CONTRIBUIÇÃO DA (CITO)GENÉTICA PARA O CONHECIMENTO DA BIOPATOLOGIA DAS GLÂNDULAS SALIVARES HUMANAS Carmo Martins

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Orientador: Prof. Doutor Jorge Araújo
Co-orientador: Prof. Doutor Jorge Soares

"Esta tese não inclui as críticas e sugestões feitas pelo júri"

Évora, 2003

Carmo Martins

Contribuição da (cito)genética para o conhecimento da biopatologia dos tumores das glândulas salivares humanas

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À memória de meu pai
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1.

Introdução

1.1 (Cito)genética e cancro

A diferença crucial entre uma célula neoplásica e uma célula normal é que a primeira sofreu alterações no seu património genético que lhe conferiram a possibilidade de escapar aos mecanismos de controlo da proliferação e da diferenciação celular. Desvendar essas alterações, o porquê do seu aparecimento e quais as suas consequências, tem sido um dos maiores desafios na investigação em oncologia humana.

A teoria actualmente prevalecente postula que o cancro é, ao nível celular, essencialmente uma doença genética, que resulta da acumulação de alterações somáticas adquiridas ou, por vezes, herdadas nos genes. De acordo com esta teoria, o processo neoplásico é sequencial, desencadeia-se por etapas, como resultado de uma série de mutações que ocorrem no genoma de uma célula somática normal e, tendo como consequência final, a emergência de uma neoplasia. Numa etapa inicial da transformação neoplásica, de entre as mutações que ocorrem de forma espontânea, aleatória, geralmente sem consequências, uma determinada alteração irá conferir à célula vantagem proliferativa, de forma que esta se expande num clone celular. Nas células desse clone devido à instabilidade genómica adquirida há a possibilidade de ocorrência de mais mutações que, por sua vez vão promover o crescimento e a progressão tumoral e aumentar o potencial maligno do tumor.

Assim, a transformação neoplásica não é da responsabilidade de uma única mutação, mas requer uma multiplicidade de acontecimentos genéticos (Volgstein & Kinzler, 1993). Contudo, permanece por esclarecer se é a acumulação progressiva ou, pelo contrário, a ordem pelo qual as alterações genéticas se sucedem o factor determinante na tumorigénese. A informação proveniente da análise citogenética e molecular sugere que alguns tumores se desenvolvem devido a uma complexa combinação e conjugação de vários eventos mutagénicos, cuja ordem de ocorrência seria pouco significativa, enquanto que outros tumores se desenvolvem através de uma cadeia sequencial e bem definida no tempo de alterações genéticas (Kinzler & Volgstein, 1996; Höglund et al, 2000).

De uma forma geral, os estudos citogenéticos e moleculares corroboram este modelo multifactorial de desenvolvimento neoplásico ao demonstrarem a existência de anomalias genéticas recorrentes, específicas, na maior parte dos tumores humanos (Rabbits, 1994; Heim & Mitelman, 1995).

A recorrência e a especificidade de certo tipo de alterações associadas a determinados tipos de tumores têm sido explicadas por um fenómeno de mutagénese preferencial (Heim & Mitelman, 1995). Várias hipóteses têm sido propostas: a actuação preferencial de um agente carcinogéneo sobre determinadas

sequências genómicas, a eficácia diferencial do sistema responsável pela integridade genómica ao longo de todo o genoma, a proximidade de certos segmentos cromossómicos no núcleo, que poderá favorecer a ocorrência de trocas recíprocas e, finalmente, as sequências homólogas que podem promover rearranjos estruturais de certos segmentos cromossómicos (Heim & Mitelman, 1995). Neste contexto, a citogenética do cancro tem sido um instrumento fundamental na investigação oncológica. Reconhecer e distinguir as anomalias citogenéticas mais relevantes em cada tipo tumoral, decifrar e avaliar os seus efeitos ao nível celular, tem contribuído, de forma decisiva, para a nossa compreensão da oncogénese humana.

Anomalias cromossómicas ocorrem frequentemente durante o desenvolvimento neoplásico, como é testemunhado pela grande quantidade de alterações, tanto numéricas como estruturais, que têm sido descritas nos tumores humanos cariotipados (Mitelman F, Johansson B, Mertens F: Mitelman Database of Chromosome Aberrations in Cancer, http:/cgap.nci.nih.gov/Chromosomes/ Mitelman.2001). As alterações cromossómicas numéricas podem incluir ganho ou perda de determinados cromossomas ou alterações na ploidia. As alterações estruturais incluem amplificações de DNA, delecções, inversões e translocações. Muitas destas anomalias ocorrem de forma recorrente, não aleatória, e estão associadas a um determinado tipo de tumor.

De acordo com critérios de ordem biológica, as anomalias cromossómicas podem, ainda, ser subdivididas em primárias e secundárias. As anomalias cromossómicas primárias ocorrem usualmente em estádios precoces do processo tumorigénico, o que lhes confere um papel determinante na transformação neoplásica, enquanto que, as anomalias secundárias estão geralmente associadas a fases mais tardias do desenvolvimento, provavelmente relacionadas com a progressão tumoral (Heim & Mitelman, 1995). Estas alterações secundárias são consideradas consequência da instabilidade cariotípica, que se pensa estar associada ao desenvolvimento neoplásico.

Há que ressalvar, porém, que mesmo as alterações classificadas como primárias, essenciais ao processo neoplásico de determinado tipo de tumor, podem ser precedidas por mutações submicroscópicas. No entanto, face aos conhecimentos disponíveis, integrando a informação relativa aos mecanismos moleculares subjacentes a estas anomalias, parece não ser descabido afirmar que as alterações cromossómicas específicas associadas a um tipo de tumor ocorrem nos

primeiros estádios da tumorigénese e são uma "condição sine qua non" para todo o processo (Heim & Mitelman, 1995).

O tipo de anomalia cromossómica e o seu nível de complexidade variam consideravelmente entre diferentes tipos de tumores. De uma maneira geral, dois tipos de padrões cariotípicos podem ser reconhecidos. As alterações estruturais simples, específicas, e geralmente equilibradas, são frequentemente detectadas em leucemias, linfomas e alguns tipos de tumores sólidos, e têm como exemplos, a translocação t(9;22)(q34;q11) associada à leucemia mielóide crónica, a translocação t(2;5)(p23;q35) em linfomas anaplásicos de células grandes e a translocação t(X;18)(p11;q11) em sarcomas sinoviais (Heim & Mitelman, 1995). Os cariotipos muito mais complexos, com várias alterações cromossómicas, tanto numéricas e estruturais, estão geralmente presentes na maior parte dos tumores sólidos malignos, em particular nos de origem epitelial.

A aquisição de padrões alterados de ploidia (aneuploidia) tem sido recentemente discutido como um mecanismo promotor de instabilidade genómica que tem papel determinante na carcinogénese (Duesberg et al, 1999). Foi sugerido que as mutações em genes reguladores do ciclo celular e em genes reguladores da função dos centrossomas provocam instabilidade cromossómica e, consequentemente, favorecem a ocorrência da transformação neoplásica (Cahill et al, 1998; Zhou et al, 1998; Lengauer et al, 1998).

Consequências patogénicas das anomalias cromossómicas

O uso de técnicas moleculares tornou possível desvendar as consequências das anomalias cromossómicas, ao nível genético e celular. Verificou-se que diferentes tipos de rearranjos cromossómicos podem ter consequências patogénicas também diferentes a nível molecular.

Os dois primeiros tipos de genes a serem reconhecidos com influência directa nos mecanismos de proliferação e diferenciação celular foram os oncogenes e os genes supressores de tumor (Weinberg, 1994). Os proto-oncogenes são genes celulares normais responsáveis pelo crescimento celular, controlando-o de uma forma equilibrada. Codificam um grupo diverso de proteínas que podem actuar como factores de crescimento, factores de transcrição e reguladores da transcrição do DNA. Quando alterados (oncogenes) por mutação, amplificação ou aumento de expressão, provocam uma desregulação nos mecanismos de crescimento celular, promovendo o desenvolvimento neoplásico. O primeiro exemplo descrito de um proto-oncogene activado por uma translocação cromossómica foi o oncogene *MYC*, que ocorre no linfoma de Burkitt (Klein, 1989).

Pelo contrário, os genes supressores de tumor actuam como travões ao crescimento e divisão celular. O seu papel exerce-se no controlo da proliferação e da diferenciação celular normal pelo que, as mutações ocorridas nestes genes, conduzem, potencialmente, ao desenvolvimento neoplásico (Winford-Thomas, 1991). Em contraste com os oncogenes que exercem o seu efeito de forma dominante, o mecanismo de actuação dos genes supressores de tumor ocorre de forma recessiva a nível celular, ou seja, é necessária a inactivação de ambas as cópias do gene para que a função supressora do crescimento celular seja eliminada. Assim, a participação destes genes no processo neoplásico requer a sua inactivação, geralmente por mutação ou delecção. Foram identificados vários genes supressores de tumor, especialmente em neoplasias sólidas do tipo epitelial, sendo o gene *RB* do retinoblastoma o paradigma desta classe de genes (Knudson, 1997).

Cada classe de genes parece desempenhar um papel distinto em diferentes tipos de neoplasias humanas. Os oncogenes têm aparentemente um papel crucial na iniciação de alguns tumores, nomeadamente em leucemias, linfomas e alguns sarcomas (Vogelstein & Kinzler, 1993). Em contraste, na maioria das células epiteliais, as vias neoplásicas parecem estar preferencialmente vigiadas por genes supressores de tumor. Ambas as classes de genes estão aparentemente envolvidas na progressão e na metastização tumoral (Knudson, 1993)

Mais recentemente, os genes que regulam sistemas de reparação do DNA (genes reparadores) têm sido encontrados mutados nalguns tipos de tumores e em síndromes hereditários com predisposição para o cancro, como o carcinoma colorectal hereditário não poliposo, a ataxia telangiectesia e o xeroderma pigmentosum (Hanawalt, 1994; Estelman & Markowitz, 1996; Dasika et al, 1999). Estes genes não regulam de forma directa o crescimento celular, mas controlam a taxa de mutações de outros genes, incluindo oncogenes e genes supressores de tumor. Como consequência, a desregulação desta maquinaria de reparação de DNA pode causar uma taxa elevada de mutações que, por sua vez, pode acelerar a progressão tumoral. Outra classe importante de genes com intervenção no processo tumorigénico (genes de susceptibilidade para o cancro) regula a integridade e a estabilidade do genoma durante a divisão celular, morte celular (apoptose) e diferenciação celular (Cahill et al, 1998; Chao & Korsmeyer, 1998; Dean, 1998) pelo que a desregulação destes genes é potencialmente oncogénica.

Na maior parte dos tumores estudados, os rearranjos cromossómicos equilibrados, como translocações, inversões ou inserções, provocam a activação de proto-oncogenes localizados nos pontos de quebra. A activação qualitativa de proto-oncogenes pode resultar da formação de genes de fusão, enquanto que a

activação quantitativa de proto-oncogenes pode ocorrer por interferência nas suas sequências reguladoras de DNA. Destes dois mecanismos propostos, o primeiro é encontrado em todos os tipos de neoplasias, enquanto que o segundo parece ocorrrer preferencialmente em neoplasias dos sistemas hematopoiético e linfoide (Rabbit, 1994; Mitelman et al, 1997).

A criação de genes de fusão origina proteínas quiméricas, geralmente com uma função nova e anormal na regulação da transcrição, exercendo dessa maneira o seu efeito patogénico. O primeiro gene de fusão a ser identificado foi o gene *BCR/ABL*, que resulta da translocação t(9;22), na leucemia mielóide crónica (Shtivelman et al, 1985). A translocação t(14;18) associada aos linfomas foliculares é um exemplo clássico de desregulação oncogénica por alteração dos níveis de expressão. Devido a esta translocação, o gene *BCL2*, regulador da apoptose no cromossoma 18, fica posicionado sob o controlo das sequências reguladoras do gene *IGH* no cromossoma 14, o que resulta em níveis aumentados de expressão da proteína BCL2 (Tsujimoto et al, 1985; Cleary et al, 1986). Embora raramente, as alterações cromossómicas equilibradas podem igualmente inactivar genes supressores de tumor ou genes de reparação de DNA que estão localizados nos pontos de quebra, por fenómenos de interrupção directa nas suas sequências codificantes (Santos & Geurts, 1999 para revisão).

A identificação de rearranjos cromossómicos específicos, associados a determinados tipos de tumores e os estudos posteriores dos produtos proteicos resultantes permitiram a compreensão dos seus efeitos no funcionamento celular e, consequentemente, no processo neoplásico. Todavia, o impacto dos desiquilíbrios genómicos, que se traduzem por ganho ou perda de sequências génicas, é praticamente desconhecido.

Os desiquilíbrios genómicos mais comuns nas células tumorais são os que resultam do ganho ou da perda de um só cromossoma ou de parte de um cromossoma, adquiridos na maior parte dos casos através de rearranjos cromossómicos estruturais, como sejam isocromossomas, duplicações ou delecções, ou através de alterações numéricas, polissomias (trissomias ou monossomias). Quando o ganho de material genético ocorre através de polissomias ou de multiplicação de determinadas regiões cromossómicas, a principal consequência esperada é um efeito na dosagem de proto-oncogenes. Os níveis elevados de amplificação de DNA contendo oncogenes são a consequência molecular das anomalias cariotípicas de tipo *double minutes* (dmin) e *homogenously staining regions* (hsr). A sua alta incidência, principalmente em tumores sólidos, e a sua associação com a agressividade tumoral e mau prognóstico

demonstram, indubitavelmente, o seu papel fundamental na génese de algumas neoplasias. Um exemplo ilustrativo é o enorme ganho de cópias do proto-oncogene *MYCN* nos neuroblastomas, que se associa à formação de dmin e hsr, com consequências dramáticas no controlo do ciclo celular (Schwab & Amler, 1990).

Quando é perdido material cromossómico pensa-se que o efeito patogénico associado é a perda de genes supressores de tumor ou de genes envolvidos no sistema de reparação de DNA. Embora estas anomalias possam ser encontradas em todos os tipos de neoplasias, algumas encontram-se associadas de forma específica a alguns tumores como é o caso da perda total ou parcial do cromossoma 7 em leucemias mielóides, ou do cromossoma 22 nos meningiomas (Heim & Mitelman, 1995). O significado destas anomalias cromossómicas ao nível molecular é difícil de avaliar, porque há centenas de genes envolvidos, e porque o desvio ao número de cópias é, no geral, relativamente pequeno. Apesar disso é, geralmente aceite, que a consequência essencial da perda de um segmento cromossómico ou mesmo de um cromossoma inteiro é a perda de um ou mais genes supressores de tumor.

1.2 Tumores das glândulas salivares – aspectos gerais

Sob a designação geral de glândulas salivares incluem-se três pares de glândulas "major", as parótidas, as submandibulares e as sublinguais e as várias centenas de glândulas "minor" que se localizam na submucosa da parte superior do tracto aerodigestivo.

Os tumores das glândulas salivares são pouco frequentes, correspondendo a cerca de 3% de todos os tumores da cabeça e pescoço (Rosenberg et al, 1997). A sua incidência anual varia com as diferentes séries, de 0,4 a 13,5 casos por 100000 indivíduos (Ellis et al, 1991). Perto de 85% dos tumores salivares ocorrem nas glândulas "major" e, nestas, 90% têm origem nas parótidas, 10% nas glândulas submaxilares sendo muito raros nas glândulas sublinguais. Quanto aos que ocorrem nas glândulas "minor", mais de metade (55%) localiza-se no palato, cerca de 20% no lábio, distribuindo-se os restantes por sítios vários na cavidade bucal e nas vias áreas respiratórias superiores.

A etiologia das neoplasias salivares é praticamente desconhecida. Os factores que têm sido discutidos como podendo contribuir para a etiopatogénese são as radiações ionizantes, alguns agentes químicos, o tabaco, o vírus de Epstein Barr e certas hormonas (Rosenberg et al, 1997).

A classificação dos tumores das glândulas salivares, tal como a da maioria das neoplasias, é baseada, essencialmente, na sua histogénese e diferenciação. No

entanto, a multiplicidade de fenótipos morfológicos dos tumores das glândulas salivares, bem como a sua relativa raridade e dificuldade de sistematização contribuíram para o aparecimento de diversas classificações.

A última classificação da Organização Mundial de Saúde, de 1991 (Seifert & Sobin, 1991) é a utilizada no Serviço de Anatomia Patológica do Instituto Português de Oncolgia Francisco Gentil, Centro de Lisboa e, como tal, é a adoptada neste trabalho. Esta classificação identifica 9 tipos de adenomas, 18 tipos de carcinomas, 5 tipos de tumores não epiteliais e 2 tipos de linfomas (Tabela 1).

Pelo menos 80% das neoplasias salivares são benignas, sendo o adenoma pleomórfico a forma tumoral mais comum (60%). Este tumor benigno é particularmente relevante pela sua tendência para recidivar, para progredir para a malignização e, embora raramente, para metastizar (Seifert et al, 1990).

No grupo das neoplasias malignas, que corresponde a 20% da totalidade dos tumores, o carcinoma adenoide-cístico, o carcinoma mucoepidermóide, o carcinoma de células acinares e o carcinoma ex-adenoma pleomórfico são os mais frequentes (Ellis & Auclair, 1996).

O carcinoma adenoide-cístico constitui cerca de um quarto de todos os carcinomas salivares. É relativamente mais comum nas glândulas "minor" do que nas "major" (Rosenberg et al, 1997). A maior parte dos casos apresenta um comportamento biológico agressivo, com uma grande potencialidade para recidivar e para metastizar (Ellis et al, 1991). Já o comportamento biológico do carcinoma mucoepidermóide é mais imprevisível. Na maioria dos casos, os tumores crescem lentamente e apresentam bom prognóstico, mas, em alguns, ocorrem recidivas locais e, mais raramente, metástases. Neste tipo de tumor a subclassificação baseada em características histológicas constitui um factor independente de prognóstico e é utilizado na opção terapêutica mais adequada (Clode et al, 1991; Seifert et al, 1990).

Em cerca de 3-4% dos adenomas pleomórficos podem desenvolver-se tumores malignos a partir da sua componente epitelial, designados por isso, carcinomas ex-adenoma pleomórfico ou carcinomas originados em tumor misto (Ellis et al, 1991). São relativamente raros, compreendendo cerca de 12% do total dos tumores salivares malignos. O risco de malignização aumenta de 1,6% em doentes que apresentam uma história clínica de 5 anos de existência de um tumor benigno para 9,6% em tumores em que essa história tem mais de 15 anos (Ellis & Auclair, 1996). A componente maligna é, geralmente, do tipo adenocarcinoma pouco diferenciado ou de carcinoma indiferenciado (Ellis et al, 1991).

Tabela 1 - Classificação histológica dos tumores das glândulas salivares segundo a OMS (1991)*

1-Adenomas	**3-Tumores não epiteliais**
Adenoma pleomórfico	Angiomas
Mioepitelioma	Lipomas
Adenoma de células basais	Tumores neurais
Tumor de Warthin	Outros tumores benignos mesenquimatosos
Oncocitoma	Sarcomas
Adenoma canalicular	
Adenoma sebáceo	**4-Linfomas**
Papiloma ductal	Linfoma da zona marginal (MALT) extranodal
Cistadenoma	Linfoma nodal
2-Carcinomas	**5-Tumores secundários**
Carcinoma de células acinares	
Carcinoma mucoepidermoide	**6-Tumores não classificáveis**
Carcinoma adenoide cístico	
Adenocarcinoma polimórfico de baixo grau	**7-Lesões pseudo-tumorais**
Carcinoma epitelial-mioepitelial	Sialoadenite
Adenocarcinoma de células basais	Oncocitose
Carcinoma sebáceo	Sialometaplasia necrotizante
Cistadenocarcinoma papilar	Lesão linfoepitelial benigna
Adenocarcinoma mucinoso	Quistos da glândula salivar
Carcinoma oncocítico	Sialoadenite esclerosante da glândula submandibular
Carcinoma dos ductos salivares	Hiperplasia linfoíde quística associada a SIDA
Adenocarcinoma NOS (not other specified)	
Carcinoma mioepitelial	
Carcinoma ex-adenoma pleomórfico	
Carcinoma indiferenciado	
Carcinoma pavimento-celular	
Carcinoma de células pequenas	
Outros carcinomas	

*extraído de Seifert et al (1991)

Tumorigénese das glândulas salivares

A maioria dos tumores das glândulas salivares é caracterizada pela presença de células epiteliais e mesenquimatosas, facto que se repercute na grande diversidade de padrões histomorfológicos que apresentam. A presença de duas populações celulares distintas levanta a questão pertinente quanto à sua origem histogenética: terão origem num único tipo celular ou, pelo contrário, serão de origem multicelular?

Diversos estudos têm abordado esta questão tomando como modelo o adenoma pleomórfico, que constitui o exemplo paradigmático de um tumor com fenótipo misto. No entanto, a sua histogénese permanece envolta em controvérsia sendo duas hipóteses avançadas: uma que os considera como resultando de expansões clonais de uma única célula pluripotencial ("stem cell") derivada dos ducto salivares (Batsakis et al, 1989; Batsakis & El-Naggar, 1999), e outra que propõe que os tumores bidiferenciados das glândulas salivares, incluindo os adenomas pleomórficos, se desenvolvem a partir do crescimento coordenado das populações celulares presentes (Dardick, 1998).

Os poucos estudos de clonalidade efectuados até à data em adenomas pleomórficos, favorecem a hipótese da existência de uma célula pluripotencial, designada por célula mioepitelial modificada, na origem dos adenomas pleomórficos (Debiec-Rychter et al, 2001; Lee et al, 2000; Noguchi et al, 1996). O alargamento do estudo, não só a um número maior de casos de adenomas pleomórficos, como a outros tumores das glândulas salivares de composição bifásica, bem como a utilização de metodologias mais adequadas poderão elucidar esta questão e, consequentemente, contribuir para um melhor entendimento das vias de desenvolvimento neoplásico e dos mecanismos de diferenciação celular.

Alterações genómicas em tumores das glândulas salivares 1.3

Ploidia do DNA nuclear

A aneuploidia tumoral, que pode ser determinada através da quantificação do DNA nuclear, é considerada um indicador importante na avaliação das alterações genómicas que ocorrem num tumor. Um padrão tumoral aneuplóide é frequentemente observado em tumores sólidos, principalmente nos malignos, e está geralmente associado a estádios tumorais tardios. Este fenómeno é considerado ser uma consequência de mecanismos diversos que incluem instabilidade genómica, não-disjunção mitótica, endoreplicação e/ou fusão celular.

Alguns estudos dedicaram-se ao estudo da ploidia do DNA nos tumores das glândulas salivares (Pinto et al, 2000 para revisão). Em contraste com outras neoplasias, os tumores das glândulas salivares, tanto benignos como malignos, revelaram, no geral, a existência de um padrão diplóide do DNA. Em relação aos tumores benignos, nomeadamente os adenomas pleomórficos, a maior parte é diplóide. Contudo, em alguns casos, foi detectada aneuploidia, geralmente associada a tumores recorrentes (Martin et al, 1994), tendo sido advogado que a aquisição desta alteração genómica desempenharia um papel potencialmente importante na malignização do adenoma pleomórfico (Pinto et al, 1999).

Relativamente aos tumores malignos salivares, foi igualmente detectada uma baixa frequência de aneuploidia e foi possível estabelecer para alguns carcinomas uma relação entre a ploidia do DNA tumoral e o seu grau histológico. De uma forma geral, todos os carcinomas das glândulas salivares classificados histologicamente de baixo grau são diplóides, enquanto que os tumores aneuplóides pertencem à categoria neoplásica de alto grau de malignidade (Pinto et al, 2000).

Citogenética

De todos os órgãos e tecidos do corpo humano as glândulas salivares apresentam, provavelmente, o conjunto de tumores com maior heterogeneidade histológica. No entanto, exceptuando o adenoma pleomórfico, a informação citogenética disponível é, comparativamente com a de outras neoplasias humanas, escassa e pouco conclusiva.

A análise citogenética de tumores das glândulas salivares demonstrou que, diferentemente do que ocorre em muitos tumores sólidos, o cariotipo é geralmente pouco complexo, sendo, na maioria dos casos, diploíde ou perto da diploidia, em perfeito acordo com os resultados das medições da ploidia do DNA nuclear. Verificou, ainda, a presença de padrões cariotípicos aparentemente distintos em tumores benignos e malignos. Os tumores benignos, nomeadamente os adenomas pleomórficos, caracterizam-se preferencialmente por translocações recíprocas equilibradas, que afectam regiões cromossómicas específicas, enquanto que os tumores malignos têm preferencialmente perda de material genético, frequentemente no mesmo cromossoma (Sandros et al, 1990).

Desde a primeira descrição sobre alterações cromossómicas em adenomas pleomórficos feitas por Mark et al, em 1980, mais de 470 casos foram cariotipados por dois grupos independentes: na Suécia (Göteburg) e na Alemanha (Bremen)

(Bullerdiek et al, 1987, 1988, 1989, 1993; Mark et al, 1986, 1997; Stenman et al, 1983). A informação obtida permitiu constituir, nesta entidade, subtipos citogenéticos bem definidos.

A par de um subgrupo de adenomas com cariotipo normal (entre 20 a 50% conforme as séries) existiriam mais 3 subgrupos (Sandros et al, 1990; Bullerdiek et al, 1993; Mark et al, 1997):

1) tumores com anomalias do cromossoma 8, geralmente translocações afectando o braço longo na banda 8q12 (~60% dos casos). A anomalia mais comum (~30% dos casos) é uma translocação recíproca, equilibrada, entre o braço curto do cromossoma 3 e o braço longo do cromossoma 8 sob a forma t(3;8)(p21;q12) (Figura 1). Neste subgrupo estão também incluídos aqueles casos que apresentam trissomia 8, a anomalia numérica mais frequente no adenoma pleomórfico;

2) tumores com anomalias do cromossoma 12, preferencialmente em 12q13-15 (~10%);

3) tumores com anomalias não recorrentes (grupo menos representativo).

As anomalias em 8q12 e 12q13-15 parecem excluir-se mutuamente, porquanto só foram encontradas conjuntamente num número reduzido de casos (Sahlin et al, 1995; Röijer et al, 1999). Estes rearranjos cromossómicos são frequentemente as únicas alterações cariotípicas detectadas, o que favorece a interpretação de poderem ter um papel crucial na génese dos adenomas pleomórficos.

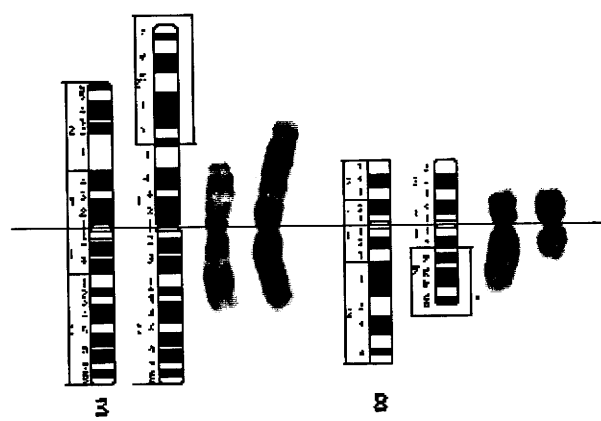

Fig. 1- Esquema representativo da translocação t(3;8)(p21;q12) em adenomas pleomórficos

O estabelecimento de subgrupos citogenéticos pode ser usado para determinar o comportamento dos tumores neles incluídos quanto ao seu potencial para recidivar e/ou sofrer tansformação maligna, e também para estabelecer uma correlação entre os subtipos histológicos e os grupos citogenéticos (Bullerdiek et al, 1988). A classificação em subgrupos baseada nos achados citogenéticos provou ser muito útil, por exemplo, em neoplasias do tecido adiposo ao permitir correlacionar esses achados com a histologia e, mais relevante ainda, com o comportamento clínico (Fletcher et al, 1991).

Têm sido feitas tentativas para correlacionar os subgrupos citogenéticos dos adenomas pleomórficos com as características clinico-patológicas dos tumores. Bullerdiek et al (1993) encontrou diferenças quanto à idade dos doentes, ao subtipo histológico e à morfologia celular *in vitro*. Os tumores com rearranjos em 8q12 ocorrem geralmente em indivíduos mais jovens, são adenomas de fenótipo tipo clássico, possuem 30-50% do seu volume representado por estroma segundo a classificação de Seifert (Seifert, 1976 em Bullerdiek, 1993) e as células cultivadas apresentam morfologia de tipo epitelial. Os tumores com cariotipo normal e que têm rearranjos em 12q13-15 ocorrem em indivíduos mais velhos, e são constituídos por mais de 80% de componente mesenquimatoso. A morfologia predominante das células em cultura é do tipo fibroblastóide nos tumores em que há alterações em 12q13-15 e também na maioria dos tumores com cariotipo normal. Assim, parece haver uma diferença significativa nos adenomas pleomórficos quanto a estes parâmetros, o que pode sugerir vias de diferenciação celular distintas. Até à data ainda não foi possível correlacionar o padrão cariotípico com as características clinico-patológicas dos adenomas pleomórficos, de forma a estabelecerem-se indicadores de prognóstico, nomeadamente quanto ao seu potencial para recidivar e em sofrerem transformação maligna.

A análise citogenética referente a outros tumores salivares benignos limita-se aos seguintes subtipos histológicos: tumor de Warthin (23 casos), oncocitoma (1 caso), linfadenoma sebáceo (1 caso) e mioepitelioma (1 caso) (Mark et al, 1990, 1991, 1994; Nordkvist et al, 1994; El-Naggar et al, 1999).

Nos tumores de Warthin, a par da perda clonal do cromossoma Y, a translocação t(11;19)(q21;p13) revelou ser a única alteração estrutural recorrente (Bullerdiek et al, 1988; Mark et al, 1989, 1990; Nordkvist et al, 1994). No linfadenoma sebáceo e no oncocitoma foram detectadas somente anomalias numéricas, respectivamente +9 e +7 (Mark et al, 1991,1994) . O mioepitelioma tem anomalias estruturais várias sendo de notar o rearranjo do braço longo do cromossoma 12 (El-Naggar et al, 1999), anomalia frequente nos adenomas

pleomórficos, entidade com a qual os mioepiteliomas se relacionam, já que podem considerar-se o limite celular do espectro de diferenciação daquelas neoplasias.

Por razões que se prendem com a baixa incidência e com a dificuldade em obter a sua cultura *in vitro* é muito escassa a informação sobre os tumores malignos salivares. Só existem 64 casos com anomalias que se distribuem de uma maneira geral pelos subtipos histológicos mais frequentes, (Mitelman F, Johansson B, Mertens F: Mitelman Database of Chromosome Aberrations in Cancer, http:/cgap.nci.nih.gov/Chromosomes/Mitelman.2001). No entanto revelaram-se suficientes para definir um padrão característico para estas neoplasias.

Os cariotipos estabelecidos são predominantemente pseudodiplóides, ou quase diplóides, com delecções de material cromossómico. As anomalias cromossómicas numéricas mais frequentes são a perda do cromossoma Y e as trissomias 7 e 8. A anomalia estrutural mais comum envolve o braço longo do cromossoma 6 e corresponde geralmente a delecções na região terminal com pontos de quebra que se situam em 6q21-25 (Jin et al, 1994). Estas alterações são comuns a todos os subtipos histológicos com excepcção do carcinoma ex-adenoma pleomórfico. Neste carcinoma, que corresponde à malignização de uma neoplasia benigna, descreveram-se rearranjos clonais envolvendo as regiões 12q13-15 e 8q12-13 (Bullerdiek et al, 1990; Mark et al, 1991, 1992; Jin et al, 1994).

A análise citogenética demonstrou ainda haver anomalias específicas associadas a subtipos histológicos particulares dos carcinomas salivares: carcinoma adenóide-cístico e carcinoma mucoepidermóide. No primeiro foi identificada a translocação recorrente t(6;9)(q23;p21) (Nordkvist et al, 1994) e, no segundo, verificou-se uma translocação entre o cromossoma 11 e o cromossoma 19 sob a forma t(11;19)(q21-22;p13) (Nordkvist et al, 1994). Curiosamente, esta translocação detectada no carcinoma mucoepidermóide, que tem pontos de quebra em 11q21 e 19p13, parece citogeneticamente idêntica à que foi descrita no tumor de Warthin (Bullerdiek et al, 1988; Mark et al, 1989, 1990; Nordkvist et al, 1994).

Genética molecular

A identificação de anomalias cromossómicas específicas associadas a certos tipos de neoplasia tem fornecido indicações fundamentais para a localização de genes envolvidos na tumorigénese.

A caracterização citogenética das neoplasias salivares tornou este grupo de tumores potencialmente informativo para análise molecular por duas razões: a existência de alterações cromossómicas recorrentes específicas e a aparente

diferença quanto aos padrões citogenéticos observados, entre tumores benignos e malignos. Nesse sentido o adenoma pleomórfico revelou-se um modelo de estudo preferencial. A regularidade e especificidade dos rearranjos cromossómicos em 8q12 e 12q13-15 sugeria a existência, nessas regiões, de genes fortemente associados ao desenvolvimento neoplásico.

Alterações em 12q13-15, para além de ocorrerem no adenoma pleomórfico, foram igualmente descritas em outros tumores benignos, como os lipomas (Mandahl et al, 1994) e os leiomiomas uterinos (Pandis et al, 1991) o que evidenciou esta região como potencialmente implicada na sua génese. Os estudos moleculares efectuados com o objectivo de caracterizar estes rearranjos e identificar o(s) gene(s) afectados conduziram à identificação do gene *HMGA2* (anteriormente designado por *HMGIC*) localizado nesta região, pertencente à família dos genes de proteínas de elevada mobilidade, que têm papel na regulação da estrutura e organização da cromatina (Shoenmakers et al, 1995; Ashar et al, 1995).

São vários os cromossomas parceiros nas translocações com 12q13-15, mas o envolvimento de regiões genómicas particulares e, consequentemente, de determinados genes, parece estar associado a tipos específicos de tumores. Um exemplo disso é o gene *LPP*, que se localiza em 3q27, e que é o parceiro do *HMGA2* na translocação t(3;12)(q27-28;q15), a anomalia citogenética mais comum em lipomas (Petit et al, 1996). Nos adenomas pleomórficos, os genes identificados como parceiros do *HMGA2*, foram, até à data, os genes *FHIT*, em 3p14.2, e *NFIB*, em 9p23 (Geurts et al, 1997, 1998).

Foi sugerido que o *HMGA2* intervem na regulação da proliferação celular. Os estudos de expressão demonstraram que o gene tem níveis altos de expressão em tecidos em que há proliferação activa como os tecidos embrionários e as neoplasias malignas, mas em tecidos adultos normais a sua expressão só foi detectada em pulmão e rim (Rogalla et al, 1996; Gattas et al, 1999).

Por forma a avaliar o seu envolvimento em tumores benignos de natureza mesenquimatosa, nomeadamente em lipomas e em leiomiomas uterinos, Tallini et al (2000) procederam ao estudo da expressão do *HMGA2* e correlacionaram os dados obtidos com as alterações cariotípicas identificadas nesses mesmo tumores. Os resultados demonstraram haver expressão aberrante do *HMGA2* nos tumores com rearranjos em 12q14-15, nos quais o ponto de quebra se encontrava no próprio gene ou na sua proximidade.

Outros tumores benignos, como os hamartoma da mama e do pulmão, o angiomixoma e o fibroadenoma da mama (Shoenmakers et al, 1995) com

rearranjos em 12q13-15 têm também envolvimento do *HMGA2*. Todas estas neoplasias benignas que incluem também o adenoma pleomórfico partilham um padrão histológico bifásico com diferenciação epitelial e mesenquimatosa, na maior parte dos casos estando a desregulação do *HMGA2* confinada ao componente estromal (Tallini et al, 1999).

A anomalia cromossómica mais comum em adenomas pleomórficos das glândulas salivares é a translocação recíproca t(3;8)(p21;q12), observada em cerca de 30% dos casos que têm anomalias do cromossoma 8. A análise molecular do ponto de quebra desta translocação permitiu identificar os genes responsáveis, nomeadamente, o gene *PLAG1*, em 8q12, que codifica um factor de transcrição e o gene *CTNNB1*, em 3p21, que codifica a beta-catenina (Kas et al, 1997). Como resultado desta translocação há uma troca dos promotores entre os genes *PLAG1* e *CTNNB1*, mecanismo que coloca o gene *PLAG1* sob o controlo do promotor do gene *CTNNB1* e vice versa, o que preserva as sequências codificantes de ambos os genes. Este mecanismo molecular, que se designa por "promoter swaping", implica a activação do gene *PLAG1* e o aumento da sua expressão, bem como a redução dos níveis de expressão do gene *CTNNB1* nas células tumorais. Por sua vez, a expressão anormal do *PLAG1* condiciona a desregulação dos genes-alvo do *PLAG1*, como o gene *IGF-II* que se pensa ter uma função fundamental nos mecanismos de diferenciação celular (Voz et al, 2000).

O alargamento do estudo a outros adenomas salivares com translocações variantes, como a t(5;8)(p13;q12), confirmou o compromisso do gene *PLAG1* e levou à identificação do outro gene parceiro na translocação, o gene *LIFR* em 5p13 (Voz et al, 1998). Também a análise de adenomas pleomórficos com cariotipo normal permitiu verificar que o gene *PLAG1* se encontra rearranjado nalguns destes casos, tendo como genes "parceiros", para além do gene *CTNNB1*, o gene *SII* que tem igualmente localização na região cromossómica 3p21.3-22 (Åström et al, 1999).

Assim, a activação da expressão do *PLAG1*, que ocorre sob o controlo de promotores de diferentes genes, parece ser um factor decisivo no mecanismo patogénico dos adenomas pleomórficos. No entanto, num estudo por hibridização *in situ* com fluorescência (FISH) realizado em três casos de adenoma pleomórfico que tinham anomalias na região 8q12-13, foi demonstrado que o *PLAG1* não estava rearranjado, o que levanta a hipótese de nessa região haver mais genes com intervenção na tumorigénese salivar (Röijer et al, 1999).

A existência de oncogenes diferentes envolvidos nestes tumores, o gene *PLAG1* em 8q12 e o gene *HMGA2* na região 12q14-15, parece indiciar duas vias

de desenvolvimento neoplásico independentes para os adenomas pleomórficos. Continua, no entanto, por esclarecer se a activação desses oncogenes é reflexo da progressão tumoral ou, se pelo contrário, é responsável *per se* pela iniciação neoplásica.

As delecções do cromossoma 6 (6q25-qter) são as alterações estruturais que ocorrem com mais frequência nos carcinomas salivares. Foram realizados estudos de perda de heterozigotia com o objectivo de caracterizar molecularmente estas delecções cromossómicas (Queimado et al, 1998). A análise de uma série de 19 casos permitiu delimitar duas regiões de delecção mínima neste tipo de tumores, 6q21-23.3 e 6q27, o que sugere a inactivação de genes onco-supressores aí localizados, o que se relacionaria com o processo de cancerigénese das glândulas salivares (Queimado et al, 1998). Não são conhecidos os mecanismos moleculares das translocações específicas t(11;19) e t(6;9) associadas, respectivamente, a carcinomas mucoepidermóides e a carcinomas adenoide-císticos.

Para além dos estudos sobre a identificação dos genes e dos mecanismos moleculares que estes regulam sugeridos pela análise citogenética, há outros trabalhos que incidiram sobre a intervenção potencial de outros genes, nomeadamente os proto-oncogenes *H-RAS*, *c-ERBB2* e *MYC* e genes supressores de tumor como o *TP53* e o *RB*, aos quais é reconhecida a participação na oncogénese de outras neoplasias humanas (Stenman et al, 1991; Sugano et al, 1992; Deguchi et al, 1993; Birek et al, 1994; Yamamoto et al, 1996; Yoo et al, 2000; Shintani et al, 2000).

A maior parte dos estudos efectuados em tumores das glândulas salivares incidiram principalmente sobre os genes *c-ERBB2* e *TP53* (Pinto et al, 2000 para revisão). As mutações no gene *TP53* ocorrem mais frequentemente em tumores malignos do que em benignos o que favorece uma possível acção deste gene no desenvolvimento e progressão tumoral (Rosa et al, 1997; Yamamoto et al, 1996). Em relação ao *c-ERBB2* foi demonstrada a sua sobre-expressão associada a um prognóstico desfavorável em várias neoplasias humanas, nomeadamente o carcinoma ductal da mama (Hanna et al, 1999). Nos tumores das glândulas salivares há vários estudos que sugerem que as alterações que afectam o gene *c-ERBB2* desempenham um papel importante no desenvolvimento e progressão em vários carcinomas (Félix et al, 1996, Giannoni et al, 1995; Hellquist et al, 1994; Press et al, 1994). Por isso se admite que as mutações que ocorrem em ambos os genes podem influenciar os mecanismos de desenvolvimento e progressão tumoral das glândulas salivares, com consequências na gravidade do comportamento biológico da doença.

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2.

Objectivos

Objectivos do estudo

O presente trabalho pretendeu utilizar a caracterização (cito)genética de algumas formas histológicas de neoplasias das glândulas salivares para elucidar sobre as vias de desenvolvimento neoplásico bem como sobre mecanismos de diferenciação celular.

Considerados estes objectivos gerais identificaram-se como objectivos específicos desta investigação:

- caracterizar alterações cromossómicas recorrentes por forma a reconhecer regiões genómicas-alvo com potencial papel na tumorigénese das glândulas salivares

- contribuir para o esclarecimento da histogénese das neoplasias salivares com diferenciação bi-celular, nomeadamente usando como modelo o adenoma pleomórfico, através da identificação das células epiteliais e mioepiteliais portadoras das anomalias cromossómicas específicas

3.

Trabalhos realizados

3.1

Benign Salivary Gland Tumors: a Cytogenetic Study of 21 cases

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Benign Salivary Gland Tumors: A Cytogenetic Study of 21 Cases

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Cytogenetic findings of 21 benign salivary gland tumors, including 14 pleomorphic adenomas, 5 Warthin's tumors, 1 myoepithelioma, and 1 cystadenoma, are reported. The present study confirms that pleomorphic adenomas characteristically have highly specific rearrangements involving only a few chromosome regions (3p21, 8q12 and 12q13-15) which suggests their specific role in the mixed tumors genesis. Warthin's tumors also show nonrandom numerical and structural alterations that were concurrent in one of the cases studied. To our knowledge no cytogenetic data are available in myoepitheliomas and cystadenomas. The former reveals a normal karyotype and the latter shows only clonal numerical alterations (gain of chromosomes 2 and 18).

KEY WORDS: **salivary gland neoplasms, cytogenetics, pleomorphic adenoma, Warthin's tumors**

INTRODUCTION

Cytogenetic analysis of human solid tumors identified consistent and recurrent abnormalities in a large proportion of neoplasms [1–3]. They have been largely demonstrated in malignant neoplasms but benign tumors, namely lipomas, uterine leiomyomas, meningiomas, and adenomas of the salivary glands also have been shown to be associated with karyotypic aberrations [2,4,5].

The correlation between specific karyotypic alterations and histological types of the tumors strongly suggests the crucial role of specific chromosomal regions in the tumorigenesis of certain neoplasms [1,2].

Cytogenetic information on benign salivary gland tumors is almost confined to pleomorphic adenomas [2,6]. Rearrangements in 3p12, 8q12, and 12q13-15 regions were described in different studies in this group of neoplasms [6–12]. Cytogenetic data on the other benign salivary tumor types are very scarce or even absent [5,13].

This study describes the chromosomal findings of a series of 21 benign salivary gland tumors. It attempts to provide further cytogenetic information on these infrequent tumors including two histological types (myoepithelioma and cystadenoma) without previous cytogenetic information.

MATERIALS AND METHODS

Study Population

Twenty-one benign salivary gland tumors from 20 patients were processed for cytogenetic study. They include 14 pleomorphic adenomas, 5 Warthin's tumors, 1 myoepithelioma, and 1 cystadenoma. One patient had two synchronous tumors: pleomorphic adenoma (case 7) and Warthin's tumor (case 15).

The patients' ages ranged between 11 and 77 years (Table I). Seventeen of the tumors were located at the major salivary glands: parotid gland (n = 14) and submandibular gland (n = 3) and the remaining four cases originated in the minor salivary glands of the oral cavity.

Methods

All tumors were histologically classified according to the World Health Organization criteria [14] by two pathologists.

Fresh tumor samples were processed under sterile conditions for short-term cultures as described by Gibas et al.

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TABLE I. Summary of the Clinical and Cytogenetic Findings of the 21 Benign Salivary Gland Tumors

Case	Age/sex	Classification	Karyotype
1	35/F	Pleomorphic adenoma	46,XX
2	60/F	Pleomorphic adenoma	46,XX
3	19/F	Pleomorphic adenoma	46,XX
4	57/F	Pleomorphic adenoma	46,XX
5	36/F	Pleomorphic adenoma	46,XX
6	69/F	Pleomorphic adenoma	46,XX
7	72/M	Pleomorphic adenoma	46,XY
8	44/F	Pleomorphic adenoma[a]	47,XX,+8[3]/46,XX[11]
9	11/F	Pleomorphic adenoma	46,XX,t(3;8)(p21;q12)[7]/46,XX[4]
10	70/F	Pleomorphic adenoma	46,XX,t(3;8)(p21;q12)[9]/46,XX[1]
11	77/F	Pleomorphic adenoma	46,XX,t(3;8)(q29;q13)[7]
12	39/F	Pleomorphic adenoma	46,XX,t(8;9)(q12;p22)[3]/46,XX[4]
13	47/F	Pleomorphic adenoma	46,XX,t(8;9)(q12;p22)[5]
14	25/F	Pleomorphic adenoma	46,XX,t(6;12)(q23;q15)[10]
15	72/M	Warthin's tumor	46,XY
16	64/M	Warthin's tumor	45,X,−Y[4]/46,XY[6]
17	69/M	Warthin's tumor	45,X,−Y[5]/46,XY[3]
18	52/M	Warthin's tumor	47,XY,t(6;8)(p23;q22),+7[2]/45,X,−Y[2]/46,XY[10]
19	63/F	Warthin's tumor	45,X,−X[2]/47,XXX[1]/46,XX[8]
20	46/F	Myoepithelioma	46,XX
21	71/F	Cystadenoma	47,XX,+2[2]/47,XX,+18[7],46,XX[5]

[a]Recurrence.

[15]. The mitotic activity and morphology of the cultured cells were daily assessed using a phase-contrast microscope.

Chromosomes were analyzed using G-banding techniques and karyotypes were described according to the International System for Human Cytogenetic Nomenclature (ISCN) [16].

The constitutional karyotypes of all but cases 13 and 21 were determined by routine lymphocyte cultures.

In 16 of the 21 tumors, flow cytometric DNA analysis was performed, using a modification of the method of Thornthwaite et al. [17]. The DNA index of the tumor tissue was compared with the modal chromosome number obtained by the cytogenetic analysis in culture, for the purpose of assessing the representativity of the original neoplasm sample.

RESULTS

The constitutional karyotypes of all the cases were cytogenetically normal.

Table I summarizes the clinical data and the cytogenetics findings of 21 benign salivary gland tumors including pleomorphic adenomas (n = 14; Fig. 1A), Warthin's tumors (n = 5; Fig. 1B), myoepithelioma (n = 1; Fig. 1C), and cystadenoma (n = 1; Fig. 1D).

Seven of the 14 cases of pleomorphic adenoma, 1 of the 5 cases of Warthin's tumor, and 1 case of myoepithelioma showed metaphases with normal karyotypes.

Clonal numerical alterations were demonstrated in one pleomorphic adenoma (case 8), three Warthin's tumors (cases 16, 17, and 19), and the cystadenoma case (case 21). They were represented by gain of chromosomes 2, 8, 18, and X as well as by loss of Y and X.

The other six cases of pleomorphic adenoma exhibited reciprocal translocations as the sole cytogenetic change, involving chromosomes 3, 6, 8, 9, and 12 (Fig. 2).

One case of Warthin's tumor (case 18) presented not only numerical alterations (gain of chromosome 7 and loss of Y) but also a translocation t(6;8)(p23;q22) (Fig. 3).

DISCUSSION

Pleomorphic adenoma is the most frequent histological type of salivary gland tumor comprising about 70% of all neoplasms originating in these structures [18,19]. In recent years, a large amount of information has been accumulated regarding its cytogenetical pattern [1,2,4–13,20–22].

In our series of benign salivary gland tumors, pleomorphic adenoma was largely predominating, being represented by 14 cases from which 7 had normal karyotype. From the other 7 cases showing chromosomal deviations, 6 were characterized by numerical and structural alterations of chromosome 8: case 8 with trisomy 8, cases 9 and 10 with a reciprocal translocation t(3;8)(p21;q12), case 11 with a translocation t(3;8)(q29;q13) and cases 12 and 13 with a translocation t(8;9)(q12;p22). Rearrangements in 12q13-15 were found in one tumor.

These results confirm the data already collected in previous cytogenetic studies of pleomorphic adenomas [4,6–12]. The karyotypic characteristics of these neo-

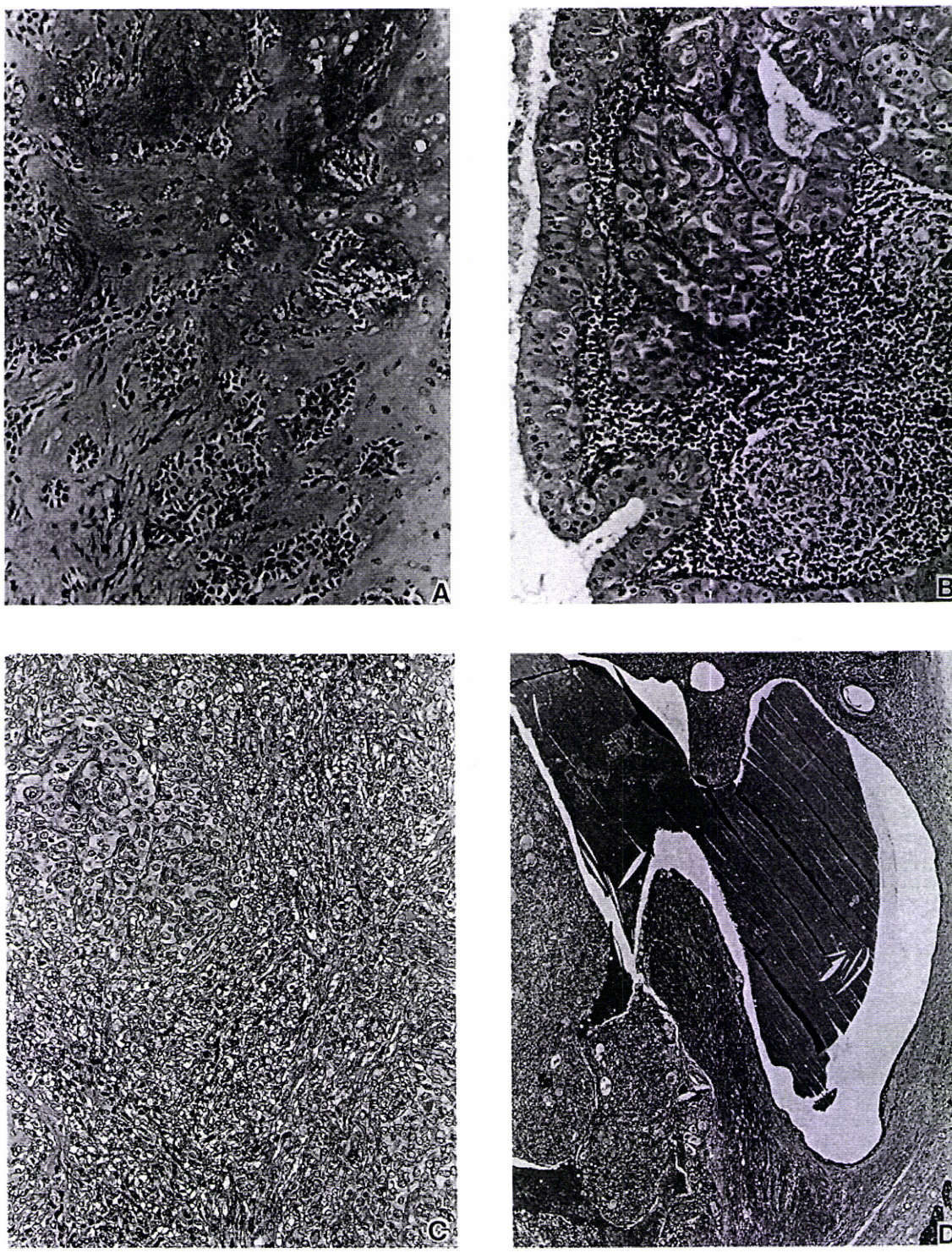

Fig. 1. Histological appearance of the studied cases: pleomorphic adenoma (A), Warthin's tumor (B), myoepithelioma (C), and cystadenoma (D).

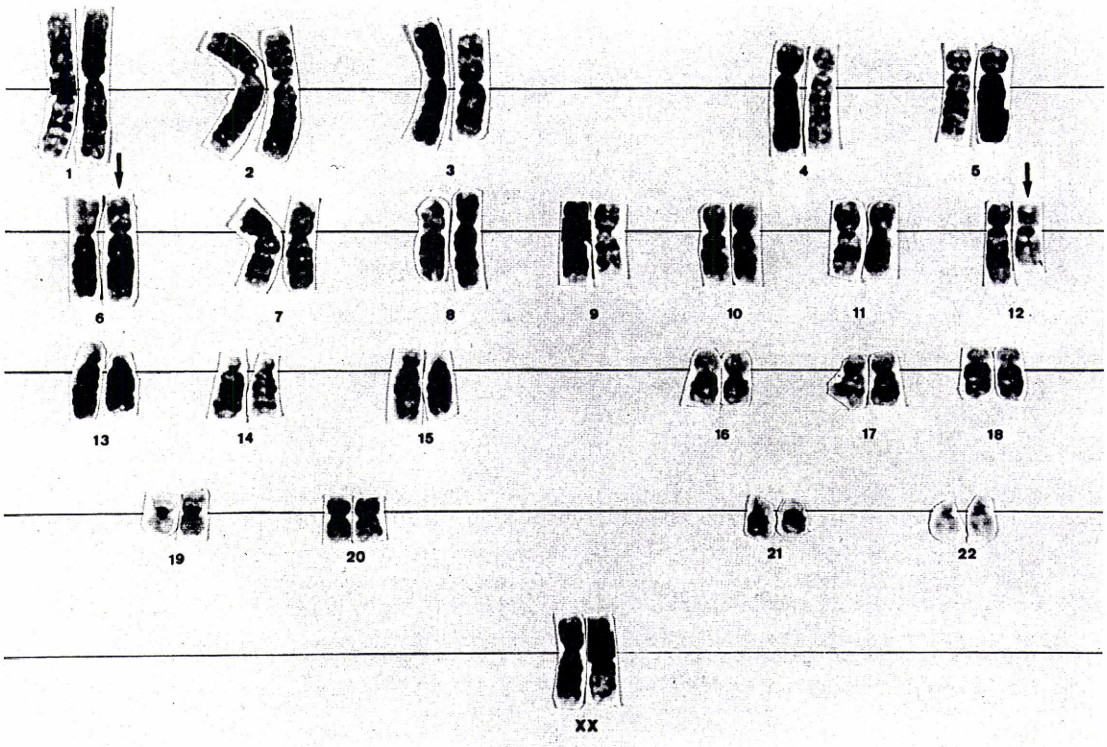

Fig. 2. Karyotype of a metaphase from case 14. Karyotypic description is shown in Table I.

plasms were described in detail by Bullerdiek et al. [8–12] and others [4,6,23]. It has been demonstrated that pleomorphic adenomas present highly specific rearrangements that preferentially affect three chromosomal regions: 3p21, 8q12, and 12q13-15 [4,6–12]. Rearrangements of 3p21 and 8q12 usually involve the same reciprocal translocation t(3:8)(p21:q12) [4,6,12].

Three cytogenetic subtypes were described in pleomorphic adenoma by Bullerdiek et al. [10–12]: 1) with normal karyotype; 2) with 8q12 rearrangement; 3) with 12q13-15 rearrangement. The cases that do not fall into these categories constitute an heterogeneous group of tumors presenting diverse nonrecurrent clonal abnormalities. Table II summarizes the data of the largest series published thus far [4,6,12] and our own data.

We verify that our results corroborate the existence of the aforementioned cytogenetic subtypes and, therefore, they strongly support the hypothesis that chromosomes 8 and 12 carry genes of relevance for the tumorigenesis of pleomorphic adenoma.

Warthin's tumor is the second most represented tumor type in this series. The cytogenetic information available in the literature is scarce [13,21–22], and was recently increased with the publication of Nordkvist et al. [22]. Similarly to pleomorphic adenomas Mark et al. [13] proposed three distinct cytogenetic categories: 1) with a normal karyotype, 2) with numerical changes only, and 3) with structural changes only. Our study of five additional cases of Warthin's tumor does not entirely support this oversimplified view since one case (case 18) evidenced both clonal numerical aberrations (+7) and structural rearrangements with a reciprocal translocation t(6;8)(p23;q22). Nordkvist et al. [22] also referred one case showing a sideline with a reciprocal translocation and loss of one chromosome.

Breakpoints at 6p23 have been already reported in a case of Warthin's tumor by Mark et al. [13] and this fact makes this abnormality a recurrent change in this group of tumors involving that particular region. It is therefore conceivable that a gene located at that locus might be potentially related to the development of these neoplasms.

To our knowledge there is no information regarding the cytogenetic characteristics of myoepitheliomas and salivary gland cystadenomas. The two entities represent uncommon histological lesions among benign salivary gland tumors. The analysis of the cystadenoma included in the present series revealed clonal numerical deviations

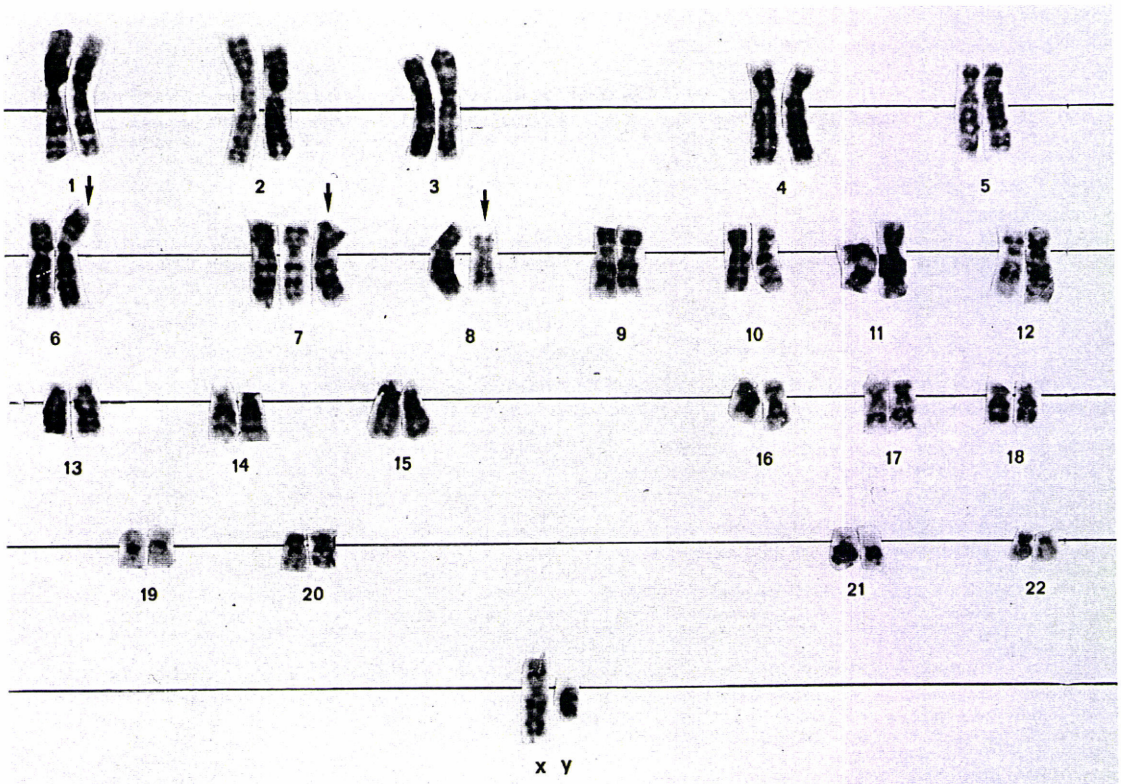

Fig. 3. Karyotype of a metaphase from case 18. Karyotypic description is shown in Table I.

TABLE II. Benign Salivary Gland Tumors: Cytogenetic Results of Chromosome Analysis in Pleomorphic Adenoma Include the Present Series and the Bullerdiek et al. and Mark et al. Series

Cytogenetic subtypes	Present study		Mark et al. [23]		Bullerdiek et al. [11]	
	n	(%)	n	(%)	n	(%)
Normal karyotype	7	50.0	53	53.0	111	50.5
8q12 rearrangements	5	35.7	22	22.0	56	25.5
12q13–15 alterations	1	7.1	10	10.0	29	13.1
Other clonal deviations	1	7.1	15	15.0	24	10.9
Total	14	100	100	100	220	100

with gain of chromosomes 2 and 18 but without structural abnormalities. The myoepithelioma studied revealed a normal karyotype. Myoepitheliomas are neoplasms that present a quite monotonous cellular composition with elements exhibiting a terminally differentiated myoepithelial cell phenotype [14,18]. Being considered as one end spectrum of differentiation of pleomorphic adenoma, it is conceivable that myoepithelioma might also share the cytogenetic characteristics described in benign salivary tumors.

CONCLUSIONS

In summary, our results confirm the existence of distinct cytogenetic patterns of chromosomal alterations among benign salivary gland tumors, particularly pleomorphic adenomas. Cytogenetical abnormalities are not limited to this heterogeneous histological type and do also occur in commonless benign salivary tumors. More data are needed to gain a better understanding of salivary

gland tumorigenesis and the morphogenetic relationship between the distinct histological types of salivary neoplasms.

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3.2

Cytogenetic Characterisation of Warthin's Tumor

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Cytogenetic Characterisation of Warthin's Tumour

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Warthin's tumour is a peculiar subtype of monomorphic adenomas of the salivary glands, frequently cystic, and that characteristically associates an epithelial glandular cell component to a dense lymphoid infiltrate. Short-term cultures from 12 Warthin's tumours of salivary glands, including 5 previously reported cases were successfully karyotyped and clonal numerical and/or structural changes were detected in 7 of them (58%). 3 cases showed numerical abnormalities with loss of chromosomes Y (2 cases) and X (1 case). The remaining 4 abnormal cases presented the following structural changes: complex translocation t(11;19;16)(q21;p12;p13.3); reciprocal translocations t(6;8)(p23;q22) and t(6;15)(p21;q15) (2 cases); and 1p22, 3p26, 11p13 changes. In 1 case, clonal numerical deviations (+7 and –Y) were concurrent with the structural rearrangement t(6;8). Two of these aberrations are suggested to be Warthin's tumour-associated: 11q;19p translocation has already been described in 3 cases, and structural rearrangements of 6p23 have also been reported in another case. Our study extends the cytogenetic information about Warthin's tumour and identifies two recurrent abnormalities — 6p rearrangements and t(11;19) — specific for this salivary neoplasm. © **1997 Elsevier Science Ltd**

Key words: salivary gland neoplasms, Warthin's tumour, cytogenetics, chromosome 6, t(11;19)

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INTRODUCTION

Warthin's tumour is a rather peculiar type of salivary gland adenoma with a well-defined clinicopathological profile, characteristically male preponderance, almost exclusive location at the parotid gland, typical histology and a uniformly benign course.

The categorisation of the lesion whether a 'true' neoplasm or a reactive pseudotumoral hyperplastic process has been a matter of controversy [1], which reinforces the potential utility of its cytogenetic study to clarify the issue.

The cytogenetic information available on this tumour type is rather scarce, amounting to less than 30 cases [2–5]. In pleomorphic adenomas, highly specific chromosome rearrangements allowed the identification of distinctive cytogenetic subgroups [2, 6]. By analogy, Nordkvist *et al.* [5] proposed three cytogenetic subgroups in Warthin's tumour: (1) with an apparently normal stemline; (2) with normal stemlines and abnormal sidelines; and (3) with abnormal stemlines.

The purpose of the present study was to report additional cytogenetic information of Warthin's tumour by describing the chromosomal pattern of a series of 12 cases, that includes 7 new cases, further contributing to the discussion concerning its relationship to the genesis of the neoplasm.

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MATERIALS AND METHODS

12 cases of Warthin's tumours of the parotid gland were successfully processed for cytogenetic study. The patients' age ranged between 28 and 72 years (mean: 58.1 ± 13.6 years) (Table 1). All but 2 patients were males.

Fresh tumour samples were processed, under sterile conditions, for short-term cultures as described by Gibas *et al.* [7]. The mitotic activity and morphology of the cultured cells were assessed daily using a phase contrast microscope.

Chromosomes were analysed using G-banding techniques and karyotypes were described according ISCN guidelines [8]. The constitutional karyotypes were checked by routine lymphocyte cultures except for cases 6, 10, 11 and 12 (Table 1), where there was no available peripheral blood.

Flow cytometric DNA analysis, using a modification of the method of Thornthwaite *et al.* [9], was performed in all but cases 2 and 7 to compare the DNA index of the tumours with the modal chromosome number found by cytogenetic analysis in culture. Flow cytometric DNA analysis revealed a diploid histogram (DNA index = 1.0) for the 10 tumours assessed.

RESULTS

The constitutional karyotypes analysed were cytogenetically normal. Table 1 summarises the clinical and cytogenetic findings of the 12 cases. 5 of the 12 cases only showed

metaphases with normal karyotypes. Clonal numerical deviations, as the sole cytogenetic abnormality, were detected in 3 cases and corresponded to loss of chromosomes Y (case 2 and 3) and X (case 5). The remaining 4 cases showed structural alterations: case 4 displayed a reciprocal translocation t(6;8) concurrent with clonal numerical deviations (+7, –Y), case 9 (Fig. 1) presented a reciprocal translocation t(6;15)(p21;q15) and case 10 had a complex translocation t(11;19;16)(q21; p12;p13.3) (Fig. 2).

Case 8 exhibited several clonal structural abnormalities affecting 11p22, 3p26 and 11p13 chromosome regions; in this case some variant cells were also observed.

Table 1. *Summary of the clinical and cytogenetic findings of 12 Warthin's tumours*

Case	Age/sex	Site	Follow-up (months, status)	Karyotype	Karyotype No clonal findings
1	72/m	lp	20,a&w	46,XY[17]	
2	64/m	lp	11,a&w	45,X,-Y[4]/46,XY[6]	
3	69/m	lp	5,doc	47,X,-Y,[5]/46,XY[3]	
4	52/m	lp	15,doc	47,XY,t(6;8)(p23;q22),+7[2]/45,X,-Y, [2]/46,XY[10]	
5	63/f	rp	52,a&w	45,X,-X[2]/47,XXX[1]/46,XX[8]	
6	67/m	rp	–	46,XY[10]	
7	63/m	lp	—	46,XY[15]	
8	67/m	lp	–	40-46,XY,add(3)(p26),del(11)(p13)[2]/45,XY, t(1;6)(p22;p11.2),-8,del(4)(q31.1)[1]/43,XY, t(1;12)(p22;q24),-13,-18-22[1]/46,XY[8]	-6,+13,der(21)t(6;21)(p13;p12)/-2,-4, der(6)del(6)(p21.3) add(6)(p21.3), -2/-2,-10, -13, t(14;15)(q2?4;p?11.2);t(16;17)(q13;p13)
9	45/m	rp	9,a&w	45-47,xy,t(6;15)(p21;q15)[5]/46,xy[7]	
10	28/f	rp	17,a&w	37-47,XX, der(11)del(11) (q21) t(11;19;16) (q21;p12;p13.3), der(16)del(16)(q22) add(16) (p13.3)t(11;19;16) (q21;p12;p13.3),der(19)del(19)(p12) t(11;19;16) (q21;p12;p13.3)[20]	
11	41/m	lp	–	46,XY[22]	t(1;18)(q21;q23)/t(2;3)(p1?3;p21)
12	67/m	lp	8,a&w	46,XY[31]	

m = male; f = female; lp = left parotid; rp = right parotid; a&w = alive and well; doc = dead from other causes.

Fig. 1. Karyotype of a metaphase from case 9 (karyotypic description is shown in Table 1).

Fig. 2. Karyotype of a metaphase from case 10 (karyotypic description is shown in Table 1).

DISCUSSION

Cytogenetic data on Warthin's tumours are, to our best knowledge, available from 28 cases reported by Bullerdiek *et al.* [2], Nordkvist *et al.* [5] and Martins *et al.* [10]. Our present series reports on twelve tumours, that comprise previously described cases [10] and 7 additional cases. 5 of the 12 cases (42%) displayed normal karyotypes which is in agreement with the studies of Nordkvist *et al.* [5] and similar to what is found in pleomorphic adenomas, the most common benign salivary gland tumour, that presents a cytogenetically defined subgroup with a normal karyotype [6, 11]. As generally referred it cannot be excluded that these cases possess alterations at a molecular level which are undetectable by conventional cytogenetic analysis.

The finding of clonal alterations in almost half of Warthin's tumour cases further supports that this lesion is a 'true' neoplasm rather than an autoimmune or hypersensitivity-related tumour-like proliferation, as previously suggested by Ogawa *et al.* [12] and Allegra *et al.* [13].

Seven out of the 12 tumours (58%) displayed clonal aberrations, four being structural changes; the remaining three were characterised as clonal numerical abnormalities (loss of chromosomes Y and X). Loss of chromosome Y was also previously reported in 3 cases of Warthin's tumour [4, 5] but the significance of this cytogenetic finding as a marker of clonal change is rather controversial, since loss of chromosome Y can occur in the normal bone marrow of elderly males and it is generally agreed that it is not pathologically meaningful [14]. Therefore, one might admit that a missing Y is most probably an age-related phenomenon instead of an intrinsic tumour-associated change.

Four neoplasms of this series presented clonal structural aberrations. Cases 4 and 9 showed reciprocal translocations involving the 6p region. In the former, a t(6;8)(p23;q22), the breakpoint was identified in 6p23, which makes this chromosomal abnormality a recurrent alteration in Warthin's tumour [4]. In the latter, a t(6;15)(p21;q15), the breakpoint was defined at 6p21. In case 8, in addition to clonal structural alterations involving the 1p22, 3p26 and 11p13 regions some variant cells with breaks in the region 6p11.2-21.3 were also identified. These alterations, taken together, point to admitting that the short arm of chromosome 6 contains a region consistently involved in the origin of Warthin's tumour.

Abnormalities of these chromosomal regions identified in distinct benign solid tumour types may suggest that they contain genes involved in tumour growth regulation [15].

A complex translocation involving the 11q21 and 19p12 regions was detected in case 10. A similar translocation, t(11;19)(q21:p12-13), was previously reported on 2 Warthin's tumour cases and a t(11;16)(q13-15;q23) also reported in another neoplasm of this type [2, 3, 5]. Interestingly, a similar t(11;19)(q14-21;p12) was also documented in mucoepidermoid carcinomas of the salivary glands and lung [16–20]. Mucoepidermoid carcinoma and Warthin's tumour do not share clinicopathological similarities, being apparently unrelated neoplastic entities both originating on salivary glands tissues [21]. We hypothesise that the above mentioned cytogenetic lesion they have in common may be associated with the histogenesis of both neoplasms anatomically related to the proximal duct system of salivary glands [22].

Our study validates the proposal of Nordkvist *et al.* [5] for the cytogenetic categories of Warthin's tumour. However,

although informative for the understanding of the pathology of tumour development, it seems to be of no clinical relevance since the neoplasm is always benign and curable by surgery.

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3.3

Malignant Salivary Gland Tumors: a Cytogenetic Study of 19 cases

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Malignant Salivary Gland Neoplasms: a Cytogenetic Study of 19 Cases

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A group of 19 malignant salivary gland neoplasms of various histological types (mucoepidermoid carcinoma, acinic cell carcinoma, adenoid cystic carcinoma, epithelial–myoepithelial carcinoma, myoepithelial carcinoma, basal cell adenocarcinoma, carcinoma ex-pleomorphic adenoma, ductal carcinoma, adenocarcinoma not otherwise specified and undifferentiated carcinoma) were cytogenetically investigated. Previous karyotypic information revealed deletion of the long arm of chromosome 6, loss of chromosome Y and the gain of chromosome 8 as the most recurrent deviations found in these neoplasms. Clonal chromosome aberrations were detected in 11 cases of this series. In 7 of them there were only numerical deviations (gain of chromosomes 2, 7, 8, 10 and X and loss of chromosomes 18, 21 and Y) without concomitant structural anomalies. Structural rearrangements such as t(2;7), t(6;16), t(6;9) and t(1;1) translocations were found in two mucoepidermoid carcinomas, one adenoid cystic carcinoma and one ductal carcinoma, respectively.

The wide spectrum of changes found in this group of neoplasms may reflect the diversity in their histogenesis and differentiation phenotypes. Copyright © 1996 Elsevier Science Ltd

Keywords: salivary gland neoplasms, cytogenetics, adenoid cystic carcinoma, mucoepidermoid carcinoma

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INTRODUCTION

Malignant salivary gland tumours are infrequent neoplasms that present various morphological patterns as well as diverse clinical behaviour [1, 2]. Cytogenetic studies on salivary gland tumours are almost restricted to benign tumours, especially pleomorphic adenomas [3–5]. The information regarding the chromosomal patterns of malignant salivary gland neoplasms is rather limited [4–17]. Structural rearrangements involving the long arm of chromosome 6, loss of chromosome Y and gain of chromosome 8 are recurrent clonal deviations that have been demonstrated in these tumours [4–17].

We present herein additional cytogenetic information on the frequency and specificity of chromosomal deviations found in a series of 19 consecutive malignant salivary gland tumour types of diverse histological type.

MATERIALS AND METHODS

Study population

Nineteen malignant salivary gland tumours were successfully processed for cytogenetic study. The patient's age ranged between 12 and 79 years (Table 1). All the tumours were located at the major salivary glands (parotid gland $n=15$; submaxillary gland $n=3$), but case 8 originated in the minor salivary glands of the oral cavity.

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Methods

The neoplasms were classified according to the World Health Organization classification [2] into the following categories: mucoepidermoid carcinomas ($n=5$), adenoid cystic carcinomas ($n=3$), carcinomas ex-pleomorphic adenoma ($n=3$), epithelial–myoepithelial carcinomas ($n=2$) (Fig. 1); acinic cell carcinoma ($n=1$), adenocarcinoma NOS (not otherwise specified) ($n=1$), basal cell adenocarcinoma ($n=1$), undifferentiated carcinoma ($n=1$), myoepithelial carcinoma ($n=1$) and ductal carcinoma ($n=1$) (Fig. 2).

Pieces of fresh tumour tissue were used to set up short-term cultures from each neoplasm. The methods for tissue culture were described by Gibas *et al.* [18]. The mitotic activity and morphology of the cultured cells were assessed daily using a phase-contrast microscope.

Chromosomes were analysed using G-banding techniques and karyotypes were described according to the International System for Human Chromosomes Nomenclature (1991) [19].

Table 1. *Summary of the clinical and cytogenetic results of* 19 *malignant salivary gland tumours*

Case	Age/sex	Classification	Karyotype
1	50/M	Mucoepidermoid ca	46, XY
2	12/F	Mucoepidermoid ca	46, XX
3	54/F	Mucoepidermoid ca	45, XX, −21[2]/47, XXX[2]/46, XX[9]
4	63/M	Mucoepidermoid ca	46, XY, t(2;7)(q23;p22)[5]/46, XY[6]
5	47/M	Mucoepidermoid ca	46, XY, +7, −21[2]/47, XY, +7[2]/94, XXYY, +7, +7[2]/46, XY, t(6;16)(q21;q22)[7]/46, XY, del(6)(q23)[1]/46, XY[3]
6	51/M	Acinic cell ca	45, X, −Y[3]/46, X, −Y, +7[1]/47, XY, +8[2]/46, XY[7]
7	41/F	Adenoid cystic ca	46, XX
8	46/F	Adenoid cystic ca	46, XX
9	32/M	Adenoid cystic ca	46, XY, t(6;9)(q23–25;p22–24)[8]/45, XY, t(6;9)(q23–25;p22–24), −20[4]
10	44/M	Epithelial–myoepithelial ca	46, XY
11	66/F	Epithelial–myoepithelial ca	48, XX, +2, +8[7]/45, X0, −X, −10, +20[2]/46, X0, −X, +20[1]/46, XX[6]
12	79/F	Myoepithelial ca	41, XX, −3, −7, −14, −17, −18[1]/43, XX, −8, −18, −19[1]/43, XX, −12, −18, −21[1]/46, XX[8]
13	31/F	Basal cell adenoca	46, XX
14	68/M	Ca ex-pleomorphic adenoma	46, XY
15	58/M	Ca ex-pleomorphic adenoma	46, X, −Y, +7[10]
16	72/F	Ca ex-pleomorphic adenoma	47, XX, +10[14]/46, XX[2]
17	65/M	Ductal carcinoma	46, XY, t(1;1)(p36;q12)[2]/47, XY, +7[1]/46, XY[9]
18	72/F	Adenocarcinoma NOS	47, XX, +7[1]/42, XX, −4, +7, −13, −15, −18, −20[1]/46, XX[4]
19	66/F	Undifferentiated ca	46, XX

ca, carcinoma; NOS, not otherwise specified.

Fig. 1. Epithelial–myoepithelial carcinoma exhibiting a biphasic composition with an inner layer of dark, epithelial cells surrounded by a layer of clear, myoepithelial-type cells (case 11) (haematoxylin and eosin).

Fig. 2. Salivary duct carcinoma, showing a predominantly cribriform architecture (case 17) (haematoxylin and eosin).

The constitutional karyotypes were checked by routine lymphocyte cultures, except for cases 3, 4, 12, 14 and 19.

For DNA cytometric analysis, tumour samples were processed according to a modification of the method of Thornthwaite *et al.* [20]. Histograms were defined as diploid (DNA index = 1.0) if they had a single G0/G1 peak and as aneuploid if there was evidence of more than one distinct G0/G1 peak. Normal counterpart cells were used as a standard control. Cell cycle analysis was performed using the Multicycle Software Program (Phoenix Flow Systems, Inc., San Diego, California, U.S.A.).

RESULTS

Table 1 summarises the clinical data and the cytogenetic findings in 19 malignant salivary gland tumours. Flow cytometric DNA analysis was performed to compare the DNA index of the tumours with the modal chromosome number found by cytogenetic analysis in culture. The results allowed the acceptance of the cytogenetic analysis as representative of the original tumours. Flow cytometric DNA analysis revealed diploid histograms (DNA index = 1.0) for all tumours with the exception of cases 5, 14, 16 and 17 which exhibited a DNA index of 1.76, 1.24, 2.18 and 1.95, respectively.

All constitutional karyotypes analysed were cytogenetically normal. Eight of the 19 neoplasms had metaphases with normal karyotypes. They corresponded to two mucoepidermoid carcinomas, two adenoid cystic carcinomas, one epithelial–myoepithelial carcinoma, one carcinoma ex-pleomorphic adenoma, one basal cell adenocarcinoma and one undifferentiated carcinoma.

All the cases with cytogenetic abnormalities also displayed normal karyotypes, except cases 9 and 15. The former presented a t(6;9)(q23-25;p22-24) and the latter showed loss of chromosome Y and gain of chromosome 7 in all metaphases analysed.

Cases 3, 6, 11 (Fig. 3), 12, 15, 16 and 18 exhibited numerical alterations (gain of chromosomes 2, 7, 8, 10 and X and loss of chromosomes 18, 21 and Y), without concomitant structural anomalies. Cases 4 and 17 (Fig. 4) had reciprocal translocations as the sole abnormality, respectively, t(2;7)(q23;p22) and t(1;1)(p36;q12) and cases 5 and 9 showed both numerical and structural abnormalities with involvement of chromosomes 6, 7, 9, 16, 20 and 21.

DISCUSSION

Malignant tumours of the salivary glands are uncommon neoplasms. Their incidence is much lower than their benign counterpart and this fact may justify the rather limited karyotypic information available.

Our series of 19 malignant salivary gland neoplasms constitute a heterogeneous group of tumours, including ten different histopathological subtypes: mucoepidermoid carcinoma, acinic cell carcinoma, adenoid cystic carcinoma, epithelial–myoepithelial carcinoma, myoepithelial carcinoma, basal cell adenocarcinoma, carcinoma ex-pleomorphic adenoma, ductal carcinoma, adenocarcinoma NOS and undifferentiated carcinoma. This histological diversity reflects the wide vari-

Fig. 3. Karyotype of a metaphase from case 11 (karyotypic description is shown in Table 1).

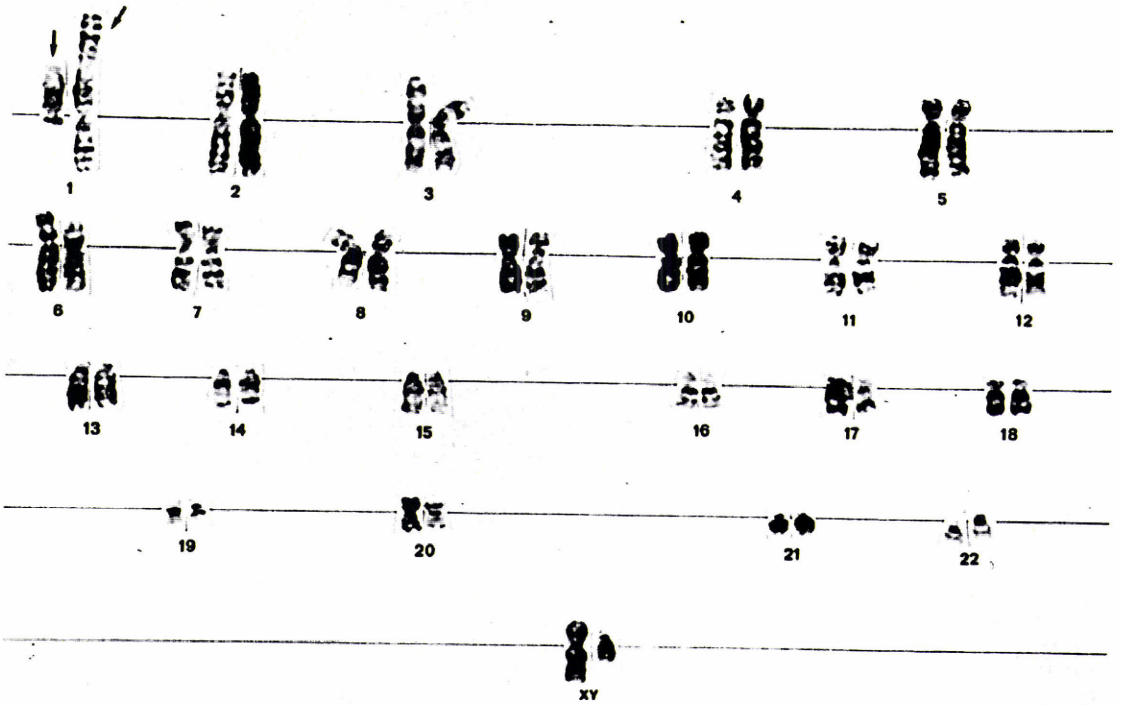

Fig. 4. Karyotype of a metaphase from case 17 (karyotypic description is shown in Table 1).

ation in the microscopical appearance of the malignant salivary gland tumours.

Flow cytometric results agreed well with the modal chromosome number found in cultured tumour tissue. For all tumours, except in cases 5, 14, 16 and 17, diploid histograms were in accordance with the modal chromosome number of the karyotypes. The aneuploid histograms obtained in cases 5, 14, 16 and 17 (1.76, 1.24, 2.18 and 1.95 DNA index, respectively), disagree with the modal chromosome number 46–47, found by cytogenetic analysis which can be explained by the loss of aneuploid cells in culture.

Eight cases revealed normal karyotypes and this finding is in part unexpected, considering the high frequency of clonal changes described in human malignancies [21, 22]. Due to the important role of the stromal mesenchymal cells in the composition of salivary neoplasms, one might argue that the apparently normal karyotypes are related to the high capacity of the stromal cells to grow *in vitro*. However, we can also admit that malignant neoplasms displaying normal karyotypes have alterations at a molecular level, without evidence of chromosomal rearrangements being, therefore, undetectable by conventional cytogenetical analysis.

Among salivary gland neoplasms, mucoepidermoid carcinoma is the best cytogenetically characterised subgroup with 18 cases already reported [11–15]. Numerical changes (loss of Y chromosome, trisomies as +7, +8) and structural abnormalities (6q22-25 and 11q14-24 rearrangements) are recurrent deviations found in these tumours. In our series, mucoepidermoid carcinoma was also the most frequent neoplasia ($n=5$). Three cases had clonal abnormalities: case 3, numerical deviations (loss of chromosome 21 and gain of chromosome X); case 4, a reciprocal translocation t(2;7)(q23;p22) and case 5, both numerical and structural deviations (loss of chromosome 21, gain of chromosome 7 and 6q rearrangements). Rearrangements of 11q14-24, namely the previously reported t(11;19)(q14-21;p12-13) [13–15] were not found in our cases.

Karyotypic information on acinic cell carcinoma includes some consistent clonal deviations: 6q rearrangements, loss of chromosome Y and gain of chromosomes 7 and 8 [14]. Apart from 6q rearrangements, case 6 also displayed trisomies 7, 8 and loss of chromosome Y. The pathogenic significance of −Y and +7 in short-term cultured neoplasms has been a matter of debate, since these aberrations were also found in non-neoplastic cells [14]. Recently, it has been demonstrated in kidney tumours and surrounding tissues that trisomies 7 and 10 characterise subpopulations of tumour-infiltrating lymphocytes [23]. The exact significance of these aberrations remains poorly understood. However, it is tempting to hypothesise a similar phenomenon occurring in the case we reported, since acinic cell carcinoma is a neoplasia characteristically containing aggregates of lymphoid cells.

The cytogenetic data of the 16 cases of adenoid cystic carcinoma recorded in the literature show 6q rearrangements, loss of chromosome Y and gain of chromosome 8 [4, 5, 14, 16]. One out of the three adenoid cystic carcinomas studied (case 9) revealed a reciprocal translocation t(6;9)(q23-25;p22-24) similar to the recurrent t(6;9)(q21-24;p13-23) found in three published cases of the parotid gland [4, 14, 17] as well as

to the t(6;9)(q23;p22) described in a case occurring at the lacrimal gland [15]. These findings indicate t(6;9) as a specific, and possibly primary non-random chromosomal abnormality in adenoid cystic carcinoma [14]. The other recurrent deviation already reported in adenoid cystic carcinoma, as well as in all major types of malignant salivary gland tumours, except in carcinomas ex-pleomorphic adenoma is the loss of genetic material from the long arm of chromosome 6, del(6)(q23-25) [4, 14, 16]. This aberration was not present in our series but the limited number of cases of this histological type do not allow definitive conclusions. However, we may admit that the normal karyotypes verified in two adenoid cystic carcinoma cases are due either to *in vitro* loss of neoplastic cell populations or to rearrangements at the molecular level, which are undetectable by chromosomal analysis.

Only 7 cases of adenocarcinomas of salivary glands were previously karyotyped, being identified loss of chromosome Y, gain of chromosome 8 and 6q deletions [4–7, 14]. The adenocarcinoma NOS of our series (case 18) only exhibited clonal numerical deviations represented by the gain of chromosome 7. Deletions of 6q, the most consistently recurrent aberration described in salivary gland adenocarcinomas, were not found in this particular case.

Case 15 displayed numerical deviations (−Y, +7) in all metaphases analysed and case 16 trisomy 10. To the best of our knowledge, only 6 cases of carcinoma ex-pleomorphic adenoma have been karyotyped: one case shared with one we described a trisomy 7, in addition to a 12q13-15 translocation [9, 10].

There is no cytogenetic information regarding salivary ductal carcinomas, basal cell adenocarcinomas, epithelial–myoepithelial carcinomas and myoepithelial carcinomas. In the present series, the malignant myoepithelioma (case 12) and one of the two epithelial–myoepithelial carcinomas (case 11) showed clonal numerical deviations. The former tumour displayed loss of chromosome 18 and the latter two unrelated clones: one had gain of chromosomes 2 and 8 and the other had loss of chromosomes 10, 20 and X. This cytogenetic biclonality may reflect the histologically biphasic composition of these low-grade adenocarcinomas. The fact that trisomy 8 was also detected in another carcinoma type suggests that chromosome 8 may be implicated in salivary gland tumorigenesis.

The salivary ductal carcinoma is a very rare and aggressive malignancy. The case studied showed the reciprocal translocation t(1;1)(p36;q12) and this is the first report on the involvement of this region in salivary carcinomas [24].

We conclude from our results and accumulated studies that the wide variation of cytogenetic findings may reflect heterogeneity in the histological types of malignant salivary gland neoplasms, a peculiar group of tumours known to have various morphogenetic pathways and diverse behaviour.

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3.4

Cytogenetic similarities between two types of salivary gland carcinomas: adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma

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Cytogenetic similarities between two types of salivary gland carcinomas: adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma

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Abstract Adenoid cystic carcinoma (ACC) and polymorphous low-grade adenocarcinoma (PLGA) are low-grade adenocarcinomas of salivary glands with a putative common histogenesis from the intercalated ducts but featuring distinct histological appearances. Hybrid tumors containing areas with histological patterns of both neoplasms have been reported but, to our knowledge, the question of their genotypic similarity has not yet been approached. As part of an ongoing study on cytogenetic characterization of salivary gland tumors, from a group of 24 malignant neoplasms, three out of five cases of ACC and three of four cases of PLGA were selected for their similar karyotypic changes. All of them displayed chromosome 12 abnormalities, affecting the 12q12–q13 region in four (all ACC cases and one PLGA case), 12q22 in one PLGA case, and 12p12.3 in the remaining. From this group of neoplasms, one PLGA and one ACC showed the same t(6;12)(p21;q13). Our findings favor the concept that tumors of salivary glands displaying epithelial and myoepithelial phenotypes share a common histogenesis.

1. Introduction

Low-grade salivary gland adenocarcinomas with myoepithelial derivation constitute a morphologically heterogeneous group of tumors in which four main histological subtypes are recognized, as well as a common histogenesis from the intercalated duct [1]. Adenoid cystic carcinoma (ACC) (Fig. 1a) and polymorphous low grade adenocarcinoma (Fig. 1b) are two of these neoplasms, that, although distinguishable by their histological appearance, share phenotypical characteristics, namely the co-expression of epithelial and myoepithelial markers.

Previous cytogenetic studies demonstrated that ACC frequently has structural alterations in chromosomes 6 and 9, 6q deletions and t(6;9)(q21–q25;p21–p22) translocation being the most common [2,3].

Cytogenetic alterations in PLGA were described in four cases [4–6], with two of the reports pertaining to cases where PLGA was diagnosed as part of the malignant component of carcinomas ex-pleomorphic adenoma [4,5]. Rearrangements at 8q12, 12q13–q15 were the recurrent chromosome aberrations found in these cases [4,5]. In the two remaining cases, one showed a clonal t(6;9)(p21;p22) [6] and the other a monosomy 22 [5].

We herein present the cytogenetic findings of three cases of ACC and three cases of PLGA, all showing chromosome 12 rearrangements, further contributing to the hypothesis that salivary adenocarcinomas with myoepithelial participation share a common histogenesis.

2. Materials and methods

In this study, three out of a series of five cases of ACC and three out of four cases of PLGA were retrieved from a consecutive series of 24 karyotyped malignant salivary tumors, and selected based on the shared finding of chromosome 12 abnormalities.

The clinical, karyotypic, and follow-up data are summarized in Table 1. All but one patient were females and the patients ranged in age from 31 to 84 years. PLGA case 1 and ACC case 4 were localized at the parotid gland and PLGA case 3 and ACC case 6 at the submandibular gland. PLGA case 2 originated at the palate and ACC case 5 in the tongue.

Follow-up information, available in all cases, ranged from 12 to 146 months. Three patients are alive without evidence of disease. The remaining have recurrent tumor in cervical lymph nodes (case 5) and lung (cases 3, 4, and 5) (Table 1).

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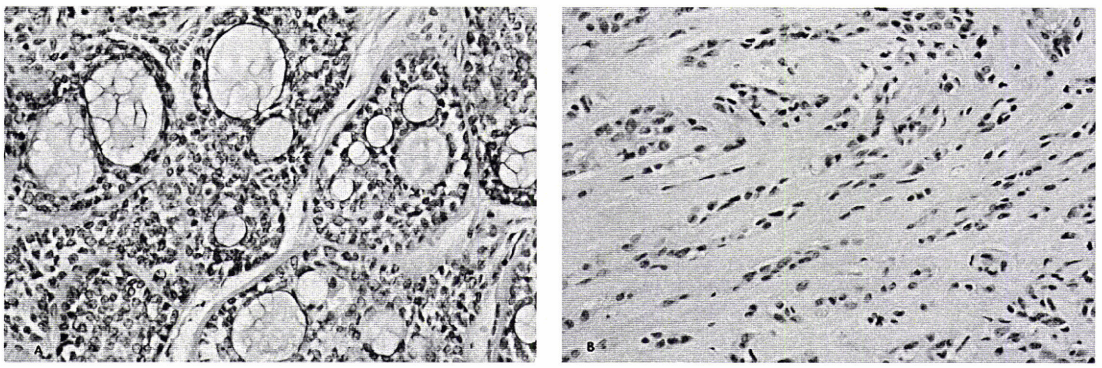

Fig. 1. Histological appearance of ACC (a) and PLGA (b). In the latter, the classical "Indian file" pattern of growth is the predominant characteristic, while in ACC, cribriform architectural pattern is readily apparent (H&E, original magnification ×600).

All tumors were short-term cultured as described by Jin et al. [7]. The cultures were harvested after 5–20 days using conventional techniques. Chromosomes for cytogenetic analysis were GTG-banded and karyotypes were described following the ISCN [8].

In PLGA cases 1 and 3, fluorescence in situ hybridization (FISH) analysis was performed with whole chromosome painting probes (Cambio, UK) for chromosomes 6, 12, and 15 to further elucidate chromosome 12 rearrangements, respectively the t(6;12) and t(12;15), according to the method described by Höglund et al. [9].

The results of GTG banding and FISH were processed and recorded with a Cytovision System (Applied Imaging, UK).

3. Results

Full karyotypic description of the six salivary carcinomas and FISH results of cases 1 and 3 are given in Table 1. Complete karyotypes of PLGA case 1 and ACC case 4 are presented in Figs. 2 and 3, respectively.

All cases displayed chromosome 12 abnormalities affecting the q arm in five tumors (two PLGA and three ACC) and the p arm in one case of PLGA (Fig. 4).

All ACC cases showed 12q12–q13 rearrangements, in cases 4 and 6 as del(12)(q12q13) and in case 5 involving a translocation with chromosome 6 t(6;12)(p21;q13). Other recurrent abnormalities were also detected in ACC cases at the following regions: 9p22 (cases 5 and 6), 6q23–q25 (cases 4 and 5) and 17p12–p13 (case 4).

Two distinct 12q regions were rearranged: 12q13 (case 1) and 12q22 (case 2) on two PLGA cases. Fluorescence in situ hybridization investigation of PLGA case 3 revealed a reciprocal translocation t(12;15)(p12.3;q?23). The 12q13 region on PLGA case 1 was the chromosome partner for the translocation with chromosome 6 originating a t(6;12)(p21;q13), which was confirmed by FISH analysis (Fig. 5). This

Table 1
Clinical and cytogenetic information from polymorphous low-grade adenocarcinoma (PLGA) and adenoid cystic carcinoma (ACC) cases

Case	Sex/age	Localization	Diagnosis	Follow-up	Days in vitro	Total cells counted/karyotyped	Karyotype
1	F/74	Parotid gland	PLGA	12 mo, A&W	5–20	20/7	46,XX,t(6;12)(p21;q13)[b][7]
2	F/84	Soft palate	PLGA	48 mo, A&W	20	46/10	45–46,XX,t(12;22)(q22;p11–p12)[cp2]/46,XX[8]
3	M/63	Submandibular Gland	PLGA	12 mo, AWD (lung met)	5–9	50/20	46,XY,t(12;15)(p12.3;q?23)[c][12]/46,XY,inv(9)p13.3p24.1),t(12;15)(p12.3;q?23)[3]/46,XY[5]
4	F/31	Parotid gland	ACC[a]	146 mo, AWD (lung met)	5–15	24/6	44–46,XX,add(1)(p34–p35),t(5;9)(q11;p22),del(6)(q25),−8,add(8)(p11),t(10;12)(p10;p10),del(12),(q12q13),t(13;19)(p11;q11),−16,del(17)(p12),+mar1,+mar2[cp6]
5	F/73	Tongue	ACC	19 mo, AWD (ln and lung met)	19–27	15/11	46,XX,der(6)t(6;12)(p21;q13)del(6)(q23),add(9)(p22)[cp4]/46,XX[7]
6	F/61	Submandibular Gland	ACC	19 mo, A&W	7–15	21/7	44–51,XX,del(1)(q42),t(8;9)(q12;p22),del(12)(q12q13),del(14)(q22–q23)[cp4]/46,XX[3]

Abbreviations: F, female; M, male; mo, months; A&W, alive without disease; AWD, alive with disease; ln, cervical lymph node; met, metastases.
[a]The analyzed tissue was from the fifth reccurrence of a tumor first diagnosed five years before.
[b]This translocation was confirmed by fluorescence in situ hybridization analysis with whole chromosome painting probes for both chromosomes 6 and 12.
[c]This translocation was confirmed by fluorescence in situ hybridization analysis with whole chromosome painting probes for both chromosomes 12 and 15.

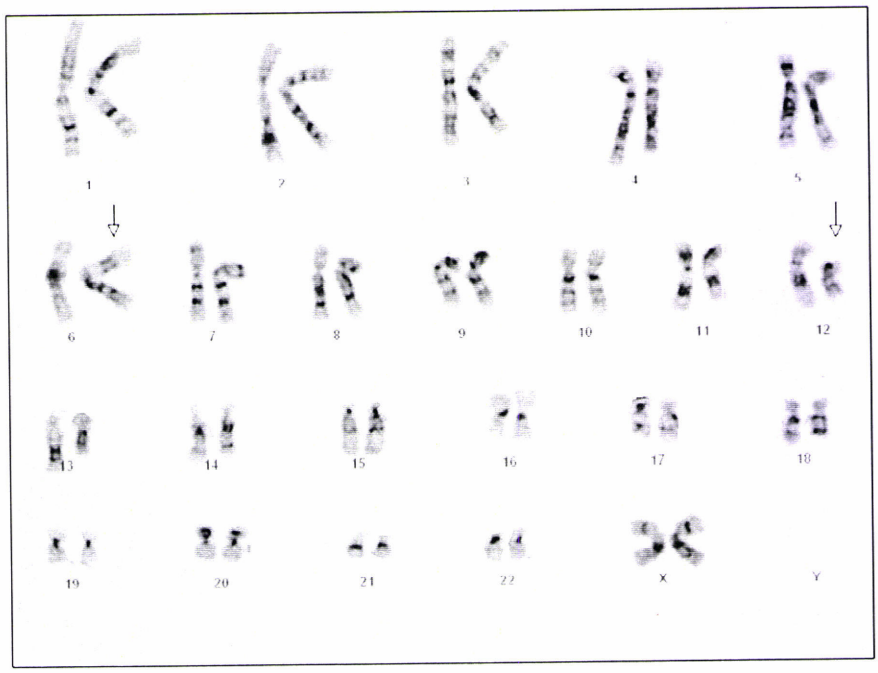

Fig. 2. Representative karyotype of PLGA case 1 with t(6;12)(p21;q13) (arrows).

translocation seems cytogenetically identical to the one described for ACC case 5. The PLGA case 3 also showed 9p alterations, as an inv(9)(p13.3p24).

4. Discussion

Malignant salivary tumors are rare neoplasms representing about 20% of all salivary gland neoplasms [10]. Despite being so uncommon, accumulated information on their cytogenetic characteristics is rather vast, for both benign and malignant tumors [2].

Deletion of the long arm of chromosome 6, usually within 6q21–q25 region is, by far, the most common karyotypic structural alteration [11–15]. This aberration is shared by all malignant tumor subtypes, except carcinoma ex-pleomorphic adenomas, that show 8q12 and 12q13–q15 rearrangements as the most common deviations [4,5,14,16–18]. More specific tumor type-associated alterations have been described in adenoid cystic carcinoma, the t(6;9)(q23–q25; p21–p22) [15,19,20], and, in mucoepidermoid carcinoma, the t(11;19)(p14–p21;p13) [14,21,22].

In the present study, we report the findings of chromosome 12 rearrangements in PLGA and ACC, two salivary glands neoplasms whose histogenesis has been claimed to be related [1].

Except for the 6q deletion, the karyotypic alteration that is common to most malignant types of salivary gland tumors, there is, to our knowledge, no other cytogenetic alteration reported to be shared by histologically distinct salivary carcinomas.

The cytogenetic information on ACC results from the karyotypic analysis of a total of 29 cases; 19 from the salivary glands and 10 from extra-salivary sites such as lachrymal gland, breast, larynx, sphenoid sinus, and lung [3,11,14, 15,17–20,23–27]. The recurrent structural abnormalities identified were 9p and 6q rearrangements, dominantly as a t(6;9)(q21–q25;p21–p22) (23%) and 6q deletions (20%). Other chromosome regions recurrently affected are 17p12–p13 and Xp21.

The cases of ACC herein reported displayed complex karyotypes with two or more chromosomal abnormalities, with 12q12–q13 rearrangements being present in all of them—as del(12)(q12q13) in two cases (cases 4 and 6) and involved in a translocation with chromosome 6p21, t(6; 12)(p21;q13) in the remaining case (case 5).

The translocation t(6;9) was not observed in the present series, but case 5 showed a t(6;12)(p21;q13) associated with a del(6)(q23) and addition of unknown material to 9p22 (Table 1). These findings indicate that the t(6;9) is indeed present; however, the limited number of metaphases available, as well as the lack of tissue sample left with which to perform the necessary FISH analysis, precluded its confirmation. Other recurrent deviations that are commonly present in ACC cases, such as del(6q), rearrangements of 9p22, and 17p12–p13, were also found in our series (Table 1), confirming that these regions are associated with ACC tumorigenesis.

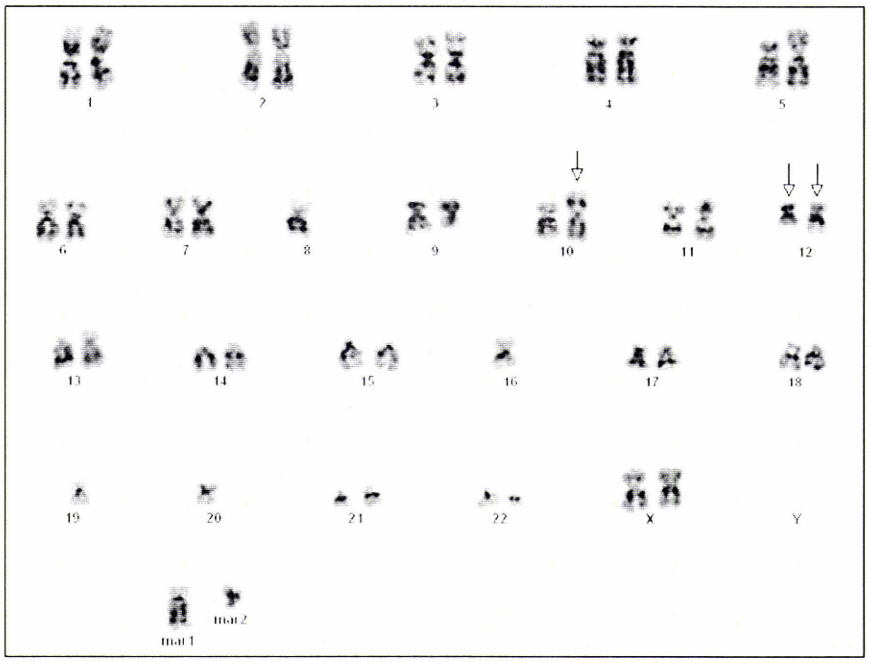

Fig. 3. Representative karyotype of ACC case 4 showing most of the aberrations included in the composite karyotype (see Table 1), except the der(19) that results from t(13;19)(p11;q11). Arrows indicate chromosome 12 rearrangements t(10;12)(p10;p10) and del(12)(q12q13).

Two previous studies reported 12q12 rearrangements [20,23], which fits with our results and strongly implicates 12q12–q13 in the pathogenesis of ACC. Interesting to note is the fact that the long arm of chromosome 12, particularly the 12q13–q15 region, is frequently rearranged in pleomorphic adenomas [28,29], as well as in a variety of other human benign solid tumors such as lipomas, lung chondroid hamartomas, endometrial polyps, and uterine leiomyomas [2]. The *HMGIC* gene, a member of the high-mobility group protein family, was identified as a participant in 12q13–q15 chromosome rearrangements in all of those tumors [30,31]. It has been associated with benign tumor growth [32], but its role in the pathogenesis of malignant tumors remains unknown.

The resolution of conventional cytogenetic techniques may explain why 12q12–q13 and 12q13–q15 were not clearly identified by the banding system used. Putative pinpointing of the association of rearrangements of 12q12–q13 with lesions of the *HMGIC* gene may eventually be clarified by molecular genetic analysis.

Two of the four cases of PLGA previously reported represent the malignant component of parotid carcinomas expleomorphic adenoma [4,5] and the remaining two cases were primary tumors of the minor salivary glands [5,6].

Monosomy 22 and a reciprocal t(6;9)(p21;p22) were the only deviations found in the two primary PLGA cases previously reported [5,6]. The three cases of PLGA of the present series displayed chromosome 12 rearrangements affecting the q arm in two different regions—12q12–q13 (case 1) and 12q22 (case 2) by translocations with 6p21 and 22p11–p12, respectively, and affecting the p arm through a reciprocal t(12;15)(p12.3;q?23) in case 3. Case 1 displayed a t(6;12)(p21;q13) that seems, at the cytogenetic level, identical to the one observed in ACC case 5. These findings, undoubtedly, illustrate that these two chromosomal regions, 12q12–q13 and 6p21, have common alterations in the two types of salivary adenocarcinomas.

Furthermore, it is interesting to note that the t(6;12)(p21;q13) in case 1 involves the 6p21 regions, as in the PLGA case reported by Dahlenfors et al. [6] with t(6;9)(p21;p22). Rearrangements in this region occur in mesenchymal tumors, including lipomas, pulmonary chondroid hamartomas, and uterine leiomyomas [2]. Molecular analysis of 6p21 breakpoint identified in these tumors the *HMGI(Y)* gene that belongs to the high mobility group protein family [33–36].

Cytogenetic rearrangements of this region were also described in pleomorphic adenoma [28,29] and Warthin tumor [37]. Molecular cytogenetic studies performed on pleomorphic adenoma by Rohen et al. [38] revealed that the 6p breakpoint maps distal to *HMGI(Y)*, not affecting the gene or its closer vicinity. The significance of p21 rearrangements in this series of salivary adenocarcinomas remains to be clarified.

In addition to 12q12–q13 and 6p21 rearrangements, other chromosome deviation was found in the two types of carci-

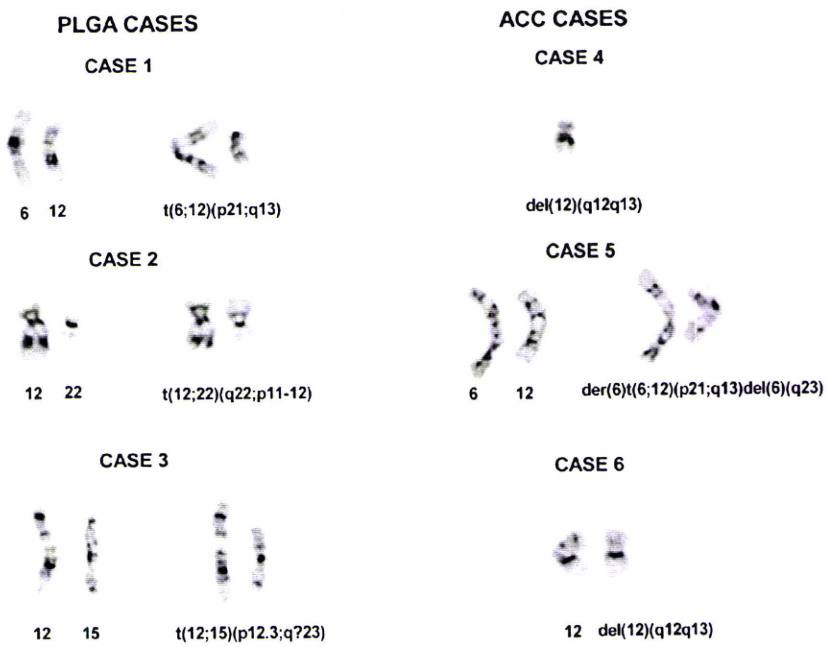

Fig. 4. Partial karyotypes from PLGA and ACC cases showing chromosome 12 rearrangements. On the left, normal chromosomes of the same metaphase. Arrows indicate breakpoints. For complete karyotype description, see Table 1.

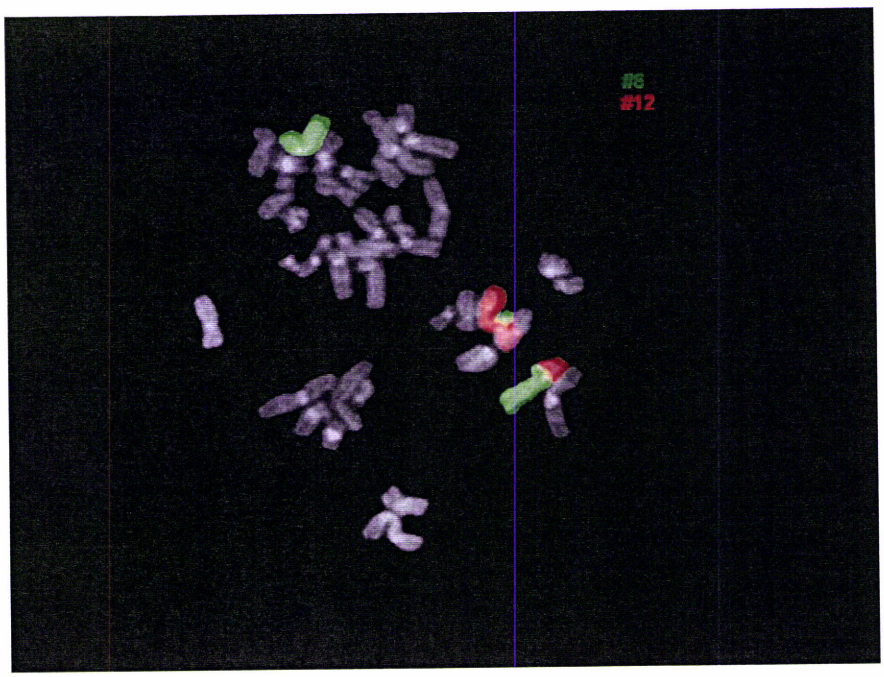

Fig. 5. Fluorescence in situ hybridization analysis of a metaphase from PLGA case 1 with whole chromosome painting probes for both chromosomes 6 (green signal) and 12 (red signal) confirming the t(6;12)(p21;q13).

nomas, at 9p22 in ACC cases 5 and 6 and at p13.3p24.1 in PLGA case 3. In the latter case, due to an inv(9)(p13.3 p24.1), the most likely explanation is the acquisition of a secondary abnormality during clonal expansion, the stemline only displaying inv(12). However, another PLGA case [6] with t(6;9)(p21;p22) also showed involvement of the same region (9p22) that is rearranged in ACC, mainly as the chromosome partner of characteristic t(6;9)(q21–q25;p21–p22).

In conclusion, taking together previous data and our own results, 12q12–q13 and 6p21 rearrangements are clearly nonrandom in PLGA and ACC, which supports that these two regions may harbor genes that can be relevant for their carcinogenesis. Moreover, these chromosomal rearrangements give genotypic support to the concept of a shared histogenesis for these two type of bidifferentiated salivary gland adenocarcinomas, as has been suggested before, mostly based on morphological and immunophenotypical observations [1,39].

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3.5

Characterization of chromosome aberrations in salivary gland tumors by FISH, including multicolor COBRA-FISH

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Characterization of Chromosome Aberrations in Salivary Gland Tumors by FISH, Including Multicolor COBRA-FISH

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Fluorescence in situ hybridization (FISH), including COBRA-FISH, was used to characterize 11 salivary gland tumors that had been investigated by banding analysis. Five cases were pleomorphic adenoma (PA), three were adenoid cystic carcinoma, and one case each was mucoepidermoid carcinoma, carcinoma ex-pleomorphic adenoma (CaPA), and adenocarcinoma. All 11 cases were selected on the basis that they had shown rearrangement of 6q or 9p or had unresolved aberrations after karyotyping. The COBRA-FISH and FISH analyses led to a revised karyotype in all informative cases and made it possible to clarify almost all chromosomal rearrangements occurring in the tumors. Of particular note were the confirmation of the existence of 6q deletions, a common change in salivary gland carcinomas, and the demonstration that a seemingly balanced t(6;9) resulted in del(6q). Other rearrangements that were revealed by FISH included amplification of 12q sequences (*MDM2* and *CDK4*) in one PA. We also investigated the status of the *PLAG1* gene in four cases (one PA, one CaPA, one adenoid cystic carcinoma, and one mucoepidermoid carcinoma) with 8q12 rearrangements. Only in the former two cases were the FISH results compatible with intragenic rearrangements. Overall, the results of the study show that, even with good banding quality and in karyotypes of modest complexity, much new information will be gained by supplementing the banding analysis with a multicolor FISH approach, such as COBRA-FISH.

INTRODUCTION

Tumors arising from the salivary glands are highly heterogeneous; among the benign lesions, the predominant entity is pleomorphic adenoma (PA), whereas the malignant lesions are more diverse, including tumor types such as mucoepidermoid carcinoma, adenoid cystic carcinoma, acinic cell carcinoma, carcinoma ex-pleomorphic adenoma (CaPA), and adenocarcinoma.

PAs have been extensively analyzed by cytogenetic techniques, and two major chromosomal subgroups have been recognized (Bullerdiek et al., 1993). One is characterized by structural rearrangements of 8q12, often in the form of a t(3;8)(p21;q12), constituting 60% of the PAs with an abnormal karyotype. Another subset displays 12q13–15 rearrangements, identified in 20% of karyotypically aberrant tumors. Recently, the molecular genetic consequences of these rearrangements have been clarified. The t(3;8) results in promoter swapping between the *PLAG1* gene in 8q12 and *CTNNB1* in 3p21. Rearrangement and overexpression of *PLAG1* have also been found in a large fraction of PAs with other types of rearrangement of 8q12, as well as in some cases without cytogenetically detectable 8q12 aberrations (Kas et al., 1997; Voz et al., 1998, 2000; Åström et al., 1999). The target gene for 12q13–15 rearrangements is *HMGIC*, which is also implicated in a variety of benign mesenchymal tumors (Ashar et al., 1995; Schoenmakers et al., 1995).

Although the cytogenetic information on malignant salivary gland neoplasms is restricted to 74 karyotypically aberrant tumors, characteristic karyotypic features have been identified (Mitelman, 1998). One common finding is deletion of 6q, observed in all subtypes except CaPA. More specific rearrangements have been identified in ade-

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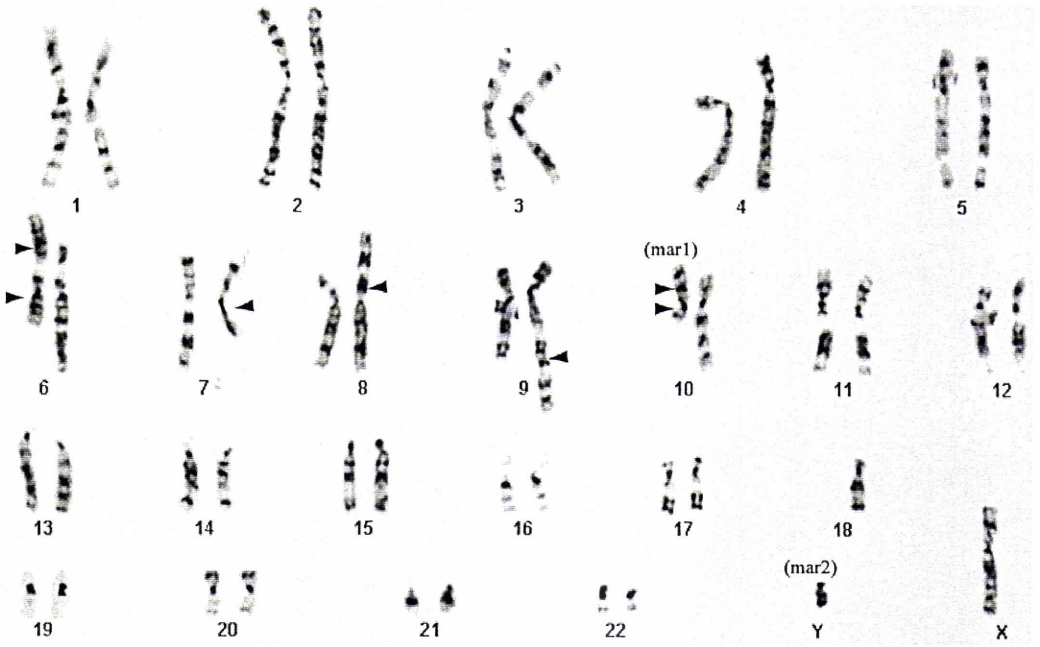


Figure 1. Karyogram from a pleomorphic adenoma (case 1). Arrowheads indicate breakpoints in clonal aberrations (see Table 1 for karyotype description).

noid cystic carcinoma and mucoepidermoid carcinoma, namely, t(6;9)(q21–23;p13–23) and t(11;19)(q14–21;p12), respectively. Furthermore, structural rearrangements of 8q12–13 are strongly associated with CaPA, in line with the notion that these tumors develop through malignant transformation of PA. Whereas the *PLAG1* gene may be suspected to be rearranged in CaPA with 8q12 rearrangements (Åström et al., 1999), the molecular targets for 6q deletions and translocations t(6;9) and t(11;19) remain unknown.

In order to delineate further the spectrum of chromosome 6 and 9 rearrangements in salivary gland neoplasms, we selected for fluorescence in situ hybridization (FISH) studies a series of 11 cases that had been analyzed cytogenetically and had shown rearrangement of chromosome 6 or 9, or had unresolved karyotypic features such as rings and marker chromosomes, i.e., chromosomes that could potentially harbor sequences from chromosomes 6 and 9. In order to obtain complete karyotypic information on these tumors, we used combined binary ratio labeling FISH (COBRA-FISH), providing individual and simultaneous staining of all 24 chromosomes, and FISH with specific DNA probes to delineate breakpoints.

MATERIALS AND METHODS

The present study included 11 salivary gland tumors. Five were PA, three were adenoid cystic carcinomas, and one case each was classified as mucoepidermoid carcinoma, CaPA, and adenocarcinoma (Table 1). Cases 8 and 9 were local recurrences, whereas the remaining nine were primary lesions. None of the patients had received radio- or chemotherapy prior to cytogenetic examination.

Samples obtained from diagnostic biopsies or at surgery were processed for chromosome analysis as described (Jin et al., 1995). The cultures were harvested after 5–10 days. The clonality criteria and the description of chromosome abnormalities were according to the International System for Human Cytogenetic Nomenclature (ISCN, 1995). Metaphase cells used for FISH analysis had been preserved for up to 5 years at –20°C.

COBRA-FISH was performed as described by Tanke et al. (1999) with minor modifications. All whole chromosome-specific painting probes used for COBRA-FISH were supplied by Cytocell (U.K.). In brief, slides with metaphase chromosomes were pretreated with RNAse A and pepsin according to Wiegant et al. (1991). The chromo-

TABLE 1. Characterization of Chromosome Abnormalities in 11 Salivary Gland Tumors by Banding Analysis, COBRA-FISH, and FISH

Case number	Age/sex	Diagnosis[b]	Karyotype after banding analysis[c]	FISH analysis	Revised karyotype after COBRA-FISH and FISH[d]
1	38/M	PA	46,X,−Y,inv(6)(p23q15), t(7;8)(q11;p11), der(9)t(9;10)(q32;q21),−10, +mar1,+mar2[3]/45,idem,−18[5]	COBRA cen7, cen8, cen10, wcp8 wcp9, wcp10	46,X**Y**,inv(6)(p23q15),**der(7)t(7;10)(q11;p11), der(8)t(7;8)(q11;p11),**der(9)t(9;10)(q32;q21), **der(10)t(9;10)(q32;p11)t(8;10)(p11;q11)**/ 45,idem,−18
2	78/F	PA	46,XX,−13,+mar[2]/46,XX,add(3)(q21), −5,+r[3]/45,idem,−19[3]	COBRA pcp12p, wcp12, wcp13, 831g11, 787f10, 751a4, 984a2	46,XX,**del(12)(q14q15),der(13)ins(13;12) (q14;q14q15)hsr(12)(q14q15)**/46,XX,**der(3) ins(3;12)(q21;q14q15)hsr(12)(q14q15),r(5), del(12)(q14q15)**/45,idem,−19
3	72/M	PA	46,XY,del(8)(q12q21),add(14)(q31)[4]	COBRA wcp8, wcp14, PLAG1	46,XY,**ins(8;14)(q12;q?24q?32),der(14) t(8;14)(q12;q?24)** [split *PLAG1*]
4	48/M	PA	44–45,Y,−X,dic(1;11)(p12;q11),der(2)t(2;?11) (q31;q21),del(3)(p21),der(8)t(X;8)(q13;q11)ins(X; ?)(q13;?),der(9)t(9;11)(p22–23; q11),?del(11)(q11),+mar[4]	COBRA	44–45,Y,−X,dic(1;11)(p12;q11),+**del(2)(p11),** del(3)(p21),**der(8)t(3;8)(p23;q24),**der(9)t(9;11) (p22–23;q11),del(11)(q11)
5	62/F	PA	46,XX,del(4)(q22q27),add(5)(q11) ,der(11)t(11;12)(q21;q15),der(12)t(11;12)(q21;q13)[4]	COBRA 831g11, 790f7, 787f10, 751a4	46,XX,del(4)(q22q27),**del(5)(q13),t(11;12)(q21;q15)**
6	74/F	AdCC	49,XX,der(6)t(6;11)(q25;q23),del(11)(q21),+15,+16,+22[3]	COBRA	49,XX,der(6)t(6;11)**(q23–24;q21),**del(11)(q21),+15,+16,+22
7	35/M	AdCC	89–90,XXYY,t(6;9)(q23;p22)×2,der(7)t(7;15) (q11;q15),add(7)(q11),−8,del(8)(p11),−12, −15,−15,add(16)(q23),add(22)(q11–12), +mar1×2[cp4]/81–87,idem,+der(7)t(7;15) (q11;q15),-add(7)(q11)[cp4]	COBRA wcp8, wcp9, wcp7, wcp15, cen15	89–90,XXYY,**der(6)t(6;9)(q23;p22),t(7;15)(q22;q22),** add(7)(q11),**der(9)t(8;9)(?;p22),**−12,**der(16)t(7;16) (q31;q12),der(22)t(15;22)(q15;q13)**/81–87,idem,+**t(7; 15)(q22;q22),**-add(7)(q11)
8[a]	31/F	AdCC	44–46,XX,add(1)(p34–35),t(5;9)(q11;p22), del(6)(q25),−8,add(8)(p11),t(10;12)(p10;p10), del(12)(q12q13),t(13;19)(p11;q11),−16, del(17)(p12),+mar1,+mar2[cp6]	COBRA wcp8, wcp16, wcp9, cen9 831g11, 790f7, 787f10, 12qter, PLAG1	44–46,XX,**t(1;8)(p35;p21),t(5;9)(p10;p10),** del(6)(q25),**t(8;16)(q12;q23),**t(10;12)(p10;p10), del(12)(q12q13),t(13;19)(p11;q11),del(17)(p12)
9[a]	79/F	MuC	45,XX,der(1)t(1;1)(p13;q11),−4,der(6)t(6;?15) (q15;q22),dup(8)(q12q24),del(9)(p22),−12, −15,−17,+18,der(18)t(6;18)(p11;p11)×2,+r,+mar[cp7]	COBRA wcp18, wcp17, pcp17p, pcp18p, cen17, cen18, PLAG1	45,XX,der(1)t(1;1)(p13;q11),−4,der(6)t(6;15) (q15;q22),dup(8)(q12q24),del(9)(p22),−12, **del(15)(q11),der(17)r(17;18)(q?;p?),** +18,**psudic(18)t(17;18)(q?;p11)×2**
10	71/F	CaPA	105–111,XXXXX,−1,i(1)(q10),del(2)(q13),+3, der(3)t(3;8)(p21;q12)×4,+6,+7,+7,+7,−8,−9,−10, −11,−11,der(11)t(11;11)(p15;q13)×3,del(12) (p12),−13,−14,−15,−16,del(16)(q22)×2,−17,−18, −18,−18,?i(18)(q10),+19,−21,+22,+der(?;1) (?;p22)×2,+marx2[cp8]	COBRA wcp1, wcp9, cen9, wcp3, wcp8, PLAG1	105–111,XXXXX,−1,i(1)(q10),del(2)(q13),+3,der(3)t(3;8) (p21;q12)×4,+6,+7,+7,+7,**der(8)i(8)(q10)t(3;8)(p23; q12)×2,+der(?9)t(1;9)(p22;?)×2,**−10,−11,−11, der(11)t(11;11)(p15;q13)×3,del(12)(p12),−13,−14, −15,−16,del(16)(q22)×2,−17,−18,−18,−18, ?i(18)(q10),+19,−21,+22, [split PLAG1]
11	74/M	AD	53,XY,+i(1)(q10),+der(1)t(1;1)(p13;q11),+2,der(2) inv(2)(p21q21)del(2)(q11q13)×2,+add(6)(q15),+7, del(8)(p21),+del(8)(p21)×2,−9,+14,+20[cp5]	COBRA 942H6, 959C4, 886D7, 947C9	53,XY,i(1)(q10),+**i(1)(q10),**+2,der(2)inv(2) (p21q21)del(2)(q11q13)×2,+7,del(8)(p21), +del(8)(p21)×2,**inv(9)(p13q13),**+14,+20

[a]Local recurrence.
[b]PA = pleomorphic adenoma; AdCC = adenoid cystic carcinoma; MuC = mucoepidermoid carcinoma; CaPA = carcinoma ex-pleomorphic adenoma; AD = adenocarcinoma.
[c]The underlined rearrangements were revised after FISH.
[d]Abnormalities in bold face indicate changes compared with the karyotype after G-banding.

some preparations were denatured by incubating them for 2 min at 72°C in 60% formamide, 2 × SSC, pH 7, on a hot plate, followed by dehydration in an ethanol series. Then, a 10-μl probe mixture (Tanke et al., 1999) was applied, and hybridization was performed for 48–72 hr at 37°C in a humid chamber. After hybridization, the slides were washed in 0.4 × SSC at 72°C for 2 min, followed by a 5-min wash at room temperature in TNT (0.1 Tris HCl, pH 7.4, 0.15-M NaCl, 0.05% Tween 20). Chromosomes were counterstained with DAPI by submersion of slides in TNT + DAPI (2–10 μl, 0.5-mg/ml DAPI) for 10 min. The slides were embedded in Citifluor prior to microscopic evaluation. An Axioplan 2 microscope (Zeiss, Oberkochen, Germany) coupled to a cooled charge-coupled device (CCD) camera and a 12-position filter wheel was used for analysis. The acquired images were evaluated using the Cyto Vision ChromoFluor System (Applied Imaging, Newcastle, U.K.), according to the principles outlined in Szuhai et al. (2000).

Further FISH analysis was performed on metaphase cells from 10 cases. The following probes were applied: whole chromosome painting (wcp) probes (Vysis, Downers Grove, IL) for chromosomes 3, 7–10, and 12–18, partial chromosome painting (pcp) probes (AL Technologies, Arlington, VA) for chromosomal arms 12p, 17p, and 18p, and centromere-specific probes (cen) for chromosomes 7–10, 15, and 17 (ATCC). For characterization of 12q rearrangements, five YAC clones (CEPH) spanning the region 12q12–15 and band 12q23 were used: 831g11 (12q12), 790f7 (12q13), 787f10 (12q14), 751a4 (12q15), and 984d2 (12q23). To characterize the localization of breakpoints in pericentromeric rearrangements of chromosome 1 (case 11), four YAC clones (CEPH) spanning 1q10–p13 (942H6, 959C4, 886D7, and 947C9) were used. In order to examine whether *PLAG1* was involved in 8q12 rearrangements, three PAC clones (kindly provided by Jörn Bullerdiek, Bremen, Germany) containing inserts of different parts of *PLAG1* were used in five tumors. All DNA probes were labeled with biotin-dUTP or digoxigenin-dUTP (Boehringer Mannheim, Germany), dUTP-fluorogreen, dCTP-Cy3 (Amersham, Amersham Place, U.K.), or dUTP-diethylaminocoumarin (NEN, Boston, MA) using randomly priming monomernucleotides (Amersham). In situ hybridization and detection were carried out as described by Höglund et al. (1996). The hybridizations were analyzed with the Cyto Vision Probe System (Applied Imaging).

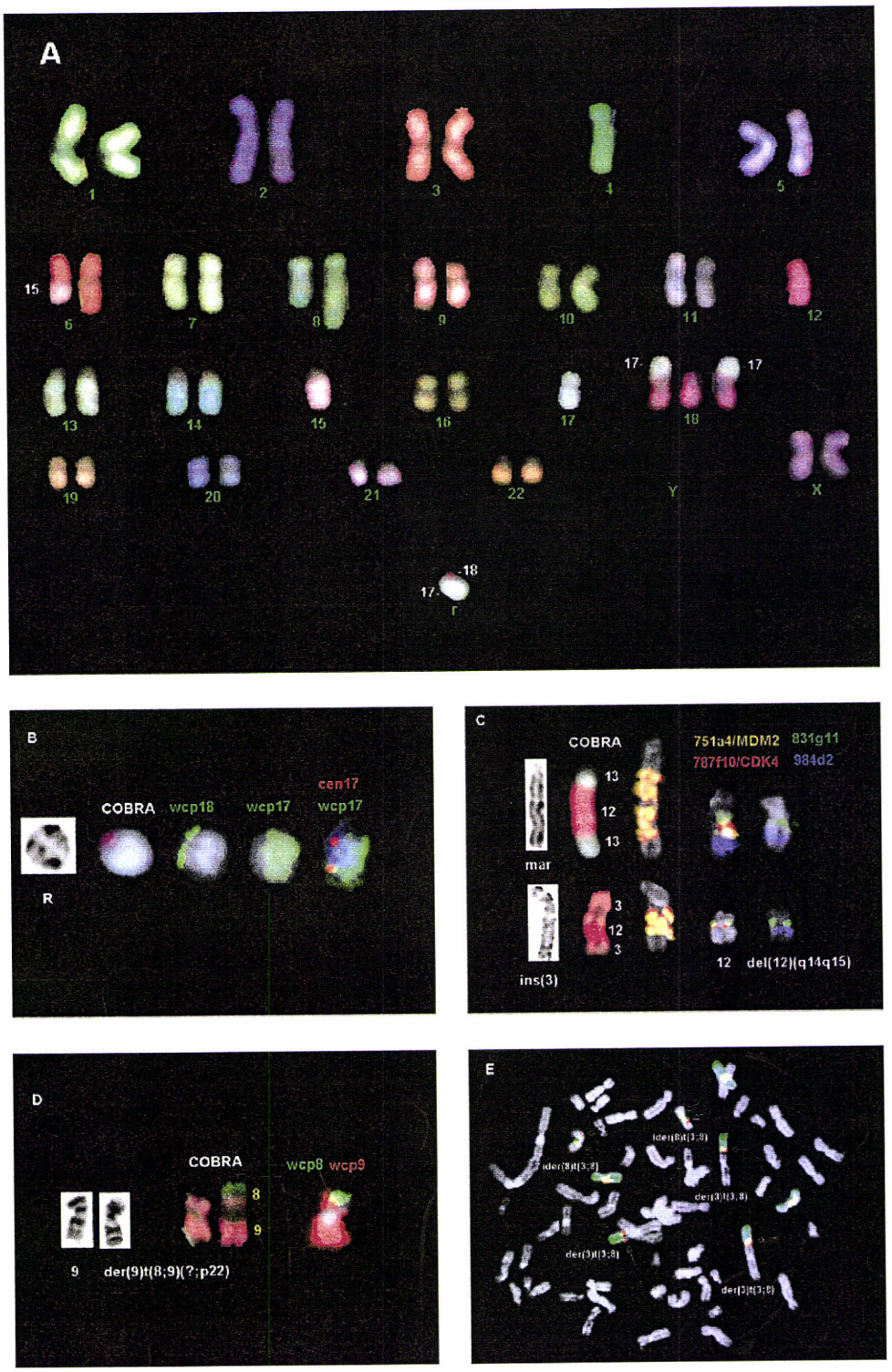
RESULTS

The karyotypes obtained after G-banding alone and after combining the banding pattern with results from COBRA-FISH and FISH with specific probes are shown in Table 1. The COBRA analysis led to a revised karyotype in all cases. Furthermore, almost all marker chromosomes and rearrangements that had been characterized only partly by banding analysis could be clarified by COBRA-FISH (Fig. 1, Fig. 2A and B). The COBRA analysis also revealed that some rearrangements had been cytogenetically misinterpreted, e.g., a rearranged chromosome 6 in case 11 was shown by COBRA-FISH to be an inverted chromosome 9. The FISH analyses with chromosome-specific, chromosome arm-specific, and centromere-specific probes verified and further clarified the rearrangements. In addition, in one tumor (case 2), the use of locus-specific probes to clarify an amplification of 12q sequences in a marker chromosome unexpectedly revealed a cryptic deletion of 12q14–q15 in the homologue that had appeared normal on banding analysis (Fig. 2C).

According to the G-banding karyotype, six cases had 6q aberrations and five (one of which also showed involvement of 6q) had rearrangements of 9p. Except for the add(6)(q15) in case 11, the FISH analyses verified the presence of each aberration in all cases. Furthermore, what appeared to be a balanced t(6;9) in one adenoid cystic carcinoma (case 7) was shown to be an unbalanced rearrangement involving chromosomes 6, 8, and 9, leading to a net loss of distal 6q. Unsuspected involvement of chromosome 9 was also identified in case 10 (Table 1).

The *PLAG1* gene was investigated by the use of three PAC clones in the four tumors (cases 3 and 8–10) showing rearrangement of bands 8q12–13. In two of these cases, the results were compatible

Figure 2. **A:** COBRA-FISH multicolor karyogram of the mucoepidermoid carcinoma of case 9. **B:** The ring chromosome in case 9 further characterized by COBRA-FISH and FISH with wcp18 (green), wcp17 (green), and cen17 (red). **C:** The marker chromosome and the add(3) of case 2 investigated by COBRA-FISH, locus-specific FISH probes for *MDM2* (YAC 751a4, yellow) and *CDK4* (YAC 787f10, red), and YAC clones 831g11 (12q12, green) and 984d2 (12q23, blue). Amplification was found on both derivative chromosomes by hybridization of YACs 787f10 and 751a4. The corresponding sequences were deleted from one of the copies of chromosome 12. **D:** In the adenoid cystic carcinoma of case 7, banding analysis suggested the presence of a balanced reciprocal translocation, t(6;9). By COBRA-FISH, it was found to be an unbalanced translocation involving chromosomes 6, 8, and 9, an interpretation confirmed by the use of FISH with wcp8 (green) and wcp9 (red; the results for chromosome 6 are not shown). **E:** Metaphase cell from the carcinoma ex-pleomorphic carcinoma (case 10) hybridized with wcp8 (green) and the *PLAG1*-specific PAC 234 (red). The intensity of the signal was weaker on the ider(8)t(3;8) and on der(3)t(3;8), compared to the signals on normal chromosomes 8, indicating that the break had occurred within the region covered by PAC 234.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der(3)t(3;8)

Figure 2.

with intragenic rearrangements; in one PA (case 3) with a complex exchange of material between chromosomes 8 and 14 and in the CaPA (case 10) with translocation between chromosomes 3 and 8, the probes hybridized to the rearranged chromosomes 8 as well as to their translocation partners (Fig. 2E). In one adenoid cystic carcinoma (case 8) with a t(8;16)(q12;q23) and in the mucoepidermoid carcinoma (case 9) with a dup(8)(q12q24), no intragenic rearrangements were observed. In case 9, the FISH results indicated that the entire *PLAG1* gene was duplicated, as determined by use of PACs 234 and 235, with a breakpoint 3′ (centromeric) of the gene. A breakpoint 3′ of *PLAG1* was also seen in case 8, where both PACs 234 and 235 hybridized to the derivative chromosome 16.

DISCUSSION

In the present study, we have utilized a recently developed technique, COBRA-FISH, to characterize a series of benign and malignant salivary gland tumors, all of which had been analyzed by G-banding after short-term culture. The main aim of the study was to delineate the spectrum of 6q and 9p rearrangements. Hence, a further prerequisite for inclusion in this study was that all cases should display rearrangement of 6q or 9p, or have one or more aberrations that could potentially involve these chromosome arms, i.e., aberrations that remained unresolved after karyotyping. Furthermore, in cases where breakpoints were detected near the locus for the *PLAG1* gene, which in a large subset of PA is rearranged through translocations, we used gene-specific FISH probes to assess whether it was involved or not.

Structural rearrangements of chromosome 6, often in the form of deletions, are the most common changes in salivary gland carcinomas. In addition to del(6q), a subset of adenoid cystic carcinoma displays apparently balanced translocations t(6;9)(q21–24;p13–23). In the present analysis, net loss of 6q material was suspected in two carcinomas after banding analysis (cases 8 and 9). By FISH, it could be shown that not only these aberrations, but also the seemingly balanced t(6;9) in case 7 resulted in deletion of distal 6q. However, because no other adenoid cystic carcinoma with a t(6;9) has been investigated by FISH or molecular techniques, it is premature to conclude whether this translocation is frequently associated with 6q deletions.

The COBRA-FISH analysis also provided information on other chromosomal rearrangements that had not been resolved by banding analysis. Ring chromosomes have been described repeatedly in salivary gland tumors, but it is unknown whether the rings may preferentially contain certain DNA sequences, as is the case in some low-malignancy mesenchymal tumors (Gisselsson et al., 1999). In the present study, ring chromosomes could be characterized by FISH in two tumors. In one of them, a mucoepidermoid carcinoma, the ring was composed of material from 17q and 18p, with a centromere from chromosome 17, whereas the other, a PA, had a ring originating from chromosome 5. The involvement of chromosome 5 in ring formation has been described in three other PAs that were studied by G-banding alone (Mark et al., 1983, 1997). In common with a benign subset of adipose tissue tumors, PA with 12q rearrangements often shows involvement of the *HMGIC* gene. Another feature shared by these two entities was identified in the present study. In one PA (case 2), both a long marker chromosome and the unknown chromosomal segment added to the long arm of one chromosome 3 were shown to contain amplified 12q14–15 sequences, including *MDM2* and *CDK4*, which is similar to the composition of the giant marker chromosomes that are characteristic for atypical lipomatous tumors (Gisselsson et al., 1999). To the best of our knowledge, a similar 12q amplification has not been reported before in PA. However, double minutes of unknown chromosomal origin have previously been detected in one PA (Mark et al., 1982), suggesting that gene amplification is a rare but recurrent event in this tumor type.

A large proportion of PAs display translocations involving chromosome band 8q12, resulting in replacement of the promoter region of *PLAG1* by that of another gene, such as *CTNNB1* in the t(3;8)(p21;q12) or *LIFR* in the t(5;8)(p13;q12) (Kas et al., 1997; Voz et al., 1998). Our results support previous molecular findings that rearrangements of *PLAG1* are detected mainly in PA with 8q12 rearrangement, and rarely in salivary gland carcinomas without relation to PA (Åström et al., 1999). Among the four tumors with 8q12 rearrangements that could be investigated in the present study, *PLAG1* involvement was found only in a PA and the CaPA, and not in the remaining two carcinomas (one mucoepidermoid carcinoma and one adenoid cystic carcinoma).

In summary, the present study shows that, even with good banding quality and in karyotypes of modest complexity, much new information will be gained by supplementing the banding analysis with a multicolor FISH approach, such as COBRA-FISH.

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3.6

Mapping the 19p12-13 breakpoint in a mucoepidermoid carcinoma of the parotid gland with the translocation t(11;19)(q21;p12) to a 3,3 Mb interval

Carmo Martins, Piere Rogalla, Isabel Fonseca, Norbert Drieschner, Martina Klee, Catarina Ribeiro, Jorge Rosa Santos, Jörn Bullerdiek, Jorge Soares

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MAPPING THE 19p12-13 BREAKPOINT IN A MUCOEPIDERMOID CARCINOMA OF THE PAROTID GLAND WITH THE TRANSLOCATION t(11;19)(q21;p12) TO A 3.3 MB INTERVAL

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Abbreviated title: 19p12-13 breakpoint in a MEC

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ABSTRACT

The translocation t(11;19)(q14-q21;p12-13) is the most frequent chromosomal aberration in mucoepidermoid carcinomas. So far neither the underlying genes have been cloned nor the breakpoints have been narrowed down.

In the present paper, cytogenetic analysis of a primary mucoepidermoid carcinoma of the parotid gland revealed a t(11;19)(q21;p12) as the sole karyotypic abnormality. In order to narrow down the breakpoint on the short arm of chromosome 19 we performed fluorescence *in situ* hybridization (FISH) experiments using three PAC clones established by PCR and hybridization screening of STS markers derived from the chromosomal region 19p12-13.1.

In a region of roughly 33 Mb assigned to the chromosomal region 19p12-13 we found the breakpoint to be localized in an interval of 3.3 Mb flanked by two markers SHGC-12528 and D1S2081 or by two corresponding PAC clones, respectively.

Thus, the molecular mapping of the breakpoint on 19p12-13 described herein will facilitate the characterization of the t(11;19)(q14-21;p12-13) in further mucoepidermoid carcinomas as well as cystadenolymphomas also known to be affected by that translocation.

TEXT

Cytogenetic studies of salivary gland neoplasms revealed specific and/or characteristic chromosomal alterations in both benign and malignant tumors. Pleomorphic adenomas clearly constitute the most intensively studied salivary gland neoplasms, with almost 500 cases karyotyped [Bullerdiek et al.,1993;Mark et al.,1997]. Consequently, the underlying molecular mechanisms have been resolved by the identification of the affected genes, i.e., *HMGIC* and *PLAG1*. [Schoenmakers et al., 1995; Kas et al., 1997]. In addition, other less frequent benign tumors, such as Warthin's tumor have been examined cytogenetically as well [Bullerdiek at al.,1988;Mark et al.,1990; Martins et al.,1997].

Malignant salivary neoplasms, though less well characterized, also show recurrent chromosomal abnormalities, some of them associated with particular tumor entities [Heim&Mitelman,1995]. An example are mucoepidermoid carcinomas (MECs) where chromosome deviations at 11q14-21 and 19p12-13 regions, usually as a reciprocal translocation t(11;19)(q14-21;p12-13), have been detected in about 36% of the cases with clonal abnormalities [Mitelman,1998]. The same abnormality has been found in mucoepidermoid carcinomas of the lung [Johansson et al., 1995] and seems to characterize a subset of Warthin's tumors [Bullerdiek et al.,1988; Mark et

al.,1990; Martins et al.,1997]. Despite the high frequency of that alteration within mucoepidermoid carcinomas neither the underlying genes have been cloned nor the breakpoints have been narrowed down. In order to characterize the 19p12-13 breakpoint of this translocation at the molecular level and, ultimately, to facilitate the identification of gene(s) affected we performed a molecular cytogenetic analysis of a primary MEC of the parotid gland with a t(11;19)(q21;p12) as the sole karyotypic deviation.

A 68-year old woman presented with a rapidly growing left parotid mass, measuring 9x6 cm. Histologically, the excised tumor was composed of medium-sized cysts, lined by goblet, mucin producing cells, and intermediate cells and focal squamous cell differentiation. It was classified as a grade I mucoepidermoid carcinoma. The patient received no further therapy and 12 months after surgery is well without evidence of recurrence or metastazation.
Short-term cultures from a tumor sample were prepared as described for other head and neck carcinomas by Jin et al (1993). Cytogenetic analysis was performed after conventional G-banding. A total of 20 metaphases were analyzed all of them displaying the same karyotype: 46,XX,t(11;19)(q21;p12). Description of karyotype followed the recommendations of ISCN,1995 [ISCN,1995].
Molecular characterization of the breakpoint on chromosome 19 of the t(11;19)(q21;p12-13) was started by choosing STS markers using the NCBI genome database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/). Within the approximately 10 Mb region 19p12-p13.1 the markers WI-12471 (GenBank accession number G13425), D1S2081 (also known as Wi-5611; GenBank accession number G03635), and SHGC-12528 (also known as STSG-12528, GenBank accession number L20968) were selected roughly overspanning 5 Mb. PAC clones containing these markers were obtained by PCR screening of a human PAC library (Genome Systems, St. Louis, USA) using the primer sets (WI-12471up) 5′-TTT GGG TTT TGT GAC ATA TTG C-3′ and (WI-12471do) 5′-AAG AAG ACA GCC ACA TCT CTT AGG-3′, and (D1S2081up) 5′-CTA CAC CAC TCC CAG GGC TA-3′ and (D1S2081lo) 5′-GTT GGT GGA GAA AAC AGG TTG-3′ or hybridization screening of a human PAC library (Resource Center of the German Human Genome Project, Berlin, Germany) using a probe generated by PCR with the primer set (SHGC-12528up) 5′-ACT GCA GGG GCA ATG GAT-3′ and (SHGC-12528do) 5′-GAC AGA TGT GAC TCA AGA GTG ACC-3′.

DNAs from positive PAC clones (clone addresses: 15H10, 141/H5, and RPCIP704B24355Q2) were isolated following the instruction of the manufacturer (Genome Systems, St. Louis, USA) with some modifications as recently published (Rogalla et al., 1998). DNAs of PAC clones were used as probes for FISH analyses. The probes were labelled with digoxigenin-11-dUTP by nick translation (Roche Diagnostics, Mannheim, Germany). After removing of unincorporated nucleotides by Sephadex G50 chromatography (Pharmacia, Uppsala, Sweden) probes were ethanol precipitated. FISH was performed according to Kievits et al. (1990) using anti-digoxigenin-fluorescein (Roche Diagnostics, Mannheim, Germany). For a bi-color-FISH the second probe was labelled with biotin-16-dUTP as decribed above. Detection of this probe was done using Cy3-streptavidin (Dianova, Hamburg, Germany). Chromosomes were counterstained with DAPI (0.025 µg/ml). The slides were analyzed using a Zeiss Axioplan fluorescence microscope (Zeiss, Oberkochem, Germany). Results were processed and recorded with the McProbe v4.0 software (PSI, Halladale, Great Britain).

First of all, FISH analyses on the primary mucoepidermoid carcinoma of the parotid gland with t(11;19)(q21;p12) have been performed using single probes. Probes for the markers D1S2081 and WI-12471, respectively, revealed signals on chromosome 19 and derivative chromosome 19, whereas the probe for the marker SHGC-12528 resulted in signals on chromosome 19 and derivative chromosome 11. Both derivative chromosomes, i.e., der(11) and der(19) clearly have been identified on same metaphases using a chromosome 11 specific painting probe (Oncor, Heidelberg, Germany) after re-hybridization (data not shown). In addition, these data have been confirmed by a bi-color-FISH using PAC-DNA of SHGC-12528 and D1S2081 resulting in single signals on both derivative chromosomes and a double-signal on the normal chromsome 19 (Fig 1a,b). The FISH results clearly show that the breakpoint is within the interval flanked by the selected markers. In Fig. 2 the localization of the markers within the chromosomal region 19 p12-p13.1 is shown based on their presentation in the NCBI genome database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) using the Map View. Thus, in a region of roughly 33 Mb assigned to the chromsomal region 19p12-13 affected in MECs we found the breakpoint to be localized in an interval of 3.3 Mb.

So far only 25 MEC cases with clonal chromosome abnormalities were described [Mitelman,1998]. Out of these seven showed a specific t(11;19)(q14-21;p12-13)

[Dahlenfors et al,1994,1995; Horsman et al,1994; Nordkvist et al,1994; El-Naggar et al,1996]. In three cases this was the sole anomaly and in the remaining four cases the t(11;19) was found either as a more complex translocation involving other chromosomes or together with other abnomalities. In addition, this particular cytogenetic lesion was also found in a case of bronchial MEC [Johansshon et al.,1995] further supporting the important role of t(11;19) in MEC tumorigenesis [Nordkvist et al.,1994].

Interestingly, a similar t(11;19)(q13-21;p12-13) has been reported in Warthin's tumors [Bullerdiek et al,1988; Mark et al,1990; Martins et al,1997] indicating an intriguing cytogenetic similarity between a benign and a malignant salivary gland tumor that do not share clinicopathological features and on a first glance apparently are also histogenetically different [Seifert,1990]. However, if they share exactly the same chromosomal abnormality this might suggest a common histogenetic pathway for both tumor types. This prompted us to a more detailed characterization of this translocation.

Herein, as a first intriguing result we were able to map the 19p12-13 breakpoint of the translocation t(11;19)(q14-q21;p12-13) in a mucoepidermoid carcinoma of the parotid gland to a 3.3 Mb interval.

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Figure 1:
Results of a bi-color-FISH analysis of a primary mucoepidermoid carcinoma of the parotid gland with a t(11;19)(q21;p12) as the sole karyotypic abnormality using PAC probes specific for D1S2081 and SHGC-12528. Normal chromosome 19, derivative chromosome 11, and derivative chromosome 19 are indicated by arrows.

(a) As the result of FISH analysis a green signal (D1S2081) on the derivative chromosome 19, a red signal (SHGC-12528) on derivative chromosome 11 and double-color signals on normal chromosome 19 were obtained.

(b) The same metaphase is shown after DAPI-Banding inverted using the McProbe v4.0 software (PSI, Halladale, Great Britain).

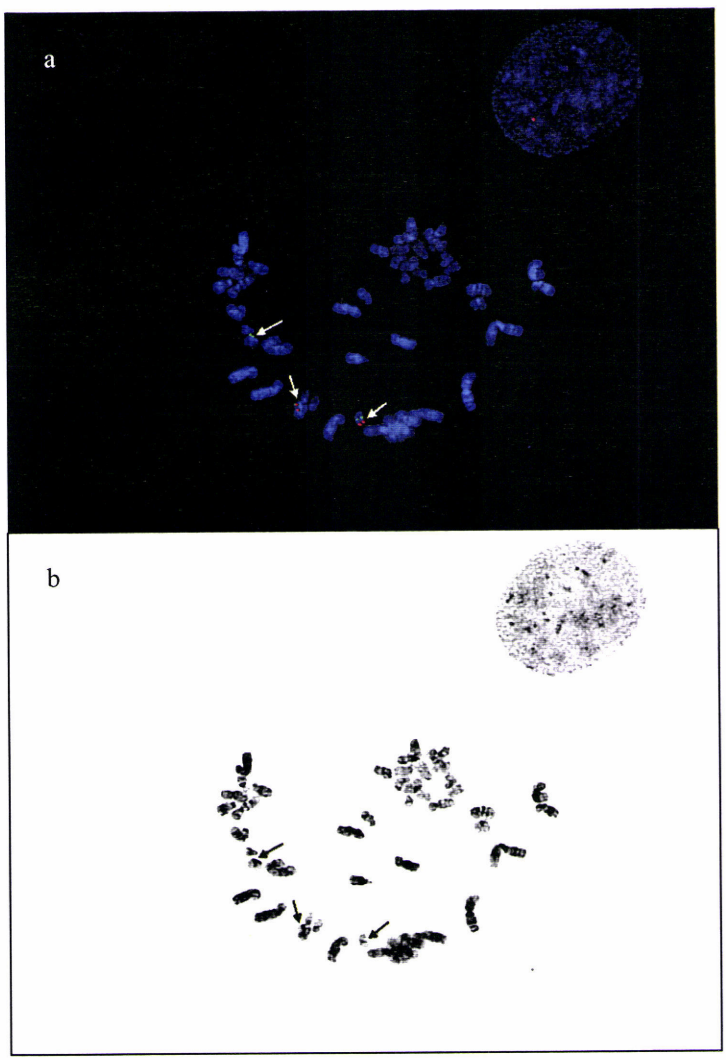
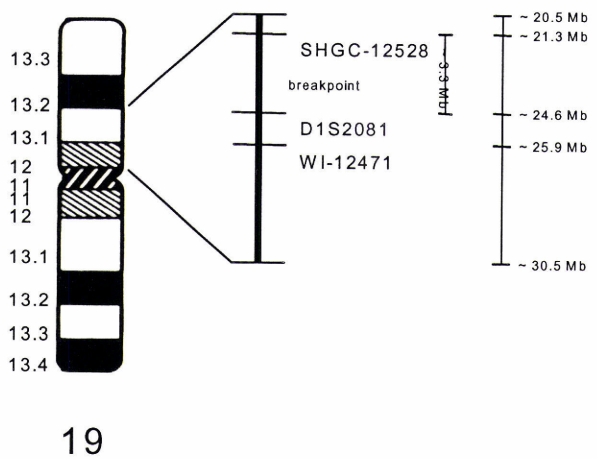


Figure 2:

Localization of STS markers within the chromosomal region 19p12-p13.1 selected to narrow down the 19p12-13 breakpoint in a mucoepidermoid carcinoma of the parotid gland with a t(11;19)(q21;p12) as the sole karyotypic abnormality. Positions are based on the presentation of markers in the NCBI genome database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/) using Map View.

The breakpoint of the primary parotid mucoepidermoid carcinoma with a simple t(11;19)(q21;p12) is between the two markers D1S2081 and SHGC-12528 overspanning a region of roughly 3.3 Mb.



Evaluation of PLAG1 gene alterations in salivary glands pleomorphic adenoma, myoepithelioma and carcinoma ex-pleomorphic adenoma using combined chromosome banding, *in situ* hybridization and immunocytochemistry

Carmo Martins, Isabel Fonseca, Lúcia Roque, Teresa Pereira, Catarina Ribeiro, Jörn Bullerdiek, Jorge Soares

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EVALUATION OF *PLAG1* GENE ALTERATIONS IN SALIVARY GLANDS PLEOMORPHIC ADENOMA, MYOEPITHELIOMA AND CARCINOMA EX-PLEOMORPHIC ADENOMA USING COMBINED CHROMOSOME BANDING, *IN SITU* HYBRIDIZATION AND IMMUNOCYTOCHEMISTRY

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Running Title: PLAG1 gene alterations in salivary gland tumors

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ABSTRACT

Pleomorphic adenoma is the most common benign tumor of the salivary glands. It shows marked histological diversity with epithelial, myoepithelial and mesenchymal-type cells arranged in a variety of architectural and differentiation patterns. Pleomorphic adenoma gene 1 (PLAG1) has been shown to be consistently rearranged in pleomorphic adenomas, being activated by chromosomal translocations involving 8q12, the chromosome region that is most frequently affected in these tumors. In this study we evaluated PLAG1 involvement in salivary gland tumorigenesis by determining the frequency of its alterations in a selected group of 20 histogenetically related salivary gland tumors: 15 pleomorphic adenomas (PA), 1 myoepithelioma and 4 carcinomas ex-pleomorphic adenoma (caPA), all sharing karyotypic chromosome 8 deviations, either structural, with 8q12 rearrangements, or numerical, with gain of chromosome 8. PLAG1 status was analyzed using *in situ* hybridization techniques, on metaphase cells, by fluorescence *in situ* hybridization (FISH) and/or interphase cells in paraffin sections, by chromogenic *in situ* hybridization (CISH). Except for one PA case (5%) that lacked PLAG1 involvement, seventeen tumors (85%), (13 PA, 3 caPA and the myoepithelioma) showed intragenic rearrangements of PLAG1 and the remaining two cases (10%), (one PA and one caPA), had chromosome trisomy 8 only. To further investigate the role of PLAG1 on pleomorphic adenomas tumorigenesis, as well as the involved morphogenetic mechanism, we attempted to identify cell types carrying 8q12/PLAG1 abnormalities by a combined phenotypic/genotypic analysis in two cases (1 PA and 1 caPA), both characterized by 8q12 translocations and PLAG1 rearrangement. In these cases, both cells populations, showing either epithelial or myoepithelial phenotypes, carried 8q12/PLAG1 rearrangements. This finding further supported the single pluripotent cell theory, that postulates that one initiated, modified myoepithelial cell, originates the variety of somatic cell phenotypes present in PA, and reinforces the role of PLAG1 on the tumorigenesis of benign and malignant pleomorphic adenoma.

Key words: PLAG1 gene – cytogenetics – *in situ* hybridization – immunocytochemistry - salivary gland tumors

List of abbreviations: caPA-Carcinoma ex-Pleomorphic Adenoma; CISH-Chromogenic In Situ Hybridization; CTNNB1-β-catenin gene, FISH-Fluorescence In Situ Hybridization; ICC- ImmunoCytoChemistry; ISH- In Situ Hybridization; PA-Pleomorphic Adenoma; PLAG1-Pleomorphic Adenoma Gene 1

INTRODUCTION

Pleomorphic adenomas, that are most common salivary gland tumors, show a marked histological diversity that reflects epithelial and mesenchymal differentiation (Dardick et al, 1991; Ellis et al, 1991). Extensive cytogenetic studies have revealed that these tumors consistently have highly specific chromosome abnormalities such as 3p21, 8q12 and 12q13-15 rearrangements and trisomy 8 (Bullerdiek et al, 1993; Mark et al, 1997; Martins et al, 1995; Sandros et al, 1990). 8q12 deviations constitute the largest cytogenetic subgroup, the reciprocal translocation t(3;8)(p21;q12) being the most frequent structural aberration. Molecular characterization of t(3;8) allowed the identification of affected genes, PLAG1 at 8q12 and CTNNB1 at 3p21 (Kas et al, 1997). The former, a developmentally regulated zinc-finger gene, is activated by a promoter swapping mechanism, in which the PLAG1 promoter is replaced by the one from CTNNB1 gene (Kas et al, 1997). Rearrangement and overexpression of PLAG1 were also detected in a large fraction of pleomorphic adenomas with other types of rearrangement of 8q12, as well as in cases without cytogenetically detectable 8q12 aberrations (Åström et al,1999; Kas et al, 1997; Voz et al, 1998, 2000).

Involvement of PLAG1 in salivary gland tumors is almost limited to benign pleomorphic adenoma, and to carcinomas ex-pleomorphic adenoma, the latter deriving, in a high percentage of cases, from benign PA (Åström et al, 1999; Jin et al, 2001). This has led to the designation of PLAG1 as a "benign oncogene" (Kas et al, 1997). We investigated the status of PLAG1 on a selected group of 20 salivary gland tumors comprising 15 pleomorphic adenomas (PA), 1 myoepithelioma and 4 carcinomas ex-pleomorphic adenomas (caPA), all sharing karyotypic chromosome 8 deviations, either structural (8q12 rearrangements) or numerical gains. This study was performed using *in situ* hybridization (ISH) techniques, on metaphase cells, by fluorescence *in situ* hybridization (FISH), and/or in interphase cells from formalin-fixed, paraffin embedded tissue, using chromogenic *in situ* hybridization (CISH). CISH analysis was aimed at verifying intragenic rearrangements of PLAG1 in uncultured tumor cells, *in situ*, and at comparing the results obtained with both ISH approaches.

The histogenesis of PA remains a controversial issue. Two main hypothesis are discussed, the one considering these tumors to be clonal expansions of a single pluripotent cell from the intercalated duct (Batsakis et al, 1989; Batsakis & El-Naggar, 1999), and the other considering that biphasic salivary tumors arise from the coordinated growth of cell populations derived from cells with maintained proliferating capability in salivary tissue (Dardick, 1998). Clonality in PA has been

demonstrated by three previous studies (Debiec-Rychter et al, 2001; Lee et al, 2000; Noguchi et al, 1996), pointing to the common pluripotent cell theory for salivary neoplasms. The study of Debiec-Rychter et al (2001) used PLAG1 genetics and immunohistochemistry in PA cases and demonstrated that the gene lesions were present in all tumor cell populations, though more intensely in cells maintaing a mesenchymal phenotype. They postulate that cells with a clearly demonstrated epithelial phenotype probably evolve through the normal pathways of differentiation (Debiec-Rychter et al, 2001). Aiming at further clarifying this issue, and at the elucidation of the morphogenetic role of PLAG1 in PA, we studied, by combined genotypic and phenotypic analysis, whether the (cyto)genetic aberrations were present in one or in both, epithelial and myoepithelial, PA cell types in two cases – one PA and one caPA, both characterized by 8q12 translocations.

MATERIAL AND METHODS

Case selection

Twenty karyotyped cases of salivary gland tumors were selected: 16 benign tumors (1 myoepithelioma and 15 pleomorphic adenomas) and 4 carcinomas ex-pleomorphic adenoma, all of them displaying chromosome 8 deviations, either as structural rearrangements affecting 8q12 region (90% of cases), or as numerical gain of chromosome 8 (10% of cases). Clinical, histopathological and karyotypic informations are provided on Table 1.

Conventional cytogenetic analysis

Chromosome metaphases of tumor cells were obtained from short term primary cultures as described previously (Jin et al, 1995). Chromosomes were GTG-banded, and karyotypes were established according to the ISCN rules (ISCN, 1995). Complete description of karyotypes can be retrieved from Table 1.

In situ hybridization – FISH and CISH analysis

FISH analysis

In order to evaluate PLAG1 alterations, FISH analysis was performed on metaphase chromosomes using a set of three PAC clones covering the PLAG1 gene. PAC clones DNAs were isolated as described by Rogalla et al (1998) and labeled, concurrently, with biotin by random octamer priming, using the Bioprime DNA labeling system (Life Technologies Inc). FISH protocol was as described by Höglund et al (1996) except for denaturation. PLAG1 probe and chromosome preparations were co-denatured at 80°C for 2-3 min and hybridized overnight at 37°C. Biotinylated PLAG1 probe was detected by Cy3-avidin (Jacksons Lab) and

chromosomes were counterstained with DAPI-Vectashield mounting solution (Vector). Fluorescence hybridization signals were analyzed and recorded with a Cytovision System (Applied Imaging).

CISH analysis

Chromogenic *in situ* hybridization (CISH) was performed on 4μm formalin-fixed paraffin-embedded tissue sections, containing both normal salivary gland and neoplastic tissue, with PLAG1 probe and a (peri)centromeric probe for chromosome 8 (D8Z2), purchased from ATCC (ATTC, USA). Centromeric 8 probe was used to verify trisomy of chromosome 8 and as a control probe.

CISH analysis was performed as previously described by Alers et al (1995) with minor modifications. Briefly, the slides were deparaffinized in xylene for 3x15 minutes, followed by washing in 100% ethanol for 4x2min. A pre-treatment was performed by immersing the slides on 2xSSC for 30min at 72°C, washed on PBS, and pepsin digested (4mg/ml in 0.2N HCl) at 37°C for 30-50min. The duration of pepsin digestion was optimized for each case. CISH probes were labeled with fluorescein (Amersham Pharmacia), by random priming, as described above. Tissue sections were then denatured in 70% formamide/SSC, for 2-3min at 74°C, and probes at 80°C, for 10min. For PLAG1 probe, an additional pre-hybridization period of 45 min at 37°C was done. Hybridization was performed overnight at 37°C. Probe detection was done using horseradish peroxidase anti-fluorescein (Roche) and H_2O_2-diaminobenzidine. Tissue sections were counterstained with hematoxylin and mounted with Entellan (Merck).

In each case, per tissue section, both probes (cen8 and PLAG1) were evaluated at tumor and normal areas. For PLAG1 probe, the number of cells analyzed varied between 69-237 (mean=197) in tumor area (15 cases) and between 65-218 (mean=174) in normal area (12 cases). For cen8 probe in tumor area (16 cases) the range was 183-218, (mean=209) and in normal area (16 cases) 115-221 (mean=195). The number of spots per nucleus was scored as 1, 2, 3, 4 or >4 in all cases, except in one aneuploid caPA case, where the range used was 1 to ≥10.

In normal areas, the mean percentage of nuclei (M) ± standard deviation (SD), containing 1, 2 and 3 hybridization signals per nucleus, was, respectively, 30.46±15.6, 68.71±15.3, and 0.67±1.1 for cen8 probe, and 28.0±32.8, 70.4±28.7 and 1.67±1.1, respectively, for PLAG1 probe.

Significant abnormal losses (monosomy) or gains (polisomy) with centromeric probes is considered to be present when the percentage of samples with one or ≥3 signals per nucleus is greater than the M±3SD of the controls (Poddighe et al, 1991). We followed the more conservative criteria proposed by Jenkins et al (1998)

for paraffin-embedded tissue sections, interpreting ≥10% of nuclei with 3 signals from cen8 and PLAG1 probes, respectively, as trisomy 8 or split of the PLAG1 gene.

Combined immunocytochemistry (ICC) and *in situ* hybridization (FISH) analysis

To investigate the cell types that carried the specific cytogenetic lesions, a combined approach of phenotypic and genotypic analysis was performed, using, respectively, immunocytochemical (ICC) and fluorescence *in situ* hybridization (FISH) techniques. ICC procedures preceded FISH, since several steps of FISH (such as enzymatic digestion, denaturation at high temperatures, and hybridization on formamide), destroy cellular antigens needed for immunophenotyping. The phenotypes of cell populations were established using mouse monoclonal antibodies against calponin (dilution 1:300) (Dako, Denmark) for myoepithelial phenotype, and low molecular weight cytokeratin (MNF116; dilution 1:250) (Dako, Denmark) for epithelial phenotype, respectively.

Primary tumor cells were cultured *in situ* on glass slides and grown for 3 to 8 days. Whenever a satisfactory number of dividing cells was detected, slides were *in situ* colcemid-treated. To preserve cytoplasmic and cell membrane morphology, a mild hypotonic treatment was applied with 0.0375 M (KCl) for 25min at room temperature (Henn et al, 1990). Subsequently, a standard immunocytochemical protocol using a complex of avidin-biotin peroxidase and AEC for chromogenic detection was applied.

Controls used immunohistochemical analysis on 4μm paraffin-embedded tissue sections from the same cases and from control tissues, using both anti-calponin and anti-cytokeratin antibodies.

At least 10 positive immunoreactive cells for each antibody (anti-calponin and anti-MNF116) were recorded, using a CCD cool camera, and the exact localization at the slide surface was registered. Subsequently, FISH protocols were performed at the same slides, using commercial painting probes (Cambio, UK) to detect the chromosome alterations identified by cytogenetic analysis on previously immunophenotyped and registered cells.

RESULTS

Evaluation of PLAG1 gene alterations by *in situ* hybridization (FISH and CISH analysis)

PLAG1 gene evaluation was feasible in all 20 tumors by metaphase FISH (18 cases) and/or paraffin-section interphase CISH (16 cases) using the PLAG1 probe and the (peri)centromeric probe for chromosome 8 (Table 2).

Intragenic rearrangement of PLAG1 was observed in 9 PA and 3 caPA cases by FISH analysis, verified by hybridization signals of PLAG1 probe on rearranged chromosomes 8 and their translocation partners (split signal). In PA cases 10, 11 and 12 no split signal could be detected. In cases 10 and 11, both carrying a classical t(3;8)(p21;q12), rearrangement of PLAG1 gene was expected, but only two signals were observed: one at der(8) and one at the normal chromosome 8. No signal was detected at the translocation partner der(3). However, the signal on der(8) appeared smaller than the signal on normal chromosome 8, indicating that a deletion of 3p sequences along with the t(3;8) presumably occurred. In both cases with trisomy 8 (PA case 15 and caPA case 19), three copies of PLAG1 were detected without PLAG1 region rearrangement. In case 1 (myoepithelioma) and case 9 (PA) it was impossible to verify PLAG1 status due to lack of metaphases.

By CISH analysis PLAG1 could be evaluated in 15 cases, confirming split of PLAG1 by a significant % of nuclei displaying 3 signals on FISH positive cases and allowing the demonstration of PLAG1 rearrangements on unprobed PLAG1 cases 1 (20.1%) and 9 (29.5%) (Table 2). With centromeric 8 probe, all cen8 cases positive-probed by CISH were disomic with the exception of case 19 that showed 21.6% of nuclei with 3 signals/copies confirming trisomy 8.

In cases 10 and 11, which were "split PLAG1 negative" by FISH, the 3 signal counts per nucleus were, respectively, 9.7% and 14%. These values fulfilled our criteria of ≥10% for PLAG1 split, thoroughly for case 11, and on the lower limit for case 10. These two cases were interpreted as positive for PLAG1 rearrangement. In case 12, CISH confirmed that there was no intragenic rearrangement of PLAG1. It was characterized by a t(8;9)(q12;p22-23) with breakpoint at 8q12. FISH analysis of this case with painting probes (data not show) confirmed the translocation, excluding involvement of other(s) chromosome(s).

PA case 3 unexpectedly displayed a more complex rearrangement of PLAG1. G-band analysis revealed what appeared to be a balanced translocation t(5;8)(p13;q12~13), that was confirmed by FISH using painting probes for chromosome 5 and 8 (Figures 1A and 1B). However, when PLAG1 status was

investigated, instead of the expected PLAG1 split with signals on both der(5) and der(8), two small signals from PLAG1 were observed on der(8), and none was identified on der(5), indicating that a complex cytogenetic rearrangement had probably occurred (Figure 1C). Notably, the same pattern was clearly detected in paraffin-sections by CISH (Figure 1D).

Identification of cell types carrying 8q12 rearrangements by combined genotypic/phenotypic analysis

Immunocytochemical techniques to assess calponin and cytokeratin immunoreactivity were applied to assess myoepithelial and epithelial immunophenotypes of primary tumor cells growing in vitro from PA case 4 and caPA case 17. In both cases, immunopositive calponin and cytokeratin mitotic cells were identified and recorded. To establish the cell genotypes, the same slides were processed for FISH analysis. Cytogenetically, both cases were characterized by 8q12 translocations, t(8;10) and t(3;8), respectively, for case 4 and 17. To verify the presence of the translocation in immunopositive cells, whole chromosome painting probes for chromosomes 3, 8 and 10 were chosen. Knowing by chromosome metaphase FISH analysis the status of PLAG1 on these cases (both showed split signals), PLAG1 probe was also tried, but the background was too high for signal evaluation. In both cases, the translocation was found in both immunopositive calponin and cytokeratin mitotic cells demonstrating that the (cyto)genetic lesion was shared by myoepithelial and epithelial cells. Figures 2A and 2B illustrate translocation t(8;10) on a cytokeratin immunopositive cell from case 4.

DISCUSSION

Our study describes to our knowledge the largest series of pleomorphic adenomas, characterized by 8q12 deviations with PLAG1 involvement, assessed by *in situ* hybridization techniques (ISH). Both metaphase and interphase tumor cells were evaluated and this represents a pioneering study on paraffin-embedded sections. Additionally, it provides information on other related salivary gland tumors, carcinomas ex-pleomorphic adenoma (caPA) and myoepitheliomas, where very scarce or even no information about the status of PLAG1 is available.

In our series seventeen tumors (85%), that includes 13 PA, 3 caPA and the myoepithelioma case, were interpreted as having intragenic rearrangements of PLAG1. Two tumors (10%), PA case 15 and caPA case 19, had trisomy for chromosome 8 without molecular cytogenetic evidence of PLAG1 rearrangement. Only PA case 12 lacked PLAG1 involvement at all. It was characterized by a t(8;9)(q12;p22-23), with breakpoint at 8q12. FISH analysis of this case with painting probes confirmed the translocation, excluding involvement of other chromosome(s). ISH analysis on both metaphase and interphase cells revealed only two signals/copies of PLAG1 indicating no disruption of PLAG1 gene. In a recent study, Röijer et al (1999) reported three PA cases with 8q12-13 rearrangements but without PLAG1 alteration. Further characterization of these cases will possibly allow disclosing the mechanisms behind these rearrangements.

PLAG1 involvement has been mainly reported in PA associated with 8q12 deviations, but it was also found to be activated in PAs with normal karyotypes and 12q13-15 abnormalities (Åström et al, 1999; Kas et al, 1997; Voz et al, 1998, 2000). In contrast, in malignant tumors, rearrangement of PLAG1 was only observed in a limited number of cases, mainly caPA cases (Åström et al, 1999; Jin et al, 2001).

In the present study, except for one tumor (case 12), all PA with 8q12 deviations had an intragenic rearrangement of PLAG1 (93%), which indicates that PLAG1 rearrangement is a very frequent genetic event in these tumors, and confirming its crucial role in salivary gland tumorigenesis.

Carcinomas ex-pleomorphic adenoma are uncommon neoplasms representing about 12% of all malignant salivary gland tumors (Gnepp, 1993). They typically develop in the context of previous pleomorphic adenoma and pursue an aggressive course. Histologically these neoplasms are characterized by the concomitant presence of benign and malignant components, the latter usually being adenocarcinoma NOS (Ellis, 1991). Previous cytogenetic and molecular studies revealed recurrent rearrangements at 8q12 and 12q13-15, the same chromosome

regions affected in pleomorphic adenomas, and consistent alterations at 17p loci (see El-Naggar, 2000 for review). Consequently, PLAG1 gene rearrangement is expected to occur, at least, in caPA with 8q12 deviations. We found that all 8q12 rearranged caPA cases showed PLAG1 rearrangement confirming the involvement of this gene.

Myoepithelioma is a rare benign salivary gland neoplasm that presents a monomorphous cellular composition with cells exhibiting a terminally differentiated myoepithelial cell phenotype (Seifert et al, 1990; Dardick et al, 1987) being considered by some authors of representing one end of the morphological spectrum of pleomorphic adenoma. Therefore it is conceivable that myoepithelioma might also share the (cyto)genetic features already described for pleomorphic adenomas. The only abnormal karyotyped case described in the literature displayed a t(1;12)(q25;q12) thus sharing a 12q rearrangement with pleomorphic adenomas (El-Naggar et al, 1999). Investigation conducted on myoepithelioma case 1 showed intragenic rearrangement of PLAG1. It seems therefore reasonable to conclude that salivary gland myoepitheliomas share (cyto)genetic characteristics with pleomorphic adenomas and as such PLAG1 also plays an important role in these tumors, at least the cases with 8q12 deviations.

Trisomy 8 is the most frequent numerical deviation in salivary gland tumors being shared by benign and malignant tumors (Mitelman F, Johansson B, and Mertens F: Mitelman Database of Chromosome Aberrations in Cancer. http:/cgap.nci.nih.gov/ Chromosomes/Mitelman.2001). In the present series gain of chromosome 8 was the sole karyotypic aberration found in one PA case and in one caPA case. FISH analysis allowed us to verify that PLAG1 region was not structurally affected, since in these tumors there was only a copy number increase (3 copies) of the gene. The pathogenic mechanisms associated with gain of chromosomes are still unclear, the most plausible explanation for the fact being gene dosage alterations. A novel oncogenic mechanism involving PLAG1 copy number gain was recently suggested in a study of lipoblastomas that present polisomy for chromosome 8 without PLAG1 rearrangement (Gisselsson et al, 2001). In these tumors, the 8q12 chromosomal rearrangements result in upregulated expression of PLAG1 transcription factor. It was hypothesized that in polisomy 8/nonrearraged PLAG1 cases, PLAG1 could be transcriptionally upregulated, in a mechanism similar to that detected in lymphoid neoplasms, such as Burkitt lymphoma, where point mutations on the promoter-region of MYC oncogene were detected in fusion gene negative cases (see Gisselsson et al, 2001 for review). If an analogous mechanism is present in salivary gland tumors remains to be investigated.

One of our aims was to compare ISH techniques on metaphase tumor cells with interphase tumor cells on archival material with a specific-gene probe for detection of gene rearrangements. Karyotyping mandates the establishment of a cell culture *in vitro* from a tumor fragment. In contrast, with ISH on paraffin sections, it is possible to evaluate the genetic alterations *in situ*, in uncultured tumor cells, avoiding *in vitro* manipulation. We found a good agreement between the two techniques. ISH on paraffin sections was shown to be a very sensitive technique as demonstrated in case 3. Karyotypically, this case displayed what suggested being a simple translocation between chromosome 5 and chromosome 8 in the form of t(5;8)(p13;q12) was confirmed by FISH with painting probes for chromosome 5 and 8. However, when PLAG1 status was evaluated on metaphase cells by FISH, two small spots from PLAG1 on der(8) were observed and none on der(5) meaning that a more complex rearrangement had occurred, probably an inversion on chromosome 8, which initially splits the PLAG1 locus, along with a translocation with chromosome 5. A similar situation was observed in pulmonary hamartomas of the lung, where hidden chromosomal inversions occurring together with simple translocations were disclosed by FISH analysis (Kazmierczak et al, 1999). Remarkably, CISH analysis on paraffin sections of this case revealed exactly the same pattern, demonstrating that ISH analysis is a powerful technique to assess PLAG1 rearrangements on paraffin embedded archival material from salivary gland tumors.

The second question addressed on this study was to investigate the role of PLAG1 in tumorigenesis and differentiation of pleomorphic adenomas.

Pleomorphic adenomas are morphologically characterized by a biphasic pattern with epithelial and mesenchymal areas. Controversy remains on whether PA arises from a single pluripotent cell or if more then one "stem cell" is involved in clonal expansion (Dardick,1998). There is increasing evidence that epithelial and myoepithelial cell populations share phenotypical and genotypical characteristics supporting the "modified myoepithelial cell model" for PA histogenesis (Batsakis and El-Naggar, 1999; Dardick, 1998).

Identification of cell types carrying PLAG1 rearrangements could help to clarify this issue. This prompted us to perform a combined phenotypic/genotypic analysis on PA case 4 and caPA case17, both characterized by 8q12 translocations and PLAG1 intragenic rearrangement. Immunocytochemical techniques were used for phenotypic characterization of tumor cells. In both cases, mitotic cells that shared immunopositivity for calponin and keratin were identified. The genotype of the cells was established using FISH techniques, allowing the localization of 8q12

abnormalities in both immunopositive calponin and keratin mitotic cells which points to accept that the cytogenetic lesion is shared by epithelial and myoepithelial cell lineages. Further evidence came from a recent study on two pleomorphic adenomas characterized by 8q12 translocations (Debiec-Rychter et al, 2001). In this study, the authors investigated which cells types expressed PLAG1 protein and demonstrated that all cells that were strongly immunoreactive for PLAG1 carried the specific chromosomal translocations, and that they could be both epithelial or myoepithelial, phenotypically. Our results and previous data show, therefore, that pleomorphic adenomas cells share a single cell origin probably from a pluripotent cell capable of differentiating into a variety of somatic phenotypes, as proposed by Batsakis & El-Naggar (1999). This potentially attributes to PLAG1 a significant role in the basic tumorigenic process or, at least, in a significant subset of salivary gland pleomorphic adenomas.

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Table 1 - Clinical, hispathological and karyotypic data from 20 salivary gland tumors with chromosome 8 deviations

CASE	CLASSIFICATION	Age/Sex	Location	Karyotype
1	Myoephitelioma	57/F	parotid	45~46,XX,t(2;8)(p23;qq22),t(8;12)(q12;p13)[cp8]/46,XX[3]
2	Pleomorphic adenoma	45/M	parotid	46,XY,t(8;9)(q12;p22-23)[7]
3	Pleomorphic adenoma	68/F	palate	46,XX,t(5;8)(p13;q12~13)[8]
4	Pleomorphic adenoma	51/M	parotid	46,XY,t(8;10)(q12;q22)[13]
5	Pleomorphic adenoma	78/M	parotid	46,XY,t(3;8)(p21;q12)[11]/45,XY,t(3;8)(p21;q12),der(13;15)(q10;q10)[2]/46,XY[2]
6	Pleomorphic adenoma	37/F	submandibular	46,XX,t(8;15)(q12;q26)[13]
7	Pleomorphic adenoma	22/F	parotid	46,X,t(X;2)(q26;q21),t(3;8;9)(p21;q12;p21)[9]
8	Pleomorphic adenoma	86/F	submandibular	46,XX,t(3;8)(p21;q12)[16]
9	Pleomorphic adenoma	72/F	palate	46,XX,t(3;8)(p21;q12)[6]
10	Pleomorphic adenoma	24/F	parotid	46,XX,t(3;8)(p21;q12)[7]/46,XX[11]
11	Pleomorphic adenoma	73/F	parotid	46,XX,t(3;8)(p21;q12)[8]
12	Pleomorphic adenoma	40/F	parotid	46,XX,t(8;9)(q12;p22-23)[8]
13	Pleomorphic adenoma	34/M	parotid	45~46,XY,t(3;8)(p21;q12)[cp11]
14	Pleomorphic adenoma	68/F	parotid	44~46,XX,t(3;8)(p21;q12)[cp6]
15	Pleomorphic adenoma	56/F	parotid	47,XX,+8[11]/46,XX[15]
16	Pleomorphic adenoma	33/F	parotid	46,XX,t(5;8)(p15;q12)[6]/46,XX[10]
17	Carcinoma ex-pleomorphic adenoma	48/M	parotid	46,XX,t(3;8)(p21;q12)[10]
18*	Carcinoma ex-pleomorphic adenoma	71/F		105-111,XXXXX,-1,i(1)(q10),del(2)(q13),+3,der(3)t(3;8)(p21;q12)x4, +6,+7,+7,+7,der(8)i(8)(q10)t(3;8)(p23;q12)x2, +der(?9)t(1;9)(p22;?)x2,-10,-11, -11, der(11)t(11;11)(p15;q13)x3,del(12)(p12),-13,-14,-15,-16,del(16)(q22)x2,17,-18, -18, -18,i(18)(q10),+19,-21,+22[cp8]/46,XX[3]
19	Carcinoma ex-pleomorphic adenoma	58/M	parotid	47,XY,+8[10]/45,X,-Y[6]/46,XY[6]
20	Carcinoma ex-pleomorphic adenoma	60/F	parotid	46,XX,ins(3;8)(p21;q12qter)[10]

*case previously published (Jin et al, 2001)

Table 2- Cytogenetic and *in situ* hybridization data from 20 salivary gland tumors with chromosome 8 deviations

Case/diagnosis	Cytogenetic	FISH PLAG1	CISH / N°of signals from PLAG1	CISH / N°of signals from cen8
1/Myoepithelioma	t(8;12)	not done	1(26,9%):2(50,4%):3(20,1%):4(2,4%) *split*	1(32,4%):2(66,2%):3(0,93%):4(0,46%)
2/PA	t(8;9)	*split*	1(11,1%):2(48%):3(38,3%):4(1,45%):>4(0,97%) *split*	1(28,1%):2(70,4%):3(1,32%)
3/PA	t(5;8)	*split*	1(19,4%):2(24,8%):3(50,7%):4(0,49%) *split*	1(33,8%):2(65,3%):3(0,9%)
4/PA	t(8;10)	*split*	1(23,8%):2(44,7%):3(30,3%):4(0,99,%) *split*	1(32,4%):2(66,6%):3(1%)
5/PA	t(3;8)	*split*	no results	no results
6/PA	t(8;15)	*split*	1(24,8%):2(44,6%):3(28,7%):4(1,28%):>4(0,42%)*split*	1(33,6%):2(64,5%):3(1,4%):4(0,46%)
7/PA	t(3;8;9)	*split*	1(19,5%):2(39%):3(38,5%):4(3,5%) *split*	1(30%):2(69%):3(1%)
8/PA	t(3;8)	*split*	1(17,4%):2(63,7%):3(17,4%):4(1,44,%) *split*	1(30%):2(68,5%):3(1,09%)
9/PA	t(3;8)	not done	1(26,5%):2(43%):3(29,5%):4(1%) *split*	1(32%):2(66,5%):3(1,47%)
10/PA	t(3;8)	no *split*	1(18,9%):2(70,8%):3(9,7%):4(0,42%) *split*	1(32,2%):2(67,3%):3(0,483%)
11/PA	t(3;8)	no *split*	1(21,5%):2(61,5%):3(14%):4(3%) *split*	1(38%):2(62%)
12/PA	t(8;9)	no *split*	1(25,7%):2(70,7%):3(3,46%):4(1,48%) no *split*	1(37,5%):2(62,5%)
13/PA	t(3;8)	*split*	1(9,6%):2(46,8%):3(41,5%):4(1,9%) *split*	1(22,5%):2(75,6%):3(1,83%)
14/PA	t(3;8)	*split*	1(18%):2(45,3%):3(33,8%):4(2,73%) *split*	1(30,5%):2(67,5%):3(2%)
15/PA	+8	trisomic	no results	no results
16/PA	t(5;8)	*split*	no results	no results
17/caPA	t(3,8)	*split*	1(11,3%):2(50%):3(34,1%):4(4,45%) *split*	1(32,3%):2(63,7%):3(3%):4(1%)
18/caPA	der(8q12)	*split*	1(2,3%):2(13,3%):3(6,9%):4(9,6%):5(13,8%) :6(14,7%): 7(11,98%):8(10,5%):9(9,2%) :10(3,22%):>10(4,6%) *split*	1(7,2%):2(18,9%):3(18,4%):4(16,2%):5(19,8%) :6(16,2%):7(3,15%)
19/caPA	+8	trisomic	no results	1(18,75%):2(47,6%):3(21,6%):4(12%) trisomic
20/caPA	ins(3;8)	*split*	no results	no results

PA-pleomorphic adenoma; caPA-carcinoma ex-pleomorphic adenoma

Figure 1:
G-band and *in situ* hybridization analysis of a pleomorphic adenoma (case 3) with a t(5;8)(p13;q12-13). (A) Representative karyotype of the case; (B) FISH analysis with painting probes for chromosome 5 (green) and chromosome 8 (red), confirming the translocation; (C) FISH analysis with PLAG1 probe (red) with signal on normal chromosome 8 (arrow head) and with two small signals on the der(8) (arrow); (D) CISH analysis on a tumor paraffin section, with PLAG1 probe; there are two adjacent brown spots, and a third spot confirming FISH observations

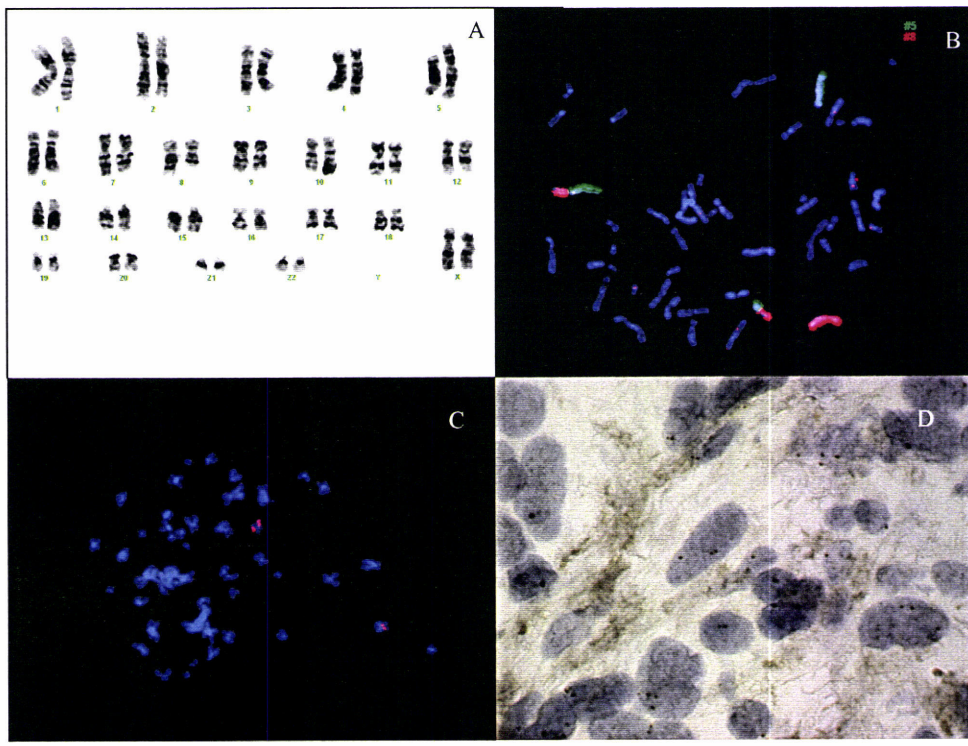
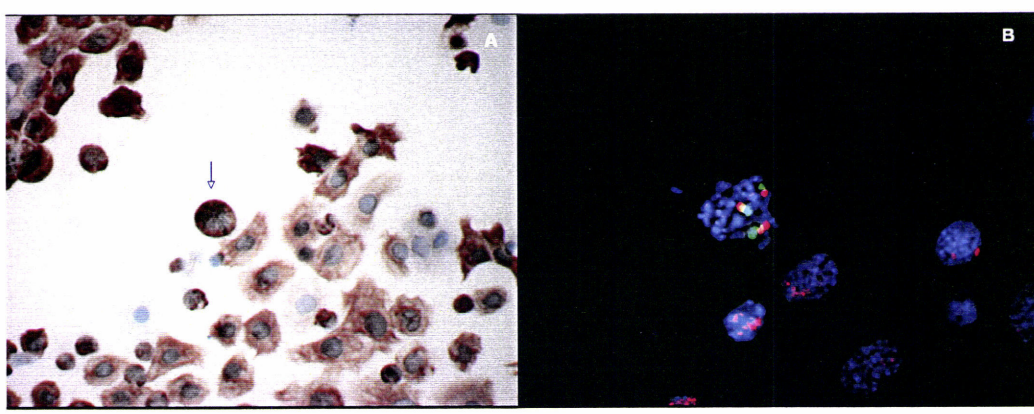


Figure 2:
Combined immunocytochemistry and FISH analysis of a pleomorphic adenoma (case 4) with a t(8;10)(q12;q22). (A) Cell undergoing mitosis with cytoplasmic immunopositivity for MNF116 antibody (arrow); (B) FISH analysis of the same cell using painting probes for chromosome 8 (red) and for chromosome 10 (green). The t(8;10) is evidenced.



4.

Discussão e Conclusões

4.1 As alterações (cito)genéticas em tumores das glândulas salivares

As alterações citogenéticas identificadas em tumores benignos e malignos parecem diferir quer quanto às regiões génicas envolvidas quer quanto ao tipo de anomalia cromossómica em causa, sugerindo a possibilidade de haver padrões de alterações citogenéticas com associação específica a distintos tipos histológicos. Foi nosso objectivo identificar e caracterizar as alterações cromossómicas recorrentes num grupo vasto de tumores salivares, por forma a contribuir para alargar o conhecimento quanto ao padrão de alterações citogenéticas e à definição das regiões genómicas-alvo com papel potencial na tumorigénese. Pretendemos, ainda, avaliar o comprometimento do gene *PLAG1* considerado como um dos oncogenes envolvidos em neoplasias das glândulas salivares, tanto benignas como malignas, e que têm em comum alterações citogenéticas do cromossoma 8.

4.1.1 Padrão de alterações citogenéticas em tumores benignos

Em tumores salivares benignos, a maior parte da informação citogenética respeita ao adenoma pleomórfico, o tumor mais frequente (Rosenberg et al, 1997; Ellis et al, 1991). A análise citogenética de outros tumores salivares benignos circunscreve-se, quase exclusivamente, ao tumor de Warthin, sendo escassa ou mesmo inexistente em outros tipos histológicos.

Adenoma pleomórfico

O estudo cariotípico de 77 adenomas pleomórficos (capítulos 3.1, 3.5, 3.7 e resultados não publicados) demonstrou que há um padrão de alterações cromossómicas que é sobreponível ao que já foi descrito nestes tumores (cf capítulo 1.3). Assim, as alterações do cromossoma 8 através de rearranjos estruturais que afectavam o braço longo ou alterações numéricas, sobretudo por trissomia, foram identificadas em 35 casos (45,5%), os rearranjos em 12q13-15 em 11 casos (14,3%), e outras alterações várias, não recorrentes, em igual número de casos (14,3%). Os tumores restantes apresentavam um cariotipo normal (25,9%).

Ao analisar a informação citogenética obtida, verifica-se que as alterações que afectam o cromossoma 8 são as mais frequentes tornando-se o subtipo citogenético mais representativo, que engloba cerca de 50% dos casos. As alterações cariotípicas encontradas neste subgrupo são maioritariamente de tipo estrutural (33 casos), sendo somente numéricas em 2 casos, ambos com trissomia 8. Os cromossomas parceiros nas anomalias estruturais que ocorrem em 8q12 são

vários sendo, no entanto, o 3p21 aquele que é preferencial, originando a translocação típica, mais comum, dos adenomas pleomórficos t(3;8)(p21;q12), a qual foi detectada em 15 tumores. Em relação aos casos caracterizados por alterações do cromossoma 12, excepto em um que possuia trisomia 12, as anomalias eram do tipo estrutural, resultantes de translocações e inversões. No caso das translocações (7 tumores) o ponto de quebra em 12q era q13-15, com diferentes parceiros cromossómicos. Nos restantes 3 casos esta região era afectada por inversões, quer do tipo pericentromérica quer do tipo paracentromérica. A diversidade de parceiros cromossómicos bem como os diferentes tipos de alteração estrutural detectada apontam para que o mecanismo molecular subjacente a estes rearranjos seja a activação do gene *HMGA2*, por desregulação da sua expressão, e não que tenha ocorrido a formação de uma proteína anómala (Åman, 1999).

Nesta série de adenomas pleomórficos, 20 casos tinham um cariotipo normal. A natureza das células cultivadas *in vitro* com cariotipo normal constituiu desde sempre um problema na citogenética de tumores sólidos. É impossível saber o que é que as células cariotipadas normais representam: serão células neoplásicas?, serão células constituintes do estroma do tumor? ou, ainda, serão células epiteliais normais de glândula salivar? Para além disso, as técnicas de citogenética convencional têm uma resolução limitada o que não possibilita detectar alterações submicroscópicas. Após a identificação dos genes envolvidos nos rearranjos mais comuns, o *PLAG1* em 8q12 e o *HMGA2* em 12q14, Åström et al (1999) verificaram, por exemplo, que 13 dos 17 adenomas pleomórficos cujo cariotipo era normal tinham aumento de expressão do gene *PLAG1*. Por isso, não deverá ser excluída a hipótese de que os casos cariotipicamente normais possuam de forma indetectável as alterações génicas implicadas na sua origem.

Perante a existência nos adenomas pleomórficos de um padrão específico, bem definido, de alterações genéticas e, face à sua diversidade fenotípica, foram feitas tentativas de verificar se existe alguma relação preferencial entre as anomalias cariotípicas e as características histológicas dos adenomas pleomórficos. Foi demonstrado que os tumores com rearranjos em 8q12 são adenomas de fenótipo tipo clássico, que contém 30-50% de estroma na sua composição, em contraste com os tumores com cariotipo normal e têm rearranjos em 12q13-15, que são constituídos por mais de 80% de estroma (Bullerdiek et al, 1993).

Com o mesmo objectivo procedemos à classificação dos adenomas pleomórficos desta série em dois grupos: aqueles em que havia predomínio celular e os que tinham predomínio de estroma, com base na classificação de Foote & Frazell (1956). Em 72 dos 77 tumores, 20 casos tinham predomínio celular e 52

casos predomínio de estroma. Integrando a informação cariotípica com estes subgrupos histológicos, verificámos que havia uma associação estatisticamente significativa entre o grupo de predomínio celular e ausência de alterações do cromossoma 12 (p=0.043), e entre este mesmo grupo e alterações do cromossoma 8 (p=0.001). Parece-nos, portanto, ser legítimo concluir que existe uma correlação entre a composição histológica dos adenomas pleomórficos e o cariotipo, visto que o grupo de neoplasias com predomínio celular apresentava predominantemente alterações do cromossoma 8 e não tinha alterações do 12, enquanto que o grupo com predomínio de estroma não mostrava nenhum padrão específico de alterações. O facto do cromossoma 12 só se encontrar rearranjado no grupo com predomínio de estroma sugere que o gene *HMGA2* envolvido nestes rearranjos poderá estar preferencialmente associado a adenomas pleomórficos com este fenótipo. O gene *HMGA2*, como atrás referido (cf capítulo 1.3), está relacionado com a origem de vários tipos de tumores benignos sendo a maior parte deles mesenquimatosos ou tendo um padrão histológico bifásico com diferenciação epitelial e mesenquimatosa e em que, maioritariamente, a desregulação do *HMGA2* está confinada ao componente estromal (Tallini et al, 1999). Vários estudos têm sido efectuados com o objectivo de investigar a associação entre as lesões do gene *HMGA2* e os tumores benignos de origem mesenquimatosa (Ashar et al, 1995; Kasmierczack et al, 1996; Tkachenko et al, 1997; Battista et al, 1999; Arlotta et al, 2000) tendo sido atribuído ao gene *HMGA2* um papel importante nessa diferenciação.

Para o adenoma pleomórfico, que constitui um exemplo paradigmático de um tumor bifásico, é geralmente aceite que ambos os componentes têm origem numa célula percursora pluripotente com capacidade divergente de diferenciação, epitelial e mesenquimatosa. Assim sendo, a desregulação do *HMGA2* induziria estas células a seguirem um determinado programa de diferenciação, que fenotipicamente se traduziria no predomínio estromal dos adenomas pleomórficos.

A contastação desta diferença significativa entre os subgrupos histológicos leva-nos a considerar vias de diferenciação celular distintas para os adenomas pleomórficos. Os rearranjos genéticos em 8q12 e 12q13-15 parecem poder representar acontecimentos primários directamente relacionados com a iniciação tumoral o que, na nossa opinião, favorece a interpretação de haver duas vias de desenvolvimento neoplásico independentes nos adenomas pleomórficos.

Mioepiteliomas

O mioepitelioma é um tumor benigno das glândulas salivares, composto quase na totalidade por células com fenótipo mioepitelial (Seifert et al, 1990; Dardick et al, 1987). É considerado um dos extremos do espectro de diferenciação morfológica dos adenomas pleomórficos e, como tal, morfologicamente relacionado com este tipo de neoplasia (Ellis et al, 1996). O único caso publicado com cariotipo alterado revelou uma translocação t(1;12)(q25;q12). Embora o ponto de quebra descrito não seja exactamente o mesmo que está referenciado em adenomas pleomórficos (12q13-15), os autores consideram que mioepiteliomas e adenomas pleomórficos partilham anomalias em 12q (El-Naggar et al, 1999). A análise cariotípica de dois mioepiteliomas por nós efectuada demonstrou que o cariotipo era normal num caso e em outro havia alterações em 8q12, através de uma translocação recíproca t(8;12)(q12;p13) (capítulos 3.1 e 3.7) Assim, com base na informação disponível parece poder concluir-se que o mioepitelioma e o adenoma pleomórfico das glândulas salivares, partilhando anomalias citogenéticas possuem uma histogénese comum.

Tumor de Warthin

A informação citogenética disponível sobre o tumor de Warthin está limitada a 35 casos, incluindo os 12 analisados por nós no IPOFG, Lisboa (Bullerdiek et al, 1988; Mark et al, 1989, 1990; Nordqvist et al, 1994; capítulo 3.3). Em 16 tumores (45,7%) os cariotipos estabelecidos são normais; os restantes 19, a par de um clone normal apresentavam anomalias clonais numéricas e/ou estruturais.

Quanto às alterações numéricas encontradas, a perda clonal do Y foi a única anomalia recorrente e, quanto a alterações estruturais, parecem estar consistentemente envolvidas 3 regiões cromossómicas diferentes: 11q21, 19p13, geralmente sob a forma t(11;19)(q21;p13) e 6p21-23.

A translocação 11;19 parece ser uma anomalia específica associada ao tumor de Warthin ou, pelo menos, comum a um subgrupo destes tumores. Tal como já foi referido, uma translocação t(11;19) idêntica foi descrita em carcinomas mucoepidermóides das glândulas salivares e do pulmão (Dahlenfors et al, 1994, 1995; Horsman et al, 1995; Johansson et al, 1995; Nordqvist et al, 1994).

As alterações cromossómicas que envolvem a região 6p21-23 são recorrentes no tumor de Warthin e são comuns em outras neoplasias benignas humanas, como o lipoma (Mandhal, 1996) e o leiomioma uterino (Pandis et al, 1991). Poder-se-á estar perante uma situação de natureza idêntica à verificada para

a região 12q13-15, em que se admite que o gene *HMGA2* esteja relacionado com a patogénese de tipos distintos de neoplasias benignas, incluindo o adenoma pleomórfico (Schoenmaker et al, 1995). Recentemente, através de estudos moleculares efectuados em hamartomas e leiomiomas uterinos, citogeneticamente caracterizados por anomalias em 6p21, verificou-se que havia um gene localizado nessa região, pertencente à mesma família do gene *HMGA2*, e como tal designado *HMGA1* [anteriormente designado por *HMGI(Y)*], que estava rearranjado (Kasmierczak et al, 1996). Assim, este gene que tem estrutura e função semelhantes às do *HMGA2*, parece ter igualmente participação no desenvolvimento neoplásico de um subgrupo de tumores benignos com origem mesenquimatosa ou com componente relevante de diferenciação mesenquimatosa. Não se sabe, ainda, se este gene estará relacionado com a patogénese dos tumores de Warthin, que possuem anomalias cromossómicas em 6p21-23.

Os resultados que obtivemos confirmam haver rearranjos cromossómicos preferenciais nos tumores de Warthin, os quais reflectem o envolvimento recorrente das regiões 11q21, 19p13, geralmente sob a forma t(11;19)(q21;p13), bem como também da região 6p21-23. Embora o significado destes rearranjos permaneça desconhecido, estes resultados contribuem, na nossa opinião, para o esclarecimento da tumorigénese destas neoplasias. A detecção de alterações cromossómicas específicas, recorrentes, em cerca de 50% dos casos, constitui um argumento decisivo para considerar o tumor de Warthin como uma neoplasia "verdadeira", afastando a hipótese defendida por alguns autores (Ogawa et al, 1990; Allegra, 1971) de que se trataria de um processo reaccional neoplasiforme. As alterações cromossómicas associadas de forma tão específica a um tipo de tumor são geralmente consideradas primárias, e com um potencial papel decisivo na tumorigénese, constituindo mesmo uma condição *sine qua non* para a ocorrência do processo (Heim & Mitelman, 1995).

Conclusão 1

Os estudos citogenéticos efectuados numa série de adenomas pleomórficos e em dois mioepiteliomas confirmam um padrão de alterações citogenéticas característico, em que há envolvimento recorrente das regiões cromossómicas específicas 8q12 e 12q13-15, facto que faz considerar que o mioepitelioma tem histogénese comum com o adenoma pleomórfico.

A correlação do padrão cariotípico com a composição histológica dos adenomas pleomórficos sugere que os rearranjos genéticos em 8q12 e 12q13-15 se

relacionam com o subtipo microscópico, o que parece indiciar vias de desenvolvimento neoplásico distintas para os adenomas pleomórficos.
O tumor de Warthin associa, de forma inequívoca, anomalias nas regiões cromossómicas 11q21, 19p13 e 6p21, confirmando que esta lesão é uma neoplasia verdadeira e afastando a interpretação de se tratar de uma lesão pseudotumoral hiperplásica.

4.1.2 Padrão de alterações citogenéticas em tumores malignos

Os tumores salivares malignos constituem um grupo muito heterógeneo de neoplasias, que inclui 18 tipos histológicos diferentes (cf capítulo 1.2). Os estudos citogenéticos efectuados, embora escassos e limitados aos subtipos histológicos mais frequentes, permitiram identificar um padrão de alterações cromossómicas que sugere especificidade para os carcinomas salivares (cf capítulo 1.3).

Carcinoma ex-adenoma pleomórfico

Podem desenvolver-se carcinomas em cerca de 3-4% dos adenomas pleomórficos a partir do seu componente epitelial, pelo que os tumores malignos são designados por carcinomas ex-adenoma pleomórfico (Ellis et al, 1991). Os estudos citogenéticos efectuados revelaram, na maior parte dos casos, cariotipos complexos, aneuplóides, com alterações estruturais recorrentes em 8q12-13 e em 12q13-15 e anomalias numéricas com ganho do cromossoma 8 (Bullerdiek et al, 1990; Mark et al, 1991, 1992; Jin et al, 1994; Mark, HFL et al, 1994; Hrynchak et al, 1994, Röijer et al, 2002).

Na análise de 7 neoplasias, verificámos haver em 6 um cariotipo alterado (capítulos 3.2, 3.5 e 3.7). Exceptuando um tumor que possuia um cariotipo complexo e aneuplóide, os outros casos tinham cariotipos relativamente simples, o que levanta a questão de o cariotipo obtido resultar de um defeito de amostragem, correspondendo ao adenoma pleomórfico pré-existente. As alterações do cromossoma 8 caracterizavam 4 neoplasias, quer sob a forma de ganho de material genético (1 caso), quer por rearranjos a nível da região 8q12 (3 casos), o que reforça a informação existente na literatura. A partilha de anomalias cromossómicas com os adenomas pleomórficos, seus equivalentes benignos, associa citogeneticamente, de forma indubitável, estes tumores entre si e aponta para que haja um padrão cariotípico evolutivo, correspondente à sequência histológica adenoma-carcinoma. No entanto, estão por esclarecer quais as

alterações (cito)genéticas que são determinantes para a evolução maligna dos adenomas pleomórficos.

Carcinoma mucoepidermóide

Os rearranjos cromossómicos estruturais prevalecentes nos carcinomas mucoepidermóides afectam as regiões 11q14-24 e 19p12-13, estas geralmente sob a forma t(11;19)(q14-21;p12-3), e a região 6q21-25.

A análise de 8 carcinomas mucoepidermóides (capítulos 3.2; 3.4; 3.6 e resultados não publicados) revelou que havia rearranjos em 6q por um mecanismo de translocação com outros cromossomas, havendo envolvimento da região 6q15-21 em 2 casos. Ambos apresentavam igualmente delecção de material cromossómico 6q que, em um caso, era concomitante com a translocação 6q e, no outro, ocorria num clone independente. A translocação t(11;19)(q21-23;p12) foi a única alteração presente em dois casos, confirmando-se a associação desta anomalia a carcinomas mucoepidermóides (capítulo 3.6 e resultados não publicados). Nunca foram detectados rearranjos em 6q conjuntamente com a t(11;19), o que parece indicar que podem haver duas vias carcinogénicas distintas para estes tumores.

Para além da análise citogenética de carcinomas mucoepidermóides originados nas glândulas salivares, a análise de um carcinoma mucoepidermóide localizado no pulmão também revelou a mesma anomalia t(11;19)(q21;p11) (Johansson et al, 1995). A presença da mesma alteração no mesmo tipo histológico de carcinoma, embora com localização anatómica diferente, torna-a uma lesão primária específica desta neoplasia, com um potencial papel na carcinogénese.

Com o objectivo de caracterizar esta translocação a nível molecular e de identificar potenciais gene(s) envolvido(s) iniciámos um projecto de mapeamento molecular do ponto de quebra 19q12-13 num dos casos de carcinoma mucoepidermóide em que se verificou haver t(11;19). Através de análise por hibridização *in situ* com fluorescência (FISH), com a utilização de sondas específicas para a região cromossómica 19p12-13, foi já possível delimitar a área onde se localiza o ponto de quebra desta translocação, que corresponde a um intervalo genómico de cerca de 3,3Mb (capítulo 3.6). Curiosamente, esta translocação t(11;19), com pontos de quebra em 11q21 e 19p13, e detectada nos carcinomas mucoepidermóides, parece citogeneticamente idêntica à que foi encontrada em tumores de Warthin. No entanto, estes dois tipos de neoplasia não partilham características clinico-patológicas, sendo consideradas entidades não

relacionadas. Se os estudos moleculares demonstrarem estarmos em presença de um rearranjo genético semelhante e afectando provavelmente o(s) mesmo(s) gene(s) poder-se-á colocar a hipótese de uma via patogenética partilhada para estas duas neoplasias no seu processo de desenvolvimento, mas cujo resultado final (morfologia e comportamento clínico) é muito distinto.

Carcinoma adenoide-cístico e
adenocarcinoma polimórfico de baixo grau

No carcinoma adenoide-cístico foram identificadas várias alterações estruturais, sendo a região cromossómica 6q16-25 a mais frequentemente envolvida, o que ocorreu através de delecção - del(6)(q16-25) -, ou por rearranjo, numa translocação recíproca com o cromossoma 9, sob a forma t(6;9)(q21-25;p21-23).

A translocação t(6;9) foi também detectada em carcinomas adenoide-císticos localizados nas glândulas lacrimais (Hrynchack et al, 1994) e na cavidade nasal (Higashi et al, 1991). Em um caso de carcinoma adenoide-cístico do pulmão, embora não tenha sido detectada a translocação t(6;9), foram encontradas duas outras translocações, t(9;17)(p13;p13) e t(X;6)(p22;q23), com pontos de quebra situados nas mesmas regiões, - 9p13 e 6q23 (Higashi et al, 1991) -. Esta observação é um argumento adicional no sentido de dar reforço ao papel fundamental daquela anomalia citogenética neste tipo histológico de neoplasia.

Foram analisados 8 carcinomas adenoide-císticos, tendo sido detectadas em 6 deles anomalias citogenéticas clonais (capítulos 3.2 e 3.4). Em 5 casos, os rearranjos afectavam o braço longo do cromossoma 6: em 2 casos em 6q23-25, sob a forma de t(6;9) e, nos restantes casos, por delecção ou por translocações com outros parceiros cromossómicos que não o 9.

Estudos adicionais realizados por FISH e COBRA-FISH descritos no capítulo 3.4 permitiram ainda verificar que, num dos casos, a translocação t(6;9) era acompanhada de delecção 6q23-ter. Não podemos esclarecer com segurança se esta situação é esporádica ou, pelo contrário, é uma anomalia recorrente nestes tumores. Parece-nos indispensável para se poder interpretar o seu verdadeiro significado conseguir ampliar a casuística. Neste tipo histológico de carcinoma, as anomalias em 6q aparecem sob duas formas: delecção e translocação. Os mecanismos moleculares subjacentes a estes dois tipos de alterações são geralmente diferentes: as delecções envolvem perda de genes supressores de tumor, enquanto que as translocações sem perda de material cromossómico relacionam-se, preferencialmente, com a activação de oncogenes. No caso específico do

carcinoma adenoide-cístico das glândulas salivares, os mecanismos moleculares associados a estas anomalias permanecem por identificar.

Há uma outra região cromossómica afectada de forma recorrente nestes tumores - a região 12q12-13 -, e tal ocorre por delecção (2 casos) e por translocação com o braço curto do cromossoma 6, t(6,12)(6p21;q13) (1 caso) (capítulo 3.5).

Estudos recentes por *hibridização genómica comparada* (CGH), realizados numa série de 24 carcinomas adenoide-císticos, demonstraram haver perdas em 6q23-qter e uma região mínima comum de delecção em 12q12-13 (El-Rifai et al, 2001). Estes factos confirmam o envolvimento destas regiões, e sugerem ainda que um ou mais genes aí localizados possam desempenhar um papel determinante na origem dos carcinomas adenoide-cístico.

Sob a designação geral de adenocarcinomas salivares com participação mioepitelial incluem-se as seguintes entidades: o carcinoma adenoide-cístico, o carcinoma epitelial-mioepitelial, o adenocarcinoma de células basais e o adenocarcinoma polimórfico de baixo grau (Seifert et al, 1990). Embora constituindo entidades histológicas distintas, este grupo de tumores partilha semelhanças fenotípicas, tendo sido sugerido que têm uma histogénese comum (Batsakis et al, 1989).

Os estudos citogenéticos que efectuámos em adenocarcinomas polimórficos de baixo grau identificaram a ocorrência da translocação t(6;12) que também foi detectada num caso de carcinoma adenoide-cístico. Em um outro caso havia alterações em 12q12-13 o que confere suporte genotípico à hipótese de haver uma histogénese comum para estas neoplasias (capítulo 3.5).

No que respeita ao carcinoma epitelial-mioepitelial e ao adenocarcinoma de células basais a informação cariotípica é muito escassa. No primeiro tipo histológico a informação disponível deriva do estudo de três casos (El-Naggar et al, 1998; capítulo 3.2) e, no segundo tipo, de apenas um caso (capítulo 3.2). Em nenhum deles foram identificadas anomalias em 12q12-13.

A raridade destes tipos histológicos de neoplasia dificulta a confirmação da hipótese que propõe ser plausível haver anomalias cariotípicas partilhadas em ambas as entidades.

Pode sumarizar-se a informação obtida no decurso deste trabalho e a que se encontra publicada sobre os carcinomas salivares do modo seguinte: as alterações numéricas recorrentes são representadas por perda do cromossoma Y e trissomia

dos cromossomas 7 e 8, enquanto que a região cromossómica 6q23-25 é a alteração estrutural mais frequente, em geral por um mecanismo de delecção.

A trissomia do cromossoma 8 é uma anomalia partilhada por neoplasias salivares benignas e malignas. Foi encontrado ganho do cromossoma 8 em vários tipos de tumores, especialmente em neoplasias hematológicas, mas também, e com elevada incidência, em carcinomas (Mertens et al, 1995). De uma maneira geral, a monossomia cromossómica é interpretada como identificando perda de genes oncosupressores, enquanto que o mecanismo patogénico associado a uma trissomia terá hipoteticamente como consequência uma alteração quantitativa na dosagem de produtos oncogénicos. O mecanismo molecular que se encontra relacionado com a trissomia 8, que é partilhado por tumores benignos e malignos, e o seu significado patogénico são questões ainda sem resposta.

A trissomia 7 é um achado citogenético comum em várias neoplasias sólidas, entre outras, do pulmão (Lee et al, 1991) do rim (Elfving et al, 1990) e da tiroideia (Roque et al, 1998). Contudo, também foi descrita a sua presença em tecidos não neoplásicos (Cin et al, 1992; Johansson et al, 1993). Inicialmente, a explicação para este facto atribuía o significado do ganho do cromossoma 7 a uma anomalia adquirida durante a cultura *in vitro*, todavia estudos posteriores, por exemplo, os efectuados em tecido tiroideu e em que foram utilizadas técnicas de hibridização *in situ* em núcleos interfásicos, confirmaram que havia células neoplásicas trissómicas para o cromossoma 7 (Roque et al, 1999). O significado da trissomia 7 nas neoplasias humanas não se encontra, por isto, ainda esclarecido.

Têm sido descritas delecções intersticiais e terminais do braço longo do cromossoma 6 em neoplasias humanas de histogénese diversa, designadamente melanomas (Trent et al, 1989), linfomas não-Hodgkin (Gaidano et al, 1992), carcinoma da mama (Devilee et al, 1991) e carcinomas do ovário (Saito et al, 1992), o que sugere que essas anomalias desempenham um papel crucial mas não específico no desenvolvimento neoplásico.

A delecção de material cromossómico 6q21-25 que tem sido observada em todos os tipos histológicos de carcinomas salivares analisados, com excepção do carcinoma ex-adenoma pleomórfico, tornam-na também fortemente implicada na carcinogénese salivar. A ocorrência da mesma alteração em subtipos histológicos diferentes significa, provavelmente, que o mecanismo patogénico implicado na transformação neoplásica seja basicamente o mesmo (Sandros et al, 1990).

Contudo, a inespecificidade da del(6q) nos carcinomas salivares, ou seja, o facto desta anomalia não estar associada a nenhum subtipo histológico particular, levou Sandros et al (1988) a considerar que não se trataria de uma anomalia

primária implicada directamente nos processos de iniciação tumoral, mas de uma anomalia secundária ligada à progressão neoplásica. A detecção de anomalias específicas claramente associadas a um subtipo histológico, como acontece nos carcinomas mucoepidermóide e adenoide-cístico, as duas formas de carcinoma salivar cariotipicamente melhor caracterizadas, parece fortalecer essa hipótese.

Conclusão 2

Os estudos citogenéticos, confirmam a ocorrência de um padrão de alterações que se caracteriza por rearranjos em 6q em todos os tipos histológicos de tumores malignos das glândulas salivares, excepto no carcinoma ex-adenoma pleomórfico. Neste último, tanto pela ausência de delecções ou translocações em 6q, como por rearranjos recorrentes envolvendo as regiões cromossómicas 8q12-13 e 12q14-15, que são partilhados com os adenomas pleomórficos, reforça-se a ideia de que à sequência histológica adenoma-carcinoma corresponde, paralelamente, um padrão característico de evolução cariotípica.

Os nossos resultados reforçam ainda a especificidade das translocações t(6;9) e t(11;19) no carcinoma adenoide-cístico e no carcinoma mucoepidermóide, o que confere a estas anomalias potencial valor de marcador tumoral, e sugere que nestes tumores há ocorrência de delecções de material genético associadas às translocações 6q. Aqueles resultados permitiram, ainda, associar, a região cromossómica 12q12-13 ao carcinoma adenoide-cístico e ao adenocarcinoma polimórfico de baixo grau, o que sugere haver genes relevantes para a carcinogénese salivar nestas regiões, e reforça a hipótese de haver uma histogénese comum para estas neoplasias.

4.1.3 O papel do gene PLAG1 nos tumores das glândulas salivares

Nos adenomas pleomórficos, a frequência e a especificidade dos rearranjos em 8q12 permitiram a identificação do gene *PLAG1* como o gene afectado por estes rearranjos. O envolvimento do gene *PLAG1* em tumores salivares malignos só foi demonstrado em carcinomas ex-adenoma pleomórfico (Åström et al, 1999). Foi, por isso, inicialmente sugerido que o gene *PLAG1* seria um "oncogene" benigno, potencialmente útil como biomarcador para o diagnóstico diferencial entre tumores salivares benignos e malignos (Kas et al, 1997), conclusão que veio, posteriormente, a ser contrariada por Queimado et al (1999).

Com o objectivo de avaliar o papel do gene *PLAG1* no processo tumorigénico salivar, analisámos a sua presença/ausência num grupo diverso de

tumores das glândulas salivares constituído por 23 casos (1 mioepiteloma, 16 adenomas pleomórficos, 4 carcinomas ex-adenoma pleomórfico, 1 carcinoma mucoepidermoide e 1 carcinoma adenoide-cístico), utilizando técnicas de hibridização *in situ* (capítulos 3.5 e 3.7). Os tumores tinham, em comum, rearranjos em 8q12 ou trissomia do cromossoma 8. Todos os adenomas pleomórficos, exceptuando um caso, todos os carcinomas ex-adenoma pleomórfico e o mioepitelioma revelaram rearranjos intragénicos do *PLAG1* ou aumento de cópias nos tumores com +8, ao contrário do que foi verificado nos restantes tipos histológicos, em que não foram observadas alterações do *PLAG1*. A frequência elevada de rearranjos do *PLAG1* encontrada, reforça o seu papel fundamental na tumorigénese dos adenomas pleomórficos e de neoplasias relacionadas, e a sua potencial utilidade como biomarcador para distinguir entre tumores salivares benignos e malignos, em casos em que histologicamente se coloque tal questão, fazendo disso excepção o carcinoma ex-adenoma pleomórfico.

Conclusão 3

A frequência elevada de rearranjos do PLAG1, principalmente em tumores benignos salivares atribui um papel fundamental ao gene PLAG1 na génese dos adenomas pleomórficos e de tumores relacionados, nomeadamente os mioepiteliomas e os carcinomas ex-adenoma pleomórfico. De igual modo reforça a sua potencial utilidade como biomarcador genético para estes tipos histológicos específicos, particularmente quando os tumores são caracterizados por rearranjos em 8q12.

4.2 Análise combinada fenótipo-genótipo no adenoma pleomórfico - modelo de abordagem da origem histogenética dos tumores salivares com participação mioepitelial

O adenoma pleomórfico caracteriza-se, histologicamente, pela sua composição bicelular, epitelial e mioepitelial, apresentando grande diversidade morfológica. Este facto levanta a questão da sua origem histogenética: terá origem num único tipo celular ou, pelo contrário, será de origem multicelular? Estudos de clonalidade efectuados em adenomas pleomórficos sugerem que a sua histogénese se relacione com uma célula pluripotente, designada por célula mioepitelial modificada (cf capítulo 1.2). Com o objectivo de elucidar esta questão, procedemos à identificação das células portadoras de anomalias citogenéticas específicas num

caso de adenoma pleomórfico e em outro de carcinoma ex-adenoma pleomórfico, ambos caracterizados por serem portadores de alterações em 8q12 e de terem rearranjos do *PLAG1* (capítulo 3.7).

As anomalias genéticas partilhadas por diferentes tipos celulares no mesmo tumor poderão ser indicativas de haver rearranjos genéticos ao nível de uma "stem cell", decisivos na iniciação do processo de transformação neoplásica, enquanto que, as alterações restritas a uma determinada linhagem sugerem que essa transformação poderia ocorrer em células mais diferenciadas, constituindo um evento temporalmente mais tardio, e relacionado com os mecanismos de diferenciação tumoral.

As técnicas convencionais de citogenética não permitem a identificação do fenótipo da célula cariotipada. Assim, ao cariotipar um tumor constituído por uma ou mais populações celulares, é impossível associar as anomalias cromossómicas detectadas aos diferentes tipos celulares nele presentes. A utilização de técnicas combinadas de análise do fenótipo (caracterização imunocitoquímica e morfológica) e do genótipo (caracterização citogenética e hibridação *in situ*) poderá permitir a identificação da célula portadora de determinada anomalia genética.

Nos dois casos estudados, a identificação de rearranjos 8q12/*PLAG1* tanto em células de linhagem epitelial como de filiação mioepitelial sugere a existência de uma "stem cell" com capacidade de diferenciação bidireccional, tal como foi proposto por Batsakis & El-Naggar (1999). Esta constatação permite ainda sugerir que o gene *PLAG1* desempenha um papel decisivo na iniciação do processo neoplásico dos adenomas pleomórficos e dos tumores relacionados, pelo menos no subgrupo caracterizado por rearranjos 8q12/*PLAG1*, ao demonstrar a sua localização em ambas as populações celulares.

Conclusão 4

A análise combinada genótipo-fenótipo evidenciou a localização concomitante das anomalias genéticas específicas em células de linhagem epitelial e mioepitelial. Esta observação conferiu suporte genotípico à hipótese de os tumores salivares com participação mioepitelial, nomeadamente os adenomas pleomórficos, terem origem numa célula "stem" com capacidade para se diferenciar de modo polimórfico, exprimindo fenótipos derivados dos componentes epitelial e mioepitelial. Adicionalmente, esta demonstração permitiu reforçar o conceito de que o PLAG1 desempenha um papel decisivo na iniciação do processo tumorigénico.

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5.

Resumo

Resumo 5

As células neoplásicas são, por definição, caracterizadas por alterações genéticas que podem ter expressão a nível cromossómico, pelo que a caracterização citogenética de tumores ao permitir reconhecer e distinguir as anomalias citogenéticas mais relevantes em cada tipo tumoral e posteriormente decifrar e avaliar os seus efeitos a nível celular tem contribuído de forma decisiva para a nossa compreensão da oncogénese humana.

O presente trabalho pretendeu elucidar as vias de desenvolvimento neoplásico e os mecanismos de diferenciação celular das neoplasias das glândulas salivares através da sua caracterização (cito)genética.

Como primeiro objectivo identificámos e caracterizámos o padrão de alterações cromossómicas das neoplasias salivares e avaliámos o envolvimento do gene *PLAG1* (pleomorphic adenoma gene 1). Através do estudo citogenético de uma série de tumores benignos que inclui adenomas pleomórficos, mioepiteliomas e tumores de Warthin verificámos que há um padrão de alterações citogenéticas característico em adenomas pleomórficos e mioepiteliomas com o envolvimento recorrente das regiões cromossómicas específicas 8q12 e 12q13-15, facto que aponta para a histogénese comum destas neoplasias. A correlação do padrão cariotípico com a composição histológica dos adenomas pleomórficos sugere que os rearranjos genéticos em 8q12 e 12q13-15 condicionam o subtipo de adenoma pleomórfico o que parece indiciar vias de desenvolvimento neoplásico independentes para os adenomas pleomórficos. No tumor de Warthin associam-se, de forma inequívoca, anomalias nas regiões cromossómicas 11q21, 19p13 e 6p21 confirmando esta lesão como uma neoplasia verdadeira e não uma lesão pseudotumoral hiperplásica.

A análise cariotípica de um grupo de carcinomas salivares carcinoma adenoide-cístico, carcinoma mucoepidermóide, carcinoma ex-adenoma pleomórfico, adenocarcinoma polimórfico de baixo grau, adenocarcinoma, carcinoma epitelial-mioepitelial, carcinoma mioepitelial, carcinoma de células acinares, adenocarcinoma de células basais, carcinoma ductal e carcinoma indiferenciado permitiu-nos igualmente confirmar que nos carcinomas salivares existe um padrão de alterações citogenéticas com rearranjos em 6q em todos os tipos analisados, excepto em carcinomas ex-adenoma pleomórfico. No caso destes últimos tumores tanto pela ausência de delecções ou translocações envolvendo 6q como pela partilha de rearranjos recorrentes com o adenoma pleomórfico envolvendo as regiões cromossómicas 8q12-13 e 12q13-15, reforça-se a ideia de

que à sequência histológica adenoma-carcinoma corresponde um padrão característico de evolução cariotípica. Os nossos resultados reforçam ainda a especificidade das translocações t(6;9) e t(11;19) presentes respectivamente em carcinomas adenoide-císticos e carcinomas mucoepidermóide, o que lhes confere potencial valor de marcador tumoral. Também sugerem a ocorrência nestes tumores de delecções de material genético associadas às translocações 6q. Permitiram, ainda, associar, de forma inequívoca, a região cromossómica 12q12-13 a dois tipos histológicos -o carcinoma adenoide-cístico e o adenocarcinoma polimórficos de baixo grau - sugerindo haver nestas regiões genes com potencial papel crucial na carcinogénese salivar, o que reforçaria a hipótese de uma histogénese comum para estas neoplasias.

Quanto ao papel do gene PLAG1, a frequência elevada de rearranjos verificada, principalmente em tumores benignos salivares, confere um papel fundamental ao gene PLAG1 na génese dos adenomas pleomórficos e tumores relacionados, o mioepitelioma e o carcinoma ex-adenoma pleomórfico e reforça a sua potencial utilidade como biomarcador genético para estes tumores, particularmente para os que são portadores de rearranjos em 8q12.

Como segundo objectivo quisemos contribuir para o esclarecimento da origem histogenética das neoplasias salivares com diferenciação bi-celular, nomeadamente usando como modelo o adenoma pleomórfico. Os resultados obtidos permitiram-nos concluir que a análise combinada genótipo-fenótipo, ao localizar as anomalias genéticas específicas em células das linhagens epitelial e mioepitelial confere suporte genotípico à hipótese de os tumores salivares com participação mioepitelial, nomeadamente os adenomas pleomórficos, terem origem numa célula "stem" única, que tenha capacidade genotípica de diferenciação fenotípica em ambos os sentidos, epitelial e mioepitelial, referenciando o PLAG1 como gene decisivo na iniciação do processo tumorigénico.

Summary

Acquired genetic alterations, of which many are visible on the chromosome level, are believed to be necessary for tumorigenesis and most tumors display characteristic patterns of recurrent structural and numerical aberrations.

This study aims to contribute to the understanding of the tumorigenic processes and differentiation mechanisms of salivary gland tumors through their (cyto)genetic characterization.

As a first goal, we identified and characterized the cytogenetic pattern of chromosome aberrations of a group of salivary gland neoplasm and evaluated the role of *PLAG1* gene (pleomorphic adenoma gene 1) in these tumors.

The cytogenetic studies performed in a series of benign tumors (pleomorphic adenoma, myoepithelioma and Warthin's tumor) allowed the identification of specific chromosome changes in pleomorphic adenomas and myoepitheliomas affecting the 8q12 and 12q13-15 chromosome regions, this fact suggesting a common histogenesis for these tumors. The correlation found between the karyotypic pattern and the morphologic subtypes of pleomorphic adenomas suggests that the 8q12 and 12q13-15 genetic rearrangements may imply divergent tumor phenotypes which substantiate the hypothesis that propose the existence of independent pathways for development and progression of these neoplasms. For Warthin's tumor the finding of clonal alterations, involving 11q21, 19p13 and 6p21 chromosome regions, supports that this lesion is a "true" neoplasm rather than an autoimmune or hypersensitivity-related tumor like proliferation, as previously proposed.

Similar to benign tumors the cytogenetic analysis of a group of malignant salivary gland neoplasm that includes adenoid cystic carcinoma, mucoepidermoid carcinoma, carcinoma ex-pleomorphic adenoma, polymorphous low-grade adenocarcinoma, myoepithelial carcinoma, epithelial-myoepithelial carcinoma, acinic cell carcinoma, adenocarcinoma, basal cell carcinoma, ductal carcinoma and undifferentiated carcinoma revealed a specific pattern of chromosome changes with rearrangements of long arm of chromosome 6 in all types excepted in carcinomas ex-pleomorphic adenoma. For the latter ones either by the absence of deletions or translocation affecting 6q or by the recurrent presence of 8q12 and 12q13-15 rearrangements shared with pleomorphic adenomas, it's reinforced the idea that to the histological sequence adenoma-carcinoma correspond a characteristic pattern of karyotypic evolution. Our results confirmed the specificity of the translocations t(6;9) and t(11;19) associated, respectively, to adenoid cystic

and mucoepidermoid carcinomas, therefore, validating their role as tumor markers. Furthermore, our findings allowed us to associate, undoubtedly, the 12q12-13 chromosome region to two histological types - adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma - suggesting the existence of genes in that particular region that might play a significant role in salivary gland carcinogenesis and support the hypothesis of a common histogenesis for these carcinomas.

Concerning the role of *PLAG1* gene, the significant high frequency of cases with gene rearrangement, particularly in benign tumors, attribute to this gene a crucial role in the genesis of pleomorphic adenomas and related tumors (myoepitheliomas and carcinomas ex-adenoma pleomorphic) and emphasize its potential utility as a genetic biomarker for these neoplasms.

The second objective of this study was to shed some light on the genesis of salivary gland tumors with bi-cellular differentiation, using as a model the pleomorphic adenoma. Our results allowed us to conclude that the combined genotype-phenotype analysis, trough the identification of the cell types (epithelial and myoepithelial) carrying specific genetic aberrations, further supports the hypothesis that pleomorphic adenomas share a single cell origin, probably from a pluripotent cell capable of differentiating into a variety of somatic phenotypes and consequently, attributes to PLAG1 a decisive role in the basic tumorigenic process of these tumors.