UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

JOÃO FIGUEIRA SCARINI

PERFIL DAS ALTERAÇÕES GENÔMICAS ENCONTRADAS NOS DIFERENTES ESTÁGIOS DE DESENVOLVIMENTO DO CARCINOMA EX-ADENOMA PLEOMÓRFICO: UMA ANÁLISE ABRANGENTE POR SEQUENCIAMENTO COMPLETO DO EXOMA E HIBRIDIZAÇÃO GENÔMICA COMPARATIVA BASEADA EM ARRAY

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

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Oral Medicine and Oral Pathology, in Pathology area.

Orientador: Profª Drª Fernanda Viviane Mariano Brum Corrêa

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO JOÃO FIGUEIRA SCARINI, E ORIENTADA PELA PROFA DRA FERNANDA VIVIANE MARIANO BRUM CORRÊA.

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UNIVERSIDADE ESTADUAL DE CAMPINAS
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A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 02 de fevereiro de 2024, considerou o candidato JOÃO FIGUEIRA SCARINI aprovado.

PROFª. DRª. FERNANDA VIVIANE MARIANO BRUM CORRÊA

PROF. DR. ROGÉRIO DE OLIVEIRA GONDAK

PROFª. DRª. ERIKA SAID ABU EGAL

PROF. DR. ROGÉRIO MORAES DE CASTILHO

PROF. DR. PABLO AGUSTIN VARGAS

A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

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Sou extremamente grato por tudo.

EPÍGRAFE

21 de setembro de 1995.
Madalena, Rio de Janeiro.
Que a força esteja com você!
Iniciar: no esquema escola, cinema, clube, televisão.
“Olhei para além: para o João, o do passado, o da rede – aquele visionário sonhador”.
Vestibular. Graduação. Evolução.
Último ano.
“Reconheci-me em meio aos jalecos espalhados por cabides pelos corredores da faculdade”.
“No dia em que decidiu partir não pensou em como seria voltar”.
Paradoxos.
“O vazio cheio de tudo do meu coração”.
Deus: “Garçom dos Céus, ei, Você mesmo. Venha, coloque algo neste copo! É uma ordem”. Testes. De caráter, paciência, bravura.
“Nestes raros atos de bravura, meu cerne regurgita a impulsividade que se manifesta. Tranquilidade nunca foi uma característica”.
Ideologia. Reflexão.
“Será uma nova vida, com um novo jeito de encarar as coisas e ver o mundo”.
Obstáculos, curvas, desvios.
“Como dizem por aí: nada é, tudo está”.
Uma ferida que dói e não se sente.
“Quente quando tem que ser quente, frio quando tem que ser frio”.
Equilíbrio? Não, excesso. 8 E 80.
Um contentamento descontente.
“Em círculos, em repetições constantes de uma instabilidade multissensorial”.
Interrupção.
Superação.
“Foi com um frio na barriga que tudo começou e é com um frio na barriga que tudo vai terminar”.
Aplausos. Abraços.
Bem-vindo a premiação.
“Lembrarei do sorriso banhado de lágrimas enquanto meus gestos desarticulados e empolgados tentavam segurar o celular em minhas mãos trêmulas”. Consegui!

Piracicaba, São Paulo.
Expressão. Mutação.
Uma Morada ao Sol.
“...sofri convosco a todo descongelamento, manuseio e dosagem”.
Um avião alcança a América Central.
Retornar. Deus é bom o tempo inteiro.
Campinas, São Paulo.
1301. 13, Transformação. 1, Movimentação.
Alma lavada até um vírus me trancafiar...
O alto da Torre. Cinderela?
Não, Rapunzel.
Célula mioepitelial, uma “vizinha boa”.
Aquela ligação inesperada.
Introspecção. Medo. Isolamento.
Uma voz veio e disse: *“All Too Well”*.
O jardim floriu.
O mundo renasceu.
E eu falhei.
“Confie em Mim! Eu tenho algo melhor para você”.
Culpa. Mudar a rota.
Repensar... Confiar...
Welcome. Go Blue!
Iniciar (again).
Tentar. Controlar. Querer ficar.
“Basta que saiba domar seu coração”.
O que é uma máscara para você?
Asas. Cortar? Não. Voar. Não parar.
Um filamento de actina e miosina me avisou que tudo podia mudar...
Uma frase. Depois de todo esse tempo, continua sendo Guimarães Rosa: “A vida é assim: esquenta e esfria, aperta e daí afrouxa.... O que ela quer da gente é coragem”.
Meta alcançada. Suor. Muito suor.
“You’re Losing Me”.
Dia 16 nunca mais será o mesmo.
Eu vou superar...
Fico com 2, número par. 8, par também.
28 anos. 2024.
Piracicaba. Manhã de 2 de fevereiro.
Orgulho. Continuar. Parabéns!
“— Você conseguiu, garoto”.
“Onde há um desejo, há um caminho”.
Albert Einstein.

João Figueira Scarini

RESUMO

O adenoma pleomórfico (AP) é propenso a alterações genéticas que levam à sua contraparte maligna, o carcinoma ex-adenoma pleomórfico (CXAP), um tumor raro e agressivo com comportamento e prognóstico distintos. Utilizando o sequenciamento completo do exoma (WES), examinamos o DNA de 7 pacientes com AP, 13 com CXAP e 5 com AP residual. Polimorfismos de nucleotídeo único (SNPs) foram identificadas em todas as amostras, filtradas com uma frequência alélica de doença na população (AF) ≥0,2, e esses resultados foram validados por imunofluorescência. Identificamos 823 genes com mutações não sinônimas, sendo 30,5% exclusivos para AP, 28,7% para AP residual e 18,3% para CXAP. Notavelmente, 12,6% desses genes apresentavam mutações persistentes do AP para o CXAP, enquanto 7,9% exibiam mutações ao longo da sequência adenoma-carcinoma. Os alvos-chave incluíram genes relacionados à matriz extracelular (MEC) (especialmente *TNXB*), genes supressores de tumor conhecidos (*PCDH9, LRMP, KDM5A, RB1, TP53*), e oncogenes cruciais (*USP4, MUC4, BRAF, GNAS*) em importantes vias do câncer, incluindo a mutação pontual do gene *MYB* nos três grupos analisados. A análise de imunofluorescência revelou expressão de c-Myb em todos os grupos, com redução ao longo da sequência adenoma-carcinoma, refletindo o impacto de alterações no número de cópias (CNAs) na perda deste gene ao longo da sequência adenoma-carcinoma. Esses achados foram corroborados pela análise de 27 casos de CXAP e 14 amostras de AP residual pareadas, utilizando hibridização genômica comparativa baseada em array (aCGH). Além disso, nossos resultados do aCGH, evidenciaram a persistência do ganho cromossômico de *PLAG1* tanto no AP residual quanto no CXAP. Amplificações de *HMGA2* e *RPSAP52* foram prevalentes, enquanto as amplificações de *GRB7* e *ERBB2* podem representar um passo inicial na transformação maligna de AP para CXAP. Os genes amplificados em áreas transformadas do tumor foram enriquecidos para processos biológicos relacionados à sinalização imunológica (como genes da família *INF-γ*). Em relação ao shift metabólico adquirido na transformação maligna do AP, observamos que *FASN* e *GLUT-1* apresentam alterações nos tumores malignos, conforme evidenciado por diferentes metodologias. Além disso, no desenvolvimento do CXAP, a célula mioepitelial parece desempenhar um papel de destaque em vários processos e interações, apesar de ter um papel ambíguo na literatura. Nossos achados do WES apontam mutações em

diferentes genes e vias que podem estar relacionados a célula mioepitelial, especialmente utilizando-se da MEC como palco de fundo para alterações no microambiente tumoral. Por fim, é crucial notar que as alterações genéticas encontradas em CXAP já estavam presentes na área benigna residual adjacente à área de transformação. Em resumo, este estudo lança luz sobre a intrincada coreografia de mutações pontuais e cromossômicas que ocorrem simultaneamente em oncogenes e genes supressores de tumor para orquestrar a transformação do AP em CXAP, com a célula mioepitelial sendo um possível alvo de mutações neste processo. Nossos resultados enfatizam também a presença precoce de alterações genéticas cruciais na área benigna residual, que, embora apresente características morfológicas de um AP convencional, exibe uma assinatura molecular pré-maligna.

Palavras-chave: Adenoma pleomorfo; Carcinoma ex-adenoma pleomórfico; Sequenciamento completo do exoma; Hibridização genômica comparativa; Perfil genético.

ABSTRACT

Pleomorphic adenoma (PA) is susceptible to genetic alterations that can result in its malignant counterpart, carcinoma ex-pleomorphic adenoma (CXPA), which is a rare and aggressive tumor with distinct behavior and prognosis. We used whole exome sequencing (WES) to analyze the DNA of 7 patients with PA, 13 with CXPA, and 5 with residual PA. All samples were analyzed for single nucleotide polymorphisms (SNPs) and filtered based on a population disease allele frequency (AF) ≥0.2. The results were validated using immunofluorescence. A total of 823 genes with non-synonymous mutations were identified, with 30.5% exclusive to PA, 28.7% to residual PA, and 18.3% to CXPA. Notably, 12.6% of these genes showed persistent mutations from PA to CXPA, while 7.9% showed mutations along the adenoma-carcinoma sequence. The study focused on various genes, including extracellular matrix (ECM)-related genes such as *TNXB*, tumor suppressor genes like *PCDH9, LRMP, KDM5A, RB1, TP53,* and key oncogenes such as *USP4, MUC4, BRAF, GNAS*. These genes are involved in important cancer pathways, including the *MYB* gene point mutation in all three groups analyzed. Immunofluorescence analysis showed c-Myb expression in all groups, with a decrease along the adenoma-carcinoma sequence, reflecting the impact of copy number alterations (CNAs) in the loss of this gene along the adenoma-carcinoma sequence. These findings were confirmed by analyzing 27 CXPA cases and 14 matched residual PA samples using array-based comparative genomic hybridization (aCGH). Additionally, our aCGH results demonstrate the persistence of *PLAG1* chromosomal gain in both residual PA and CXPA. Amplification of *HMGA2* and *RPSAP52* was prevalent, while the amplification of *GRB7* and *ERBB2* may represent an initial step in the malignant transformation from PA to CXPA. The genes that were amplified in the tumor-transformed areas were enriched for biological processes related to immune signaling, such as *INF-γ* family genes. In the context of the malignant transformation of PA, we observed alterations in *FASN* and *GLUT-1* in malignant tumors, as evidenced by different methodologies. Additionally, the myoepithelial cell appears to play a prominent role in CXPA development through various processes and interactions, although its role is unclear in the literature. Our WES analysis indicates that mutations in different genes and pathways may be linked to myoepithelial cells, especially in the context of changes in the tumor

microenvironment with reference to the ECM. It is important to note that the genetic alterations identified in CXPA were already present in the adjacent benign residual area adjacent to the transformation area. In summary, this study illuminates the complex interplay between point mutations and chromosomal alterations that occur concurrently in oncogenes and tumor suppressor genes to facilitate the transformation from PA to CXPA. Our results also highlight the early presence of crucial genetic alterations in the residual benign area, which, although displaying morphological characteristics of a conventional PA, exhibits a pre-malignant molecular signature.

Keywords: Pleomorphic adenoma; Carcinoma ex pleomorphic adenoma; Whole exome sequencing; Comparative genomic hybridization; Genetic profile.

Sumário

1 INTRODUÇÃO

O Projeto Genoma, lançado na década de 1990 como uma notável iniciativa científica, representou um marco significativo no avanço da genética e biologia molecular ao unir cientistas, pesquisadores e instituições ao redor do mundo. Com o objetivo principal de mapear e identificar todos os genes presentes no genoma humano, essa empreitada internacional resultou na decifração do código genético humano, o que permitiu a identificação de genes associados a predisposições genéticas para o desenvolvimento de tumores e outras doenças, marcando um avanço crucial na pesquisa biomédica (Collins and Fink, 1995; Lander et al., 2001). Esse progresso, especialmente no contexto do câncer, possibilitou não apenas a identificação de genes relacionados a predisposições genéticas, mas também o desenvolvimento de terapias direcionadas e estratégias de prevenção mais eficazes. No entanto, apesar dos incansáveis esforços de pesquisadores em todo o mundo para desvendar os verdadeiros mecanismos por trás do desenvolvimento de tumores das glândulas salivares (TGS) e avançar em estratégias de tratamento eficazes, nosso entendimento ainda é limitado, deixando muitas questões sem resposta.

Estudos indicam que aproximadamente 0,5% de todas as neoplasias que afetam o corpo humano e 3-6% de todas as que acometem os tumores de cabeça e pescoço, originam-se nas glândulas salivares (Ettl et al., 2012; Gontarz et al., 2021; Speight and Barrett, 2002). Entre as glândulas salivares maiores, as parótidas são as glândulas mais frequentemente afetadas, enquanto nas glândulas menores, a maioria dos casos ocorre no palato (Eveson and Cawson, 1985a). Não há diferenças significativas entre os sexos na ocorrência de TGS, com pico de incidência entre a quarta e a sétima década para tumores benignos e malignos, respectivamente (Alsanie et al., 2022). Tumores benignos são mais prevalentes, representando entre 75% e 85% de todos os casos (Zhan et al., 2016). Por outro lado, os carcinomas salivares, embora menos comuns, tendem a ser agressivos, apresentando alto risco de recorrência e metástases à distância (Alfieri et al., 2017). Em 2020, na última publicação da Agência Internacional de Pesquisa sobre o Câncer, foram registrados globalmente 53,583 novos casos de câncer de glândulas salivares, resultando em 22,778 óbitos relacionados à doença (Sung et al., 2021).

Na sua quinta edição mais recente, a Organização Mundial de Saúde (OMS) descreveu mais de 30 subtipos distintos de TGS (El-Naggar et al., 2023). Dentre esses subtipos, dois emergem como modelos intrigantes para o estudo dos mecanismos de transformação maligna. O primeiro, um tumor benigno denominado adenoma pleomorfo (AP), pode sofrer transformações genéticas e metabólicas, culminando em sua contraparte maligna, o carcinoma ex-adenoma pleomorfo (CXAP). Esse modelo apresenta semelhanças com a sequência adenoma-carcinoma encontrada no câncer colorretal (Scarini et al., 2021), e destaca a importância desses subtipos específicos como ferramentas valiosas para a investigação dos processos de carcinogênese e desenvolvimento de estratégias terapêuticas personalizadas em tumores raros.

1.2 Aspectos epidemiológicos, clínicos e microscópicos do AP e do CXAP

O AP, também conhecido como tumor misto, destaca-se como a lesão mais comum entre os TGS, representando aproximadamente 40-70% desses casos (Kalwaniya et al., 2023). De acordo com dados *do Armed Forces Institute of Pathology (AFIP)*, o AP constitui 66% de todos os tumores benignos nesta região, sendo responsável por 67% dos casos nas glândulas salivares maiores e 63% nas glândulas menores. Pode ocorrer em todas as faixas etárias, sendo mais prevalente entre a 5ª e 6ª década de vida, com uma média de 43 anos, e demonstra uma leve predileção pelo sexo feminino. A maioria destes tumores surge na glândula parótida, seguida pelas glândulas menores (especialmente as do palato) e glândula submandibular. Clinicamente, o AP se manifesta como nódulos discretos, geralmente assintomáticos e de crescimento lento. No entanto, quando não tratados, esses nódulos podem evoluir para grandes massas (Panoussopoulos et al., 2002).

Apesar de sua natureza benigna, aproximadamente 40% dos AP podem apresentar recorrência após o tratamento inicial, configurando-se como AP recorrentes (APR). A incidência de recorrência varia em função da técnica cirúrgica utilizada, sendo que procedimentos como enucleação com ruptura e/ou excisão incompleta durante a cirurgia podem contribuir para o reaparecimento da lesão, frequentemente manifestando-se com um crescimento multinodular. A gestão clínica dos APR representa um desafio significativo para os cirurgiões, não apenas devido à

alta taxa de recorrência, mas também devido ao aumento do risco de transformação maligna, uma ocorrência rara, mas relatada em 1,3-7,5% dos casos e que se eleva com o número de recorrências (Abu-Ghanem et al., 2016; Eveson and Cawson, 1985b; Henriksson et al., 1998; Kanatas et al., 2018; Silva et al., 2018; Singh et al., 2017; Witt et al., 2015).

CXAP, também conhecido como tumor maligno misto, é uma entidade rara, caracterizada por sua agressividade e uma patogênese ainda pouco compreendida. Sua prevalência representa aproximadamente 3% a 5% de todos os TGS (Hu et al., 2016) e constitui 11% de todas as neoplasias malignas nesta localização anatômica (Chooback et al., 2017). Geralmente, manifesta-se nas sexta e sétima décadas de vida, frequentemente afetando a glândula parótida, embora também possa acometer a glândula submandibular e glândulas salivares menores (Singh et al., 2017).

Por definição, CXAP deve surgir em associação com um componente benigno, conhecido como AP residual (Williams et al., 2017). A área transformada pode apresentar diversos fenótipos carcinomatosos, influenciando seu comportamento biológico. Entre esses fenótipos, o carcinoma do ducto salivar, o carcinoma mioepitelial e o adenocarcinoma sem outra especificação são mais frequentes (El-Naggar et al., 2023). No entanto, outros tipos histológicos podem ocorrer, ampliando a diversidade dessas neoplasias. Esses incluem carcinoma epitelial-mioepitelial, carcinoma epidermoide, carcinoma indiferenciado, carcinoma adenoide cístico, carcinoma de células claras, carcinoma mucoepidermoide, carcinoma de células acinares, carcinoma oncocítico, adenocarcinoma de células basais, carcinoma sarcomatoide e carcinoma secretório (de Lima-Souza et al., 2021; Mariano et al., 2013; Olsen and Lewis, 2001).

Os CXAP são ainda subclassificados com base na invasão, referenciando a cápsula do adenoma. Essas subcategorias incluem: (1) carcinoma não invasivo – intracapsular (contido pela cápsula); (2) minimamente invasivo (infiltração do tecido extracapsular a uma distância ≤ 1,5 mm); e (3) francamente invasivo (infiltração > 1,5 mm) (Hu et al., 2016). Embora essa classificação tenha sido estabelecida por edições anteriores ao ano de 2017, foi recentemente sugerido que uma margem de 4 mm a 6 mm, em vez de 1,5 mm, seja mais apropriada (El-Naggar et al., 2023; Seethala and Stenman, 2017). No entanto, uma revisão sistemática realizada por um grupo brasileiro demonstrou que 1,5 mm continua sendo um ponto de corte importante na

análise prognóstica de casos de CXAP (Morais et al., 2019). Independentemente da margem adotada, CXAP intracapsulares e minimamente invasivos geralmente exibem um comportamento biológico de baixo grau, semelhante ao adenoma pleomorfo (AP) com margens livres. Em contraste, o CXAP francamente invasivo apresenta um comportamento mais agressivo, podendo resultar em metástases, recidivas e óbito (Zbären et al., 2008).

1.3 Aspectos genéticos do AP e do CXAP

Nos últimos anos, a compreensão da patogênese da doença tem evoluído, sendo atribuída ao acúmulo de distúrbios genéticos em AP pré-existentes (Sedassari et al., 2017). No entanto, até o momento, não foi confirmada a existência de um gene alvo comum associado a todos os subtipos histopatológicos, nem foram elucidados os fatores decisivos para a transformação maligna em um subtipo específico (de Brito et al., 2016b). Grande proporção dos AP abriga translocações em 8q ou 12q envolvendo *PLAG1* (8q12) e *HMGA2* (12q13-15) (Bullerdiek et al., 1993; de Brito et al., 2016a; Mariano et al., 2020). Em relação ao CXAP, muitos deles apresentam alto grau de instabilidade genética, das quais destacam-se os rearranjos em *PLAG1* e *HMGA2* (Katabi et al., 2015a; Martins et al., 2005), mutações em *TP53* (Fowler et al., 2006) e ganhos em *MDM2, EGFR,* e *ERBB2* (Mariano et al., 2016b; Nishijima et al., 2015; Röijer et al., 2002).

Com a introdução da Hibridização Genômica Comparativa baseada em array (aCGH), nosso grupo de pesquisa investigou o perfil genético da carcinogênese do AP sem recorrência (Mariano et al., 2016a), o perfil genético da recorrência do AP e suas implicações na transformação maligna (Mariano et al., 2016b), a correlação do perfil genético entre AP recorrentes e aqueles sem recorrência (Mariano et al., 2020) e, mais recentemente, o papel de genes de miRNA na transformação maligna do AP (Kimura et al., 2024). No entanto, apesar dos achados inéditos fornecidos por esses estudos, ainda não tínhamos analisado as alterações genéticas no AP residual adjacente à transformação e aquelas exclusivas à área transformada de um CXAP.

A técnica aCGH é uma ferramenta poderosa que permite a identificação de alterações no número de cópias (CNAs) do DNA em todo o genoma. Estas CNAs podem ser "ganhos", "amplificações" e/ou "perdas" genômicas (Kirchhoff, 2001; Weiss

et al., 1999). Um ganho genômico ocorre quando uma região do DNA apresenta uma cópia extra de material genético em comparação com o genoma de referência. A amplificação, por outro lado, é um tipo específico de ganho em que uma região do DNA é aumentada em múltiplas cópias. Uma perda genômica ocorre quando uma região do DNA apresenta menos cópias do que o genoma de referência. Isso pode resultar da deleção de genes ou regiões genômicas inteiras.

Há seis anos, iniciamos uma pesquisa inovadora sobre a transformação maligna do adenoma pleomorfo (AP) após a aprovação do projeto *"Estudo das alterações genéticas e metabólicas do adenoma pleomorfo e carcinoma ex-adenoma pleomorfo por Exoma, Expressão Gênica e Imunoistoquímica"*. Este projeto, financiado pela Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) na modalidade Jovens Pesquisadores em Centros Emergentes (Processo 15/07304-0), tinha como objetivo investigar as mudanças no metabolismo tumoral destes tumores e comparar os achados genômicos com nossos dados prévios derivados do aCGH. Os resultados da expressão gênica e proteica desta pesquisa, parte da minha Dissertação de Mestrado, revelaram descobertas significativas, especialmente em relação à expressão de FASN e GLUT-1 durante o processo de carcinogênese do AP (Scarini et al., 2020). Esses achados foram os primeiros dados publicados na literatura sobre as alterações metabólicas que poderiam influenciar o desenvolvimento do CXAP. Validando esses dados, nosso grupo demonstrou que FASN era superexpressa em uma amostra independente de CXAP (Díaz et al., 2019) e em carcinomas de glândula salivar de alto grau, correlacionando-se com a agressividade tumoral (de Angelis et al., 2021). Ao estabelecer a expressão alterada da proteína nesses tumores, identificamos um possível alvo terapêutico, abrindo portas para o desenvolvimento de terapias direcionadas e mais eficazes.

No contexto do câncer, o Sequenciamento Completo do Exoma (WES) tornou-se uma ferramenta poderosa para identificar mutações somáticas em genes que desempenham papéis críticos na carcinogênese. Ao analisar as sequências de DNA das regiões exômicas, onde a maioria das variantes patogênicas estão localizadas, esta técnica permite a detecção de mutações pontuais, inserções, deleções e rearranjos genômicos que podem impulsionar o desenvolvimento e a progressão da doença (Bartha and Győrffy, 2019; Malhotra et al., 2014). Com isso, tornou-se possível a elucidação de interações entre oncogenes e genes supressores

de tumor, a identificação de biomarcadores genômicos para diagnóstico, prognóstico e previsão de resposta ao tratamento em diferentes tipos de câncer (Bartha and Győrffy, 2019; Malhotra et al., 2014).

Com esta Tese de Doutorado, buscamos concluir a última etapa do Projeto Jovem Pesquisador mencionado relacionado a técnica do WES e apresentar os resultados do estudo de aCGH em pacientes com AP e CXAP. No primeiro artigo, realizamos o WES de um grupo de AP e outro de CXAP, destacando as principais mutações não-sinônimas relacionadas ao DNA exômico desses tumores. Essas descobertas buscam complementar o conhecimento já adquirido pelo grupo ao longo dos anos, especialmente no que diz respeito aos resultados do aCGH, os quais são apresentados no segundo artigo. Ao final, realizamos uma análise abrangente das metodologias utilizadas, discutindo como todas essas abordagens se interligam e podem fornecer *insights* valiosos que, no futuro, têm o potencial de serem traduzidos em benefícios significativos para os pacientes.

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2 ARTIGOS

2.1 ARTIGO 1 — Whole-exome sequencing identifies somatic mutations associated with the malignant transformation of pleomorphic adenoma: A preliminary study

Submitted to the journal: Modern Pathology **(Anexo 1)**.

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Conflicts of interest/Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

2.1.1 Abstract

Pleomorphic adenoma (PA) has the potential to progress into a complex and aggressive tumor known as carcinoma ex-pleomorphic adenoma (CXPA). However, the mechanisms underlying the adenoma-carcinoma sequence are controversial and have received limited investigation. To analyze DNA from 13 CXPA, 7 PA, and 5 residual PA (rPA) cases, whole exome sequencing (WES) was utilized, revealing genetic alterations driving CXPA development. The study identified single nucleotide polymorphisms (SNPs) in all samples, which were filtered based on a population disease allele frequency (AF) of ≥0.2. Immunofluorescence was used for validation. A total of 10,008 genes with non-synonymous mutations were identified, of which 2735 (27.3%) were unique to CXPA, 2520 (25.2%) to PA, and 848 (8.5%) to rPA. During the adenoma-carcinoma transition, mutations in 1463 genes (16.4%) were observed in PA and persisted in carcinoma. In addition, 973 mutated genes were present throughout the adenoma-carcinoma sequence, accounting for nearly 10% of all genes. After filtering with AF≥0.2, a total of 823 genes with non-synonymous mutations were identified. Of these, 251 (30.5%) were unique to PA, 236 (28.7%) to rPA, and 151 (18.3%) to CXPA. Notably, 104 genes (12.6%) had mutations persisting from PA to carcinoma, and 65 genes (7.9%) exhibited mutations across the adenoma-carcinoma sequence. The study identified genes associated with the extracellular matrix, tumor suppressor genes, and oncogenes related to cancer pathways. Additionally, a novel MYB mutation was detected in the adenoma-carcinoma sequence. Immunofluorescence analysis revealed c-Myb expression in all groups, which decreased along the adenoma-carcinoma sequence. In summary, these findings highlight the intricate interplay between oncogenes and tumor suppressor genes that lead to the gradual transformation of PA into CXPA. It is important to note that, although the benign residual areas of CXPA display morphological features of a typical PA, they possess a pre-malignant molecular signature.

Keywords: Salivary gland tumors, Salivary gland carcinomas, Pleomorphic adenoma, Carcinoma ex pleomorphic adenoma, Whole-exome sequencing, Somatic mutation

2.1.2 Introduction

Similar to colon and breast cancer, a salivary gland adenoma has the potential to transform into a carcinoma. This carcinogenic process involves the progression of the most common benign tumor of the salivary gland, the pleomorphic adenoma (PA), to a complex and aggressive tumor known as carcinoma ex-pleomorphic adenoma (CXPA). The mechanisms involved in this sequence are controversial and poorly understood. Studies have identified genomic instability [1,2], metabolic changes [3], and alterations in the tumor microenvironment, primarily controlled by the myoepithelial cell [4], as the most critical molecular events for malignant transformation.

The adenoma-carcinoma sequence is characterized by a series of recurrent driver mutations in different genes that accumulate during adenoma formation and progression to carcinoma. Many CXPAs exhibit a high degree of genetic instability, with notable rearrangements in *PLAG1* and *HMGA2* [5,6], mutations in *TP53* [7], and gains in *MDM2, EGFR,* and *ERBB2* [8–10]. However, no WES studies have yet elucidated the specific molecular characteristics of these two entities or mapped the alterations involved throughout malignant transformation. Much remains unknown, and many answers may lie in sequences and regions that have not yet been explored due to the limitations of current techniques.

Carcinomas with a wide range of tumor microenvironmental heterogeneity are exhibited by CXPAs. This range can vary from carcinomas composed solely of luminal cells developing in a PA to those in which the myoepithelial cell undergoes transformation, either with or without the ductal epithelial cell [11,12]. Additionally, CXPA should have a residual benign area (rPA) by definition, which further contributes to the tumor's heterogeneity [13]. These areas, which are morphologically similar to conventional PA, may carry a pre-malignant molecular signature.

The study aims to enhance the genetic knowledge of PA and CXPA by performing whole exome sequencing (WES) on a group consisting of PA, CXPA, and rPA. The mutations acquired in PA and conserved in the benign (residual PA) and malignant portions of CXPA will be compared, and alterations specific to each will be identified. The genetic profile for each group is crucial in understanding CXPA, one of the most aggressive neoplasms of the salivary glands, with a 5-year survival rate of

approximately 63% [14]. Our results may provide insight into the development of targeted therapy, which are currently limited in the medical literature.

2.1.3 Material and Methods

Patients

Ethical approval for this study was obtained from the Research Ethics Committee of the State University of Campinas (protocol number: 2.601.457), AC Camargo Cancer Center (protocol number: 2.485.588), and the Piracicaba Dental School of the State University of Campinas (protocol number: 2.928.348). The study analyzed frozen sections of PA (n=7) and CXPA (n=13) samples. The slides of the collected fragments from the operating room morphologically represented each group. The samples collected as CXPA that showed only areas of PA were classified as a third group: residual PA (n=5) **(Fig. 1)**.

Clinical and pathological analysis

The study collected clinical and epidemiological data from the patients' medical records. For PA cases, the following data were collected: sex, age, skin color, location of the lesion, time of development, history of previous PA, clinical tumor size, type of surgery, and patient's condition at last medical record. In addition, for CXPA cases, data on staging and site of metastasis (if available), treatment, need for cervical drainage, presence of recurrence, and time of recurrence were collected. The histologic diagnosis of all cases was reviewed. Carcinomas were reclassified according to the extent of invasion beyond the PA capsule as (1) intracapsular carcinoma (contained by the capsule), (2) minimally invasive (infiltration of extracapsular tissue at a distance ≤1.5 mm), and frankly invasive (infiltration >1.5 mm). The transformed phenotype was evaluated according to the 2017 WHO histologic classification [15]. The study also evaluated the type of cell differentiation in CXPA's malignant areas, classifying them according to the proliferating cell type (luminal and/or myoepithelial). Additionally, the presence or absence of compromised margins, compromised lymph nodes (if removed), vascular, lymphatic and perineural invasion, and necrosis were observed.

DNA isolation and whole-exome sequencing

DNA was extracted from fresh tissue samples using DNeasy Blood & Tissue (Qiagen, USA) following the manufacturer's instructions for tissues weighing less than 20 mg. The concentration of DNA was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) at 260 nm, and the purity of the sample was assessed by the ratio of absorbance readings at 260 nm and 280 nm. The integrity of each sample was verified by performing 1% agarose gel electrophoresis. The DNA amount in each pool was balanced to equally represent each genome. The pools were quantified using the fluorometric method (Qubit®) and normalized to 5 ng/µl in 10 mM Tris-HCl, pH 8.5. Libraries were prepared using the Nextera® Rapid Capture Enrichment Kit (Illumina) and quantified by q-PCR using the KAPA Library Quantification Kit (KAPA Biosystems). The libraries were normalized to the same concentration in nM and combined to create an equimolar pool. The pool was then loaded into the cBot (Illumina) to cluster the libraries in the flowcell. Paired sequencing was performed on HiSeq1000 or HiScanSQ (Illumina) with read lengths of 100 bp, targeting 18x/pool coverage.

Data analysis

Quality and frequency filters were applied to ensure accuracy. The sequencing reads (FASTQ files) were aligned to the reference genome (GRCh38/hg19) using default parameters to generate a binary sequence alignment map (BAM) file. MuTect (version 1.1.6) was used for somatic nucleotide variation (SNV) calling. The control group data were obtained from the BIPMed-WES-db database, which provides information derived from WES experiments and includes 257 healthy individuals from the Brazilian population (bipmed.org). These samples were collected from patients followed at the University of Campinas (UNICAMP) hospital, representing various geographic regions across Brazil [16]. Mutations were filtered using the FilterMutectCalls software, which applies quality filters to ensure the highest probability of a mutation being true. Variants within genes (gene mutations) were then selected and annotated. The correlation analysis of the mutated genes in the groups was performed using Venn diagrams [17].

To determine the most likely biological effects of the mutated genes, we performed gene ontology (GO) analysis (p adjusted <0.05) on these WES data using

the STRING database [18]. The mutated genes in each group were classified into different functional categories according to GO term enrichment analysis for biological process (BP), molecular function (MF), and cellular component (CC). These categories, if significantly enriched, may help us to better understand the role of these mutations in the disease. To analyze the distribution profile of these genes in our samples, we used the tumor suppressor genes available in the *Tumor Suppressor Gene Database (https://bioinfo.uth.edu/TSGene/)* and the oncogenes available in the *Oncogene Database (https://ongene.bioinfo-minzhao.org/)*. To analyze the distribution profile of mutated genes common to the groups (intersections) and unique, we correlated the genes of our cohort with genes mutated in salivary gland tumor samples available in the *Catalog of Somatic Mutations in Cancer (COSMIC) (https://cancer.sanger.ac.uk/cosmic)*.

To refine the results and identify potentially more relevant genetic variants, we applied population allele frequency (AF) thresholds of ≥0.1, ≥0.2, and ≥0.5, given the presence of numerous mutated genes. We chose to use the AF≥0.2 for the analyses, as it includes both known cancer-related genes and genes not yet described. This means that only variants present in at least 20% of the gene copies or genomic region were considered.

Immunofluorescence and Imaging

Immunofluorescence was used to evaluate protein expression in formalin-fixed paraffin-embedded (FFPE) tissue samples from 10 SGN (surrounding normal tissue adjacent to a tumor), 18 PAs, 8 RPA, and 18 CXPA using a selected target. Antigen retrieval was performed with citric acid. Tissue samples were incubated with 1% BSA and 0.1% PBS, followed by exposure to primary antibody (recombinant Alexa Fluor® 488 anti-rabbit anti-c-Myb (phospho S11) antibody, 1:50). The secondary antibody (Alexa Fluor® 488 anti-rabbit, 1:50) was then added. Tissues were counterstained with Hoechst 33342 and mounted with an aqueous mounting medium. A comprehensive computational analysis of all cases was performed using Molecular Devices (Danaher Corporation, San Jose, California, USA) to generate images at 400× magnification. The images were divided into two channels: DAPI and FITC (for c-MYB). For analysis, the entire slide was scanned, and 10 fields of interest were selected. These images were saved in black and white and exported to individual folders.

The tissue sections were analyzed using QuPath software version 0.4.3 (https://qupath.github.io), following the method described by Bankhead et al. (2017) [19].

2.1.4 Results

2.1.4.1 Sample Profile

Detailed clinical characteristics of the cohorts for WES are shown in **Table 1**. The entire cohort consisted predominantly of samples from adult males in the fifth and sixth decades of life, although some peculiarities were found in each group. PA affected patients a decade earlier than its malignant counterpart and presented microscopically as a cellular bipopulation composed of epithelial and myoepithelial cells embedded in a myxochondroid stroma. Patients diagnosed with CXPA were generally in early clinical stages of disease progression, and microscopically, most carcinomas originated from the ductal epithelial cell and showed minimal invasion of the adenoma capsule. Only one case of CXPA originating from the myoepithelial cell was analyzed **(Supplementary file 1) (Figure 1)**.

2.1.4.2 Profile of somatic mutations defined by WES without allelic frequency filter

After the FilterMutectCalls analysis (**Table 2)**, variants were annotated and those within genes (gene mutations) were selected. Regarding the point gene mutations analyzed, the genes were mainly targeted with missense mutations (affecting protein formation), although silent (synonymous) mutations (with no change in the gene product) were frequently found. Missense mutations (which include stop-gain, stop-loss, start-gain, star-loss, and frameshift) were detected, and although they were mostly high-impact mutations, they were not prevalent in our cohort. A comprehensive analysis identified a total of 10,008 genes with non-synonymous mutations. Of these, 2735 (27.3%) were unique to CXPA, 2520 (25.2%) were unique to PA, and 848 (8.5%) were unique to rPA. In the adenoma-carcinoma transition, 1463 mutations (16.4%) were shared in PA and CXPA. Moreover, almost 10% of all mutated genes were shared along the entire adenoma-carcinoma sequence (973 of them) **(Fig. 2a)**.

To assess the uniformity of the genetic profile of the three groups, we performed a Principal Component Analysis (PCA), a multivariate technique that reduces the complexity of data sets and provides relevant information about the cohort's consistency. In this analysis, we identified some outliers and showed that the groups are internally highly homogeneous, especially among PAs. The rPA mutation profile appears to be moving towards the CXPA genetic profile **(Fig. 2b)**. Of the 1060 tumor suppressor genes available in the database, 48.5% (514 of them) were mutated in our cohort. Of these, 11.3% (120) were mutated in CXPA alone **(Fig. 2c)**. In the other hand, of the 789 oncogenes available in cancer databases, 43% (340 of them) were detected in our samples. Of these, 12.4% (98) were mutated in CXPA alone **(Fig. 2d)**.

Functional enrichment analysis of mutated genes

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of mutated genes

Pathway enrichment analyses of KEGG were performed (cut-off criterion: $p<0.05$) to help understand the signaling pathways involving the mutated genes in PA, rPA and CXPA. Among all the genes unique and shared in each analyzed group, only the genes shared among the three groups were enriched in KEGG. The results of the enrichment analysis showed only one enriched pathway: the extracellular-receptor matrix interaction pathway ($p< 0.0092$), whose 15 mutated genes were correlated with collagen (*COL4A2, COL6A3, COL6A5*), integrins (*ITGA10, ITGB4, ITGB7*), laminins (*LAMA1, LAMA4, LAMA5, LAMB3, LAMC1*) and other glycoproteins/proteoglycans (*HSPG2, RELN, TNR, TNXB*). Protein interactions between overlapping mutated genes were predicted using the STRING tool. A protein-protein interaction (PPI) network was constructed for subsets of mutated genes with a combined score >0.9 to determine the interaction of these genes. A total of 15 nodes and 20 edges were displayed in the PPI network with an enrichment P-value of PPI<1.0e-16 **(Fig. 2e) (Supplementary File 2 and 3)**.

GO analysis of the genes that are mutated exclusively in PA, rPA and CXPA

GO analysis of the mutated genes in PA and rPA were not significantly enriched in processes in the Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) categories. Similarly, mutated genes unique to CXPA were

not significantly enriched in BP and MF category processes. On the other hand, CC analysis showed that 2314 of these genes were enriched for the Cell subcategory (p = 0.0018).

GO analysis of mutated genes shared between PA and rPA

GO analysis on mutated genes common to PA and rPA was not significantly enriched for processes in the BP and CC category. However, MF analysis showed that 9 of these genes were enriched for the sub-category Sodium Channel Activity (*p* = 0.0053): *CACNA1H*, *GRIK2, GRIK3, GRIK4, HCN4, SCN4A, SCNN1D, TRPM4, and TRPM5.*

GO analysis of mutated genes shared between PA and CXPA

Regarding the genes common to PA and CXPA, the results of BP analysis showed that the mutated genes were significantly enriched mainly for microtubule-based process, microtubule-based movement, and regulation of cell morphogenesis **(Fig. 2f)**. MF analysis showed that they were significantly enriched for cytoskeletal protein binding, microtubule-based motor activities, and ATP binding **(Fig. 2f)**. CC analysis showed enrichment mainly in microtubule cytoskeleton, cell cytoskeleton and plasma membrane **(Fig. 2f) (Supplementary File 4)**.

GO analysis of mutated genes shared between residual PA and CXPA

GO analysis of mutated genes shared in residual PA and CXPA was not significantly enriched in processes in the BP, MF, and CC categories.

GO analysis of mutated genes shared among all groups

Regarding the mutated genes common to all analyzed groups, the results of BP analysis indicated that these genes were significantly enriched mainly for cell adhesion process (p=7.70E-08), microtubule-based process/movement (p=8.02E-08), and process related to cell motility **(Fig. 2g)**. We found mutations mainly in the cadherin (*CDH3, CDH13, CDH23, CDH24, CDHR2*), protocadherin (*PCDH7, PCDH12, PCDH15, PCDHA7, PCDHB2, PCDHB5, PCDHB10, PCDHB15, PCDHGA7, FAT1, FAT2, FAT4*), integrins (*ITGA10, ITGB4, ITGB7, ITGAX*), laminins (*LAMA1, LAMA4, LAMA5, LAMB3, LAMC1*), collagenases (*COL6A3, COL6A5, COL28A1*),

glycoproteins (*MUC4, MUC16*), and annexins (*ANXA1*). On the other hand, MF analysis showed that the mutated genes in these groups were significantly enriched for calcium ion binding (p=5.67E-08), ATP-dependent microtubule motor activities (p=3.34E-06), and microtubule motor activity (p=1.06E-05) **(Fig. 2g)**. Among the genes related to calcium ion binding, we also found mutations in the cadherin, integrin, and annexin families. Among the genes related to microtubules, we highlighted mutations in the dynamin (*DNAH*) and myosin (*MYO*) families. Finally, CC analysis revealed enrichment mainly in the primary cilium (p=7.01E-09), cytoskeleton (p=1.41E-08), and cell processes (p=2.78E-07) **(Fig. 2g) (Supplementary File 3)**.

Common alterations in human cancers

Furthermore, we correlated the mutated genes in our cohort with genes mutated in salivary gland tumor samples available in COSMIC and showed that despite the presence of common genes, the PA-CXPA transition still retains its molecular signature. In particular, when evaluating the 28 genes available in the database and related to PA and CXPA, five genes common to these tumors were exclusively identified in our PA samples (*CDH1, FGFR2, FGFR3, RET, SPEN*), three were shared between PA and CXPA (*ATM, NCoR-1, Rb1*), two were found only in residual PA (*BRAF* and *PIK3CA*), and only one was shared between residual PA and CXPA (*TP53*). On the other hand, four were unique to CXPA (*SMARCB1, FBXW7, ARID1A, Notch-1*) and five were shared between all groups (*BRIP1, FANCA, LRP1B, PALB2, TSC2*). Finally, other genes important for other cancers were not detected in our analysis (*BCORL1, CDKN2B, DDR1, DNMT3A, H-RaS, KDM6A, K-RaS, PTEN*).

2.1.4.3 Profile of somatic mutations defined by WES with allelic frequency filter

To further refine our results, we applied a population disease AF filter. We performed three filters with AF ≥0.1, ≥0.2, and ≥0.5 **(Supplementary File 5)**. We chose to highlight the results obtained with AF≥0.2. As a result, we identified 823 genes with non-synonymous mutations: 251 (30.5%) exclusively in PA, 236 (28.7%) in residual PA, and 151 (18.3%) in CXPA. Remarkably, 104 genes (12.6%) with mutations persisted from PA to carcinoma. Of these, 65 genes (7.9%) showed mutations along the adenoma-carcinoma sequence **(Fig. 3a)**. Of the 1060 tumor suppressor genes available in the database, 3.7% (39 of them) were present in our cohort. Of these, only

5 genes (*TP53, PTPRT, ARL11, STARD13, SEPTIN4*) were mutated in CXPA alone **(Fig. 3b)**. On the other hand, of the 789 oncogenes available in cancer databases, 3.2% (25 of them) were present in our samples. Of these, 12.4% (98) were mutated in CXPA alone. In the intersection of the three groups, we observed that three common target oncogenes for mutation are *CD24*, *MYB*, and *SATB1* **(Fig. 3c).** Mutated *MYB* was detected in 4 of 7 patients with PA (57.1%), 4 of 5 patients with rPA (80%), and 5 of 13 patients with CXPA (38.4%). A protein-protein interaction (PPI) network was constructed for subsets of the found tumor suppressor genes and oncogenes with a combined score >0.7 to determine the interaction of these genes. A total of 60 nodes and 16 edges were displayed in the PPI network with an enrichment P-value of PPI=0.00291 **(Fig. 3d)**

GO analysis of mutated tumor suppressor genes and oncogenes among all groups

The results of BP analysis showed that these genes were significantly enriched mainly for regulation of gene expression (p=9.6E-08), regulation of macromolecule metabolic process (p=6.95E-06), and negative regulation of biological process (p=6.95E-06) **(Fig. 3e)**. On the other hand, MF analysis showed that the mutated genes were significantly enriched for macromolecule complex binding (p=0.000336), transcriptional cofactor activity (p=0.000336), chromatin binding (p=0.000336), and protein binding (p=0.0014) **(Fig. 3e)**. Finally, CC analysis showed enrichment mainly in the neuron part (p=0.00427), neuron projection (p=0.00427), and nucleus (p=0.00427) **(Fig. 3e)**. KEGG pathway enrichment analyses were performed (cut-off criterion: p<0.05) to help understand the signaling pathways involved in these mutated genes. The results of the enrichment analysis showed that many important pathways were enriched, especially the MAPK pathway (*BRAF, MAP2K4, MAPK10, NF1, TP53*) (p=0.0242) **(Fig. 3e) (Supplementary File 6).**

The expression of c-MYB is lost throughout the adenoma-carcinoma sequence

Among all the targets, c-Myb was selected for analysis by immunofluorescence. Our results showed that neoplastic tissues did not express a percentage of expression ranging from 0.06% to 34.3% (6.27±7.8). The expression of c-Myb was significantly higher in PA (p<0.0001) and rPA (p<0.05) compared to SGN. Benign areas of CXPA expressed significantly more c-Myb than transformed areas

(p>0.05) **(Fig. 3f)**. Regarding PA samples with MYB mutation, 3 out of 4 showed expression of c-Myb with a percentage of expression ranging from 6% to 11% (7.9±7). One sample could not be evaluated by immunofluorescence. Regarding mutant rPA samples, all showed expression of c-Myb with a percentage of expression ranging from 1.7% to 17% (8.9±6.2). Regarding CXPA, 3 out of 5 samples showed expression of c-Myb with a percentage of expression ranging from 0.07% to 10.4% (3.6±5.9). Two samples could not be analyzed by immunofluorescence.

2.1.5 Discussion

It is estimated that 85% of disease-causing mutations occur within coding and functional regions of the genome. Therefore, sequencing the entire coding regions (exome) has the potential to reveal the underlying causes of many rare genetic diseases and cancers [20]. In this study, we conducted a comprehensive analysis of WES data from 7 patients with PA and the malignant component of 13 patients with CXPA. We examined the benign component (rPA) in 5 different CXPA patients. Our findings reveal a heterogeneous molecular signature throughout the transformation process. This is characterized by the absence of mutations in genes extensively studied in these tumors, such as *PLAG1* and *HMGA2*, and mutation in other important cancer targets, such as *TP53* and *MYB*. WES identified 572 genes with non-synonymous mutations, of which 190 (33.2%) were exclusively found in CXPA, 279 (48.8%) were exclusively found in residual PA, and 103 (18%) were shared by both groups, including mutations. These results suggest that 18% of the genetic alterations observed in CXPA were already present in the adjacent benign residual area. This finding indicates a premalignant molecular signature in the benign residual areas of PA, despite their morphological resemblance to conventional PA.

Our results indicate that 48.5% of tumor suppressor genes and 43% of oncogenes present in the database showed mutations in our samples of PA, rPA and CXPA when no AF filter was applied. To refine our analysis, we implemented a filter requiring an AF≥0.2. This filtering process allowed us to identify potential major mutation targets within this cohort. This highlighted the enrichment of our sample for genes associated with the MAPK pathway, including *BRAF*, *MAP2K4*, and *MAPK10*. Given the current importance of these genes in anticancer drug development [21], our data may provide novel targets for the treatment of these patients.

Our study represents the first characterization of WES of CXPA, following robust studies that have examined the molecular profiles of other major salivary gland tumors such as salivary duct carcinoma (SDC), mucoepidermoid carcinoma (MEC), and adenoid cystic carcinoma (AdCC). Kang and colleagues (2017) characterized the exome of salivary gland MEC and identified recurrently altered genes such as TP53, *IRAK1, MAP3K9, ITGAL, ERBB4, OTOGL, KMT2C, OBSCN, MAP3K9, OTOGL, ARID1A, CBL, ABL1, AR, EPHA5, FH, INSR, PRKDC, RET, HRAS, PBRM1, SMAD2, SMAD3, FBXW7,* and *HNF1A* [22]. In contrast, a comprehensive characterization of the AdCC exome by Stephens and colleagues (2013) revealed no mutations in TP53 and RB1. In our study, similar to SDC and in contrast to AdCC, we observed mutations in *TP53, PIK3CA* and *RB1*. Notably, similar to AdCC, we did not find mutations in *PTEN*, *K-Ras* or *H-Ras* [23]. However, it's worth mentioning that H-Ras has been identified as a critical common target in the WES of SDC [24] and MEC.

In cancer, uncontrolled cell proliferation can cause abnormalities in cell morphogenesis and genomic instability during cell division. These abnormalities often involve the cytoskeleton (actin) and microtubules (tubulin), which play a central role in tumor progression. Both structures are essential for chromosome segregation and cell division in the cell cycle [25]. Recent studies have highlighted the involvement of tubulin in cancer development, including metastasis, chemotherapy resistance, and cell signaling [26]. The tubulin family of proteins is a well-known target of tubulin-binding chemotherapies that disrupt mitotic spindle dynamics, resulting in mitotic arrest and cell death [27]. The results indicate that genes associated with the cytoskeleton and microtubules are important targets for somatic mutations, even in benign and preneoplastic tissues. Further research is needed to explore the role of these molecules in disease, particularly in malignant tissues, to better understand their contributions to pathology.

The transformation of a normal cell into a malignant cell one involves substantial alterations in calcium pumps, Na/Ca exchangers, and calcium channels. These changes result in heightened cell growth and decreased cell death [28]. In recent studies, calcium-permeable ion channels have been identified as crucial regulators of autophagy, and their impact is likely dependent on calcium signaling [29]. The myoepithelial cell is a central player in the malignant transformation of pleomorphic adenoma (PA). It is now recognized as a potential orchestrator of the entire

carcinogenic process, although the mechanisms behind its role remain poorly understood [4]. Autophagy leads to the disappearance of myoepithelial cells and the inhibition of their suppressive role in carcinogenesis during the malignant transformation of PA [30]. In this context, it is hypothesized that mutations in genes related to calcium signaling may contribute to this critical event in tumor progression. The BP profile results suggest a somatic mutation profile that mainly affects cell motility, while the MF profile suggests that these mutations may confer proliferative advantages to cells and help them escape cell death. The significance of calcium ions in the pathogenesis of salivary gland tumors is not well understood, and further investigation is necessary to clarify their role in this context.

Our recent narrative review exploring the dual role of myoepithelial cells during the transition from PA to CXPA highlighted their function as both suppressors and promoters of tumors [4]. PAs are characterized by a prominent extracellular matrix (ECM) that regulates tumor growth and progression [31]. In a neoplastic state, myoepithelial cells may have the capacity to increase ECM production [32]. In addition, they may maintain or even increase the synthesis of basement membrane molecules, contributing to their spectrum of tumor suppressor activity [33]. Based on these findings and our results, we propose that the enrichment of mutations in the extracellular-receptor matrix interaction pathway during the PA-CXPA transition, involving cadherins, protocadherins, integrins, laminins, glycoproteins, and annexins, suggests that mutated molecules in this pathway may drive carcinogenesis, disrupt adhesion processes, and consequently influence cell motility. Furthermore, the data indicate a possible correlation between these mutations and myoepithelial cells. This is supported by the variation in tenaxin (TN) expression observed between CXPA with only epithelial components and those with myoepithelial components in a previous study conducted by our group [34]. This variation may be attributed to the role of TN in the uncertain nature of myoepithelial cells during the malignant transformation of PA.

Upon careful analysis of our sample, we observed the significant presence of the *MYB* gene mutation in the three groups studied. Validation in FFPE tissue from paired and independent samples showed that c-MYB expression increases as normal tissue transitions to neoplastic but decreases as the phenotype becomes malignant. A similar pattern was observed for point mutations identified by WES, where the mutation was more common in patients with benign neoplasms and in benign residual areas of

CXPA than in transformed areas. Although we expected a more pronounced activation of this oncogene in malignant tissues compared to benign tissues, it is known that genetic alterations in cancer can be highly variable. This is evidenced by the knowledge that benign tumors have genomic aberrations necessary to maintain neoplasia, but these changes are not sufficient to result in malignant tissue [35]. Various regulatory controls, both pre- and post-transcriptional, can influence protein expression patterns. Copy number alterations (CNAs), in which large segments of DNA are translocated, amplified, or lost, may play a critical role in the presence of these genes and consequently in the associated mutations.

Although our findings are intriguing, it is important to acknowledge the limitations of a small sample size. CXPA is a rare tumor, and obtaining frozen tissue, particularly residual PA, is considered a challenge in studies involving next-generation sequencing. Additionally, CXPA exhibits significant heterogeneity in the tumor microenvironment due to the acquisition of various phenotypes, leading to distinct biological behaviors. The tumors analyzed in this study were primarily of luminal origin **(see Supplementary Material 1)**. To confirm these findings, a larger, more homogeneous sample would be required. However, our preliminary results, which represent the first elucidation of the genetic profile of these tumors in the scientific literature, provide a foundation for future studies that can validate these findings. Additionally, our findings may be useful in the study of other rare cancers that face similar challenges to those encountered in the CXPA study.

2.1.6 Conclusion

In summary, our findings suggest that while our efforts are only the first steps in understanding the complexities involved, we have made steady progress in unraveling the molecular changes that contribute to the pathogenesis of these tumors. This extends to unraveling both the similarities and differences in the sequence of events between PA and CXPA. The presence of common mutations among the three groups underscores the sequential nature of the adenoma-carcinoma transition, in which genetic alterations in tumor suppressor genes and oncogenes play a pivotal role in determining the phenotypic progression of the tumor. Mutations in known cancer-related genes, particularly those associated with the MAPK pathway (such as TP53,

BRAF, MAP2K4, and NF1), have been identified in these tumors. It is important to note that, although the benign residual areas of CXPA display morphological features of a typical PA, they possess a pre-malignant molecular signature. Significantly, the presence of validated point mutations in the *MYB* gene, as demonstrated by immunofluorescence, highlights the complexity of simultaneous small and large scale mutations that can alter the expression pattern of these molecules and disrupt their function.

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2.1.8 Tables

Table 1 – Clinical and epidemiological characteristics of 7 patients diagnosed with Pleomorphic Adenoma (PA), 13 diagnosed with Carcinoma Ex Adenoma Pleomorphic (CXPA) in which only the transformed area was evaluated, and 5 patients with CXPA in which the residual benign area (rPA) was analyzed

Clinical Characteristics	**PA** **N (%)**	**CXPA** **N (%)**	**rPA** **N (%)**
Sex			
Male	5 (7.4%)	8 (61.5%)	5 (100%)
Female	2 (28.6%)	5 (38.5%)	0
Age (years)			
Average	40.3±13,6 (21-56)	55±16.5 (22-84)	50.6±9.6 (41-66)
Anatomical location			
Major salivary gland	6 (85.7%)	9 (69.2%)	4 (80%)
Minor salivary gland	1 (14.3%)	2 (15.4%)	0
Lacrimal gland	0	2 (15.4%)	1 (20%)
Size (cm)			
2<4	7 (100%)	6 (46.2%)	4 (80%)
>4	0	2 (15.4%)	1 (20%)
Not informed	0	5	0
Evolution time (years)			
Average	2.6±2.8	4.3±6.5	7.2±8.2
Tumor history			
PA History	0	6 (46.2%)	1 (20%)
No PA history	7 (100%)	6 (46.2%)	4 (80%)
Not informed	0	1	0
Type of surgery			
Superficial parotidectomy	4 (57.1%)	4 (30.8%)	1 (20%)
Total parotidectomy	1 (14.3%)	6 (46.2%)	3 (60%)
Partial maxilectomy	1 (14.3%)	1 (7.7%)	0
Submandibulectomy	1 (14.3%)	1 (7.7%)	0
Orbital Exenteration	0	1 (7.7%)	1 (20%)
Patient Status			
Live with or without disease	7 (100%)	7 (53.8%)	5 (100%)
Death by disease	0	5 (38.5%)	0
Death from other causes	0	1 (7.7%)	0
Staging (T)			
T1-T2	0	0	4 (30.8%)
T3-T4	0	0	3 (23.1%)
Not Informed	0	0	6
Staging (N)			
N0	0	0	5 (38.5%)
N1	0	0	1 (7.7%)

N2	0	0	1 (7.7%)
Not informed	0	0	6 (46.2%)
Staging (M)			
M0	0	0	7 (53.8%)
M1	0	0	1 (7.7%)
Not informed	0	0	5 (38.5%)
Clinical stage			
I-II	0	0	5 (38.5%)
III-IV	0	0	4 (30.8%)
Not informed	0	0	4 (30.8%)
Radiotherapy			
Yes	0	0	9 (69.2%)
No	0	0	4 (30.8%)
Not Informed	0	0	0
Chemotherapy			
Yes	0	0	1 (7.7%)
No	0	0	12 (92.3%)
Not Informed	0	0	0
Neck dissection			
Yes	0	0	6 (46.2%)
No	0	0	6 (46.2%)
Not Informed	0	0	1

Table 2 – Variant calling after WES analysis.

	PA	**rPA**	**CXPA**
Mutect2/BIPMED	5.174.467	5.042.702	6.939.705
FilterMutectCalls	2.370.532	2.335.821	2.958.172

2.1.9 Figure legends

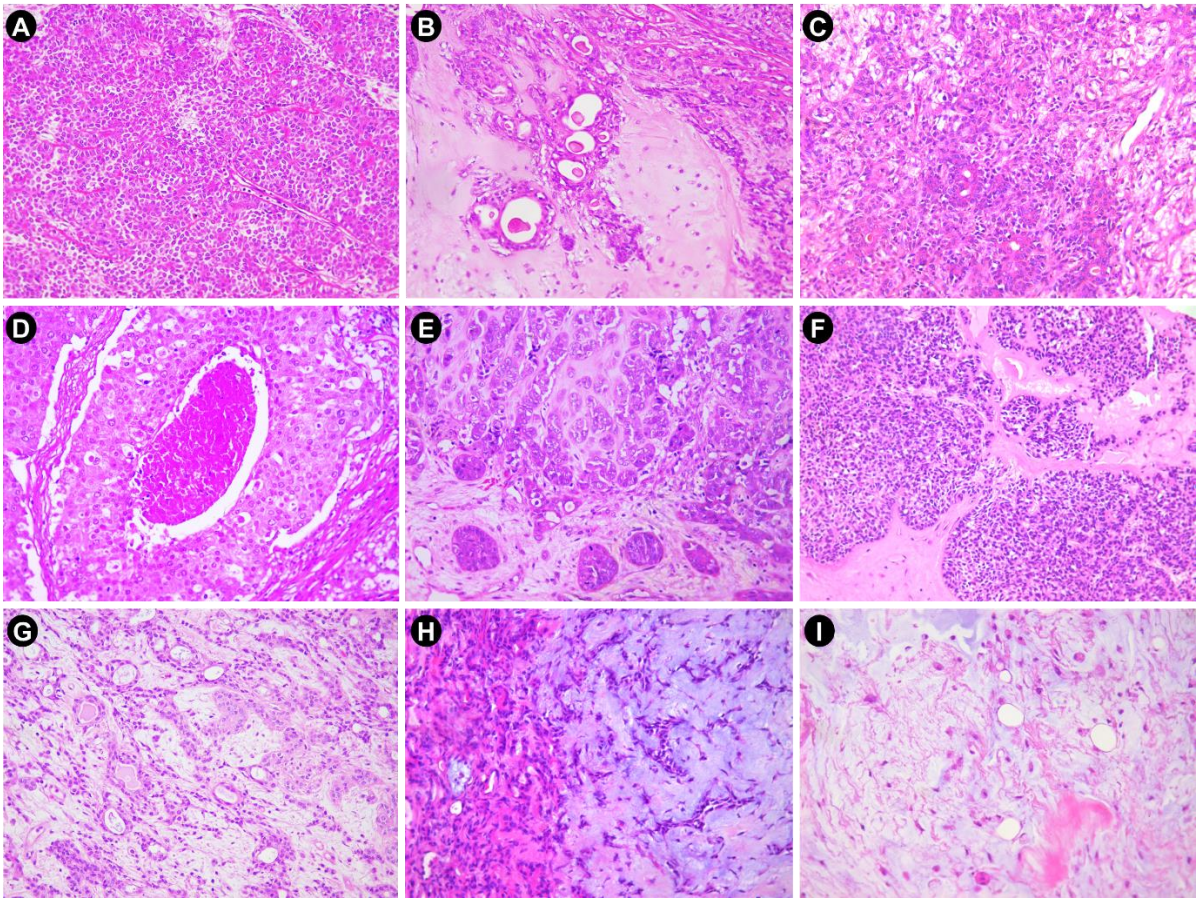

Figure 1. A, B, C (×20) – Main histopathologic features of the PA cases used in our study. Note the proliferation of epithelial and myoepithelial cells in a myxoid or mixochondroid stroma. In B and C, note the significant formation of ductile structures. **D, E, F (×20) – Main histopathologic features of the CXPA cases used in our study.** In D, salivary duct carcinoma ex-PA, showing intraductal carcinoma with cells with high cellular pleomorphism. E, Adenocarcinoma NOS ex-PA showing proliferation of pleomorphic cells reaching the pre-existing myxoid stroma of the PA. F, Proliferation of transformed myoepithelial cells into a myoepithelial carcinoma. **G, H, I (×20) – Primary histopathologic features of the rPA cases used in our study.** Proliferation of benign epithelial cells in a myxoid stroma. Note the significant formation of ductile structures in some cases and the absence of cellular pleomorphism.

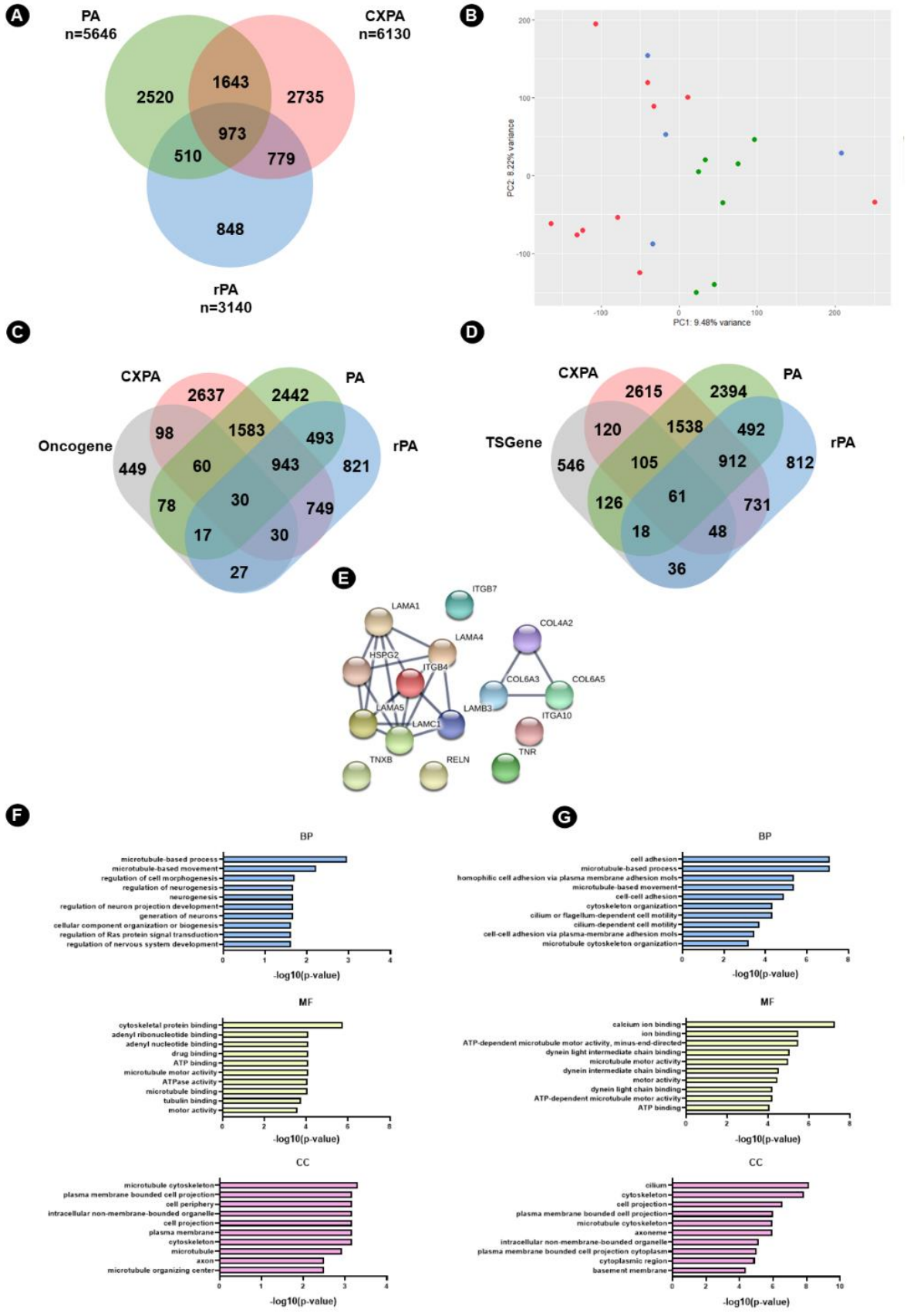

Figure 2. Profile of somatic mutations defined by WES without allelic frequency filtering.

(A) Venn diagram illustrating the overlap of mutated genes in the three groups, highlighting the presence of non-synonymous mutations, particularly prominent in CXPA**. (B)** Analysis of the uniformity of the genetic profile in the three groups, excluding four discrepant samples (three CXPA and one rPA). It is observed that the genetic profile of rPA follows the sequenced samples of CXPA, while PA remains consistent among themselves. **(C)** Correlation between genes found in our cohort and tumor suppressor genes, with only 449 genes from the database not identified in our cohort. **(D)** Correlation between genes found in our cohort and known cancer-related oncogenes available in the database, with 546 of these genes not identified in our cohort. **(E)** Based on the KEGG pathway results, we chose to construct a protein interaction network among mutated extracellular matrix-related genes using the STRING tool. **(F)** The top ten functional enrichment analyses of mutated genes shared between PA and CXPA. **(G)** The top ten functional enrichment analyses of mutated genes shared by the three groups. *PA: Pleomorphic adenoma; CXPA: Carcinoma ex pleomorphic adenoma; rPA: Residual pleomorphic adenoma; TSGene: tumor suppressor gene database; Oncogene: Oncogene database. BP: Biological process; MF: Molecular function; CC: Cellular component.*

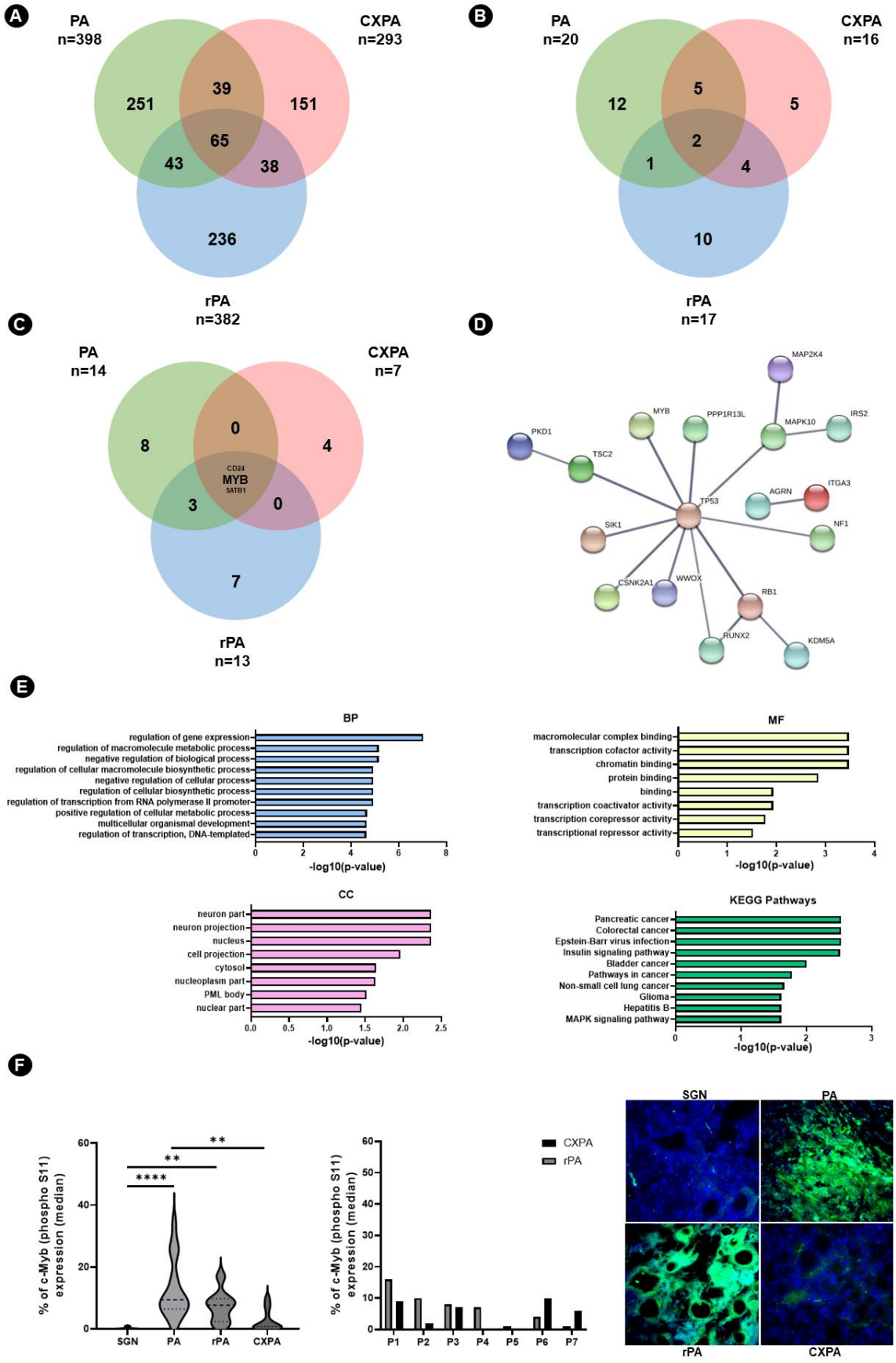
A
PA
n=398
CXPA
n=293
rPA
n=382
251
39
151
65
43
38
236
B
PA
n=20
CXPA
n=16
rPA
n=17
12
5
5
2
1
4
10
C
PA
n=14
CXPA
n=7
rPA
n=13
8
0
4
CD24
MYB
SATB1
3
0
7
D
MAP2K4
MYB
PPP1R13L
MAPK10
IRS2
PKD1
TSC2
ITGA3
AGRN
TP53
SIK1
NF1
CSNK2A1
WWOX
RB1
RUNX2
KDM5A
E
BP
regulation of gene expression
regulation of macromolecule metabolic process
negative regulation of biological process
regulation of cellular macromolecule biosynthetic process
negative regulation of cellular process
regulation of cellular biosynthetic process
regulation of transcription from RNA polymerase II promoter
positive regulation of cellular metabolic process
multicellular organismal development
regulation of transcription, DNA-templated
-log10(p-value)
MF
macromolecular complex binding
transcription cofactor activity
chromatin binding
protein binding
binding
transcription coactivator activity
transcription corepressor activity
transcriptional repressor activity
-log10(p-value)
CC
neuron part
neuron projection
nucleus
cell projection
cytosol
nucleoplasm part
PML body
nuclear part
-log10(p-value)
KEGG Pathways
Pancreatic cancer
Colorectal cancer
Epstein-Barr virus infection
Insulin signaling pathway
Bladder cancer
Pathways in cancer
Non-small cell lung cancer
Glioma
Hepatitis B
MAPK signaling pathway
-log10(p-value)
F
% of c-Myb (phospho S11) expression (median)
****
**
**
SGN
PA
rPA
CXPA
% of c-Myb (phospho S11) expression (median)
CXPA
rPA
P1
P2
P3
P4
P5
P6
P7
SGN
PA
rPA
CXPA

Figure 3. Profile of somatic mutations defined by WES with allelic frequency filtering.

(A) Venn diagram showing mutated genes in the three groups. A significant reduction in the number of mutated genes is observed, indicating a successful refinement of the results and highlighting significant mutations in our cohort. The mutation profile of CXPA may reflect the nature of our samples, where either the epithelial or myoepithelial cell transformed and gave rise to the tumor, emphasizing genetic variation originating from a single cell type, in contrast to PA, where both cells are neoplastic. **(B)** Correlation of genes found in our cohort with tumor suppressor genes. Of note, five are exclusive to CXPA: *TP53, PTPRT, ARL11, STARD13, SEPTIN4*. **(C)** Correlation of genes found in our cohort with known cancer-related oncogenes available in the database, highlighting genes found at the intersection of the groups: *MYB*, *CD24,* and *SATB1*. **(D)** Predicted protein interaction networks between tumor suppressor genes and mutated oncogenes using the STRING tool, highlighting a TP53-dependent network. **(E)** Top ten functional enrichment analyses of mutated genes shared by these groups. **(F)** Expression profile of c-Myb in paraffin-embedded tissues from the three analyzed groups. c-Myb expression was significantly higher in PA and rPA than in SGN. There was no statistical difference in c-Myb expression between SGN and CXPA. In addition, c-Myb expression was significantly higher in PA compared to CXPA. This was confirmed in an analysis of paired rPA and CXPA samples from 7 patients where P1, P2, P3, P4 and P5 showed lower c-Myb expression in the transformed tissue than in the benign residual areas of the lesion. This expression pattern is illustrated by representative immunofluorescence analysis images taken with Alexa Fluor 488 at 40× magnification. *****p<0.0001; **p=0.05; Kruskal-Wallis test with Dunn's multiple comparison. SGN: salivary gland normal; PA: Pleomorphic adenoma; CXPA: Carcinoma ex pleomorphic adenoma; TSGene: Tumor suppressor gene database; Oncogene: Oncogene database. BP: Biological process; MF: Molecular function; CC: Cellular component. P: Patient.*

2.1.10 Supplementary Materials

File 1 – Table showing the microscopic features of patients with CXPA.

https://docs.google.com/document/d/1RfvPWwYvlf9ZqoShAtAAB8MPonKYD361/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 2 – Distribution of the most important extracellular matrix genes found in this study between the patient group, without using the allelic frequency filter.

https://docs.google.com/document/d/1njM8paITqdPInoct5ui2Qh2PwjoTjYqp/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 3 – Enrichment analysis of mutant genes shared by all groups without allele frequency filtering.

https://docs.google.com/spreadsheets/d/1MS5WyG41PGpy1nygbNwEUpEdmZ3LCq7a/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 4 – Enrichment analysis of mutated genes shared between PA and CXPA without allele frequency filtering.

https://docs.google.com/spreadsheets/d/1NK7gzFfvmo4NegpCk2JDEr3rB769m0Nr/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 5 – Mutated genes found in each analysis subset according to the allele frequency filters used: ≥0.1, ≥0.2, and ≥0.5.

https://docs.google.com/spreadsheets/d/13Tn5Ksw26g9cMCxrcxG1C3nUkGa8JFgj/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 6 – Enrichment analysis of mutant genes shared by all groups with an allele frequency filter.

https://docs.google.com/spreadsheets/d/1oEcyJG5tx69MAQw0NasZY2JTC9QIotGW/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

2.2. ARTIGO 2 — Carcinoma ex pleomorphic adenoma: Distinct genetic features between transformed area and residual pleomorphic adenoma

Submitted to the journal: Scientific Reports **(Anexo 2)**.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions Statement

JFS and FVM wrote the manuscript. JFS and WLS analyzed all the data. JFF, RALS, ESAE, RG and ACV participated in sample preparation and experiments. AJT and LPK participated in the clinical selection of the sample. AA and FVM carried out the morphological analysis of the cases and designed the study. All authors reviewed the manuscript.

Data Availability

Data is provided within the manuscript or supplementary information files.

Acknowledgments

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2.2.1 Abstract

The mechanisms involved with the pathogenesis of carcinoma ex pleomorphic adenoma (CXPA) seem to be associated with the accumulation of molecular alterations in the pleomorphic adenoma (PA). In this sense, using array-based comparative genomic hybridization (aCGH) a rare series of 27 cases of CXPA and 14 residual PA (rPA) adjacent to the transformation area, we investigated the profile of the copy number alterations (CNAs) comparing benign residual and transformed areas. The main findings were correlated with the histopathological classification by histologic subtype and degree of invasion. The distribution of losses (p=0.187) and amplifications (p=0.172) was not statistically different between rPA and CXPA. The number of gains was increased in the transformed areas compared to the benign residual areas (*p*=0.005). *PLAG1* gain was maintained along the malignant transformation, as it was observed in both residual PA and CXPA samples, likely being an earlier event during transformation. The amplification of *GRB7* and *ERBB2* may also be an initial step in the malignant transformation of PA to CXPA (salivary duct carcinoma subtype). Furthermore, the amplification of *HMGA2* and *RPSAP52* were the most prevalent alterations among the studied samples. It was noteworthy that amplified genes in the transformed areas of the tumors were enriched for biological processes related to immune signaling. In conclusion, our results underscored for the first-time crucial CNAs in CXPA, some of them shared with the residual benign area adjacent to the transformation site. These CNAs included *PLAG1* gain, as well as amplification of *GRB7, ERBB2, HMGA2,* and *RPSAP52*.

Keywords: array-based comparative genomic hybridization; copy number alteration; gene; carcinoma ex pleomorphic adenoma; pleomorphic adenoma

2.2.2 Introduction

Carcinoma ex pleomorphic adenoma (CXPA), a tumor resulting from the malignant transformation of pleomorphic adenoma (PA), is a rare and aggressive tumor. Its pathogenesis has attracted considerable research interest over the past two decades [1–8]. By definition, CXPA must show histologic evidence of coexisting residual benign areas (residual PA – rPA) or pre-existing tumor (prior histologic diagnosis of PA in the patient's medical history). The diagnosis of CXPA is not self-sufficient and should include the carcinomatous phenotype developed in the transformed carcinoma and the extent of capsule invasion of the rPA [9].

Regarding the malignant area, carcinomas composed of luminal cells only are more likely to develop within a pleomorphic adenoma (PA), typically adenocarcinoma not otherwise specified (AdNOS) or salivary duct carcinoma (SDC). CXPA with a myoepithelial component has also been reported, notably myoepithelial carcinoma (MC) and epithelial-myoepithelial carcinoma (EMEC) [2,3]. Other less common subtypes include squamous cell carcinoma (SCC), sarcomatoid carcinoma (SC), mucoepidermoid carcinoma (MEC), and adenoid cystic carcinoma (AdCC) [10]. In terms of invasion, the most widely accepted classification defines intracapsular CXPA (iCXPA) as a tumor with neoplastic cells confined to the capsule, minimally invasive CXPA (mCXPA) as tumor extension up to 1.5 mm into extracapsular tissues, and frankly invasive CXPA (fCXPA) as a tumor with extracapsular invasion greater than 1.5 mm [11].

Taken together, the mechanisms involved in the pathogenesis of CXPA, regardless of histologic subtype or degree of invasion, appear to be related to the accumulation of molecular alterations in the PA. PAs are characterized by recurrent genetic alterations, particularly *PLAG1* and *HMGA2* translocations, which are widely recognized as genetic hallmarks of these tumors [12]. In CXPA, loss of heterozygosity in the 12q region and additional alterations in 17q [13], deletions of 5q23.2-q31.2, gains of *PLAG1* and *MYC*, and amplifications of *MDM2*, *ERBB2* [14], and *HMGA2* [15] are frequently reported CNAs.

With the advent of array-based comparative genomic hybridization (aCGH), our group has previously investigated the copy number alterations (CNAs) profile of PA carcinogenesis [16] and recurrent PA, with its impact on malignant transformation [17], and the correlation of the genetic profile of recurrent and nonrecurrent PA [18]. More

recently, the role of CNAs encompassing miRNAs in the CXPA development [19]. However, a comparative study of gains, losses, and amplifications in the benign residual and transformed areas of CXPA is lacking.

Therefore, in this study, we applied aCGH to analyze a rare series of 27 CXPA samples aiming to identify differences between their CNAs profile of benign residual and transformed areas. In addition, we correlated the main CAN findings with histopathologic classification according to histologic subtype and degree of invasion.

2.2.3 Material and Methods

Patients and tumor material

A total of 27 patients diagnosed with CXPA were included in this study. Formalin-fixed paraffin-embedded (FFPE) tissue sections of surgical specimens were used. The transformed area of all these cases and the benign residual area of 14 of them were analyzed. The benign residual areas of the other 13 cases of CXPA could not be analyzed by aCGH. Approval for all experimental protocols was obtained from the Institutional Ethics Committee of the Faculty of Medical Sciences of the University of Campinas (approval number: 2011/23366-5), ensuring compliance with the relevant guidelines and regulations throughout the study.

Pathological analysis

The histological diagnosis of all cases was reviewed. Carcinomas were reclassified according to the extent of invasion beyond the PA capsule as (1) iCXPA (contained by the capsule); (2) mCXPA (infiltration of extracapsular tissue at a distance ≤ 1.5 mm) and fCXPA (infiltration> 1.5 mm). The histological analysis was evaluated according to the 2017 WHO histologic classification [20].

*DNA extraction a*nd *genome-wide copy number analysis*

Tumor DNA was extracted from a 1.5-mm-diameter puncture of FFPE using the Qiagen Extraction Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations and our previous studies [17–19]. To improve DNA quality, the protocol included dewaxing with xylene, followed by methanol washes and incubation in 1 mol/L sodium thiocyanate for 24 hours. The tissue sediment was then

dried and digested in high-dose proteinase K lysis buffer for 1.5 days. The material was purified on the column and eluted in the buffer. Samples of the tumor DNA and a reference DNA (collected from different healthy donors (Promega, Madison, WI, USA) were labeled with the Enzo Genomic DNA Labeling Kit according to the manufacturer's instructions. Five hundred nanograms of test DNA and 500ng of reference DNA were co-hybridized with a 180k oligonucleotide matrix (SurePrint G3 Human CGH Microarray 4x 180K design 22060, Agilent Technologies, Palo Alto, CA, USA) according to Agilent procedures.

The genome-wide copy number analysis was realized according to Kimura et al. (2024) [19]. In summary, this work contained 24,011 exomic probes. Microarray images were acquired using the Agilent Microarray Scanner Bundle, and data were extracted using Feature Extraction (v9.1) (Agilent Technologies, Santa Clara, CA, USA). aCGH data were analyzed using Nexus Copy Number Discovery edition v7.0 software, according to Mariano et al. (2020) [18]. Genomic CNA was called based on the FASST2 segmentation algorithm (limit of significance defined in 5x10-8) with log2 limit ratios of 0.2 or 0.8 for high copy gains or losses, respectively, and -0.2 or -1.0 for homozygous losses or gains, respectively. A gain was considered when a region of DNA had one extra copy of genetic material compared to the reference genome. Amplification, on the other hand, was considered when a region of DNA was increased by multiple copies. A loss was considered when a region of DNA had fewer copies than the reference genome.

Data analysis

Correlation analysis of the amplified genes in the rPA and CXPA in different invasion phases and different histologic subtypes was performed using Venn diagrams [21]. In addition, the amplified genes identified in the study groups were subjected to enrichment analysis using the STRING database [22] to identify the major biological processes, molecular functions, and KEGG pathways associated with the amplified genes in CXPA and rPA. The Mann-Whitney U Test was used to statistically evaluate the distribution of loss, gain, and amplification between rPA and CXPA. The Kruskal-Wallis test was used to analyze the distribution of amplification across histological subtype and degree of invasion. The statistical software SPSS (version 22.0) was used, and the significance level was set at 5%.

2.2.4 Results

Clinical, microscopic and genomic profile of analyzed tumors

In this cohort, there was a higher predilection for males (13 out of 27 – 48.1%), with a mean age of 58.1 years (range 27-82, ± 13.8). The majority of cases involved the parotid gland (22 out of 27 – 81.5%), followed by the submandibular gland (2 out of 27 – 7.4%) and minor salivary glands (2 out of 27 – 7.4%). Sex information was not available for 4 patients (14.8%) and site information was not available for 1 patient (3.7%).

The microscopic profile of the samples analyzed in this study is summarized in **Table 1** and **Fig. 1**. Of the 27 CXPA cases analyzed, 13 (48.1%) carry losses in known cancer genes, whereas this was observed in 8 (57.1%) of the 14 rPA cases analyzed.

The transformed areas (220 losses – 60.4%) showed a tendency towards gene loss compared to the benign residual areas (144 losses – 39.6%) (*p*=0.187). Regargind gains, 15 (55.6%) CXPA carry losses in know cancer genes, whereas this was observed in 6 (42.9%) rPA cases. Gene gains were more frequent in the transformed areas (480 gains – 83.9%) than in the benign residual areas (92 gains – 26%) *(p=0.005)* **(Supplementary file 1-2) (Fig. 2a).** Of the 27 CXPA cases analyzed, 7 (25.9%) showed amplifications, whereas this was observed in only 3 (21.4%) of the 14 rPA cases analyzed. We identified 505 amplifications in the examined tissues **(Supplementary file 1)**. The transformed areas showed a tendency towards gene amplifications (334 amplifications – 66.1%) compared to the benign residual areas (171 amplifications – 33.9%) (*p*=0.172) **(Fig. 2b).** In the Venn diagram, repeated CNAs are not considered, so the lower total number of CNAs in the CXPA reflects more repetitions of CNAs in that group.

Gains and losses in rPA and CXPA

The majority of samples analyzed showed a profile of frequent gains and losses in known cancer-related genes **(Supplementary file 1)**. In the benign areas, losses were more frequent on chromosomes 17q (4 cases – 28.6%), 5q and 6q (3 cases each – 21.4%) **(Supplementary file 2)**. Among all identified losses, those affecting the genes *CD74*, *EBF1*, *ITK*, *NPM1*, *NSD1*, *PDGFRB*, *RANBP17*, *STL*, *TLX3*, and *TNFAIP3* were more frequently (3 losses of 144 each – 2.1%). Conversely, gains on 8p and 8q (3 cases each – 21.4%) were most frequently observed **(Supplementary**

file 2). The most frequent gains observed involved the genes *PLAG1* and *TCEA1* (4 gains of 92 each – 4.3%), *HOOK3 (*3 gains of 92 – 3.3%), and *CHCHD7 (*2 gains of 92 – 2.2%).

In contrast, losses on chromosomes 8p (5 cases – 18.5%), 2q (4 cases – 14.8%), and 5q (3 cases – 11.1%) were most frequently observed in the transformed areas **(Supplementary file 2)**. Notable losses included *WRN, PCM1* (5 losses of 220 each – 2.3%)*, BCL2, CD74, ECT2L, EZR, FGFR1OP, GOPC, MALT1, MLLT4, MYB, PDGFRB, PRDM1, ROS1, STL, TNFAIP3, and WHSC1L1* (3 losses of 220 each – 1.4%). On the other hand, gains on 6p, 8p (4 cases each – 14.8%), 3q, 8q, 17q, and 22q (3 cases each – 11.1%) were more frequent **(Supplementary file 2)**. Gene gains more frequently involving the genes *TCEA1* and *PLAG1* (7 gains of 480 each – 1.5%) although gains of *CHCHD7, MLF1* (6 gains of 480 each – 1.3%)*, BCL3, COX6C, HEY1, HOOK3,* and *NCOA2* (5 gains of 480 each – 1%) were also observed.

We grouped all cytobands with CNAs. Interestingly, we observed gains on chromosome 8p11.22q12.1 in two rPA cases (samples 1A7 and 3B1), involving the genes *PLAG1, HOOK3,* and *TCEA1*. Gains on chromosome 22q11.1-q13. 33 were observed in two cases: fCXPA (SDC) and fCXPA (EMEC), involving the genes *BCR, CHEK2, CLTCL1, EP300, EWSR1, MKL1, MN1, MYH9, NF2, PDGFB,* and *SMARCB1*. Loss on chromosome 8p23.3p11.1 was observed in an iCXPA (SDC) and in a fCXPA (SCC) with alterations in the genes *FGFR1, HOOK3, PCM1, WHSC1L1,* and *WRN*. Finally, a loss at 8p23.3-p11.23 was detected in an iCXPA (SDC) and in a fCXPA (SDC), involving the genes *PCM1, WHSC1L1,* and *WRN*.

Amplification in rPA

Among the 505 identified amplifications, 149 (29.5%) were exclusively detected in the benign residual area, whereas 22 (4.4%) were shared with the transformed area **(Fig. 2b)**. We observed amplification in rPAs adjacent to only three histologic subtypes of CXPA: SDC (132 of 171 – 77.2%), AdNOS (34 of 171 – 19.9%), and EMEC (5 of 171 – 2.9%). There was a statistically significant difference in the number of amplifications per histologic subtype (p=0.888) **(Supplementary file 3) (Fig. 2c)**. Interestingly, all amplifications identified were exclusive to each subgroup. We performed a search for recurrent amplifications among the analyzed rPA samples, but each amplification appeared only once in these tissues.

Amplification in CXPA

Among all the amplifications not recurrently detected in CXPA (unique events), 279 (55.2%) were exclusive to the transformed areas **(Fig. 2b)**. When analyzing these tumors based on their degree of invasion, there was a tendency for the number of amplified genes to increase as the degree of invasion and aggressiveness increased (*p*=0.792): 8 genes were associated with iCXPA (2.4%), 59 with mCXPA (17.7%), and 267 with fCXPA (79.9%). No common amplification was detected between iCXPA and the other two groups. Comparing mCXPA and fCXPA, 28 amplified genes were shared between these groups: *BEST3, CCT2, CNOT2, CPM, CPSF6, FRS2, HMGA2, KCNMB4, LOC100507195, LOC100507250, LOC101928002, LOC101928062, LRRC10, LYZ, MDM1, MDM2, MIR1279, MIR3913-1, MIR3913-2, MIR6074, NUP107, PTPRB, RAB3IP, RAP1B, RPSAP52, SLC35E3, SNORA70G,* and *YEATS4* **(Fig. 2d)**.

Regarding the histologic subtype of these tumors, only CXPA classified as MC subtype and CXPA classified as SC subtype did not present amplifications. CXPAs with the EMEC subtype had more genes amplified (164 – 49.1%) than the SCC subtype (97 – 29%), the SDC subtype (50 – 15%) and the AdNOS subtype (23 – 6.9%). However, there was a statistically significant difference in the number of amplifications per histologic subtype (*p*=0.134) **(Supplementary file 3) (Fig. 2e)**.

As for the rPA, we looked for reccurent amplifications among the analyzed CXPA samples **(Supplementary file 3)**. Amplifications of *HMGA2* (3 amplifications – 1%) and *RPSAP52* (3 amplifications – 1%) were more frequent. Another thirty-six amplifications were detected twice when we analyzed all malignant areas that showed alteration: *AGAP2-AS1*, *BVES-AS1, CCT2, CDK12*, *CDKN2B-AS1*, *CNTN5*, *COM, CPSF6, ERBB2*, *FGFR1*, *FRS2*, *GRB7, IFNG-AS1, JRKL-AS1, KCNMB4*, *LOC100129940, LOC100507195*, *LOC100507250*, *LOC100507420*, *LYZ*, *MDM1*, *MDM2*, *MEF2C-AS1*, *MIR1279, MIR3913-1, MIR3913-2, MIR6074*, *PTPRB, RAP1B, SLC35E3*, *SNORA70G*, *STARD13-AS*, *TMEM161B-AS1*, *TRHDE-AS1*, *WHSC1L1,* and *YEATS4*.

Correlation between amplifications of rPA and CXPA

When we compared rPA with CXPA at different stages of invasion, two amplifications were shared to rPA, mCXPA and fCXPA: *HMGA2* and *RPSAP52*.

Among all the amplifications, *GRB7* and *ERBB2* amplifications were observed in rPA and iCXPA. Interestingly, 18 genes were shared to rPA and fCXPA: *CETN3, GPR98, KL, LINC00423, LINC01339, LOC100129940, LOC102546226, LOC731157, LYSMD3, MBLAC2, MEF2C, MEF2C-AS1, MIR3660, MIR9-2, POLR3G, STARD13, TMEM161B,* and *TMEM161B-AS1* **(Fig. 2f)**.

In an attempt to observe whether the amplification of the malignant area in each histologic subtype was shared with the residual benign area, we compared the rPA alterations with CXPA separated by histologic subtype and degree of invasion. When comparing the amplifications of the benign residual area and malignant area of CXPA (SDC subtype), we observed that most of the amplifications of rPA were unique (130 – 71.4%). Six of them (3.3%) were exclusive to the iCXPA (SDC subtype): *CDC6*, *CDK12*, *HER2*, *IGFBP4, LASP1* and *MLLT6;* and two amplifications (1.1%) was shared by the rPA and the iCXPA (both SDC subtype): *GRB7* and *ERRB2* **(Fig. 3a)**. When we compared the genes amplified in the rPA adjacent to the CXPA (AdNOS subtype) with the amplified genes of CXPA (AdNOS subtype) cases, we observed that 20 amplifications (54.1%) were shared and three (8.1%) were unique to the transformed areas: *LIC00461*, *PDS5B*, and *STARD13-AS1* **(Fig. 3b)**.

There were no shared amplifications between benign residual areas and malignant areas of CXPA (EMEC subtype) **(Fig. 3c)**. Finally, since we could not evaluate the benign residual area of CXPA (SCC subtype), we decided to compare the amplifications of malignant areas of this tumor with the rPA adjacent to other histologic subtypes that showed alteration. This analysis showed that all amplifications of the malignant area of the CXPA (SCC subtype) were unique **(Fig. 3d)**.

Gene Ontology (GO) and pathway enrichment of amplified genes

To determine the most likely biological effects of the amplified genes, we performed gene ontology (GO) analysis for these aCGH data **(Supplementary file 4)**. GO analysis revealed that CXPA presented with gene enrichment involving different biological processes and molecular functions. Among the top 10 enriched biological processes, five were directly related to the tumor immune response, including the *IFNA* and *MEF2* family genes in the processes of NK cell activation (p=6.59E-16), lymphocyte proliferation (p=3E-11), and T cell activation (p=7.03E-11). Among the top 10 enriched molecular functions, the cytokine receptor binding (p=1.89E-07) and

cytokine activity (*p*= 4.94E-07) were identified as molecular functions related to the tumor immune microenvironment. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed. Among the top ten enriched pathways, the RIG-I-like receptor signaling pathway (*p*=6.53e-12), Jak-STAT signaling pathway (*p*=8.29e-11), natural killer cell mediated cytotoxicity (p=1.28e-08), Toll-like receptor signaling pathway (*p*=1.36e-08), and NOD-like receptor signaling pathway (*p*=1.36e-08) were highlighted. GO analysis of the genes amplified in rPA did not reveal any statistically significant biological processes or KEGG pathways. These genes were enriched solely in trace-amine receptor activity (*p*=1.34e-05) *(TAAR2, TAAR5, TAAR6, TAAR8, TAAR9),* according to the molecular function analysis.

2.2.5 Discussion

In this study, we used aCGH to evaluate residual benign and transformed areas in patients who developed CXPA in Brazilian individuals. Our findings represent the first study in the literature of multiple CNAs in CXPA, with some of these alterations also present in the adjacent benign tissue surrounding the transformation site. Crucially, our investigation highlights that the transition from PA to CXPA is characterized by a significant increase in the number of CNAs. This finding is supported by a previous and recent study from our group, which used aCGH to demonstrate an increase in the number of miRNA genes throughout the malignant transformation of PA [19].

Our primary results revealed several key findings: (1) The chromosomal gain of *PLAG1* persisted throughout the malignant transformation, being present in both rPA and CXPA samples. (2) Amplification of *HMGA2* and *RPSAP52* was particularly prevalent in both groups analyzed. (3) *GRB7 and ERBB2* amplification may represent an early event in the malignant progression from rPA to CXPA (SDC subtype). (4) Genes showing amplification in the transformed regions of the tumor were found to be enriched for biological processes associated with immune signaling.

A gain indicates a higher copy number of a genomic region compared to a reference sample, suggesting duplication or a few additional copies of that region. On the other hand, the term amplification indicates a particularly pronounced and often substantial increase in copy number, typically representing multiple extra copies a

given genomic segment. In our series, amplifications of *PLAG1* were not detected by aCGH; however, copy number gains of *PLAG1*, especially involving chromosomal region 8q, were observed in both residual benign areas and transformed areas. The pleomorphic adenoma gene 1 (*PLAG1*) produces a zinc finger protein with functions during embryogenesis and fetal development [23]. While specific alterations in *PLAG1* can be observed in lipoblastomas and other mixed skin and soft tissue tumors [24], the role of *PLAG1* in salivary gland tumors is largely limited to PA and the CXPA [25]. Previous studies support chromosomal gain of *PLAG1* in the 8q12 region in PA [26] and suggested that it may contribute to the malignant transformation of PA [14,23,27]. The involvement of rearrangements in this region in CXPA samples may further support the role of this gene in tumorigenesis [25]. Indeed, rearrangements in *PLAG1* have been identified as one of the most common genetic events in CXPA, regardless of histologic subtype [28].

In a recent study of our group, we showed that the expression of the PLAG1 protein, and presumably its role, was maintained in the majority of rPA cases and in some CXPA cases [4]. However, data indicated that the expression of PLAG1 was reduced after transformation. Although we observed loss of PLAG1 expression in malignant tissues, occasional discrepancies between PLAG1 protein expression and genetic status have been reported in the literature. Some investigators have suggested that a thorough investigation should be conducted to identify the underlying mechanism of this event in these tumors [12,29].

Rearrangements in 12q14.3 involving the high mobility group AT-hook 2 (*HMGA2*), also known as *HMGI-C*, have been documented during the transformation of PA to CXPA [12,15]. In fact, abnormalities in *HMGA2* and *PLAG1* have been shown to be useful in distinguishing CXPA from *de novo* counterparts [28,30], and the overexpression of these proteins may aid in the detection of CXPA, especially when a PA component is not evident [31]. *HMGA2* is recognized as a transcriptional co-regulator that is expressed at high levels during embryonic development, silenced in adult tissues, and re-expressed in several human cancers [32]. Previous observations have suggested that PAs with *HMGA2* amplification may carry an increased risk of malignant transformation [15]. Interestingly, in our study we show that although *HMGA2* amplifications were maintained in rPA, they were more prevalent in transformed areas.

In the correlation analyses between residual benign areas and transformed areas, we observed that, similar to *HMGA2*, amplification of the *RPSAP52* gene was prevalent in our CXPA samples. Surprisingly, *RPSAP52* is a transcribed RNA pseudogene that positively regulates *HMGA2* expression through the formation of an R-loop structure [33]. Under normal conditions, *RPSAP52* is also expressed in embryonic tissues and is silenced in most adult tissues [34]. In breast cancer and sarcoma, the *RPSAP52* pseudogene controls the *HMGA2/IGF2BP2/LIN28B* axis through positive transcriptional regulation of *HMGA2* and regulation of the function of the IGF2BP2 protein, which has pro-proliferative targets [34]. Thus, *RPSAP52* could be considered an oncogene whose dysregulation in CXPA could stimulate cell growth and maintain cells in a more undifferentiated state. This finding is unique in the literature, and to date there have been no reports of the presence of *RPSAP52*, especially in association with *HMGA2*, in rPA and CXPA. However, although it appears promising, methodologies that validate these findings should be applied in these tumors to understand the mechanisms that govern this process.

A comprehensive study analyzing 24 CXPA cases showed that of the 73 genomic alterations detected, 35.6% were amplifications, with *ERBB2* being particularly prevalent [35]. This finding is consistent with our study, which also demonstrated two amplifications of *ERBB2*: one in a case of iCXPA and another in a case of rPA. Amplifications in *CDK4*, *MDM2*, *ZNF703* and *FGFR1* were identified in both studies, further supporting their importance in CXPA pathogenesis.

Growth factor receptor-bound protein 7 (*GRB7*) is a multidomain adaptor protein co-opted by numerous tyrosine kinases involved in various cellular signaling pathways [36]. Its binding partner is the activated epidermal growth factor receptor (*EGFR*) [37]. The human *GRB7* gene is located on chromosome 17q12 and has been shown to be involved in the regulation of cell proliferation, migration and invasion in mammary, ovarian and esophageal tissues [38,39]. In our study, we showed that in CXPA (SDC subtype), *GRB7* and *ERBB2* genes are amplified in rPA and iCXPA. This finding suggests that these amplifications may be an initial step in the development of CXPA.

Furthermore, as a final highlight of this study, we observed that the amplified genes in CXPA were enriched for biological processes related to immune signaling. This discovery is particularly intriguing, because the amplified genes in rPA did not

show a significant process and function profile in GO analysis. The study of the immune microenvironment in CXPA is limited, and this finding underscores the need to focus our attention on this crucial aspect of the carcinogenic process [40].

Although the findings regarding *GRB7*, *ERBB2*, and *RPSAP52* amplification are significant, additional validations should be performed using different methods, such as fluorescence in situ hybridization (FISH) and/or immunohistochemistry (IHC), to further confirm and strengthen our results. This approach would allow a more comprehensive comparison and a deeper understanding of the results, especially when considering the analysis of genes such as *PLAG1* and *HMGA2*, which have been studied using these techniques. Therefore, considering that CXPA is a rare tumor arising from the accumulation of alterations in the pre-existing PA, we believe that our results highlight promising initial findings for further studies aimed at better understanding the malignant transformation of PA.

2.2.6 Conclusion

In conclusion, this study is the first to explore CNAs in the malignant transformation of CXPA. The study's results indicate that CXPA exhibited significant CNAs, some of which were also present in the adjacent residual benign area following transformation site. These CNAs included *PLAG1* gain, as well as amplification of *GRB7, ERBB2, HMGA2,* and *RPSAP52*. Furthermore, our study highlights a crucial aspect: the transformation process from PA to CXPA is associated with an increase in the number of CNAs. This underscores the stepwise development and progressive accumulation of alterations during the evolution of CXPA and sheds light on the dynamic nature of this transformation. These findings have the potential to inform more accurate diagnostic strategies and guide the development of personalized therapeutic approaches in the future. However, it is important to recognize that further studies are needed to validate these findings and translate them into tangible clinical applications.

2.2.7 References

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2.2.8 Tables

Table 1. Microscopic profile of the 27 analyzed tumors.

Microscopic features	**N (%)**
Histologic subtype	
AdNOS	6 (22.2)
SCC	1 (3.7)
EMEC	4 (14.8)
MC	6 (22.2)
SDC	9 (33.3)
SC	1 (3.7)
Degree of invasion	
iCXPA	5 (18.5)
mCXPA	5 (18.5)
fCXPA	17 (62.9)
rPA	
Present	14 (51.8)
Absent	13 (48.2)

CXPA: Carcinoma ex pleomorphic adenoma; rPA: Residual pleomorphic adenoma; AdNOS: Adenocarcinoma NOS; SCC: Squamous Cell Carcinoma; EMEC: Epithelial-Myoepithelial Carcinoma; MC: Myoepithelial Carcinoma; SDC: Salivary Duct Carcinoma; SC: Sarcomatoid Carcinoma; iCXPA: Intracapsular CXPA; mCXPA: Minimally invasive CXPA; fCXPA: Frankly invasive CXPA

2.2.9 Figure legends

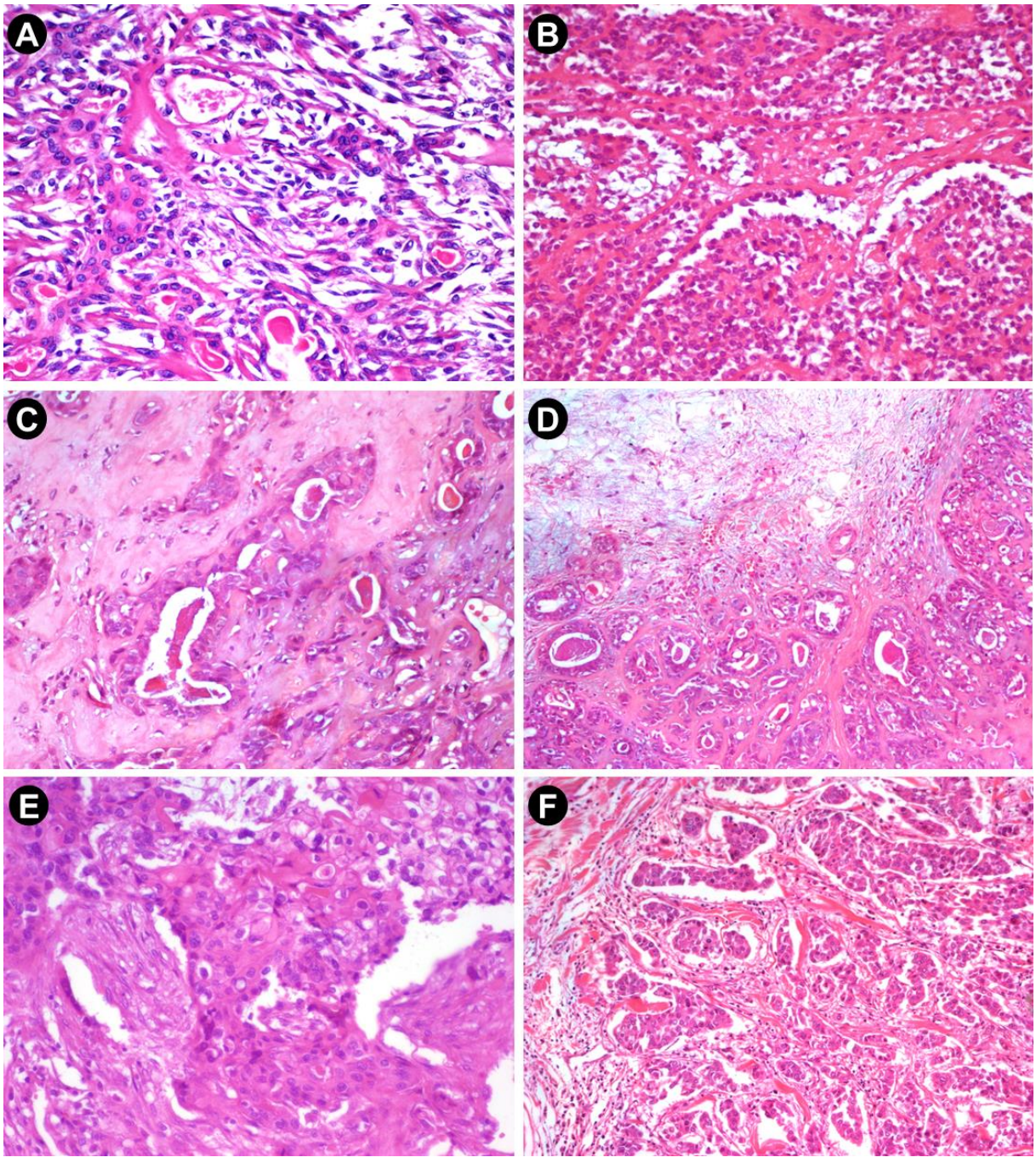

Figure 1. Representative illustration of the major histopathologic subtypes of CXPA used in this study. (A) EMEC ex-PA: Emphasizes the proliferation of transformed epithelial and myoepithelial cells within a myxoid stroma (H&E, ×20). **(B)** Growth pattern of MC ex-PA, with cells showing obvious cellular pleomorphism (H&E, ×20). **(C)** Adenocarcinoma NOS ex-PA: Tissue fragment shows cells with intense cellular pleomorphism in an invasive pattern, lacking features of other specific types. *In situ* component present (H&E, ×20). **(D)** Adenocarcinoma with residual benign area (rPA): Lower magnification shows coexistence of residual benign and transformed areas (H&E, ×10). **(E)** SCC ex-PA: Cells with epithelioid appearance, although showing pronounced cellular pleomorphism, still show the squamous nature of the tumor (H&E,

×20). **(F)** SDC ex-PA: Cells with abundant eosinophilic cytoplasm forming cords, nests, and mild cribriform structures within a desmoplastic stroma. *In situ* component present (H&E, ×10). *PA: Pleomorphic adenoma; CXPA: Carcinoma ex pleomorphic adenoma; rPA: Residual pleomorphic adenoma; EMEC: Epithelial-myoepithelial carcinoma. MC: Myoepithelial carcinoma; SCC: Squamous cell carcinoma; SDC: Salivary duct carcinoma.*

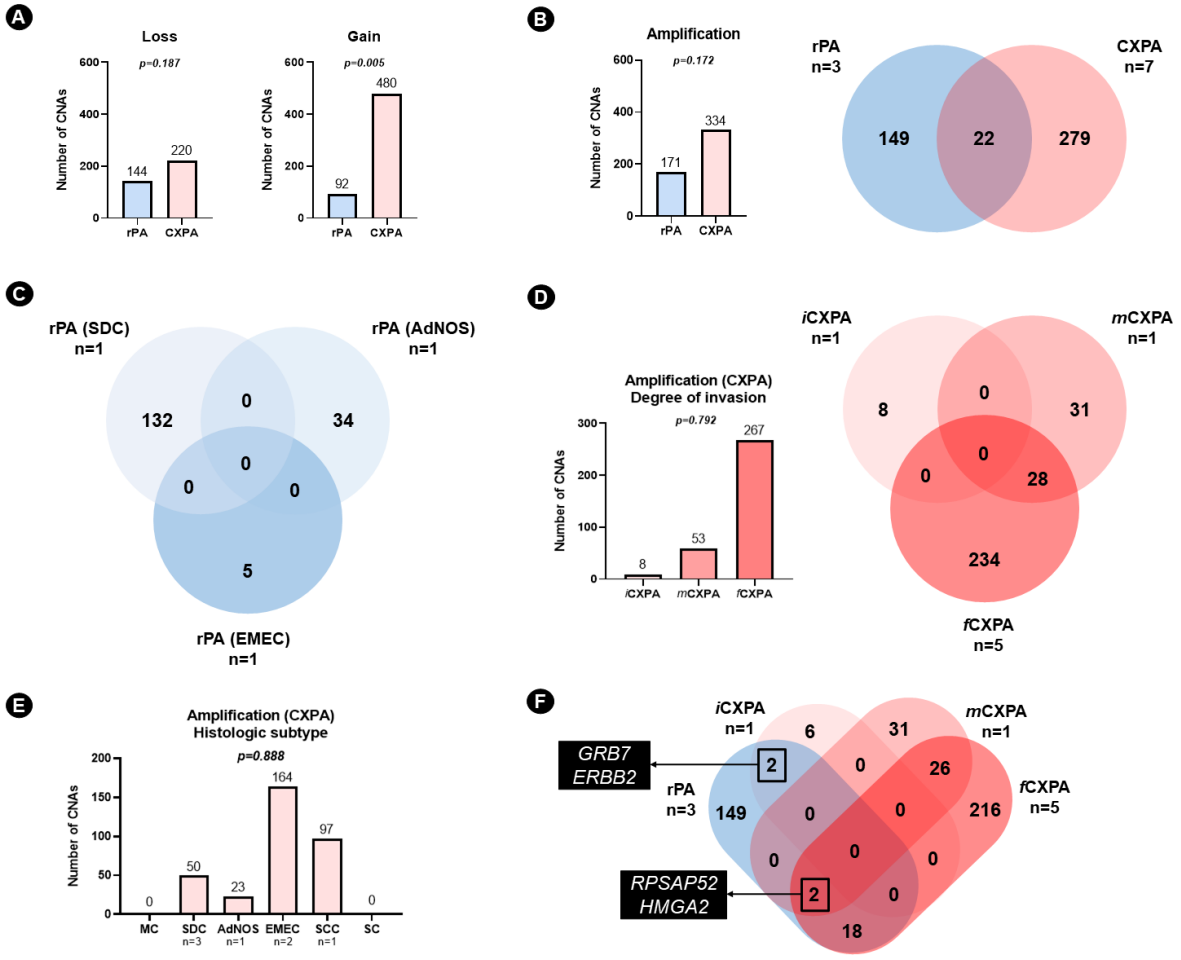

Figure 2. Profile of losses, gains, and amplifications between the bening residual area and the transformed area of CXPA included in this study. (A) Profile of gains and losses between the benign residual area (rPA) and the transformed area of carcinoma ex pleomorphic adenoma (CXPA) cases included in the study. Note that the number of gains (p=0.005) increases in the transformed area compared to the benign area. The transformed areas showed a tendency towards gene loss compared to the benign residual areas (p=0.187). *Mann-Whitney U Test*. **(B)** The transformed areas showed a tendency towards gene amplifications compared to the benign residual areas (p=0.172); Twenty-two of them were common to both groups. Note that the Venn diagram excludes repetitions of copy number alterations (CNAs). *Mann-Whitney U Test*. **(C)** Amplification of rPAs was unique to each subgroup. **(D)** There was no statistically significant difference in the number of amplifications per histological subtype of CXPA (p=0.792); some amplifications were shared between minimally invasive CXPA (mCXPA) and frankly invasive CXPA (fCXPA). No amplifications of iCXPA were shared with mCXPA and fCXPA. *Kruskal-Wallis Test.* **(E)** Among all histologic subtypes presented, more amplifications were observed in the two cases of CXPA (EMEC subtype) with identified CNAs. However, no statistically significant difference was found among the analyzed subtypes (p=0.888). *Kruskal-Wallis Test.* **(F)** To better understand the 22 shared genes between rPA and CXPA, we plotted a graph dividing the CXPA group according to the degree of invasion. Amplification of the *GRB7* and *ERBB2* genes was observed between rPA and iCXPA. *RPSAP52* and *HMGA2* amplification were shared between rPA and both mCXPA and fCXPA. *CXPA: Carcinoma ex pleomorphic adenoma; rPA: Residual pleomorphic adenoma; AdNOS:*

Adenocarcinoma NOS; SCC: Squamous Cell Carcinoma; EMEC: Epithelial-Myoepithelial Carcinoma; MC: Myoepithelial Carcinoma; SDC: Salivary Duct Carcinoma; SC: Sarcomatoid Carcinoma; iCXPA: Intracapsular CXPA; mCXPA: Minimally invasive CXPA; fCXPA: Frankly invasive CXPA; n: Number of samples included in the analysis.

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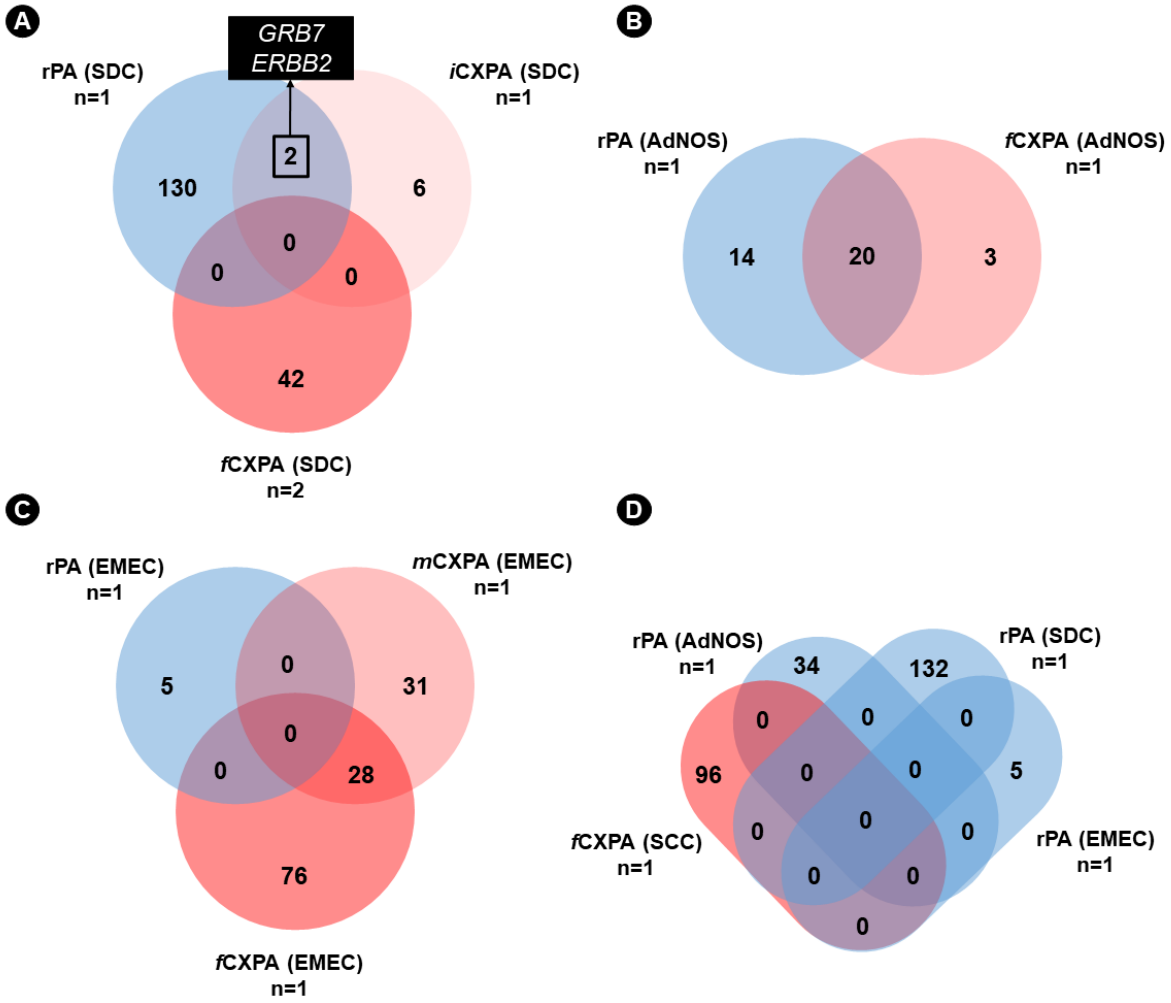

Figure 3. CNA profile in CXPA by histologic subtype. (A) CNA profile in CXPA (SDC subtype). The majority of amplifications in the benign residual area were unique, with amplifications in *GRB7 and ERBB2* being common to both rPA and intracapsular (iCXPA). **(B)** CNA profile in CXPA (AdNOS subtype). Most of the amplified genes in the malignant area were shared with the residual area. **(C)** CNA profile in CXPA (EMEC subtype). None of the amplifications in the transformed areas were shared with the rPA. **(D)** CNA profile in CXPA (SCC subtype) compared to rPA subdivided by histologic subtype. All amplifications in CXPA (SCC subtype) were unique and did not share alterations with any of the rPAs analyzed. *CXPA: Carcinoma ex pleomorphic adenoma; rPA: Residual pleomorphic adenoma; AdNOS: Adenocarcinoma NOS; SCC: Squamous Cell Carcinoma; EMEC: Epithelial-Myoepithelial Carcinoma; MC: Myoepithelial Carcinoma; SDC: Salivary Duct Carcinoma; SC: Sarcomatoid Carcinoma; iCXPA: Intracapsular CXPA; mCXPA: Minimally invasive CXPA; fCXPA: Frankly invasive CXPA; n: Number of samples included in the analysis.*

2.2.10 Supplementary Materials

File 1 – Summary of all the genetic findings by patient.

https://docs.google.com/spreadsheets/d/1ZRJ-u-bEHmLIz7Vy3nKJyYe2tNrKOtVr/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 2 – Frequencies of chromosomal copy number alterations by group.

https://docs.google.com/spreadsheets/d/1nIropSWUY1HDPPq1B6aZDjYo6MB03DLW/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 3 – List of recurrent genes by histologic subtype of CXPA.

https://docs.google.com/spreadsheets/d/1yS8g0oX_NJhYz_KE-0dLkpvLv9iWyAG_/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

File 4 – Enrichment analysis of the CXPA amplified genes in our cohort.

https://docs.google.com/spreadsheets/d/1NZxVJgO3zjWHTTGW-j9JxNXp8McuW1uO/edit?usp=drive_link&ouid=107762504631344245848&rtpof=true&sd=true

3 DISCUSSÃO

As neoplasias malignas são desencadeadas por um intricado processo de carcinogênese, resultante de mutações somáticas adquiridas e rearranjos cromossômicos que se acumulam ao longo do tempo, influenciando diversas vias e estágios dos processos biológicos. No contexto específico dos TGS, como o AP e o CXAP, a pesquisa ao longo dos anos tem se concentrado em compreender e mapear as alterações genéticas e cromossômicas presentes nessas neoplasias, explorando anomalias cromossômicas como perda de heterozigosidade, rearranjos e translocações (Katabi et al., 2015b; Mariano et al., 2015).

O avanço da tecnologia de sequenciamento de última geração, especialmente na última década, tem revolucionado a genética molecular humana, proporcionando uma avaliação abrangente de porções significativas do genoma. Essa tecnologia desempenha um papel crucial na identificação de variantes genéticas normais e patológicas, destacando-se como uma ferramenta valiosa no estudo da evolução do câncer. Ela permite a detecção da progressão tumoral, recorrências e remissão da doença, contribuindo para uma compreensão mais profunda dos eventos genômicos associados aos TGS (Moore et al., 2020; Todorovic et al., 2020).

Entretanto, os cânceres salivares, em particular, são ainda mais raros do que os tumores benignos, o que resulta na escassez de estudos com coortes significativas. Essa limitação dificulta a identificação de rotas mais seguras para tratamento. Os desafios são agravados pela natureza intrinsecamente complexa e desafiadora dos cânceres raros, que representam um obstáculo na pesquisa oncológica. A falta de caracterização genética abrangente desses cânceres, juntamente com a disponibilidade limitada de ferramentas de pesquisa, como modelos animais, linhas celulares e tamanhos de amostras de tumores, impõe obstáculos significativos ao avanço do conhecimento sobre essas neoplasias (Emerick et al., 2022).

Embora os resultados do aCGH tenham revelado descobertas inéditas, é importante ressaltar que esses dados se limitam a anomalias cromossômicas em todo o genoma, uma característica intrínseca à técnica utilizada. Como resultado, outros tipos de mutações presentes no genoma não foram avaliados. Para abordar essa lacuna, recorremos ao WES, que nos permitiu mapear as alterações associadas ao

AP e às áreas benignas e transformadas do CXAP. Ambos os métodos se complementam e nos direcionam para caminhos promissores de pesquisa futura. É importante destacar que, até o momento, não existem estudos de WES que elucidem as características moleculares específicas desses dois tumores, nem estudos de aCGH comparativos entre áreas benignas e malignas do mesmo paciente com CXAP.

Os resultados obtidos revelam uma ampla gama de descobertas, cujo maior desafio reside em entender como essas informações podem ser aplicadas da bancada para a clínica, visando o benefício dos pacientes. Portanto, nesta discussão, optamos por nos concentrar nas conclusões derivadas de todas as metodologias aplicadas a esses tumores nos últimos seis anos, lançando luz sobre possíveis alvos de estudo que, no futuro, podem resultar em avanços significativos no tratamento dos pacientes.

Ao compararmos os resultados obtidos pelo aCGH e pelo WES sem filtro de frequência alélica, observamos que 30% dos genes afetados (78 deles) por amplificações no aCGH em CXAP estão mutados pelo WES. No entanto, ao analisarmos os genes compartilhados nos AP residuais por ambas as metodologias, percebemos que apenas 13,2% dos genes afetados (19 deles) por amplificações no aCGH em AP residuais estão mutados pelo WES. A análise de *Gene Ontology (GO)* desses genes compartilhados não revelou uma significativa riqueza em processos biológicos, funções moleculares ou componentes celulares. Isso pode indicar que esses genes constituem uma coleção desconexa ou que suas interações ainda não são bem compreendidas. De qualquer forma, o aCGH serviu como um método complementar e confirmatório de nossos achados anteriores.

Uma análise de GO nos genes mutados comuns entre essas duas metodologias não foi significativamente enriquecida em processos na categoria “componente celular”. Entretanto, a análise de “função biológica” mostrou que enriquecimento para três subcategorias: regulação positiva da fosforilação de peptidil-serina da proteína STAT ($p = 0.0175$), ativação de células natural killer envolvidas na resposta imune ($p = 0.0167$) e degradação de colágeno ($p = 0.0188$). Destacam-se nas duas primeiras categorias os genes *IFNA2, IFNA7, IFNA10* e *IFNA13*. No câncer, interferon-γ (IFN-γ) desempenha um papel duplo e complexo. Enquanto participa da estimulação da resposta imune anti-tumoral, inibindo a angiogênese, induzindo a apoptose de células T reguladoras e/ou estimulando a uma resposta M1 (Jorgovanovic et al., 2020), IFN-γ também pode participar da promoção da

imunoevasão do câncer, estabelecendo um microambiente imunossupressor (Mojic et al., 2017). Estudos recentes têm mostrado que ao aumentar seus efeitos antitumorais benéficos, o IFN-γ poderia contribuir para a imunoterapia do câncer (Gocher et al., 2022; Ni and Lu, 2018). Nossos achados enfatizam que os genes amplificados no CXAP e mutados no WES foram enriquecidos para processos biológicos relacionados à sinalização imune por IFN- γ. Estudos do microambiente imunológico em CXAP são escassos (Egal et al., 2022; Lavareze et al., 2022). Outros estudos são necessários para entender melhor os aspectos imunológicos na carcinogênese do AP.

Em relação à análise de “função molecular”, houve enriquecimento para três subcategorias: ligação do receptor de interferon tipo I (p= 0.0042) (IFNA2, IFNA7, IFNA10 e IFNA13), atividade de metaloendopeptidase (p = 0.0065) e atividade de metaloproteinase (MMP) (p = 0.0089) (*MMP7, MMP8, MMP12, MMP20, MMP27, ADAM2, CPM*). Os MMPs são um grupo de endopeptidases dependentes de zinco relacionadas à degradação e remodelação da matriz extracelular (MEC). Elas podem degradar os componentes proteicos do MEC e da membrana basal, facilitando a invasão e progressão tumoral. Dados da literatura mostram que a expressão de MMPs entre o AP e diferentes tumores benignos de glândula salivar não apresenta quaisquer diferenças quantitativas (Lipari et al., 2012). O fato é que ao que parece, pelo menos no AP, MMPs (especialmente MMP-2 e MMP-9) são sintetizadas principalmente pelas células mioepiteliais (Zhang et al., 2009) e isto traz à tona, o papel desta célula ímpar na configuração de um microambiente pró-tumoral.

Em 2020, publicamos uma revisão narrativa da literatura abordando o papel ambíguo desta célula durante a transição do AP-CXAP, demonstrando que essa célula pode atuar tanto como supressora quanto como promotora tumoral (Scarini et al., 2021). Na fase inicial da carcinogênese do AP, as células do carcinoma são delimitadas por uma barreira composta por uma camada intacta de células mioepiteliais do AP pré-estabelecido, caracterizando as áreas de carcinoma *in situ* (ou carcinoma intraductal) (Cheuk and Chan, 2007). Estas células separam as células epiteliais malignas do ambiente do estroma (de Araújo et al., 2006), formando uma verdadeira barreira de retenção entre as células transformadas e a membrana basal. Em algum momento durante à evolução da neoplasia, por mecanismos ainda pouco estudados, as células do carcinoma rompem essa barreira mioepitelial e invadem o estroma circundante (Scarini et al., 2021).

Assim, podemos destacar que o estopim da progressão tumoral parece ocorrer quando as células epiteliais malignas rompem a barreira de células epiteliais benignas. De fato, estudos anteriores mostraram que a célula mioepitelial benigna se torna muito bem diferenciada e produz proteínas importantes relacionadas à função supressora de tumor (Scarini et al., 2021). Mas isso parece não ser suficiente para impedir o ataque das células epiteliais malignas, e pelo menos na transformação maligna do AP, os mecanismos supressores parecem falhar e as células mioepiteliais tendem a desaparecer à medida que o tumor progride. Ademais, tumores puramente mioepiteliais se desenvolvem no AP e os motivos pelo qual a célula mioepitelial se transforma são ainda mais enigmáticos.

Biologicamente, o AP apresenta uma célula mioepitelial proeminente que desempenha um papel crucial na regulação do crescimento e da progressão do tumor (Scarini et al., 2023). As células mioepiteliais, quando neoplásicas, têm a capacidade de influenciar a MEC, promovendo alterações significativas (Sternlicht and Barsky, 1997). Elas podem contribuir para o aumento na síntese de moléculas da membrana basal, uma característica associada à atividade supressora tumoral (Barsky et al., 1988). Nossos resultados do WES em relação à MEC desses tumores sugerem que mutações em genes associados a esse ambiente podem desempenhar um papel na carcinogênese do AP. Além disso, a análise aponta para uma possível relação íntima entre essas mutações e a célula mioepitelial. A compreensão dessas interações pode fornecer *insights* valiosos sobre os mecanismos envolvidos na transição maligna do AP para CXAP.

A interação complexa entre biologia tumoral, genômica e o microambiente desempenha um papel crucial no desenvolvimento e progressão de tumores, incluindo o AP e o CXAP. A compreensão desses fatores em conjunto pode abrir novas perspectivas para estratégias terapêuticas mais direcionadas a estes tumores. O microambiente do câncer, em particular, desempenha um papel vital, e a interação da célula neoplásica com a MEC é um evento central para a proliferação neoplásica (Hanahan and Weinberg, 2011; Liotta and Kohn, 2001). Aqui, a análise do WES revelou a presença de diversas mutações em genes associados à MEC em nossa amostra. Um achado particularmente intrigante foi a identificação de uma mutação no gene *TNXB*, responsável pela codificação da proteína Tenascina-X (TNX). A família Tenascina (TN) compreende membros com padrões singulares de expressão, e a TNX, expressa de forma ubíqua nos tecidos conjuntivos, desempenha um papel

fundamental na montagem e organização da rede de colágeno (Liot et al., 2020). A expressão de TNX durante a progressão do câncer tem sido pouco explorada e é alvo de controvérsias, sendo regulada negativamente em muitos cânceres, incluindo melanoma e leiomioma, e positivamente regulada no mesotelioma maligno e câncer de ovário (Liot et al., 2020). A análise de WES revelou mutações em *TNXB* em todos os grupos analisados, sugerindo um papel crucial deste gene ao longo da transformação maligna do AP.

Estudos prévios do nosso grupo destacaram diferenças na expressão de TN em CXAP com componentes epiteliais versus mioepiteliais, indicando uma associação entre a expressão de TN e a diferenciação tumoral (Araujo et al., 2008). A observação de *TNXB* mutado ao longo da transformação maligna do AP adiciona uma nova camada de complexidade e destaca a importância contínua da investigação genômica para compreender os mecanismos subjacentes a essas neoplasias salivares. Essa descoberta inédita sugere que as alterações em *TNXB* podem estar envolvidas na progressão tumoral, abrindo portas para investigações mais aprofundadas sobre o papel desse gene específico na tumorigênese do AP e desenvolvimento do CXAP.

O desenvolvimento do câncer é geralmente concebido como uma complexa interação de causas e efeitos, em que o incremento de alterações genéticas nos proto-oncogenes, genes supressores de tumor e genes de reparo de DNA contribui sinergicamente para uma transdução de sinal anormal. Esse processo, por sua vez, desencadeia uma proliferação celular desregulada e, em estágios avançados, facilita a disseminação metastática. Essas transformações genéticas, que podem ser desencadeadas por diversos fatores, culminam em uma cascata de eventos que comprometem o controle normal sobre o ciclo celular, impulsionando a progressão do câncer (Kontomanolis et al., 2020). Considerando o número de genes mutados, implementamos um filtro de frequência alélica de 0,2 (ou 20%). Isso significa que apenas variantes presentes em pelo menos 20% das cópias do gene ou região genômica foram consideradas. Esse filtro permite distinguir variantes germinativas, herdadas e geralmente presentes em todas as células do organismo, de variantes somáticas, que podem ocorrer apenas em células específicas e contribuir para doenças como o câncer (Solís-Moruno et al., 2023).

Utilizando esse filtro de refinamento de mutações, identificamos um total de 823 genes com mutações não sinônimas. Dentre esses, 251 (30,5%) eram exclusivos

para AP, 236 (28,7%) eram exclusivos para o AP residual, e 151 (18,3%) eram exclusivos para o CXAP. Notamos que mutações em 104 desses genes (12,6%) estavam presentes no AP e permaneceram no carcinoma correspondente, enquanto mutações em 65 genes (7,9%) estavam presentes ao longo de toda a sequência adenoma-carcinoma. Isso implica, em parte, que as alterações genéticas encontradas no CXAP já estavam presentes na área benigna residual adjacente à área de transformação. Embora essas regiões apresentem características morfológicas de um AP convencional, elas exibem uma assinatura molecular pré-maligna. Esses resultados são respaldados pelos nossos achados no aCGH, onde 4.4% das amplificações identificadas no CXAP eram compartilhadas com as áreas residuais benignas adjacentes à área transformada.

Além disso, nossos achados do WES revelam um conjunto significativo de mutações em genes supressores de tumor e oncogenes, compartilhadas nos três grupos estudados. Essa constatação reforça a ideia de que a transição do AP-CXAP é um processo sequencial, no qual as mutações genéticas desempenham um papel crucial na progressão fenotípica do tumor. Identificamos mutações em genes amplamente reconhecidos como relevantes para o câncer, especialmente aqueles associados à via de sinalização MAPK, tais como *TP53, BRAF, MAP2K4* e *NF1*. Essas descobertas oferecem perspectivas valiosas para o desenvolvimento de estratégias de tratamento para estes pacientes. É importante ressaltar que a via MAPK, especialmente a via ERK, tem se destacado como um alvo promissor e clinicamente relevante na terapia do câncer, de acordo com as evidências atuais (Lee et al., 2020).

Nossos resultados do WES evidenciaram também a potencial importância do gene *c-Myb* na alteração fenotípica do tecido glandular ao longo da sequência AP-CXAP. Notamos a presença do gene *MYB* mutado na interseção de todos os grupos estudados, sendo encontrado em 4 AP, 4 AP residuais e 5 CXAP. A família do gene *MYB*, composta por três membros em humanos (*MYB, MYBL1 e MYBL2*), regula a expressão e a atividade da quinase dependente de ciclina, desempenhando um papel essencial na duplicação celular. Em particular, o *MYB* tem sido associado à tumorigênese em carcinomas adenoides císticos (CAC), onde fusões envolvendo o *MYB* e o fator de transcrição *NFIB* são recorrentes.

Nossa escolha pelo c-Myb foi motivada pela sua implicação na tumorigênese do CAC, sendo reconhecido na literatura como um impulsionador significativo desse tipo de câncer (Humtsoe et al., 2022; Wagner et al., 2022; West et

al., 2011). Além disso, não havia dados disponíveis sobre a função específica do *MYB* na transformação maligna do AP até o momento. Para validação, utilizamos tecidos incluídos em parafina de glândulas salivares normais e AP residuais de áreas adjacentes às amostras de AP ou CXAP. Contudo, a análise enfrentou desafios devido à presença de uma considerável quantidade de matriz tumoral, sobreposição de células nos cortes histológicos, hemorragias teciduais e infiltrado inflamatório, especialmente nas amostras de CXAP. Reconhecemos que essas limitações podem influenciar nossos resultados, e a heterogeneidade das amostras de CXAP destaca a necessidade de futuros estudos com grupos mais amplos para cada fenótipo, apesar dos desafios logísticos e custos associados.

A validação por imunofluorescência revelou que a expressão do c-MYB aumenta conforme o tecido normal se torna neoplásico, porém diminui à medida que o fenótipo se torna maligno. Esse padrão foi corroborado pelas mutações pontuais identificadas por meio do WES, onde a mutação era mais prevalente em pacientes com neoplasias benignas e em áreas residuais benignas do CXAP do que em áreas transformadas. Essa descoberta complementa nossos resultados do aCGH, que, ao contrário do WES, destaca alterações no número de cópias (CNA) em grandes segmentos de DNA, os quais podem ser translocados, amplificados ou perdidos. De fato, nossos resultados de validação estão alinhados com as descobertas do aCGH, onde foram identificadas perdas nos cromossomos 2q, 5q e 8p, incluindo perdas em diversos genes, como o *MYB*. Essa observação sugere que o *MYB* pode não ter um papel crucial na manutenção do tumor, como no caso do carcinoma adenóide cístico (CAC), mas possivelmente desempenha um papel significativo nos estágios iniciais da carcinogênese, atuando como um gatilho para o processo de transformação. Além disso, ela indica a coexistência de mutações pontuais e cromossômicas orientando o processo carcinogênico desses tumores.

Finalmente, é relevante destacar os resultados obtidos anteriormente por meio das abordagens de Imunoistoquímica e Expressão Gênica, originados do projeto do Jovem Pesquisador que deu origem a esta tese de Doutorado. A integração desses achados com os resultados do WES fornece uma compreensão mais sólida e aprofundada das descobertas prévias. Essas metodologias revelaram que, embora não significativa, a expressão gênica e imunoistoquímica de FASN tendeu a aumentar ao longo da carcinogênese do AP (Scarini et al., 2020). Na análise de mutações pontuais no exoma de pacientes ao longo da transformação maligna, identificamos

duas variantes de FASN de impacto moderado (missense): uma no AP e outra no CXAP. Apesar da ausência de amplificações gênicas de FASN no aCGH, recentemente demonstramos que a análise de CNAs de genes de microRNAs revelou alterações epigenéticas no gene FASN nesses tumores (Kimura et al., 2024). Ao examinar outras amostras, nosso grupo observou que a expressão de FASN foi significativamente mais elevada nos pacientes com tumor maligno em comparação com os pacientes com tumor benigno (P <0,001). Essa superexpressão poderia estar associada à transformação maligna de células epiteliais ductais e/ou células mioepiteliais do AP (Diaz et al., 2019). Além disso, ao avaliar a expressão proteica de FASN em outros carcinomas de glândulas salivares, nosso grupo evidenciou que essa expressão está relacionada tanto com o grau de malignidade quanto com o fenótipo secretório adquirido por algumas dessas neoplasias no processo de transformação maligna (de Angelis et al., 2021).

De todos os resultados encontrados durante a análise da expressão gênica de marcadores metabólicos nesses tumores, nosso achado mais consistente parece estar relacionado ao metabolismo da glicose, regido pelo *GLUT-1*. Encontramos apenas uma mutação de *GLUT-1* de impacto moderado (missense) no CXAP. Esse resultado confirma o aumento da expressão gênica de *GLUT-1* nos tecidos malignos quando comparado com os tecidos benignos (AP e AP residual), como identificado anteriormente por nosso grupo (Scarini et al., 2020).

Portanto, embora tenhamos adquirido uma compreensão mais profunda da genética e biologia desses tumores raros por meio de nossos resultados, enfrentamos desafios que limitaram a amplitude de nossa pesquisa. O desafio do número amostral é significativo, especialmente dada a raridade do CXAP e a dificuldade em obter tecidos congelados desses tumores. Durante a análise do fragmento tumoral, identificamos que cinco amostras anteriormente consideradas como CXAP exibiam microscopicamente apenas áreas de AP residual. Devido à escassez de tecidos congelados de AP residual, optamos por incluí-los na análise comparativa com o AP convencional e o CXAP, mesmo com um número limitado de casos.

De todo modo, para uma compreensão mais robusta do papel desses genes ao longo do processo, é necessário aumentar o número de amostras, buscando perfis mais homogêneos, e validar outros achados interessantes. Apesar dessas considerações, nossos resultados contribuirão significativamente para uma compreensão mais aprofundada das alterações gênicas relacionadas ao

desenvolvimento do AP e do CXAP. É importante destacar que nossas descobertas não se aplicam apenas a esses tumores específicos, mas também têm implicações para outros cânceres salivares. Essa base científica sólida proporcionará avanços no diagnóstico e tratamento dessas doenças, contribuindo para a aplicação do conceito de "ciência de cânceres raros" na pesquisa oncológica contemporânea.

4 CONCLUSÃO

Tomados em conjuntos, os achados aqui apresentados sugerem que embora todos os nossos esforços ainda pareçam ser apenas a ponta do *iceberg*, avançamos consistentemente na elucidação de alterações moleculares contribuintes para a patogênese destes tumores, bem como nas semelhanças e diferenças entre os eventos próprios da sequência AP-CXAP e àqueles envolvidos com outros subtipos de tumores salivares. Vale salientar que na maioria dos casos, a assinatura molecular de cada tumor de glândula salivar parece ser individualizada, com um perfil de mutação único e limitado. Mesmo que muitos genes mutados tenham sido encontrados em outros cânceres humanos, muitas destas alterações foram pouco exploradas em TGS e, na transformação maligna do AP, não seria surpreendente que sejamos os primeiros a relatá-las. Dessa forma, nossas principais conclusões foram:

1) **Assinatura molecular pré-maligna do AP residual:**
    - Identificamos alterações genéticas em áreas benignas residuais pelo WES e aCGH, indicando uma assinatura molecular pré-maligna, mesmo quando as características morfológicas sejam de um AP convencional.
2) **Transição sequencial acarretada por mutações genéticas:**
    - Identificamos diversas mutações cruciais compartilhadas entre os grupos (AP, AP residual e CXAP) por WES, reforçando a transição AP-CXAP como um processo sequencial, no qual as mutações genéticas desempenham papel na progressão fenotípica.
    - Observamos a presença de genes supressores de tumor e oncogenes durante a transição AP-CXAP, indicando a aquisição de eventos mutacionais nesses tumores. Destacamos especialmente aqueles associados à via de sinalização MAPK, fornecendo *insights* sobre os mecanismos moleculares subjacentes aos tumores e possíveis alvos de tratamento.
3) **Mutação pontual no gene *MYB*:**
    - Identificamos pioneiramente mutações pontuais no gene *MYB* na sequência adenoma-carcinoma por meio do WES, as quais foram validadas por imunofluorescência.

- Observamos a perda de expressão proteica de c-Myb no CXAP, o que corrobora com os achados do aCGH. Isto destaca a simultaneidade de mutações pontuais e cromossômicas na patogênese e manutenção do CXAP.

4) **Família do INF-γ como alvo de mutações:**
    - Evidenciamos que mutações na família do INF-γ em AP e CXAP, detectadas pelas duas metodologias, sugerem um papel potencial da imunoterapia nesses tumores.
5) **Papel da célula mioepitelial e da matriz extracelular:**
    - Destacamos a relevância da célula mioepitelial no desenvolvimento do CXAP por meio dos resultados obtidos pelas duas metodologias, evidenciando mutações em diferentes genes e vias relacionadas a MEC.
    - Além disso, ressaltamos a importância da MEC nas alterações relacionadas à transformação maligna, com ênfase para o gene *TNXB*. Este gene, cuja expressão e função são tão controversos em outros tumores, deve ser alvo de estudos futuros para elucidar melhor seu papel na patogênese do AP e CXAP.
6) **Shift metabólico e potenciais alvos terapêuticos:**
    - Identificamos alterações nos genes *FASN* e *GLUT-1* nos tumores malignos por meio do WES, as quais foram confirmadas por estudos prévios de nosso grupo.
    - A presença de um shift metabólico nestes tumores revela que FASN pode ser uma via promissora de estudo para a identificação de alvos terapêuticos.

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ANEXOS

ANEXO 1 – Comprovante de submissão do artigo 1

Modern Pathology

Whole-exome sequencing identifies somatic mutations associated with the malignant transformation of pleomorphic adenoma: A preliminary study

--Manuscript Draft--

Manuscript Number:	
Full Title:	Whole-exome sequencing identifies somatic mutations associated with the malignant transformation of pleomorphic adenoma: A preliminary study
Article Type:	Research Article
Keywords:	Salivary gland tumors; Salivary gland carcinomas; Pleomorphic Adenoma; Carcinoma Ex Pleomorphic Adenoma; Whole-exome sequencing; Gene mutation; Pathway mutation
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Manuscript Region of Origin:	BRAZIL
Abstract:	Pleomorphic adenoma (PA) has the potential to evolve into a complex and aggressive tumor known as carcinoma ex-pleomorphic adenoma (CXPA). However, the mechanisms involved in the adenoma-carcinoma sequence are controversial and have

ANEXO 2 – Comprovante de submissão do artigo 2

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Carcinoma ex pleomorphic adenoma: Distinct genetic features between transformed area and residual pleomorphic adenoma

Corresponding Author: Fernanda Viviane Mariano

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COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "Estudo das alterações genéticas e metabólicas do Adenoma Pleomorfo e Carcinoma Ex-Adenoma Pleomorfo por exoma, expressão gênica e imunohistoquímica", CAAE 68157517.7.3002.5418, dos pesquisadores Fernanda Viviane Mariano, Albina Messias de Almeida Milani Altemani, Joao Figueira Scarini, Cláudia Malheiros Coutinho Camillo, Oslei Paes de Almeida, Ricardo Della Coletta, Luiz Paulo Kowalski e Leisa Lopes Aguiar, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado em Coparticipação ao CEP da Faculdade de Ciências Medicas – UNICAMP em 01/10/2018.

The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that the research project "Study of genetic and metabolic changes of Pleomorphic Adenoma and Carcinoma Ex Pleomorphic Adenoma by exome, gene expression and immunohistochemistry", CAAE 68157517.7.3002.5418, of the researchers Fernanda Viviane Mariano, Albina Messias de Almeida Milani Altemani, Joao Figueira Scarini, Cláudia Malheiros Coutinho Camillo, Oslei Paes de Almeida, Ricardo Della Coletta, Luiz Paulo Kowalski and Leisa Lopes Aguiar, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health Health, and was approved in Coparticipation to the Research Ethics Committee of the Faculty of Medical Sciences - UNICAMP in October, 01 2018.

Profa. Fernanda Miori Pascon

Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior

Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.

ANEXO 4 – Lista da produção do aluno publicada e relacionada ao projeto

- ***Diretamente relacionados a Tese***

Scarini JF, de Lima-Souza RA, Lavareze L, Ribeiro de Assis MCF, Damas II, Altemani A, Egal ESA, Dos Santos JN, Bello IO, Mariano FV. Heterogeneity and versatility of the extracellular matrix during the transition from pleomorphic adenoma to carcinoma ex pleomorphic adenoma: cumulative findings from basic research and new insights. Front Oral Health. 2023 Apr 17;4:942604. doi: 10.3389/froh.2023.942604. PMID: 37138857; PMCID: PMC10149834.

Egal ESA*, ***Scarini JF****, de Lima-Souza RA, Lavareze L, Fernandes PM, Emerick C, Gonçalves MT, Helms MN, Altemani A, Mariano FV. Tumor microenvironment in salivary gland carcinomas: An orchestrated state of chaos. Oral Oncol. 2022 Apr;127:105777. doi: 10.1016/j.oraloncology.2022.105777. Epub 2022 Feb 18. PMID: 35189584. *The authors share the first authorship.

Scarini JF, Egal ESA, de Lima-Souza RA, Crescencio LR, Emerick C, Kowalski LP, Altemani A, Mariano FV. Two sides of the same coin: Insights into the myoepithelial cells in carcinoma ex pleomorphic adenoma development. Crit Rev Oncol Hematol. 2021 Jan;157:103195. doi: 10.1016/j.critrevonc.2020.103195. Epub 2020 Dec 9. PMID: 33307199.

- ***Artigos relacionados ao Jovem Pesquisador que complementam discussão dos achados apresentados***

Scarini JF, Rosa LF, Souza RAL, Egal ESA, Tincani AJ, Martins AS, Kowalski LP, Graner E, Coletta RD, Carlos R, Gondak RO, de Almeida OP, Altemani AMAM, Bastos DC, Mariano FV. Gene and immunohistochemical expression of HIF-1α, GLUT-1, FASN, and adipophilin in carcinoma ex pleomorphic adenoma development. Oral Dis. 2020 Sep;26(6):1190-1199. doi: 10.1111/odi.13332. Epub 2020 Apr 2. PMID: 32180291.

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