HGT and in 18 ACCs the expressions of GLUT1, mitochondrial antigen (MTA), CD34 (for assessing MVD), α-SMA and P63 (for detection of myoepithelial cells) and Ki-67 (for evaluation of proliferation index) were examined.

Results: The transformed component corresponded to adenocarcinomas with frequent (4 cases) or scarce/ absent (3 cases) gland differentiation. In the latter, Ki-67 index was higher, two patients presented lymphatic metastasis and one died of disease. In the former, there was one long-term survivor and one with liver metastasis. Conventional areas of both ACC-HGT and ACC were negative for GLUT1 in most cases (83.3% and 81.3%, respectively) and exhibited low or no expression of MTA (100% and 66.7% of cases respectively). In contrast, the HGT component presented increased expression of both proteins (GLUT1+ in 50% of cases; MTA+ in 100%). However, the degree of GLUT1 expression did not correlate with clinical outcome. MVD did not differ significantly between conventional and transformed components.

Conclusions: transformation of classical ACC into ACC-HGT encompasses adenocarcinomas with variable degrees of differentiation and seems to lead to metabolic changes without reflection in tumor vasculature. Despite the tumors' higher GLUT1 expression, this protein has no utility as prognostic marker.

INTRODUCTION

Adenoid cystic carcinoma (ACC) is the second most common malignancy of the salivary glands. This tumor shows a dual cellular composition, i.e., ductal (luminal) and myoepithelial cell differentiation, and presents three major growth patterns: tubular, cribriform and solid.[1]. In terms of biological behavior, ACC is characterized by a prolonged clinical course, with frequent local recurrences, late onset of metastases, and fatal outcomes.[1] Metastases are predominantly of the sanguineous type whereas lymphatic ones are infrequent.[1]

In contrast, ACCs containing areas with a pleomorphic high-grade proliferation of tumor cells can show an accelerated clinical course with a high propensity for lymph node metastases. This phenomenon, which has been referred to as dedifferentiation or high-grade transformation (HGT), is uncommon and there have been only 26 reported cases so far. [2-10] The dedifferentiated component shows loss of myoepithelial cell differentiation and usually presents the morphologic features of a poorly differentiated adenocarcinoma or solid carcinoma. [2, 3, 7]

In cancer biology, a growing body of evidence has suggested that many of the genes presenting expression modification during tumorogenesis are intricately linked to metabolic regulation.[11] In tumor cells the best known energy metabolism alteration is an increased glycolytic capacity. For this process, glucose transporters (GLUT) have been considered key molecules regulating the transport and metabolism of glucose.[12] There are several isoforms of GLUT expressed in mammalian cells of which GLUT1 is the most common and is overexpressed in a significant proportion of human carcinomas.[13] In some of these tumors, GLUT1 expression has been reported to correlate with poor prognosis, tumor aggressiveness and lymph node metastases.[14-17]

In order to broaden our understanding of phenomena involved in progression from classical ACC to tumors with HGT (ACC-HGT), we analyzed expression of proteins linked to cellular metabolism (GLUT1, mitochondrial antigen) as well as the microvascular density (MVD) in conventional and transformed areas. MVD was evaluated because it has been considered to reflect the metabolic burden of the supported tumor cells.[18] We also compared the findings with ordinary ACC and looked at the clinical follow up of the patients.

MATERIAL AND METHODS

The present study was performed in 25 ACCs samples (7 ACC-HGT and 18 ordinary tumors) diagnosed between 1994 and 2009. The transformed areas were identified according to the recommendations of Seethala et al and in all cases at least 3 of the major criteria were present.[2] Demographic and clinical information was obtained from the patients' medical records.

Immunohistochemistry

One paraffin block from each case was chosen for the immunohistochemical study. Table 1 shows the antibodies that were used in the current series. Sections (3μm) from the paraffin blocks were deparaffinized in xylene, rehydrated through descending ethanol series and submitted to heat-induced antigen retrieval (AR) in water bath with citrate pH 6.0 buffer solution for 30 minutes, except for Ki-67. For Ki-67 AR was performed using Tris-EDTA instead of citrate. For all antibodies, sections were immersed in 0.3% hydrogen peroxide in methanol and incubated with primary antibody.

Evaluation of Staining

A) MVD and Ki-67 positive cell counting

In each CD34 and Ki-67 stained sections, three hotspot areas were chosen for counting of vessels and positive cells, respectively, at 40X magnification. MVD was considered the mean number of vessels in these areas in each sample. Ki-67 index was considered as the percentage of positive cells in relation to all tumor cells in these three areas in each sample.

B) Quantification of GLUT1 and mitochondria antigen (MTA) expressions in carcinoma cells

In each stained section, the relative numbers of neoplastic GLUT1+ and MTA+ cells were considered: a) in ordinary ACC in relation to all neoplastic cells, b) in ACC-HGT in relation to all neoplastic cells of conventional and transformed areas respectively. When the number of positive cells was more than 10%, the case was judged positive and the quantity of stained neoplastic cells were assessed using a three-tiered scale: >10% - 25%, and > 25 – 50% and >50% of positive cells.

Statistical Analysis

For comparison of MVD between: a) conventional and HGT areas of ACC-HGT and b) ordinary ACC and conventional areas of ACC-HGT, the Wilcoxon´s Signed Rank Test was used. Data were presented as mean + SD, and the results with $p< 0.05$ were considered significant. Statistical analysis was performed with the SAS System for Windows, version 8; SAS Institute Inc., Cary, NC, USA.

RESULTS

Table 2 shows the main clinical and pathological findings in the ACC-HGT group. The transformed areas presented variable degrees of gland differentiation and did not show any feature that allowed classifying them as a specific carcinoma of the salivary gland. These areas were termed as (Fig 1): moderately differentiated adenocarcinomas (MDAd) when at least 2/3 exhibited gland differentiation and poorly differentiated carcinomas (PDC) those with scarce or absent gland differentiation. Ki-67 index was higher in the transformed component (table 2). Stromal reaction of desmoplastic type and foci of tumor necrosis were found only in the transformed component in 85.7% (6/7) and 71.4% (5/7) of cases respectively.

Expressions of GLUT1 and MTA in carcinoma cells

Table 4 shows the amount of positive cells for MTA (Fig 2A and B) and GLUT1 (Fig 2 C and D) in ACC-HGT group and in ordinary ACCs. In ACC-HGT group, patients with GLUT1+ tumors (3 cases) showed clinical outcome similar to those with negative tumors (3 cases): in each subgroup one patient died and two were alive without showing evidence of disease. In ordinary ACC group, 8 out of 10 patients with GLUT1 negative tumors showed local recurrence or distant metastases whereas 1 out of 2 patients with GUT1+ tumors presented distant metastases.

MVD

Figure 3 illustrates CD34-MVD in ACC-HGT group. In all cases but one, conventional areas showed higher MVD (mean 19.28, SD 7.78) than HGT component (mean 13.05, SD 5.72), although the differences were not significant ($p=0.1563$). CD34-MVD in ordinary ACC was similar (mean 19.62, SD 8.74) to that detected in conventional areas of ACC-HGT ($p=0.9734$).

DISCUSSION

The main point of interest in ACC-HGT lies in their proposed poor prognosis and higher propensity for lymph node metastasis.[2] In the current study we found that ACC-HGT presented altered expression of proteins linked to cellular metabolism and encompassed a wide spectrum of tumors in morphological appearance and probably in biological behavior as well.

In the literature, the transformed component of ACCs has been described as poorly differentiated tumors, particularly adenocarcinomas or solid carcinomas.[2-10] In our series, the transformed component was demarcated from typical ACC according to the criteria of Seethala and collaborators and, thus, showed: a) nuclei with marked enlargement (more than 2-fold when

compared to tubular/ cribriform ACC) and size variation, b) prominent nucleoli and c) loss of myoepithelial differentiation (at least focal).[2] However, using criteria similar to those adopted to classify adenocarcinomas not otherwise specified [19], we could clearly separate ACC-HGT into two groups based on the degree of gland differentiation of the transformed component: moderately (cases 1-4) and poorly differentiated (cases 5-7) lesions (MDAd and PDC respectively). As expected, an inverse correlation was found between the degree of gland differentiation and proliferation index (Ki-67 index ranged from 12.5% to 33.6% in MDAd and from 46.7% to 72.3% in PDC).

Furthermore, despite the small number of cases and short follow-up period in some, patients' outcome suggested that the degree of differentiation of the transformed component, and consequently the proliferation index, might influence tumor biological behavior. The PDC group showed a more aggressive clinical course with lymph node metastases, reinforcing data previously reported regarding ACC-HGT.[2, 3, 7] However, the MDAd group seemed to present a slower course so that in some cases it could be comparable to that of classical ACC (see case 2).

A similar phenomenon is described in pleomorphic adenoma that undergoes transformation to carcinoma, in spite of the fact that in this entity transformation refers to progression from benign to malignant tumor. Most carcinomas arising in pleomorphic adenoma (CXPAs) are aggressive, but 15% may behave in a more indolent fashion.[20] These differences in behavior of CXPAs seem to be related to differences in malignant potential of carcinomas arising in pleomorphic adenomas and we believe that a similar phenomenon may occur in transformed ACCs. The striking differences between case 2 and 5 in terms of phenotype of the transformed component and clinical outcome lend support to this assumption. However, further molecular and cytogenetic studies are necessary to confirm this hypothesis.

Malignant cells present increased glycolytic metabolism and it has been shown that GLUT1 plays an important role in the uptake and metabolism of glucose by cancer cells.[21] In neoplasia, GLUT1 expression is believed to be a marker for tumor progression and aggressiveness as well as an indicator of increased metabolic state and enhanced utilization of energy.[14, 16, 21, 22] In the current study, the transformed areas of ACCs showed increased expressions of GLUT1 and MTA when compared to: a) conventional areas in the same tumor and b) ordinary ACCs. These findings suggest that the transformation of ACC into ACC-HGT

changed the metabolic state of cancer cells imposing an increased utilization of energy. It has been proposed that an enhanced utilization of energy may correlate with an aggressive behavior of cancer cells and in some tumors, such as colorectal, lung and hepatocellular carcinomas, expression of GLUT1 has been associated with poor prognosis.[14-16, 21, 22]

However, in our cases we did not find any correlation between GLUT1 expression in ACC-HGT and clinical outcome. For some types of carcinomas, not only the mere presence, but the degree of GLUT1 expression is important since it correlates with tumor aggressiveness. For example, patients with colorectal carcinomas featuring high GLUT1 expression (>50% of positive cells) did show significantly higher mortality and lymph node metastases.[16, 22] Therefore, we believe that the increased expression of GLUT1 in the transformed component of ACCs reflects higher energy production, but as this happens in a small proportion of cells (<50%), this ultimately has no implication in tumor aggressiveness.

Metabolic demand but not tumor growth rate has been considered a major factor contributing to vessel density in the cancer tissue [18] so that MVD would reflect the metabolic burden of the supported tumor cells. In our cases, the transformed areas did not present significant differences in relation to the conventional ones. These findings could be interpreted as unexpected since our data in the transformed component suggest an augmentation of metabolic demand. However, tumor vascularization is a complex process that is also influenced by the crosstalk between cancer and stroma cells [23] A recent study in pancreatic ductal adenocarcinoma has shown that stromal cells can amplify production of angiogenic inhibitor by cancer cells resulting in a hypovascular stroma.[23] We speculate that in the transformed areas, the crosstalk between cancer and stroma cells would be modified as a consequence of alteration in the tumor cell composition, leading to a desmoplastic stroma with low vascular density. Lower vascular density along with higher metabolic demand could be an explanation for necrotic foci (hypoxic tissue) found in the transformed areas.

In conclusion, transformation of classical ACC into ACC-HGT encompasses adenocarcinomas with variable degrees of differentiation and seems to lead to metabolic changes without reflection in tumor vasculature. Despite the tumors' higher GLUT1 expression, this protein has no utility as a prognostic marker.

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Table 1: Details of the antibodies used for immunohistochemistry.

Specificity	Clone	Dilution	Source	Buffer (AR)	Detection
MTA£	MTCO2	1:200	Abcam†	Citrate	Mitochondria
GLUT1	Polyclonal	1:400	Abcam†	Citrate	Glucose transporter 1
CD34	QBEnd 10	1:50	Dako*	Citrate	Endothelial cells
α-SMA	1A4	1:200	Dako*	None	Myoepithelial cells
P63	4A4	1:75	Neomarkers ¥	Citrato	Myoepithelial cells
Ki67	MIB1	1:150	Dako*	Tris-EDTA	Celular proliferation

† Abcam Inc., Cambridge, USA; *Dako Corporation, Glostrup, Denmark; ¥ Neomarkers, Fremont, USA.

£ MTA is an antibody against Cytochrome C oxidase subunit II, which is located in mitochondrion inner membrane and is one of the components of the mitochondrial respiratory chain.

Table 2. Clinicopathologic parameters in 7 cases of ACC-HGT

Case	Age	Sex	Site	T	Positive Margins	Treatment	Local Recurrence	Cervical LN Metastasis	Distant Metastasis	Follow-up (mo)	Outcome	Histological Features		Glandular Differentiation	KI-67 index	
												CA	TA (%)		CA (%)	TA (%)
1	44	F	Submandibular	T2	Yes	SE+RT	No	No	No	18	NA	T/C	MDAd (10)	frequent	13,7	12,5
	55	F	Palate	T4	Yes	SE+RT	No	No	No	140	NED	S	MDAd (50)	frequent	8,5	20,8
3	65	M	Paranasal sinus	T4	Yes	SE+RT	No	No	No	8	Dead	S	MDAd (50)	frequent	29	32
4	49	F	Parotid	T3	No	SE+RT	No	No	Yes (liver)	33	Alive	S	MDAd (40)	frequent	27,9	33,6
5	64	F	Submandibular	T2	Yes	SE+RT	No	Yes	Yes (liver)	7	DOD	S	PDC (60)	scarce	13	46,7
6	58	F	Lips	T2	Yes	SE	No	No	No	18	NED	T/C	PDC (80)	scarce	7,4	58,4
7	47	M	Palate	T4	Yes	SE	No	Yes	Yes (lung)	12	Alive	S	PDC (80)	absent	34,3	72,3

ACC-HGT, adenoid cystic carcinoma with high grade transformation; DOD, died of disease; F, female; LN, lymph node;M, male; MDAd, moderately differentiated adenocarcinoma; Mo, months; NA, not available; NED, no evidence of disease;PDC, poorly differentiated carcinomas; RT, radiotherapy; S, solid;SE, surgical excision; T, TNM classification, TA, transformed area; T/C, tubular/cribriform; UC, undifferentiated carcinoma.

Table 3. Clinicopathologic parameters in 18 cases of ordinary ACC

Parameters		Number of cases
Gender	Female	13
	Male	5
Tumor location	Parotid	1
	Submandibular	4
	Oral cavity	5
	Larynx	1
	Lacrimal gland	1
	Rhinopharynx	1
	Information not available	5
Treatment	Surgical excision	13
	Radiation therapy	12
	Information not available	5
Clinical outcome	Local recurrence	5
	Distant metastasis	5
	Lymph node metastasis	0
	Death	0
	Information not available	4
Tumor histological subtype	Tubular/cribriform	16
	Solid	2
TNM classification	T1	5
	T2	1
	T3	0
	T4	6
	Information not available	6

Table 4. Expressions of GLUT1 and mitochondrial antigen (MTA).
ACC-HGT, adenoid cystic carcinoma with high grade transformation; CA, conventional area; TA, transformed area.

Markers \ Amount of positive cells		<10%	10-25%	>25-50%	>50%
MTA					
Ordinary ACC		5/15 (33,4)	5/15 (33,3)	2/15 (13,3)	3/15 (20,0)
ACC–HGT	**CA**	2/6 (33,3)	4/6 (66,7)	0/6 (0,0)	0/6 (0,0)
	TA	0/6 (0,0)	0/6 (0,0)	1/6 (16,7)	5/6 (83,3)
GLUT1					
Ordinary ACC		13/16 (81,3)	1/16 (6,3)	2/16 (12,4)	0/16 (0,0)
ACC–HGT	**CA**	5/6 (83,3)	1/6 (16,7)	0/6 (0,0)	0/6 (0,0)
	TA	3/6 (50,0)	2/6 (33,3)	1/6 (16,7)	0/6 (0,0)

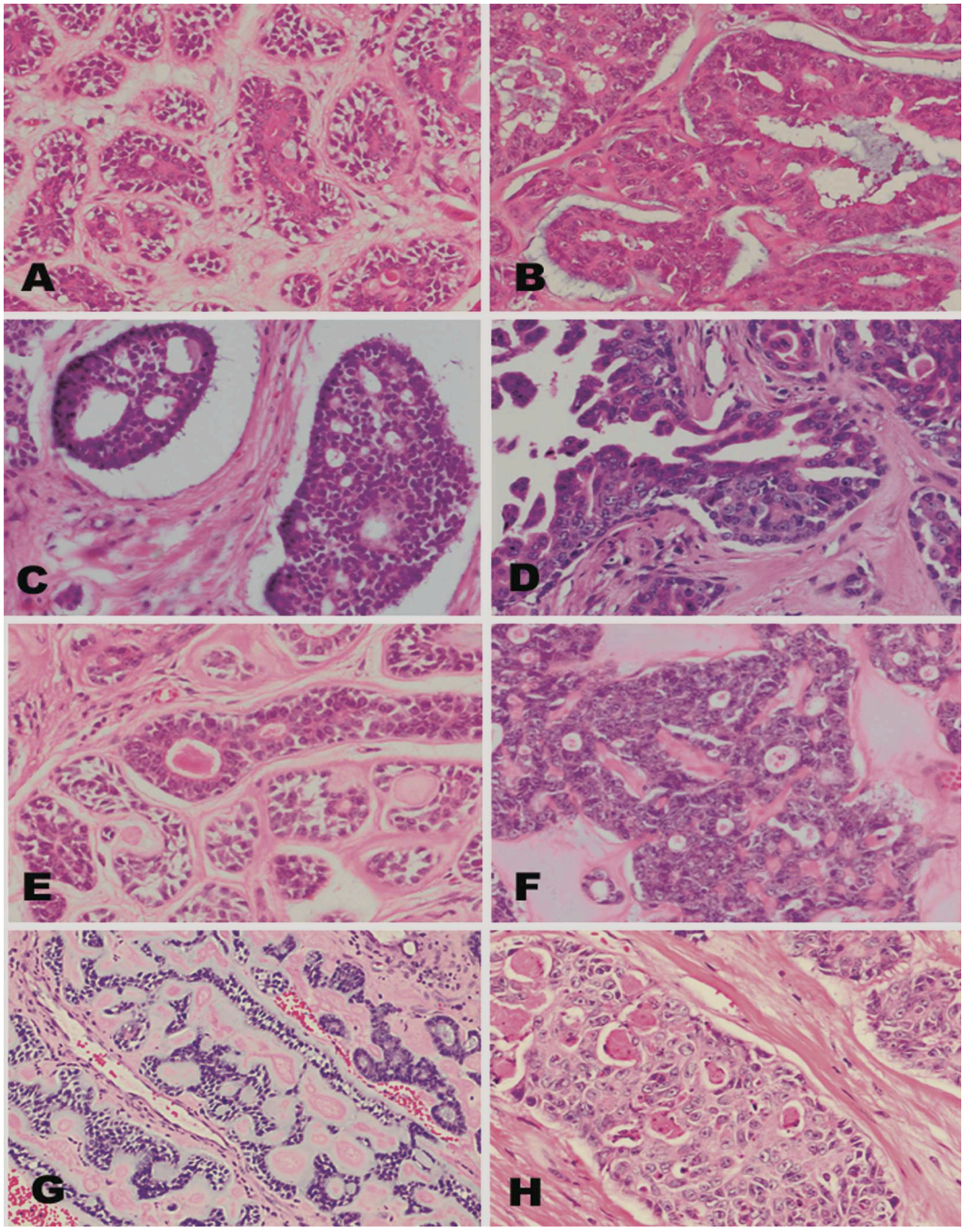

Figure 1 – Case 2 (A and B), case 4 (C and D), Case 5 (E and F) and case 7 (G and H): the transformed components are composed of moderately differentiated adenocarcinomas in cases 2 (B) and 4 (D) and of poorly differentiated carcinomas in cases 5 (F) and 7 (H). A, C, D and G show the conventional areas of these cases (H&E: original magnification 400x).

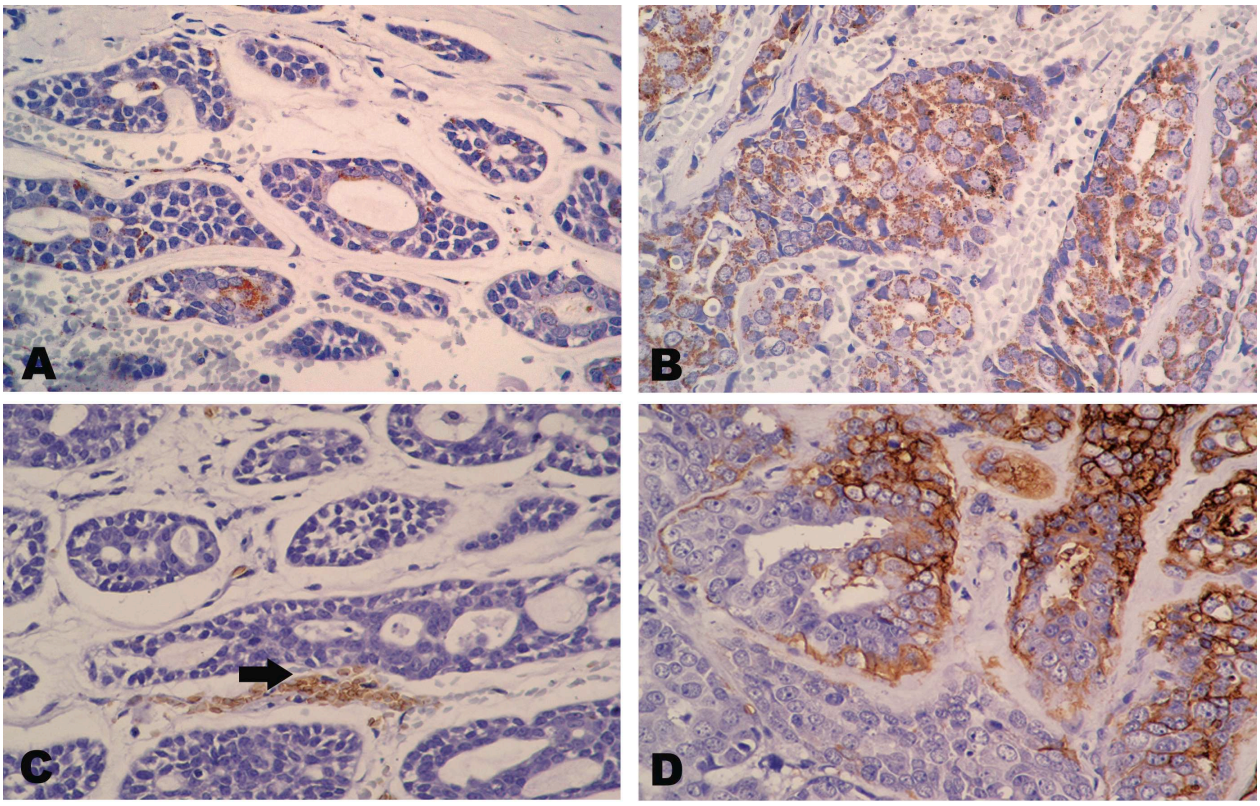

Figure 2 – Adenoid cystic carcinoma with transformation to an adenocarcinoma: the conventional component shows few positive cells for mitochondrial antigen (A) and absence of GLUT1 expression (C) in carcinoma cells (only blood red cells are stained for GLUT1- arrow). In contrast, in the transformed component there are numerous positive cells for mitochondrial antigen (B) and GLUT1 (D). (Immunohistochemistry of mitochondrial antigen and GLUT1: original magnification 400x A, B, C, D)

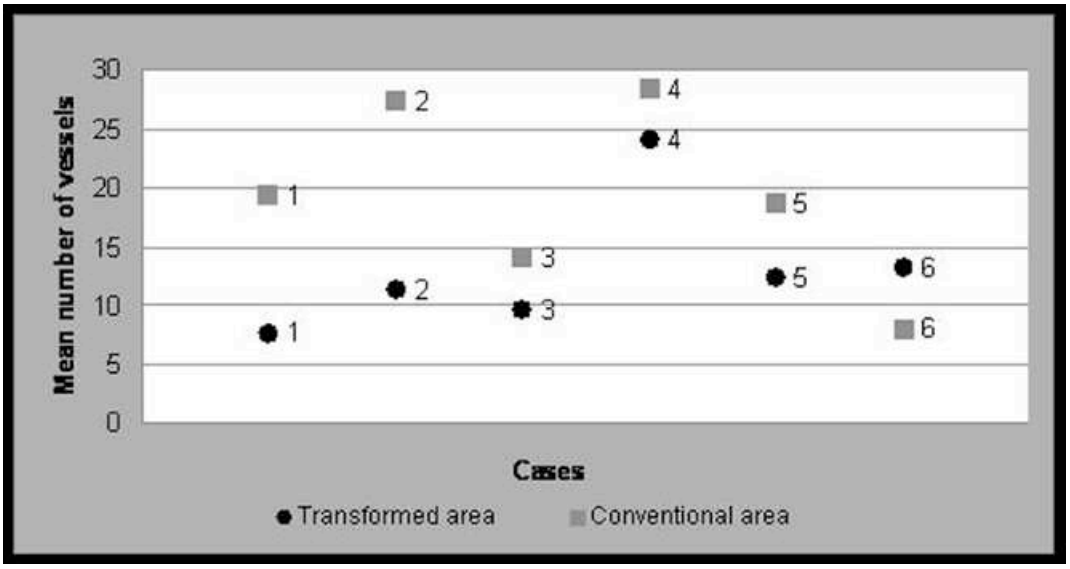

Figure 3 – MVD in ACC-Ad/UC group. In all cases but one, conventional areas showed higher MVD than Ad/UC component, although the differences were not significant (p=0.1563).

Artigo relacionado 2

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Signet-ring cell change in adenoid cystic carcinoma: a clinicopathologic and immunohistochemical study of four cases

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Abbreviations: ACC, adenoid cystic carcinoma; ACC-HGT, adenoid cystic carcinoma with high-grade transformation; ACC-SRC, adenoid cystic carcinoma with signet-ring cell; AR, antigen retrieval; ICL, intracytoplasmic lumen; PAS, periodic acid Schiff; SRC, signet-ring cell

ABSTRACT

Aims: Signet-ring cell (SRC) change has not been reported in adenoid cystic carcinomas (ACC). This study describes the clinicopathological and immunohistochemical findings in four cases of ACC with SRCs (ACC-SRC), in which the relative proportion of the SRC component ranged from 25% to 50%.

Methods and Results: The median age was 58 years (range: 48 to 81 y) and all patients were women. The involved sites were sinonasal, lip and submandibular. Two patients developed lung metastasis and one died of disease 63 months after tumor resection. Neither mucinous nor lipid substances were detected in the SRCs. These were positive for AE1/AE3, CK14 and EMA; which highlighted the intracytoplasmic vacuole borders. The SRC nests were surrounded by α-SMA and p63 positive myoepithelial cells. When compared to the conventional component, the SRCs exhibited similar p53 positivity but lower Ki-67 and mitotic indices. SRCs were C-Myb negative. Ultrastructural examination revealed that the intracytoplasmic vacuoles were lumens lined by microvilli.

Conclusions: ACC-SRC is a nonmucin and nonlipid producing phenomenon, possibly related to disturbed differentiation of ductal/luminal cells. This cellular modification in ACC apparently does not change the biological behavior of the tumor but it may cause significant diagnostic problems, particularly in incisional biopsies.

INTRODUCTION

Adenoid cystic carcinoma (ACC), the second most common salivary malignancy, is a slow-growing but widely infiltrative tumor that presents frequent local recurrence, late onset of metastases and poor long-term survival.[1-4] ACCs show a dual cellular composition (ductal and abluminal myoepithelial cells) and three major growth patterns (tubular, cribriform and solid). In the typical ACC, the more peripheral cells of tumor nests and glands show myoepithelial differentiation whereas the ductal cells line glandular lumens.[1,2] Both types of cells usually present scant amphophilic to clear cytoplasm and hypercromatic nucleus, which gives this tumor an isomorphic basaloid appearance.[1] Regarding the growth patterns, ACC with greater than 30% of solid component are classified as a solid variant, which generally pursues a more aggressive course than lesions composed of tubular and cribriform patterns.[5,6]

Signet-ring is a term used to describe the histologic appearance of a cell that presents a large cytoplasmic vacuole which compresses the nucleus into a form of a crescent. Signet-ring cell (SRC) change can be found in association with: a) carcinoma cells of the various body sites (gastro-intestinal tract, pancreas, breast, lung, urinary bladder, prostate, etc), b) noncarcinomatous neoplasms (lymphoma, malignant melanoma) and c) nonneoplastic process such as stromal nodule of prostate.[7-13] The signet ring-cell appearance can result from different mechanisms, and the content of the vacuoles is also variable (for instance, mucin, lipid or glycogen). Ultrastructural examinations have revealed that vacuoles can represent cytoplasmic lumen, cytoplasmic pseudoinclusion, intracellular edema or hydropic swelling of mitochondria.[14-17]

For a tumor to be classified as signet ring-cell variant, the required extent of SRC has varied from 5% to 90%.[9,12,18,19] In the stomach, where the great majority of signet ring cell carcinomas arises, over 50% of the tumor should be composed of SRCs to warrant this designation.[20] However, in the prostate, where this variant is also well-known, a recent review of the literature showed 84% of the patients with SRC constituting more than 20% of the specimen had been accepted as signet ring cell carcinomas.[12]

Regarding clinical significance, SRC has been associated with aggressive behavior and high-grade lesions in gastric, prostate, sinonasal and in eccrine sweat gland carcinomas.[12,17,18,21] In salivary glands, tumors described by name containing signet-ring cells have rarely been reported. A literature search (MEDLINE) has revealed two case reports of benign salivary tumors

with SRC and few malignant neoplasms. The benign tumors were an oncocytic cystadenoma with SRC and a signet ring cell tumor of the minor salivary gland, possibly originated from striated excretory ducts.[22,23] In relation to malignant neoplasms, all were mucin-producing adenocarcinomas and almost all occurred in minor salivary glands.[14,24-26] These adenocarcinomas were characterized by the marked presence of mucin-containing signet-ring cells and, unexpectedly, behaved as low-grade carcinomas.[25]

In ACC, to the best of our knowledge, SRC has not been reported. However, in our consultation practices we (AA) received a case of ACC with extensive SRC change (Fig. 1) leading us to look for such cellular alteration in tumors with diagnosis of ACC in our surgical pathology archives. Herein, we describe the clinicopathologic features and immunohistochemical findings of 4 cases of ACC with SRC (ACC-SRC) in more than 20% of cells.

MATERIALS AND METHODS

This study was approved by the Committee of Ethics of the University of Campinas, Brazil. The surgical pathology archives and consultation practices of the University of Campinas were reviewed between 1990 and 2011 and contained 64 tumors which had the microscopic features of ACC. SRC was defined when the tumor cells presented a large cytoplasmic vacuole which compressed the nucleus into a form of a crescent. In four out of 64 ACCs, SRC was identified in tumor cells and its proportion was estimated semiquantitatively by determining the amount of area involved in the available hematoxylin and eosin slides. In the four tumors with SRC, additional histopathologic features, such as: grade of the conventional ACC component[6], degree of nuclear atypia (mild, moderate and severe), mitotic activity, necrosis, tumor calcification, tumor-associated fibrocellular proliferation, margin status, perineural and vascular invasions were evaluated. A representative section was selected and stained by periodic acid Schiff (PAS) with or without diastase digestion, mucicarmine and alcian blue. Clinical details and follow-up data were obtained when possible from medical records.

Immunohistochemistry

One paraffin block from each case was chosen for the immunohistochemical study. Table 1 shows the antibodies that were used in the current series.

Single-staining for AE1/AE3, CK 7, CK 14, EMA, adipophilin, α-SMA, p63, androgen receptor, Ki-67, p53 and C-Myb were carried out using the EnVision System (DAKO, Denmark).

Briefly, 5μm sections from the paraffin blocks were deparaffinized in xylene, rehydrated through descending ethanol series and submitted to heat-induced antigen retrieval (AR) in water bath with Tris-EDTA solution for 30 minutes, except for AE1/AE3, CK14, C-Kit, Adipophilin and α-SMA. For AE1/AE3, CK14, C-Kit, AR was performed using citrate acid instead of Tris-EDTA and for α-SMA and Adipophilin, AR was not done. Subsequently, for all antibodies, sections were immersed in 0.3% hydrogen peroxide in methanol and incubated with the primary antibody and afterwards with the EnVision peroxidase system, dual link (K4061, DAKO, Carpenteria, CA, USA) for 1 h at 37°C. After washing, sections were stained for 5 min at 37°C with 3.3'-diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin. Appropriate positive and negative controls were used throughout.

Double-labelling immunohistochemical staining (EnVision doublestain, code K1395, Dakopatts S/A, Denmark) was performed for p63/EMA. Briefly, anti-p63 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 EMA 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.

Evaluation of Staining

Immunoreactivity for each antibody was assessed separately according to the tumor-cell types: ductal/epithelial, myoepithelial and with SRC changes and classified as absent (0% to 5%), focal (>5% to 50% of cells) and diffuse (>50%). In each p53 and Ki-67 stained sections, three hotspot areas were chosen in conventional and in SRC components for counting of positive cells at 40X magnification. Ki-67 and p53 indexes were considered as the percentage of positive cells in relation to all tumor cells in these three areas in each sample.

Ultrastructural Examination

Electron microscopy was carried out on formalin-fixed paraffin-embedded material from 1 case (case 2). A 3 mm^3 portion of tissue was mechanically dissected from the paraffin block, cut into smaller portions and postfixed in 2% osmium tetroxide. Thick sections were cut and stained with toluidine blue to select suitable areas for ultrastructural examination under a LEO/ 906 transmission electron microscope.

RESULTS

Clinical Findings

The incidence of ACC-SRC was 6.2% of all ACC cases. The clinical findings of ACC-SRC are summarized in Table 2. All four patients were women and the age ranged from 48 to 81 years (median 58.7). The involved sites were sinonasal, lip and submandibular. Follow-up information was available in three cases; all presented T3/T4 tumors that were treated with surgical excision without neck dissection and postoperatively received radiotherapy. Of these three patients, the surgical margins were positive in all, none presented local recurrence, two developed lung metastasis (cases 2 and 3) and two died (cases 3 and 4) 63 and 69 months after tumor resection, respectively. However, only case 3 died of disease. In case 2, the follow-up was discontinued 46 months after lung metastasis, with a total follow-up of 72 months.

Histopathological Findings

The histopathological findings of ACC-SRC are summarized in Table 3. The conventional component of all four tumors was composed of a mixture of tubular, cribriform and solid patterns but the latter represented less than 30% (Table 3). SRC was mainly observed in the tubular pattern and the proportion of the SRC component ranged from 25% to 50%. In all four tumors, SRCs showed ductal/ luminal features, forming clusters of SRCs surrounded by myoepithelial cells with appearance similar to those of the conventional areas (Fig 1 and 2). SRCs did not stain for PAS with and without diastase digestion, mucicarmine or alcian blue (Fig 3). In one tumor (case 3), necrotic cells and focal calcification were found in some clusters with squamoid appearance (Fig. 2C). Regarding nuclear atypia, an overall assessment showed slight nuclear size variation and a tendency to have more vesicular nuclei in the SRC component of 3 of the 4 tumors. The mitotic activity was almost absent in SRC component whereas in the conventional ones ranged from 0-12 per 10 high power fields.

Immunohistochemical findings

Ductal/luminal cells of the conventional component showed strong and diffuse positivity for AE1/AE3 and CK14, whereas the myoepithelial cells were selectively highlighted by α-SMA and p63 (Table 4). SRCs presented a level of positivity for AE1/AE3 and CK 14 comparable to those of the ductal/luminal cells of the conventional component and were negative for α-SMA and p63 (Fig 4A, B, C, D). In all four tumors, the clusters of SRCs were surrounded by α-SMA and p63 positive myoepithelial cells except for some areas of variable extension where this outer

layer of myoepithelial cells was lost (Fig. 4C, D). In all cases, SRCs were strongly positive for EMA that stained the vacuole borders (Fig 4E) whereas in the conventional component, ductal cells showed positivity only in their luminal aspect. Dual immunohistochemistry using EMA and p63 confirmed that SRCs containing EMA positive vacuoles were p63 negative (Fig 4F) and, thus, ductal/luminal cells. CK 7 positivity was seen in ductal cells of the conventional component as well as in SRCs, but in the latter this was observed only in one case (case 3).

Tumor cells were negative for adipophilin (antibody used for identification of intracytoplasmic lipids in formalin-fixed paraffin embedded tissue [27]) in both conventional and SRC components in all four cases. However, in the conventional component of the case 1, rare sebaceous cells were detected and they were positive for adipophilin (Fig. 3D). C-myb reactivity was absent in SRCs and strongly positive in myoepithelial cells (Fig. 4G, H). In all cases, C-Kit positivity was strong in ductal cells of the conventional component. However, only in two cases, SRCs were positive for C-Kit and the reactivity was weak. Ki-67 proliferation index was markedly lower in the SRC than in the conventional component (means 7.7% and 25.4%, respectively), whereas p53 index was similar in SRC and conventional areas (means 57.9% and 56.1%, respectively) (Fig. 5C, D and Table 3).

Ultrastructural Findings

The SRCs (case 2) showed an intracytoplasmic space lined by a layer of short microvilli (Fig. 6A, B). As the material was originally processed for paraffin sections, microvilli as well as organellar details were difficult to determine.

DISCUSSION

In the current study we demonstrated for the first time SRC change in ACC. Furthermore, we showed the cytoplasmic vacuole represented intracytoplasmic lumen (ICL) and did not contain mucin or lipid. The former finding was based on ultrastructural and immunohistochemical studies which showed ICLs lined by a layer of short microvilli and positivity of the vacuole borders for EMA, respectively. In general, ICLs are associated with production of secretory material. However, in our cases such production was not observed. This fact could be due to the removal of the luminal substance during tissue processing. Indeed, this is the hypothesis that has been proposed to explain the empty appearance of the cytoplasmic vacuoles in breast carcinomas which show ICLs similar to those found in the current series.[10,28]

SRC with empty vacuole is not a usual finding in signet-ring cell carcinomas. The majority of these carcinomas generally produces mucin which accumulates in the cytoplasm leading to signet ring appearance.[9,18,20] However, there are also tumors with SRCs that are nonmucin-producing and can contain lipid in the cytoplasmic vacuole (such as signet ring cell carcinomas of the prostate [8,29]) and others that are nonmucin and nonlipid producing (as signet-ring stromal tumor of ovary [15,30,31]). In the latter, the vacuoles have been described as invagination of the cell membrane by extracellular matrix, hydropic swelling of mitochondria or generalized edema of the cytoplasmic matrix.[15,30]

ACC is a carcinoma with a dual cellular composition (ductal and abluminal myoepithelial cells). In the current series, SRC change was observed in cells with morphology and immunoprofile consistent with ductal differentiation (AE1/AE3 and EMA positive, SMA and p63 negative). All four cases of SRC-ACCs were predominantly made up of tubular and cribriform patterns and SRC were mainly observed among ductal cells of the tubular form. Ductal cells with SRC change were p53 positive, MYB negative and clearly less proliferative than the outer layer of myoepithelial cells. The latter showed markedly high expression of Ki-67 as well as of MYB and p53. Regarding MYB (a leucine zipper transcription factor), it has been reported that the fusion of MYB and NFIB generated by t(6;9) translocation is likely to be a major oncogenic event in ACCs and leads to the deregulation of the expression of MYB.[32,33] The sensitivity and specificity of MYB protein expression for ACCs have been found to be 82 and 86%, respectively.[32] However, although MYB immunostaning is not entirely specific for this tumor it is interesting to point out the pattern of MYB protein expression. In our four cases it was similar to that recently reported in classical ACCs, i.e. strong positivity in myoepithelial/ basal cells and lack of expression in ductal /luminal cells[32-34], where SRC change was observed. As MYB activation plays a key role in the control of cell proliferation and differentiation[35], it is a matter for further investigation whether the formation of SRC and the low proliferative capacity of these cells might be related to the lack of MYB activation.

Site, sex and age distribution of the four tumors in this study were similar to those of classical ACCs, which occur predominantly in the minor salivary glands, on women and during the fifth decade of life.[3,4,36] In the current series, the age of the patients ranged from 48 to 81 years (mean 58.7), 3 out of 4 ACC-SRCs affected minor salivary glands and all patients were women. The clinical outcome of ACC-SRC was also comparable to that of classical ACC. In the

latter, 52% of the patients have been reported to die of disease, with a mean survival period of 35.4 months.[3] In ACC-SRC, one out of three patients with available follow-up information died of disease 63 months after the tumor resection. Regarding metastasis (nodal and/ or distant), two patients presented lung metastasis, which is in agreement with the high incidence and site of this event in ordinary ACC.[4] Therefore, despite the small number of cases in our study to comment accurately on prognosis, the patient outcomes suggest that SRC in ACC does not change the usual clinical course of the disease. However, there is also the possibility that the extent of the SRC component (≤ 50%) might be insufficient to influence tumor biological behavior. In some carcinomas, such as lung carcinoma with SRCs, the extent of the SRC component (<50% versus ≥ 50%) has been reported to correlate with tumor aggressiveness. [19]

Although SRC change in ACC does not appear to have prognosis implications, this is of interest because can potentially cause misdiagnosis especially in small incisional biopsies. ACC-SRC should be distinguished from other salivary tumors with SRC, metastasis of carcinomas of other sites and hybrid carcinomas. Regarding salivary tumors, in addition to signet-ring cell mucin-producing adenocarcinomas, SRC features can occasionally be seen in acinic cell carcinomas, mucoepidermoid carcinomas, salivary duct carcinomas [37] and epithelial-myoepithelial carcinoma with sebaceous differentiation. However, the presence of areas with typical features of ACC and the absence of mucin or lipid in the vacuoles would allow the recognition of the ACC-SRC variant. The same reasoning can be used to distinguish ACC-SRC from metastasis of carcinomas with SRC of other sites (such as stomach, large intestine, breast, lung, etc) to salivary glands. In relation to hybrid carcinoma, the presence the SRCs surrounded by a residual layer of myoepithelial cells and the intermixed appearance of typical ACC with the element containing SRC would differentiate ACC-SRC from such entity. In salivary glands, hybrid carcinomas represent two histologically distinct types of tumor within the same topographic area.[38, 39] ACCs may also have areas closely simulating other salivary tumors with a dual cellular composition, particularly basal cell adenocarcinoma. In this sense, in our cases few tumor nests showed organoid appearance with peripheral palisading, similar to those found in basal cell adenocarcinoma. However, this diagnosis was ruled out as this feature was occasional and mixed with typical ACC nests in which the more peripheral cells of tumor nests showed hyperchromatic angulated nuclei.

Finally, we believe that SRC change cannot be considered another example of high-grade transformation in ACC. ACC with high-grade transformation (ACC-HGT) is a phenomenon that has been associated with poor prognosis and increased tendency to nodal metastasis.[40] Furthermore, the HGT component differs significantly from the SRC change. In contrast to SRC change, the HGT component characteristically presents marked nuclear enlargement, higher Ki-67 indices and stronger p53 expression when compared with conventional ACC. [40] These alterations are consistent with high-grade tumor and can be observed in other salivary carcinomas with high-grade transformation or dedifferentiation.[41,42] However, regarding the loss of the dual ductal-myoepithelial differentiation, another important characteristic of ACC-HGT, it was also found in all cases of our series of ACC-SRC (Figure 4C and D). This finding suggests that the loss of myoepithelial cells could not be used as a morphologic indicator of high-grade transformation of ACC, leading us to speculate that the mechanisms behind this event in ACC-HGT and ACC-SRC could be different. SRC change in ACC lends support to recent hypothesis that this tumor can undergo transformation to a wide spectrum of histological forms, which are not necessarily associated with more aggressive behavior.[43]

In conclusion, SRC in ACC is a nonmucin and nonlipid producing phenomenon, possibly related to disturbed differentiation of ductal/luminal cells. This cellular modification in ACC apparently does not have prognostic implications but may cause significant diagnostic problems, particularly in incisional biopsies.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Table 1. Index of immunohistochemical stains

Antibody	Immunohistochemical dilution	Clone	Antigenic retrieval	Source
Pankeratin	1:400	AE1/AE3	Citrate	Dako
EMA	1:100	E29	Tris/EDTA	Dako
CK14	1:200	LL02/1	Citrate	Dako
CK7	1:100	OV-TL12130	Tris/EDTA	Dako
α-SMA	1:200	1A4	–	Dako
p63	1:400	4A4	Tris/EDTA	Dako
Adipophilin	1:1	AP125	–	Fitzgerald
Androgen receptor	1:100	1DS	Tris/EDTA	BioSystems
C-Myb	1:300	EP769Y	Tris/EDTA	Abcam
C-Kit	1:100	CD117	Citrate	Dako
Ki67 LI	1:150	MIB1	Tris/EDTA	Dako
p53 LI	1:100	DO-7	Tris/EDTA	Dako

α-SMA, α-smooth muscle actin; CK, cytokeratin; EMA, epithelial membrane antigen; –, no antigenic retrieval.

Table 2. Clinical findings of adenoid cystic carcinomas with signet-ring cells

Case	Age (years)	Sex	Site	T	Treatment	Local recurrence	Metastasis	Follow-up (months)	Outcome
1	81	F	Lip	T2	NA	NA	NA	NA	NA
2	48	F	Right submandibular	T3	Ex, Rx	No	Lung	72	NA
3	49	F	Right maxillary sinus	T3	Ex, Rx	No	Lung	63	DOD
4	57	F	Right nasal	T4	Ex, Rx	No	No	69	Died

DOD, died of disease; Ex, excision; NA, not available; Rx, radiotherapy.

Table 3. Histopathological findings for adenoid cystic carcinomas (ACC) with signet-ring cells (SRCs)

Case	Conventional ACC component grade	% SRC component	Necrosis	Calcifications	Desmoplasia	PNI	VI	Mitosis (per 10 HPFs)	
								C-C	SRC-C
1	2	50	–	–	+	+	+	12	0
2	2	30	–	–	–	+	+	1	0
3	2	30	+	+	–	+	–	0	1
4	2	25	–	–	+	+	–	5	0

C-C, conventional component; HPF, high-power field; PNI, perineural invasion; SRC-C, signet-ring cell component; VI, vascular invasion.

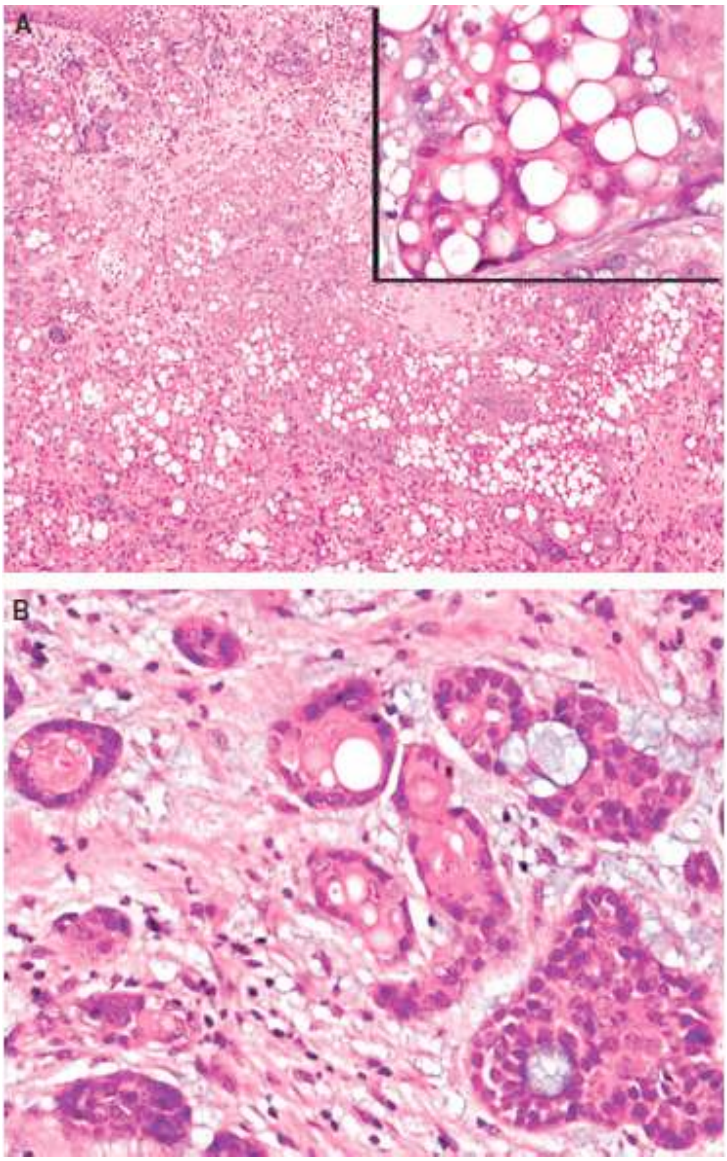

Figure 1. Case 1. **A**, Low-power view showing an área with extensive signet-ring cell (SRC) change, masking the adenoid cystic carcinoma morphological characteristics. Inset: cells with SRC change showinh a large cytoplasmic vacuole compressing the nucleou sinto the formo f a crescente. **B**, Conventional area with dual epitelial-myoepithelial differentiation and few SRCs in the ductal/epitelial cells.

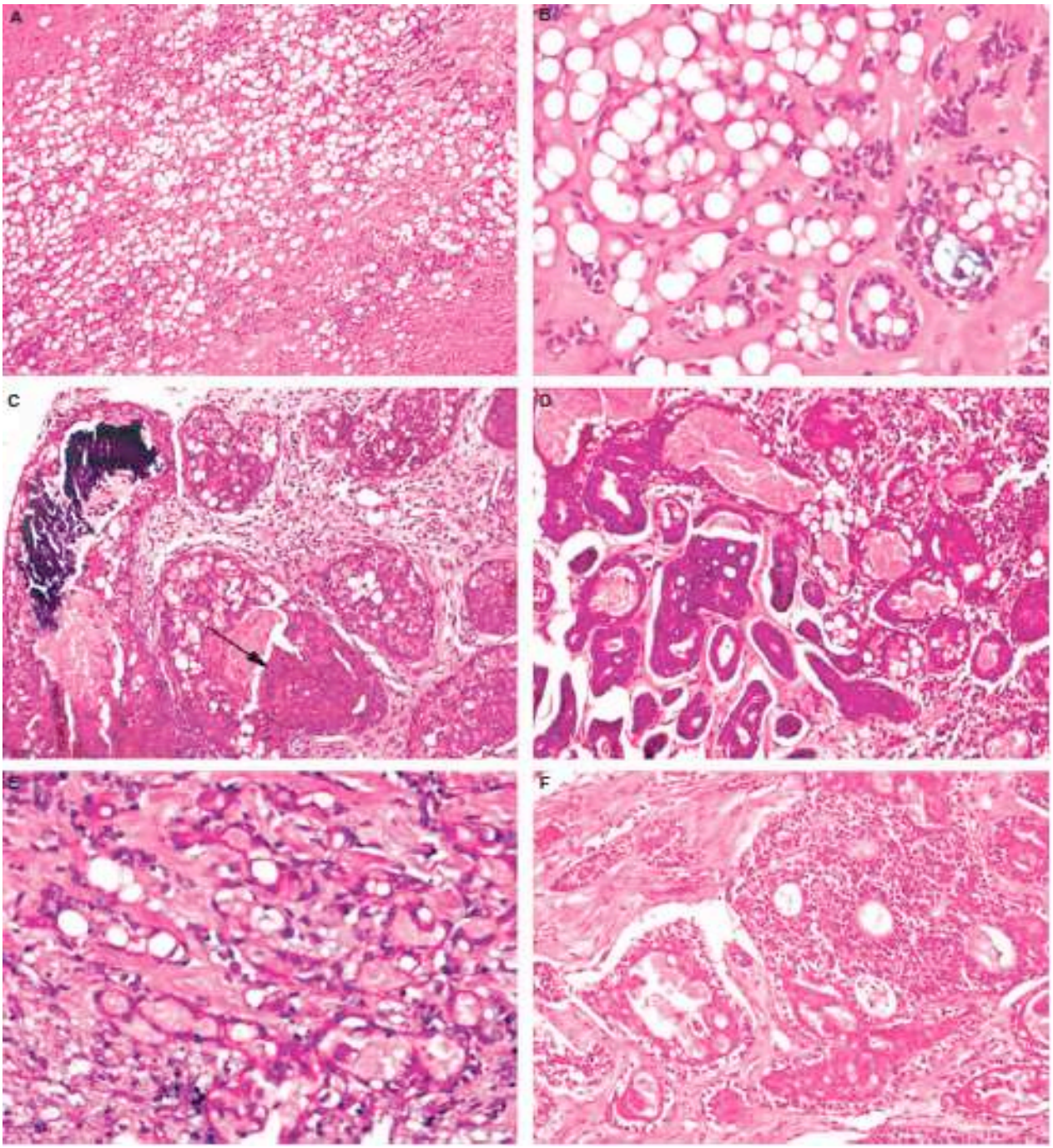

Figure 2. Histological features of adenoid cystic carcinoma with signet-ring cells (SRCs). The transitional áreas containing both SRC and conventional componentes of cases 2, 3 and 4 are illustrated in **(A)**, **(C)** and **(E)**, respectively. The transitional áreas containing both SRC and conventional componentes of cases 2, 3 and 4 area illustrated in **(B)**, **(D)**, and **(F)**, respectively. In the transitional áreas, ductal cells with SRC change are surrounded by myoepithelial cells. Case 3 **(C)** shows an área (arrow) with more densely eosinophilic cytoplasm suggestive of squamoid differentiation and focal necrosis with calcification on the left.

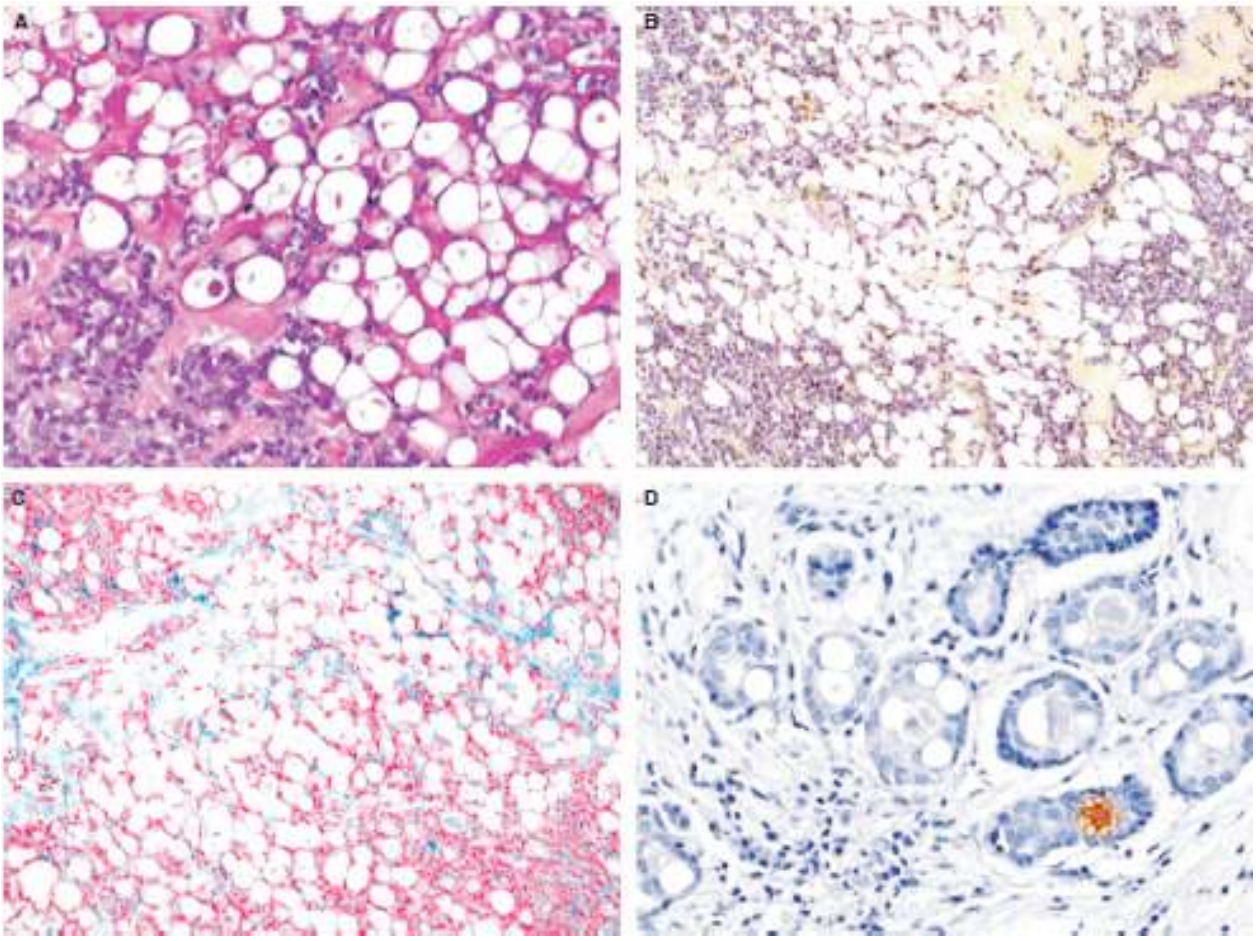

Figure 3. A-C, Histochemical staining with periodic acid Schiff (PAS) **(A)**, mucicarmine **(B)**, and alcian blue **(C)**. Signet-ring cells (SRCs) do not stain with PAS, except for a minute PAS-positive droplet of secretion in the vacuole. They also do not stain with mucicarmine or alcian blue. **D**, Immunohistochemical staining for adipophilin. SRCs do not show immunoreactivity for adipophilin. Note that the conventional componente presentes focal sebaceous differentiation that expresses adipophilin.

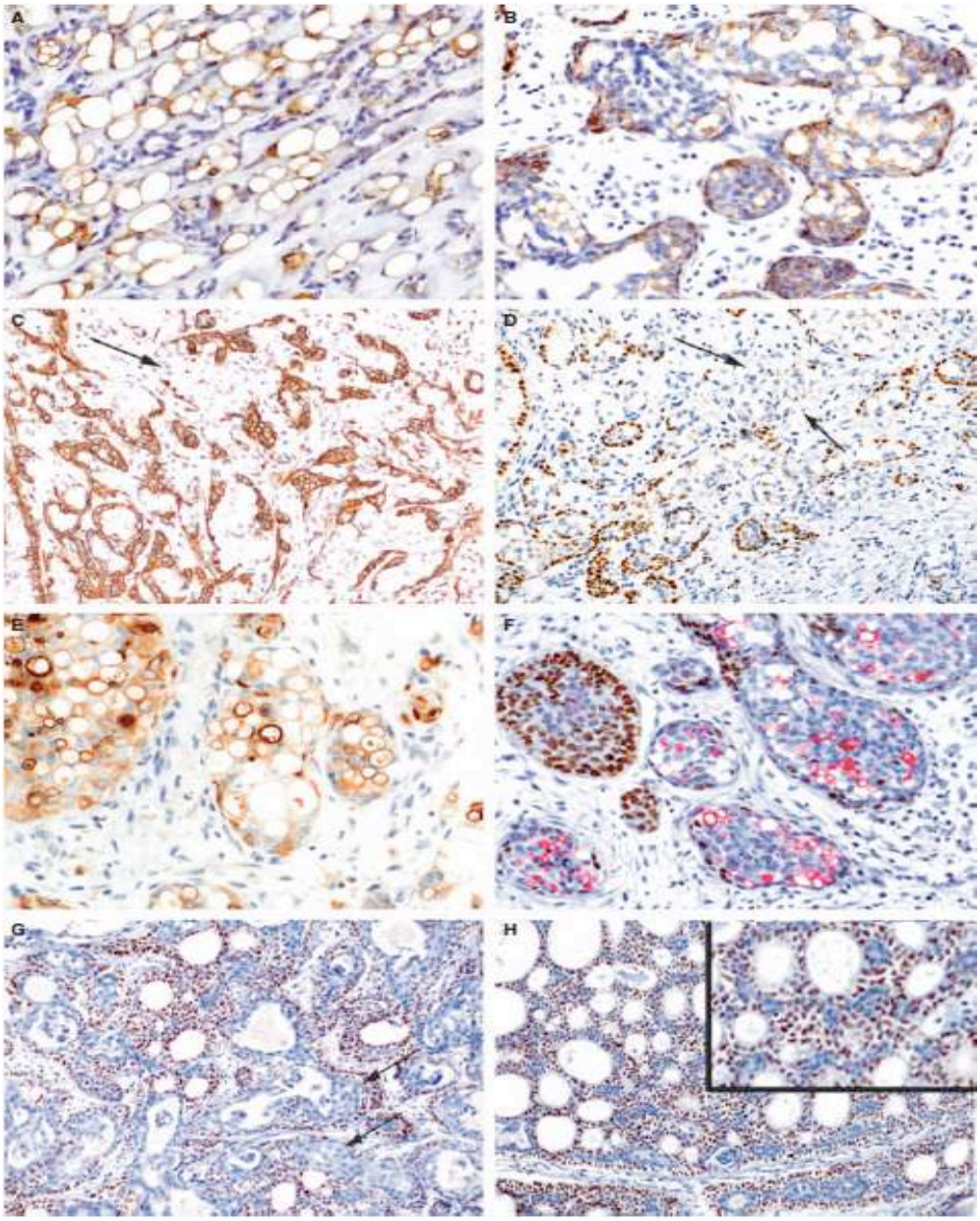

Figure 4. Immunohistochemical staining with AE1/AE3 **(A)**, CK14 **(B)**, α-smooth muscle actin (α-SMA) **(C)**, p63 **(D)**, epithelial membrane antigen (EMA) **(E)**, p63 and EMA (double-labelling) **(F)**, and c-Myb **(G,H)** antibodies. Note positive immunoreactivity of signet-ring cells (SRCs) for AE1/AE3 and CK14. α-SMA and p63 antibodies stain myoepithelial cells at the periphery of the SRC nests, but there is loss of this abluminal layer of myoepithelial cells in some áreas [arrows in **(C)** and **(D)**]. The vacuole borders of the SRCs are intensely positive for EMA, and the double-labelling for p63 and EMA shows that the SRCs are completely negative for p63. SRCs (arrows) are negative for c-Myb **(G)**, Whereas the myoepithelial cells of the conventional componente are positive **(H)**. Inset: myoepithelial cells showing Strong expression. Of c-Myb.

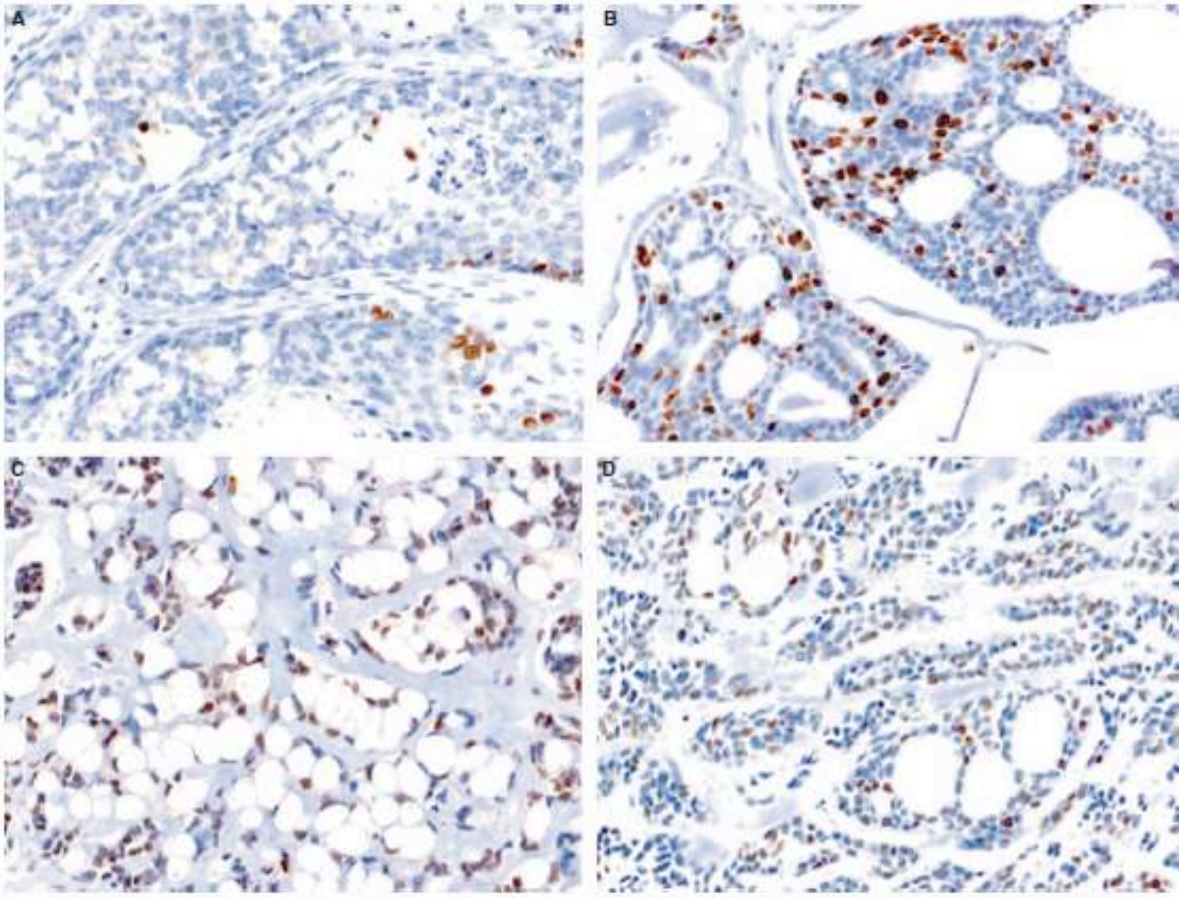

Figure 5. Immunohistochemical staining for Ki-67 **(A,B)** and p53 **(C,D).** The signet-ring cell (SRC) component shows few positive cells (0041), whereas they are numerous in the conventional componente **(B)**. SRC **(C)** and conventional **(D)** componentes show similar proportions of p53-positive cells.

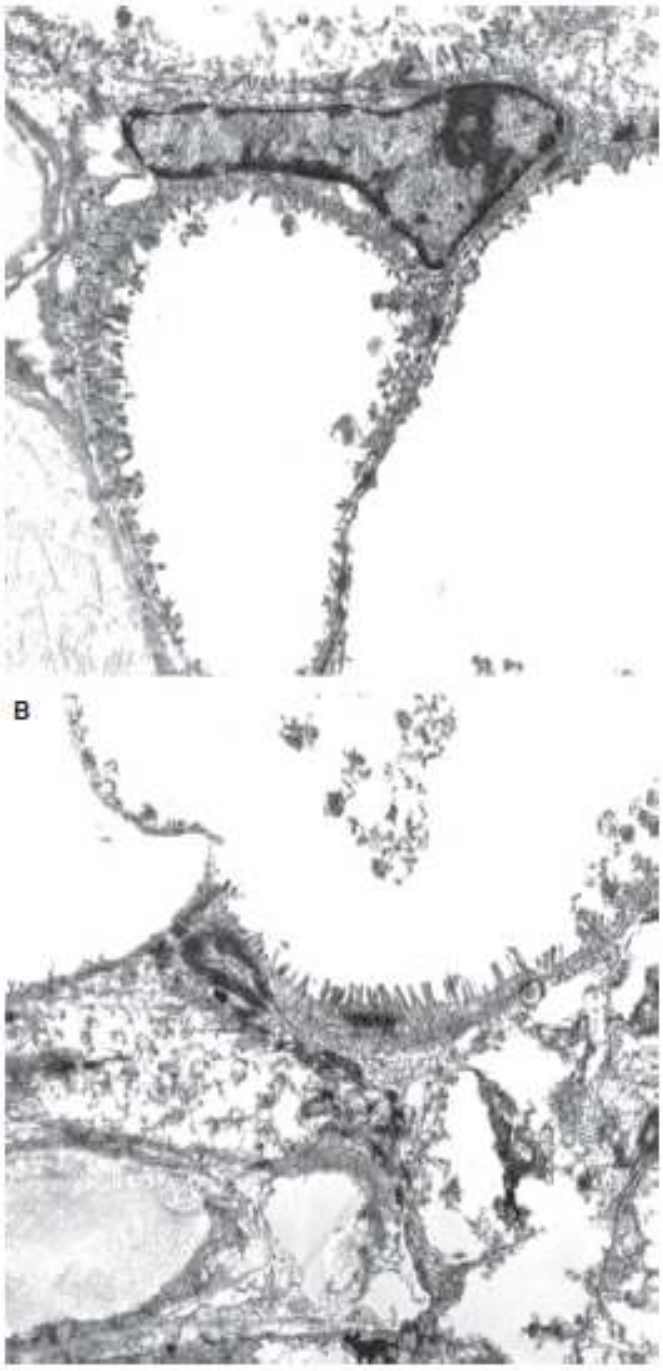

Figure 6. Case 2. Ultrastructural features of adenoid cystic carcinoma with signet-ring cells (SRCs). A, an SRC showing an intracytoplasmic space lined by short microvilli. B, detail of the microvilli in the cytoplasmic vacuole of the SRC. (Osmium tetroxide).

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Anexos

CEP, 13/11/09.
(Grupo II)

PARECER CEP: N° 955/2009 (Este n° deve ser citado nas correspondências referente a este projeto)
CAAE: 0742.0.146.000-09

I - IDENTIFICAÇÃO:

PROJETO: **"IDENTIFICAÇÃO DAS ALTERAÇÕES GENÉTICAS E EPIGENÉTICAS NO CARCINOMA ADENÓIDE CÍSTICO COM TRANSFORMAÇÃO PARA ALTO GRAU".**
PESQUISADOR RESPONSÁVEL: Ana Flávia de Mattos Costa.
INSTITUIÇÃO: Departamento de Anatomia Patológica/FCM/UNICAMP
APRESENTAÇÃO AO CEP: 09/10/2009
APRESENTAR RELATÓRIO EM: 13/11/10 (O formulário encontra-se no *site* acima)

II - OBJETIVOS

Identificar as alterações genéticas e epigenéticas relacionadas com a transformação para alto grau do carcinoma adenóide cístico.

III - SUMÁRIO

Investigar em carcinomas adenóide císticos com transformação para alto grau as alterações genéticas e a metilação do DNA através da técnica de MLPA (Multiples Ligation Probe Amplification).

IV - COMENTÁRIOS DOS RELATORES

Após respostas às pendências, o projeto encontra-se adequadamente redigido e de acordo com a Resolução CNS/MS 196/96 e suas complementares, bem como a dispensa do Termo de Consentimento Livre e Esclarecido.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar sem restrições o Protocolo de Pesquisa, bem como ter aprovado a dispensa do Termo do Consentimento Livre e Esclarecido, assim como todos os anexos incluídos na Pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

Comitê de Ética em Pesquisa - UNICAMP
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13083-887 Campinas – SP

FONE (019) 3521-8936
FAX (019) 3521-7187
cep@fcm.unicamp.br

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII- DATA DA REUNIÃO

Homologado na X Reunião Ordinária do CEP/FCM, em 27 de outubro de 2009.

Profa. Dra. Carmen Silvia Bertuzzo
VICE-PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM/UNICAMP

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COMISSÃO DE PÓS-GRADUAÇÃO

Campinas, 8 de maio de 2013

Sr(a). Coordenador(a),
Programa de Pós-Graduação em Ciências Médicas

Submeto à análise da Comissão do Programa, a composição da Banca Examinadora do **Exame de Qualificação** de **Doutorado** do projeto/aula intitulada **"Análise Genômica Integrada do Carcinoma do Ducto Salivar"** do(a) aluno(a) **Ana Flávia de Mattos Costa (RA – 065745)** regularmente matriculado(a) no Programa de Pós-Graduação em **Ciências Médicas,** cuja qualificação está agendada para o dia **20/06/2013** às **14:30** horas, no(a) **Departamento de Anatomia Patológica do Hospital de Clínicas/UNICAMP**.

Orientador(a):
Prof(a). Dr(a). Albina Messias Milani de Almeida Altemani

Co-Orientador(a) (caso haja):
Prof(a). Dr(a). Mario Hermsen

Segue a lista dos nomes indicados para escolha da Banca Examinadora:

Prof(a). Dr(a). Liliana Aparecida Lucci de Ângelo Andrade – Instituição: UNICAMP
Prof(a). Dr(a). Leandro Lopes de Freitas – Instituição: UNICAMP
Prof(a). Dr(a). Fábio Rogério – Instituição: UNICAMP
Prof(a). Dr(a). Luciana Rodrigues de Meirelles – Instituição: UNICAMP
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> Histopathology. 2013 Mar;62(4):531-42. doi: 10.1111/j.1365-2559.2013.04352.x. Epub 2013 Feb 5.
>
> Levels and patterns of expression of hypoxia-inducible factor-1α, vascular endothelial growth factor, glucose transporter-1 and CD105 in adenoid cystic carcinomas with high-grade transformation.
> **Costa AF**, Tasso MG, Mariano FV, Soares AB, Chone CT, Crespo AN, Fresno MF, Llorente JL, Suárez C, de Araújo VC, Hermsen M, Altemani A.
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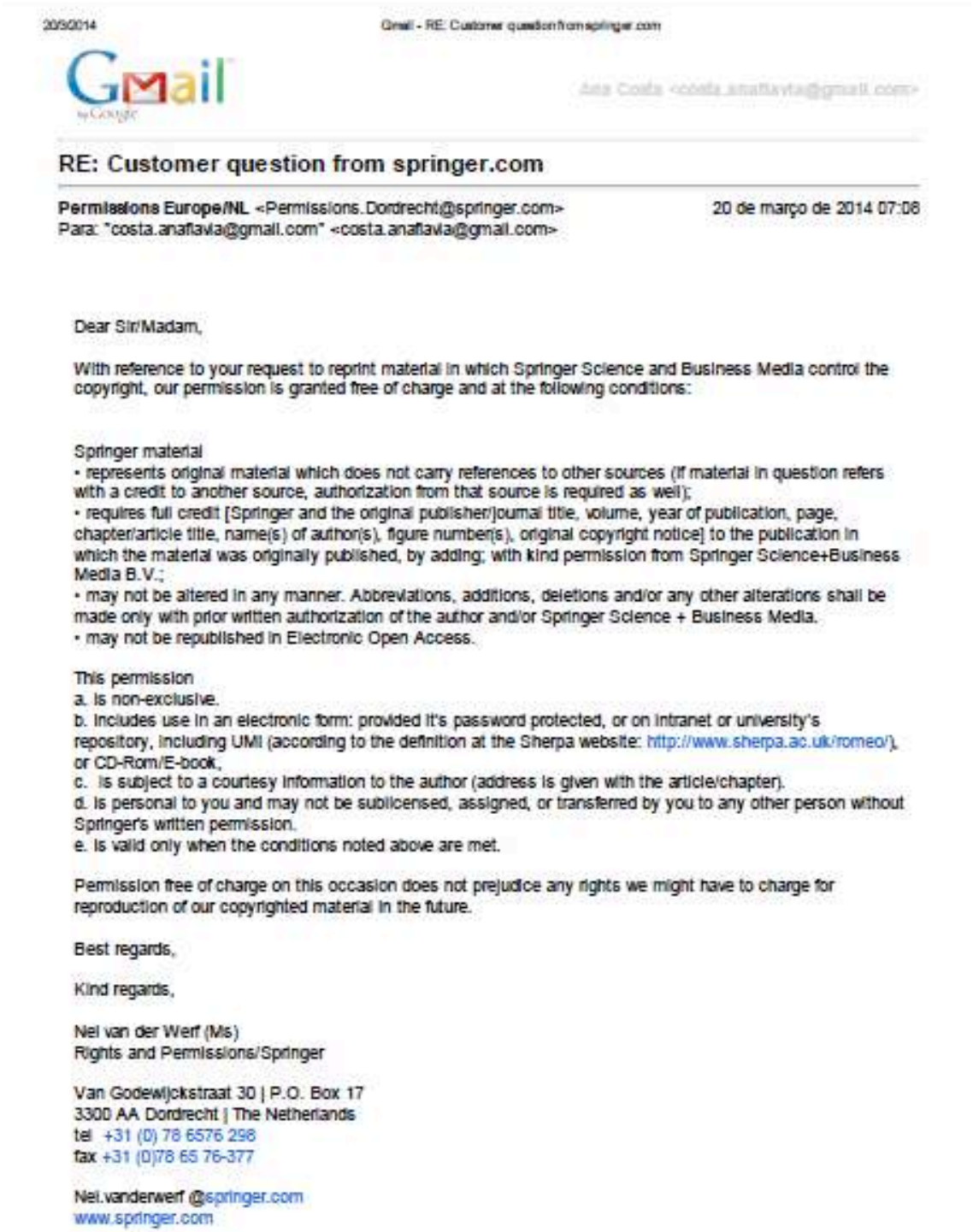

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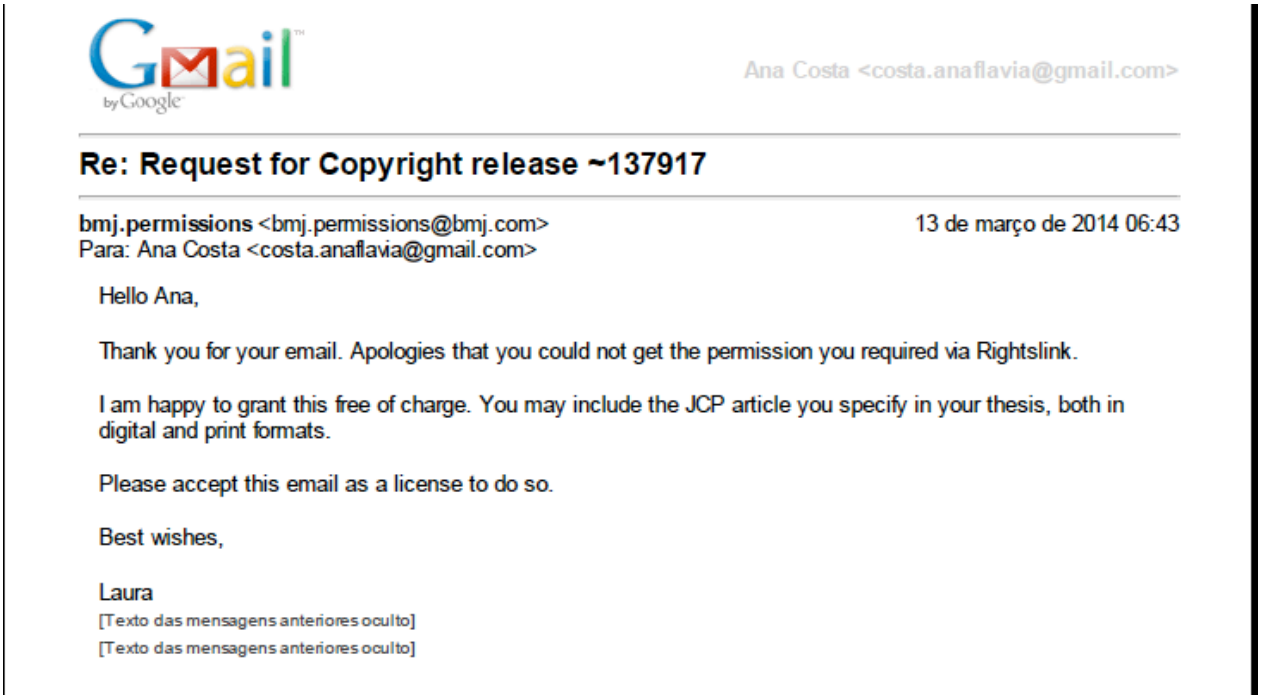

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