UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

CAROLINA EMERICK DA SILVA RANGEL

EXPANSÃO DA COMPREENSÃO DOS TUMORES DE CARCINOMA ADENOIDE CÍSTICO E DESENVOLVIMENTO DE TERAPIAS EPIGENÉTICAS INOVADORAS

EXPANDING UNDERSTANDING OF ADENOID CYSTIC CARCINOMA TUMORS AND DEVELOPING INNOVATIVE EPIGENETIC THERAPIES

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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 Doutora em Estomatopatologia, na Àrea de Estomatologia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Stomatopathology, in Stomatology area.

Orientador: Rogério Moraes de Castilho

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RESUMO

O carcinoma adenoide cístico (CAC) apresenta um cenário clínico desafiador, caracterizado por prognósticos variados, dependendo do local anatômico de envolvimento. Enquanto as glândulas salivares e lacrimais geralmente apresentam um curso clínico difícil, o CAC na mama tende a apresentar um comportamento menos agressivo. Os esforços para melhorar o prognóstico e a sobrevida livre de doença envolvem a exploração de estratégias terapêuticas para recorrências e metástases. A construção deste trabalho, motivada pelos desafios enfrentados pelos pacientes com lesões de CAC, enfatiza um foco mais amplo em perfis moleculares para decisões de tratamento, e um afastamento da ênfase tradicional em tipos histológicos. A exploração das lesões de CAC em diferentes locais anatômicos revelou perfis clínicos, genéticos, prognósticos e de tratamento distintos. Nossa revisão abrangente ressaltou como o local anatômico influencia significativamente a sobrevida do paciente. O estudo posterior investigou a função de drogas epigenéticas nas lesões de CAC salivar. Usando uma abordagem de alto rendimento, a pesquisa analisou os efeitos inibitórios de 157 medicamentos modificadores de histona em células-tronco tumorais (CTT) de CAC em células aderidas e esferas tumorais. As descobertas revelaram 11 possíveis medicamentos com diversos alvos capazes de inibir as histonas. O estudo revelou respostas diferenciadas das CTT e das células aderidas de CAC e esses medicamentos, indicando a necessidade de terapias medicamentosas personalizadas, possivelmente envolvendo uma combinação de medicamentos direcionados a ambas as populações de células cancerígenas. De modo geral, essa tese defende uma mudança de paradigma em relação ao perfil molecular para decisões de tratamento do CAC, fornecendo informações valiosas para o cenário clínico desafiador desse câncer raro.

Palavras-chave: Tumores de cabeça e pescoço. Câncer de glândula salivar. Terapia alvo. Epigenética. Células tronco tumorais.

ABSTRACT

Adenoid cystic carcinoma (ACC) presents a challenging clinical scenario, characterized by varying prognoses depending on the anatomical. While salivary and lacrimal glands generally present a difficult clinical course, ACC in the breast tends to present a less aggressive behavior. Efforts to improve prognosis and disease-free survival involve exploring therapeutic strategies for recurrences and metastases. The construction of this thesis, motivated by the challenges faced by patients with ACC lesions, emphasizes a broader focus on molecular profiles for treatment decisions, and a move away from the traditional emphasis on histological types. Exploration of ACC lesions in different anatomical locations has revealed distinct clinical, genetic, prognostic and treatment profiles. Our comprehensive review highlighted how the anatomical site significantly influences patient survival. The subsequent study investigated the role of epigenetic drugs in salivary ACC lesions. Using a high-throughput approach, the research analyzed the inhibitory effects of 157 histone-modifying drugs on cancer stem cells (CSC) of ACC in non-CSC and tumorspheres. The findings showed 11 possible drugs with diverse targets capable of inhibiting histones. The study revealed differentiated responses of CSC and non-CSC cells to these drugs, indicating the need for personalized drug therapies, possibly involving a combination of drugs targeting both cancer cell populations. Overall, this thesis advocates a paradigm shift towards molecular profiling for treatment decisions in ACC, providing valuable information for the challenging clinical scenario of this rare cancer.

Keywords: Head and neck tumors. Salivary gland cancer. Targeted therapy. Epigenetics. Tumor stem cells.

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1 INTRODUÇÃO

Os cânceres raros constituem 25% de todos os casos de câncer nos Estados Unidos e representam menos de 15 casos por 100.000 pessoas por ano no país. Apesar de raros, estes tumores geralmente apresentam uma taxa de sobrevida menor do que outros tipos mais prevalentes de câncer, e o conhecimento limitado sobre a biologia destes tumores impactam diretamente seu manejo clinico (DeSantis et al., 2017). Dentre a classificação de tumores raros, o Carcinoma Adenoide Cístico (CAC) chama atenção pois, apesar de se encaixar nos critérios que o definem como raro, é visível o aumento de estudos moleculares e de abordagem terapêutica (Emerick et al., 2022b), seja com inciativa de órgãos federais, como o National Institutes of Health (NIH), ou com fundações sem fins lucrativos, como a Adenoid Cystic Carcinoma Research Foundation (ACCRF), ambos sediados nos Estados Unidos.

O CAC é um crescimento tumoral localizado principalmente nas glândulas salivares, mas também pode afetar outras glândulas, como as glândulas lacrimais e mamárias. Ele representa 1% de todas as neoplasias malignas da cabeça e pescoço e 10% das neoplasias malignas das glândulas salivares (Andreasen et al., 2018; Fang et al., 2022). O tumor segue um curso clínico agressivo, apresentando um risco substancial de invasão local e metástase, resultando em um prognóstico sombrio. A taxa de sobrevivência em 10 anos para indivíduos com essa lesão de glândula salivar varia de 29% a 40% (Andreasen et al., 2018; Ferrarotto et al., 2021). A abordagem adequada no tratamento desses tumores é fundamental, uma vez que os CACs de glândulas salivares geralmente apresentam invasão perineural, associada a um padrão de crescimento lento e indolente (Ferrarotto et al., 2021; Jiang et al., 2019). Dessa forma, o tratamento primário consiste em excisão cirúrgica com margens livres de doença e tentativa de manutenção funcional da glândula, além de radioterapia pós-operatória (Ellington et al., 2012). Em lesões sintomáticas ou com rápida progressão, a quimioterapia deve ser realizada, mesmo que de forma paliativa (Laurie et al., 2011).

O uso de agentes quimioterápicos é muito recorrente em CAC salivar avançado, porém, não existe um protocolo específico para a lesão, e embora amplamente estudado, os resultados permanecem ruins (Almeida et al., 2017). Além disso, é sabido que a

quimiorresistência tumoral intrínseca ou adquirida limita a eficácia dos quimioterápicos, como a cisplatina, levando ao óbito do paciente poucos anos após o início do tratamento (Adelstein et al., 2012; Papaspyrou et al., 2011). Os mecanismos que levam a esta quimiorresistência estão associados à metabolização das drogas, regulação na sobrevivência e morte celular e no reparo do DNA (Shen et al., 2012).

As neoplasias malignas apresentam uma arquitetura tecidual complexa, onde há dependência da interação entre as células neoplásicas e o estroma, a fim de superar as células de defesa do hospedeiro, para progredir e se desenvolver no organismo. A heterogeneidade celular presente nesses tumores é um fator muito importante, pois está intimamente relacionado à recorrência, metástase e falha no tratamento do câncer. Neste contexto, há dois modelos que tentam explicar este padrão celular heterogêneo. O modelo estocástico explica que todas as células tumorais são capazes de se proliferar infinitamente, originando novos tumores. Em contrapartida, a hipótese de células-tronco tumorais (CTT) sugere que apenas uma parte das células na neoplasia são capazes de se proliferar e desenvolver uma nova lesão maligna (Hanahan and Weinberg, 2011). São essas células que estão presentes nos mecanismos de quimiorresistência, e contribuem amplamente na ação da radioterapia e quimioterapia de diversas neoplasias malignas, incluindo as de cabeça e pescoço (Le et al., 2014).

As CTT fazem parte de um microambiente tumoral amplo que coordena vias de manutenção e sinalização celular, e apresentam alta taxa de multiplicação, agressividade e potencial metastático (Prince et al., 2007). Além disso, são responsáveis pelos eventos epigenéticos do câncer, como por exemplo, acetilação do DNA e consequente alteração das histonas. Através de histonas acetiltransferases ou desacetilases (HDAC) a cromatina é descondensada ou condensada, respectivamente, e esta função está intimamente relacionada à vitalidade das CTT e quimiorresistência tumoral (Glozak and Seto, 2007; Khan and La Thangue, 2012). Neste caso, acetilação da cromatina dita o comportamento biológico dos tumores e a interferência com a HDAC altera o comportamento da CTT (Giudice et al., 2013).

Os estudos acerca das modificações pós-traducionais regulados pelos inibidores de histonas através do uso terapêutico de fármacos que inibem essas enzimas vem ganhando destaque em lesões de cabeça e pescoço. Giudice et al. (2013), em estudo

de linhagem celular de carcinoma espinocelular de cabeça e pescoço usando os níveis de acetilação da histona 3 como um marcador de compactação da cromatina, descobriram que a inibição química das HDAC reduz o número de CTT e inibe a formação de esferas clonogênicas. Com a mesma finalidade, Almeida et al. (2017) e Guimaraes et al. (2023), em estudo com linhagem celular e de xenoenxerto derivado de pacientes com CAC de glândula salivar, observaram que o Vorinostat e o Entinostat, fármacos que inibem a HDAC, junto à cisplatina, fármaco quimioterápico amplamente utilizado em tumores malignos, esgotaram eficientemente as CTT in vitro e in vivo e reduziram a viabilidade das células tumorais primárias por meio da senescência celular. Portanto, a inibição de HDAC pode constituir uma nova estratégia para interromper a população de CTT em tumores de cabeça e pescoço para criar uma população homogênea de células cancerosas com assinaturas biologicamente definidas e comportamento previsível (Almeida et al., 2017; Giudice et al., 2013; Guimarães et al., 2023).

Portanto, o presente trabalho teve como objetivo explorar as nuances de prognóstico e tratamento de lesões de Carcinoma Adenoide Cístico, definir sua importância como câncer raro e explorar novas possibilidades terapêuticas através da utilização de drogas modificadores de histona neste tumor.

2 ARTIGOS

2.1 ARTIGO 1: Spotlight on rare cancers

Spotlight on rare cancers

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In the United States, rare cancers have been defined as diseases with an incidence of fewer than 15 cases per 100,000 people per year. Combined, they affect about 40,000 people yearly and represent a quarter of all cancers in the United States. (DeSantis et al., 2017; Greenlee et al., 2010). The European Surveillance of Rare Cancers project (RARECARE) created a database used to classify rare cancers based on an incidence of fewer than 6 people per 100,000 per year (Casali & Trama, 2020). Similar to the U.S. statistics, close to 25% of all cancers in Europe are diagnosed as rare, presenting a lower survival rate of 49% compared with 63% for non-rare cancers (Gatta et al., 2017).

Managing rare cancers can be challenging. The lack of basic information on the etiopathogenesis, alongside the suboptimal genetic characterization and limited availability of research tools (e.g., animal models, cell lines, and tumor sample sizes), imposes serious challenges to advancing knowledge on rare cancers. Difficulties in recruiting patients for clinical trials are also a limitation, and when combined with other obstacles related to the study of rare cancers, many funding organizations choose to fund and focus on more prevalent diseases. Fortunately, research funding for rare cancers has recently gained significant traction, with increased funding opportunities from the National Institutes of Health and the Department of Defense. Moving to include rare cancers into the portfolio of major funding agencies is partially due to the push to classify tumors not by their histological morphology but by considering their molecular signature. As a result, rare cancers are not-so-rare, as previously suggested (Komatsubara & Carvajal, 2016). Lesions such as glandular secretory carcinoma and infantile fibrosarcoma, which have distinct histological features, could receive the same molecularly targeted therapy since both have NTRK fusion (Solomon et al., 2019). Advances in therapeutic strategies for a specific rare cancer, such as the adenoid cystic carcinoma (ACC) from the salivary glands (Ferrarotto et al., 2021), are likely to translate into effective therapy for ACC tumors from other organs, like the ones from the breast and lacrimal glands. Both tumors present distinct disease progression and survival rates from the salivary gland ACC and are, to date, vastly understudied due to their rarity.

It is of great importance to support the research of rare cancers. Support should come in different flavors, from federal agencies creating and expanding programs targeting rare tumors to non-profit foundations that are capable of mobilizing patients,

communities, and researchers towards the common goal of finding a cure to neglected tumors. Successful stories of foundations include the Adenoid Cystic Carcinoma Research Foundation (https://accrf.org/), which has changed the research landscape on ACC tumors by incentivizing collaboration among researchers. Foundations can play a crucial role in centralizing databases and support the development of core services to increase access to animal models and other research tools. Scientific publishers also play an important role in rare cancer research by creating special issues on rare tumors and continue to make available meaningful genomic information to the research community.

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Carolina Emerick: Conceptualization; data curation; formal analysis; methodology; project administration; writing – original draft; writing – review and editing. Fernanda Viviane Mariano: Conceptualization; writing – review and editing. Cristiane H Squarize: Conceptualization; writing – review and editing. Rogerio M Castilho: Conceptualization; data curation; formal analysis; methodology; project administration; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

None declared.

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2.2 ARTIGO 2: Adenoid Cystic Carcinoma from the salivary and lacrimal glands and the breast: Different clinical outcomes to the same tumor

Adenoid Cystic Carcinoma from the salivary and lacrimal glands and the breast: Different clinical outcomes to the same tumor

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Abstract

Adenoid cystic carcinoma (ACC) is a biphasic malignant lesion that can develop at various anatomical sites. Salivary and lacrimal ACC lesions have a high risk of local invasion, metastasis, and poor prognosis. In more distant organs, such as the breast, ACC is a rarer and less aggressive lesion. One of the major predictors of mortality of ACC is perineural invasion, which can be seen in 30 % of breast lesions, 85% of salivary lesions, and almost 100 % of lacrimal gland tumors. The biological differences between these three ACC tumors are still poorly understood. We focused on the current understanding of the genetic variations observed on ACC tumors and prognostic differences associated with distinct anatomical sites. A special effort was made to present the currently available therapies alongside the emerging strategies under development.

Introduction

Adenoid Cystic Carcinoma (ACC) is a biphasic malignant tumor comprised of a similar number of epithelial and myoepithelial cells presented in 3 distinct histological patterns, tubular, cribriform, or solid (Ferrarotto et al., 2021; Gatta et al., 2020) (Fig. 1). Most commonly found in the salivary gland, ACC presents a high risk for local invasion and metastatic dissemination, resulting in poor prognosis with a 10-year disease-free survival between 29 % and 50 % of all patients. With similar histological and behavioral characteristics, ACC from the lacrimal gland presents a 10-year survival rate of approximately 30 %. In tissues outside of the head and neck anatomical area, such as the breast, ACC is rare, less aggressive, and presents a 10-year survival rate of 90 % of all patients. Further, ACC from the mammary gland also presents a triple-negative subtype for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) (Andreasen et al., 2017; Andreasen et al., 2018; Rafizadeh et al., 2022; Liu et al., 2021; Ji et al., 2022). Although locally distinct, ACC from the salivary and lacrimal glands and the breast share genetic similarities. Yet, current therapeutic strategies differ from the anatomical location of the tumor. Here, we will discuss the unique aspects of ACC from each of these sites, focusing on impacts on the clinical outcome for patients.

A key point that draws attention to these tumors is the complexity of clinical management. Salivary and lacrimal ACC present high rates of perineural invasion and an increased propensity for intracranial invasion, both associated with slow and indolent growth. Interestingly, the same does not occur with breast ACC, which, although it presents a similar progression rate, its metastatic potential and perineural invasion are significantly reduced (Andreasen et al., 2018; Thompson et al., 2011). As for the histological pattern found in all three anatomical sites, cribiform is the most common histological pattern for all 3 ACC anatomical localizations (Fig. 1A and B). The solid pattern is known to be the most aggressive as it presents with myoepithelial dedifferentiation (Fig. 1D) (Zhang et al., 2020).

The genomic trademark of ACC is the fusion of the MYB and NFIB genes or, less often, MYBL1 and NFIB, both resulting from translocation t (6; 9) (Andreasen et al., 2018).

ACC is characterized by genomic stability, with few mutations and relatively small changes in the number of gene copies (Andersson et al., 2020). ACC from the salivary glands also presents a limited number of methylated gene regions, such as the apoptosis activator PITX1, Wnt/β-catenin pathway activator FOXL1, and the breast cancer-associated gene NR2F2 (Bell et al., 2011). Along with methylation, salivary ACC tumors have been studied in the context of histone modifications in which tumor cells present reduced acetylation levels while responding well to the administration of histone deacetylase inhibitors (Almeida et al., 2017). Research on lacrimal ACC has tackled the EGFR-RAS-RAF pathway as a potential therapeutic strategy as KRAS, NRAS, MET, and PIK3CA were found mutated (Bell et al., 2016). The oncogenic NOTCH signaling pathway has been explored and is involved with aberrant transcripts resulting from cytogenetically silent dissections in breast ACC (Stoeck et al., 2014). Other pathways have also been associated with breast ACC progression due to mutations directed to cell adhesion, chromatin remodeling, and activation of a canonical signaling pathway, such as BRAF, FBXW7, FGFR2, and mTOR (Martelotto et al., 2015).

Although much is reported on salivary gland ACC, information on lacrimal and breast ACC is limited in the literature. Genetic, molecular, and clinical data is scarce and does not fully explain the differences observed in the prognosis of these lesions. Thus, this study aims to discuss the differences and similarities of the salivary gland, lacrimal gland, and breast ACC that may be responsible for the distinct behavior of these tumors (Fig. 2).

Leading gene

ACC from the salivary and lacrimal glands and the breast present a common recurrent t (6;9) translocation due to the MYB-NFIB fusion genes (Andreasen et al., 2018). MYB gene is a proto-oncogene that encodes the c-MYB transcription factor, responsible for regulating cellular proliferation and differentiation, including progenitor and hematopoietic stem cells. The NFIB gene encodes a nuclear transcription factor I/B (NFI-B) involved in cellular division, differentiation, and viability (Andersson et al., 2020; Brayer et al., 2016). Upon MYB-NFIB fusion, the MYB DNA binding domain is joined to the C-terminal domain of NFIB, normally encoded by the last exon, which determines MYB overexpression in more than 50 % of ACC (Andersson et al., 2020; Mitani et al., 2011). MYB-NFIB fusion results in overactivation of the MYB gene, leading to increased oncogenic MYB fusion protein in ACC tumors (Wagner et al., 2022). In addition to fusion, MYB can be activated by obtaining a copy number or by juxtaposing other enhancer genes such as RAD51 or TGFBR39, and NFIB (Drier et al., 2016). Interestingly, high MYB expression is also found in fusion-negative tumors (Brill et al., 2011).

Some studies suggest that the MYB protein can also be activated in quantitative and qualitative ways, from MYB RNA splicing or the breakpoint of the gene rearrangement (Ji et al., 2022; O'Rourke and Ness, 2008; Togashi et al., 2018). Furthermore, MYB can have positive regulation even if the translocation occurs "far away" from its promoter region, requiring only physical interaction in that area, or if such a rearrangement region is very discrete and incompatible with a reading by karyotyping or FISH (Togashi et al., 2018). In other words, there are several breakpoints in the MYB and NFIB genes, and consequently, there is a vast possibility of transcriptional combinations between them. The molecular mechanism defining the activation of MYB by (6;9) translocation has not yet been elucidated. Some hypotheses are that the overexpression of the MYB protein may result from the breakpoint and consequent loss of a segment of the MYB gene or that yet the segment of the NFIB gene that joins in the fusion may be paramount in triggering this overexpression. Depending on the height of this breakpoint, the gene may lose the negative regulatory domain, responsible for negatively regulating the MYB-NFIB

translocation, interfering with the activity of the protein in question (Wagner et al., 2022; Corradini et al., 2005).

Brill et al. (2011) performed an immunohistochemical analysis of the MYB protein in 68 cases of ACC from the aerodigestive tract and other anatomic sites, such as the breast and lacrimal gland. Overall, 56 cases (82 %) were positive for this protein, with no differences in staining according to the patient's anatomic site or clinical characteristics. However, in correlation with histopathology, lesions with tubular differentiation showed epithelial cells with little immunostaining for MYB, while myoepithelial cells were mainly positive (Brill et al., 2011). Von Holstein (2013), using RT-PCR, observed MYB-NFIB fusion in 7 cases out of 14 lacrimal ACC. In addition, immunohistochemical staining revealed strong nuclear staining of MYB in 13 lacrimal ACCs, while FISH analysis found MYB rearrangements in 8 of 13 cases (Holstein, 2013). In a study by Poling et al. (2017), 100 % of the 11 cases of breast ACC had positive immunoexpression for MYB, with strong nuclear staining largely restricted to myoepithelial cells, and 89 % (8 in 9 patients) had detected MYB rearrangement with a FISH break-apart probe (Poling et al., 2017).

D'Alfonso et al. (2014) studied MYB-NFIB translocation in 2 morphological groups of breast ACC: the first with a tubular, cribriform, reticular, or solid histological growth pattern, the second with a solid pattern with basaloid characteristics. In the first group, MYB rearrangement was present in 5 (33.3 %) of 15 cases, while in the second, MYB was reported in 2 (12.5 %) of 16 cases (D'Alfonso et al., 2014). Brill et al. (2011) found no difference in the expression of MYB-NFIB in their study with immunohistochemical analysis that compared breast and salivary gland ACC (Brill et al., 2011). Therefore, it is believed that the clinical and prognostic differences between these two lesions are not related to MYB-NFIB fusion but rather due to other genetic changes or related to the organ in which they are involved (Brill et al., 2011; D'Alfonso et al., 2014).

Although common, MYB-NFIB fusion may not be present in all ACCs. While MYB activation is related to the tumor profile of ACC, some tumors show insignificant expression of this gene. This can be explained by acquired alternative somatic alteration, where another gene mimics the signaling exerted by the activated MYB (Gao et al., 2014). Rearrangements with the MYBL1-NFIB fusion associated with t(8; 9) and MYBL1-

YTHDF3 intra-chromosomal fusion, with the last two NFIB exons associated with gene fusion, have been described in the literature (Mitani et al., 2016).

Kim et al. (2018) presented data from 4 breast ACCs with MYBL1-NFIB fusion, with 100 % confirmation of the translocation by FISH, RT-qPCR, and RNA sequencing (Kim et al., 2018). MYBL1 gene, which encodes the A-MYB protein, has structural similarities with the MYB gene, and this fusion change may be supported by an alternative splicing system (Brayer et al., 2016; Mitani et al., 2016). West et al. (2011) performed FISH analysis and observed that from 37 salivary glands ACCs, 18 (49 %) tumors had MYB-NFIB translocation, 6 (16%) cases had a pattern suggestive of MYB translocation but not necessarily involving NFIB, and 13 (35 %) patients did not show involvement of the MYB gene in translocations. The same authors also observed that local recurrence rate in the salivary gland ACC was higher in lesions with MYB-NFIB fusion. There was a greater tendency to perineural invasion in tumors with MYB translocation. Another interesting finding is that two patients with metastatic ACC did not present MYB translocation, despite having strong MYB protein expression (West et al., 2011). Andreasen et al. (2018) conducted a study with ACC of the salivary gland, lacrimal gland, and breast. Of the 63 cases of salivary ACC, 37 (57.8 %) had MYB-NFIB fusion, 11 (17.2 %) had MYBL1-NFIB fusion, 3 (4.7 %) had MYB amplification, and 2 (3.1 %) presented isolated rearrangements of MYB-X and MYBL1-X, respectively. In lacrimal gland lesions, of the 9 cases of ACC, 5 (55.5 %) presented MYB-NFIB fusion, and 1 (11.1 %) presented MYB amplification. Finally, of the 11 mammary gland cases, 4 (40%) showed MYB-NFIB fusion, and 2 (20 %) demonstrated isolated NFIB-X rearrangement (Andreasen et al., 2018).

The biological outcome of MYB-NFIB translocation consists of the overexpression of MYB and protein overexpression irrespective of chimeric variants or breakpoints. MYB acts as a transcription factor, and its overexpression advent from MYB-NFIB translocation results in cellular proliferation, survival, and differentiation through the downstream activation of oncogenic genes (Ramsay and Gonda, 2008). Like NFIB, the rearrangement of enhancers from TGFBR3 and RAD51B and its relocation with the MYB gene lead to enhanced MYB mRNA expression (Drier et al., 2016). Although several reports come short in establishing a correlation between MYB and MYBL1 fusion and clinical outcomes,

the overexpression of MYB is well accepted to dictate poor clinical outcomes (Mitani et al., 2011; Mitani et al., 2010; Rettig et al., 2015).

Prognosis differences

The clinical outcome between salivary, breast and lacrimal ACC differs significantly despite genetic and histologic similarities. ACC from the salivary glands is characterized by aggressive behavior, presenting high recurrence rates compared with lacrimal glands and breast tumors. In addition, ACC from the salivary glands presents an overall survival rate of around 50% in 10 years (Kiss et al., 2015; Dos Santos et al., 2021). Distant metastases and local recurrence are common, especially in the lung; however, lymph node invasion is rare. Nonetheless, the clinical course in high-grade tumors tends to be accelerated and more prone to nodal metastasis (Sk´ alov´ a et al., 2018). Breast ACC has an overall survival rate of about 90 % in 10 years, characterizing a favorable prognosis for the patient (Kiss et al., 2015). Metastases and local invasion of axillary lymph nodes are extremely rare, representing less than 2% of all cases. Yet, some cases of breast ACC present local recurrence or metastasis to the lungs, bones, or kidneys (Agafonoff et al., 2019). ACC from the lacrimal glands presents the worst prognosis and the lowest survival rates from the 3 anatomical locations explored in this review (Holstein, 2013), with a 10-year survival rate of between 20 % and 30 % of the patients (Andreasen et al., 2017). Such a poor survival rate is related to intrinsic challenges for surgical ablation of the tumor associated with the anatomical site of the lacrimal glands (Le Tourneau et al., 2011). Also, the presence of distant metastasis and intracranial dissemination of the tumor contributes to poor survival (Andreasen et al., 2017).

Besides the anatomical localization of ACC tumors, the histopathological grading also plays a role in the patient's prognosis. High-grade tumors presenting greater pleomorphism are often associated with a more aggressive clinical course than less anaplastic tumors (Agafonoff et al., 2019). Salivary gland ACC displaying a solid histological pattern is known to proliferate more (Vargas et al., 2008), presenting an aggressive behavior and worst prognosis compared with the subtype of the cribriform and tubular tumor (Du et al., 2016). In a study with breast ACC patients, 48.4 % of all cases (31 cases in total) were classified histologically as cribriform, solid, or tubular. There was no presence of lymph node involvement prior to surgery during follow-up. Another 16 patients (51.6 %) had lesions presenting a basaloid pattern in the same study. Two

patients with basaloid tumors had axillary lymph node metastasis, and two others presented with recurrences of the primary tumor (D'Alfonso et al., 2014). Little information is available for lacrimal gland ACC. However, the presence of solid ACC tumors and the tumor's size are considered poor prognosis factors (Andreasen et al., 2017; Holstein, 2013).

Perineural invasion is considered one of the most significant predictors of ACC mortality (Fig. 1E). Of the 60 patients studied by Bhayani et al. (2012) in salivary ACC, 49 (82 %) presented with perineural invasion, and 6 (10%) were characterized as having a severe neural invasion (Bhayani et al., 2012). Li et al. (2012), in a study with multiple ACC sites, showed that primary tumors located in the breast were associated with approximately half the risk of death compared with primary tumors from the salivary glands (Li et al., 2012). In another report, Khanfir et al. (2012) detailed that of the 61 patients presenting breast ACC, only 5 (8.1%) had perineural invasion, while 7 (11.5%) tumors were described as poorly circumscribed (Khanfir et al., 2012). In a study with 14 lacrimal gland ACC, Holstein (2013) reported that 6 out of 8 patients (75 %) presenting periosteal or cortical involvement died, while 4 out of 6 patients (66 %) were still alive without evidence of disease, in a follow-up of up to 14 years after adequate treatment (Holstein, 2013). El-Sawy et al. (2012), after orbital exenteration with adjuvant radiotherapy of ACC lesions, reported that 10 of the 18 patients (55.6 %) were alive without evidence of the tumor, while eight patients (44.4 %) had died of cancer with an average follow-up time of 49 months. The same study showed that 13 out of 18 patients (72.2 %) had involvement of the periosteum or cortical bone (El-Sawy et al., 2012). In a study involving the three anatomical sites covered in this review, Andreasen et al., (2018) reported that, given the mean follow-up of 81, 92, and 123 months for breast, salivary gland, and lacrimal gland ACCs, respectively, the perineural invasion occurred in 30 %, 85 % and 100 % of cases. None of the 11 cases of breast ACC relapsed; however, 9 of the 64 cases of salivary gland (14 %) had local recurrence, 16 (25 %) had distant metastasis, and 27 (42 %) died of the disease. In lacrimal ACC, on the other hand, 5 out of 9 patients (56 %) presented relapses similar to the reported for salivary gland tumors (Andreasen et al., 2018).

To date, there is little understanding of molecular mechanisms that could explain the differences in the clinical course of ACC from the salivary, mammary, and lacrimal glands. The process by which tumor disseminates continues to be a significant focus of ACC research, particularly the process of perineural invasion (Amit et al., 2016; Liebig et al., 2009). In the head and neck anatomical area, lacrimal ACC tumors are often found to invade the trigeminal nerve's ophthalmic branch and disseminate to the central nerve system. Similarly, the maxillary and mandibular branches of the trigeminal nerve are often found positive for perineural invasion of ACC tumors from major and minor salivary glands. ACC tumors from the parotid glands also have a direct pathway for perineural invasion of the facial nerve (Amit et al., 2016). An emerging picture of head and neck ACC spread to the central nervous system involves the perineural invasion that initiates at the tumor microenvironment, moving towards all the interconnections from the trigeminal and facial nerves that include the auriculotemporal nerve, the greater superficial petrous nerve, and the vidian nerve and further involvement of the pterygopalatine fossa, the cavernous sinus and Meckel's cave (Amit et al., 2016; Maroldi et al., 2008; Ginsberg and DeMonte, 1998).

Breast lesions, on the other hand, are characterized by a lower level of perineural invasion, greater resistance to the dissemination of tumor cells advent from multiple layers of collagen and basal cells present in the nerve sheath, and reduced overall innervation of the breasts when compared with the head and neck anatomical area, and overall greater distance from vital organs like the central nervous system (Li et al., 2012; Khanfir et al., 2012; Amit et al., 2016).

Current treatments

ACC tumors from the salivary, lacrimal, and breast glands have been historically managed in different ways despite similar histological characteristics. Much of the consistency in breast ACC is also related to an overall low aggressive behavior and favorable clinical course. Nonetheless, some breast ACC tumors may present an aggressive behavior commonly associated with a basaloid tumor morphology (Foschini et al., 2017). Typically, ACC from the breast is managed with local excision, simple mastectomy, or mastectomy with axillary dissection (Thompson et al., 2011). There are few reports of recurrence in the literature, so some surgeons recommend mastectomy as the standard treatment for breast ACC (Boujelbene et al., 2012). In addition to the preferred surgical Management of breast ACC, radiotherapy is also considered an important alternative treatment. Khanfir et al. (2012) reported a five-year improvement of 12 % in the local, regional control of breast ACC in 66% of all patients with radiotherapy (Khanfir et al., 2012).

Along with radiotherapy, chemotherapy is also indicated for patients presenting lymph node involvement and patients with high-grade lesions (Boujelbene et al., 2012; Goldhirsch et al., 2011). The application of hormone therapy has also been considered, mainly due to the triple-negative characteristic of breast ACCs for estrogen receptors, progesterone, and HER2 receptors. However, initial trials failed to deliver positive results for hormone therapy (Boujelbene et al., 2012).

Unlike breast ACC, the clinical Management of ACC from the salivary glands remains challenging. Many of the limitations faced by surgeons are related to the scarcity of effective therapies, the difficulties associated with the anatomical location of the tumors, and the presence of myriad histological subtypes that dictate disease progression (Jiang et al., 2019). Typically, ACC from the salivary glands is managed by surgical excision and postoperative radiotherapy (Ellington et al., 2012). Radical neck dissection is also necessary in cases of lymph node metastasis (Papaspyrou et al., 2011). Although surgical Management of ACC tumors from the salivary glands aims at securing a disease-free margin, the presence of high rates of perivascular, perineural, and even bone invasion halts the effectiveness of surgical procedures as a single therapy approach. The

application of radiotherapy as a single therapeutic modality also fails to control disease progression beyond the loco-regional anatomical area effectively. Chemotherapy is usually reserved for symptomatic diseases or whose progression occurs quickly as a palliative benefit for a small portion of patients with salivary ACC (Laurie et al., 2011). Thus, there is no accepted standard systemic chemotherapy for salivary gland ACC tumors. The use of brachytherapy (iodine-125) has been explored in parotid gland ACC patients in combination with surgery. The side effects of using brachytherapy in 86 patients resulted in seven patients reporting ear discharge with tingling or hearing loss, five presented sensitive erythema, desquamation, or edema, one patient had another primary tumor, and another patient manifested skin ulceration and hoarseness. Overall, patients had long-term grade 1 skin toxicity, such as follicular erythema, desquamation, or decreased sweating. Nonetheless, using postoperative interstitial brachytherapy combined with surgery resulted in lower rates of nodal metastases, distant metastases, and radiological toxicities (Gao et al., 2021).

The management of ACC from the lacrimal glands is typically challenging due to the anatomical complexity of the area and greater chances for intracranial invasion. The standard of care consists of orbital exenteration and postoperative chemo-radiotherapy. Even so, it is still challenging to assert long-term control of a locally invasive lesion (Doddapaneni et al., 2019). Radical exenteration with bone removal is a mutilating surgery with functional and aesthetic complications. Some authors report the possibility of tumor excision en bloc that preserves the eyes and adjuvant radiotherapy in lesions confined to the orbit, without invasion to other tissues, with favorable local control and long-term survival outcomes (Han et al., 2018). Recently, these lacrimal lesions have been treated with intra-arterial cytoreductive chemotherapy, which consists of depositing the drug within the blood vessel to improve disease control and patient survival (Doddapaneni et al., 2019; Woo et al., 2016). This procedure also aims to reduce the tumor mass and facilitates surgical resection with the preservation of the eye. Unfortunately, the use of intra-arterial cytoreductive chemotherapy reduces patient tolerance to the chemotherapeutic agent and is often associated with increased side effects when compared with neoadjuvant intravenous chemotherapy (Woo et al., 2016).

In general, the development of cancer therapies is constantly hampered by the discrepancy between promising results from preclinical studies and their clinical translation, often marked by suboptimal outcomes (Doddapaneni et al., 2019; Lavareze et al., 2022). Given the MYB-NFIB translocation pattern in most ACCs, some authors explore the potential use of MYB inhibitors. Jiang et al., (2019) investigated the effectiveness of the peptidomimetic MYBMIM in salivary ACC using mass spectrometry (Jiang et al., 2019). They proposed that MYBMIM can suppress the growth and survival of MYB-activated neoplastic cell lines. Andersson et al., (2017) addressed the oncogenic signaling pathways of MYB that trigger the activation of the tyrosine kinase receptor and proteins such as IGF1R, EGFR, and MET and found targeting these pathways can reduce the growth of xenografted human ACCs. However, few approaches involve these transcriptional regulators and their direction regarding the silencing of gene expression (Andersson et al., 2017). Fibroblast growth factor receptor 1 (FGFR1) has also been studied as a therapeutic strategy for lacrimal ACC lesions combined with chemotherapeutic cisplatin, with favorable results compared to the individual use of FGFR1 inhibitor or cisplatin (Doddapaneni et al., 2019).

Some medications have also been studied to help fight recurrent or metastatic ACC based on injury to the salivary gland (Appendix tables 1 and 2). One example is Lenvatinib; a multi-purpose tyrosine kinase inhibitor used to treat thyroid and liver cancer. In a phase II study, Tchekmedyian et al., (2019) evaluated, among other variables, the overall response rate of patients after ingestion of 24 mg/day in 28-day cycles. Of the 32 patients who had recurrent or metastatic ACC, 5 (15.6 %) had a partial response to the medication, 24 (75 %) had stable disease, 2 (6.3 %) reported toxicity before the first examination, and one patient (3.1 %) had disease progression (Tchekmedyian et al., 2019). Another phase II study evaluated sorafenib, an oral multi-kinase inhibitor, by taking 800 mg/day in recurrent or metastatic ACC patients. It was observed that 46.2 % of the 23 patients had progression-free survival at 12 months. This drug has multiple routes of action, such as proliferative, apoptotic, and angiogenic, and can be more effective in combination with another chemotherapeutic agent (Thompson et al., 2011).

Emerging therapies

ACC from the salivary glands represents most of the published studies and clinical trials compared with ACC from lacrimal glands and the breast. The current standard of care for salivary ACC encompasses surgical resection that can be accompanied by radiotherapy. Unfortunately, the current therapeutic strategy falls short in preventing tumor progression despite the current need for new therapeutic approaches to manage ACC. These current clinical trials range from the delivery of monoclonal antibodies for PD-L1 to carbon ion radiation and a variety of multi-kinase inhibitors (Appendix Tables 3 and 4).

Much of the challenges in studying the biology of ACC tumors reside in the limitations of preclinical tools such as cell lines and animal models. The slow-growing pace of ACC makes it challenging to establish cell lines and further delays the studies (Andersson et al., 2020; Bell and Hanna, 2013). Patient-derived xenografts (PDX) are among the best tools for developing preclinical trials for new therapies. PDX can reproduce the morphology of ACCs and replicate the gene expression pattern found in patients as small tumor blocks are inserted into the flank of immunocompromised mice recapitulating the entire structure of tumors from the tumor stroma to the ACC histological architecture (Moskaluk et al., 2011). The recent development of a fusion-positive human ACC cell line has further enhanced the tools available for laboratory research and supported the preclinical testing of therapies targeting the MDM2-p53 pathway (Warner et al., 2018).

New clinical studies are often based on the drug's success with other tumors, such as squamous cell carcinoma and p53 mutations, and their regulation by MDM2. This tumor suppressor protein, for example, is a transcription factor regulating the cell cycle, DNA repair, and apoptosis of tumor cells, and mutations have already been identified in approximately 5 % of ACC cases. Prabakaran et al. (2017) studied the radiosensitizing effects of AMG232, an MDM2 inhibitor, which synergizes with p53 and enhances its signaling. This MDM2 inhibitor has already been studied in association with other cancer lesions, including salivary ACC; it showed improvement in tumor control in the isolated and combined treatment with radiotherapy (Appendix table 3 and 4) (Prabakaran et al.,

2017). Warner et al. (2016) analyzed the action of MI-773, an inhibitor of the MDM2-p53 interaction capable of enhancing the tumor suppressor function of p53, and observed tumor regression using 3 different PDX models of ACC (Warner et al., 2016). In another preclinical study, Nor et al. (2017) reported that MI-773 ablates ACC cancer stem cells and prevents tumor recurrence, suggesting that patients might benefit from MDM2-targeted therapies (Nor et al., 2017). A multicenter phase I/II Trial with APG-115 (analog of MI-773) is currently underway in p53 wild-type salivary gland carcinoma (NCT03781986).

Conclusion

ACC tumors from the salivary, lacrimal and mammary glands share many similarities yet present fundamental differences in the clinical behavior and response to therapy. The evidence found in the literature point towards the presence of perineural invasion as a critical determinant of tumor aggressiveness and patient survival. This evidence is clearly observed in ACC from the breast, characterized by a reduced incidence of the perineural invasion compared with tumors from the salivary and lacrimal glands.

In addition, because of the rarity and individuality of ACC tumors, most studies are carried out using salivary gland samples. More recently, we have been witnessing an increasing body of studies focusing on the biology of ACC tumors, mainly due to the emerging number of tumor cell lines available to the scientific community. Not only cell lines, but we are also observing an increased use of patient-derived xenografts (PDX) technology capable of exploring novel and emerging therapeutic strategies in a meaningful preclinical setting.

Finally, the study of rare diseases, including ACC tumors, has historically been scarce due to the rather limited funding opportunities to support meaningful mechanistic studies. However, in recent years, we are witnessing the expansion of salivary gland ACC research as reflected by the increasing number of publications and the current increased number of ongoing clinical trials. Such an emerging body of information is likely to lead to the development of ACC-focused therapies and the prospective improvement in managing this debilitating disease.

CRediT authorship contribution statement

Carolina Emerick: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. Rogerio M. Castilho: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. Fernanda Viviane Mariano: Conceptualization, Writing – review & editing. Jacques E. Nor: Conceptualization, Writing – review & editing. Pablo Agustin Vargas: Conceptualization, Writing – review & editing. Cristiane H. Squarize: Conceptualization, Writing – review & editing.

Conflict of interest statement

The authors have no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.critrevonc.2022.103792.

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Figures

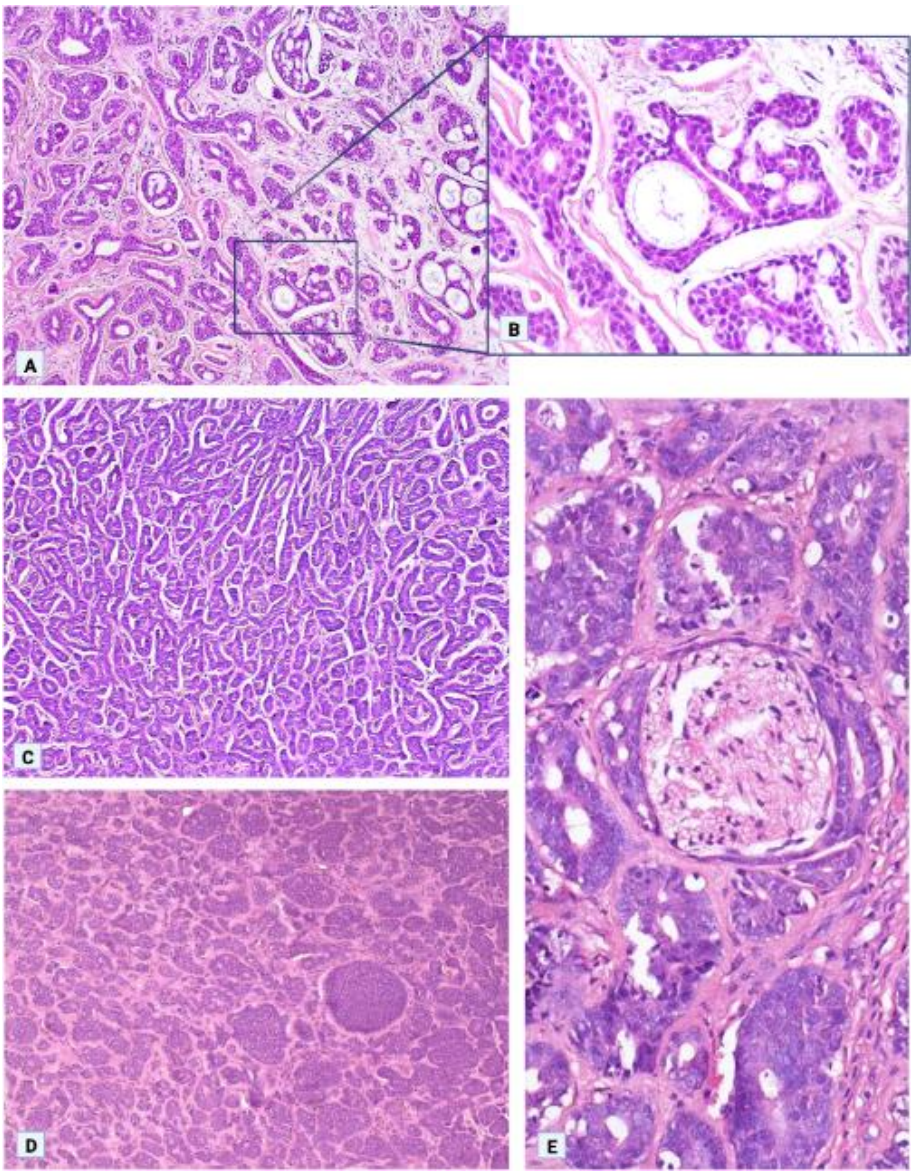

Fig. 1. Histopathologic feature of Adenoid Cystic Carcinoma (H&E). A) Cribiform patterns present cuboidal/epithelial and basaloid/myoepithelial cells with cyst-like spaces (B). C) Tubular patterns present epithelial cells with eosinophilic cytoplasm and line small lumens, while myoepithelial cells are more external and have clear cytoplasm and angular nuclei. D) Solid patterns present basaloid myoepithelial cells that form clusters of various sizes and shapes, with little tendency to structure tubular patterns or cyst-like spaces. E) Perineural invasion represented as ACC cells infiltrating the nerve.

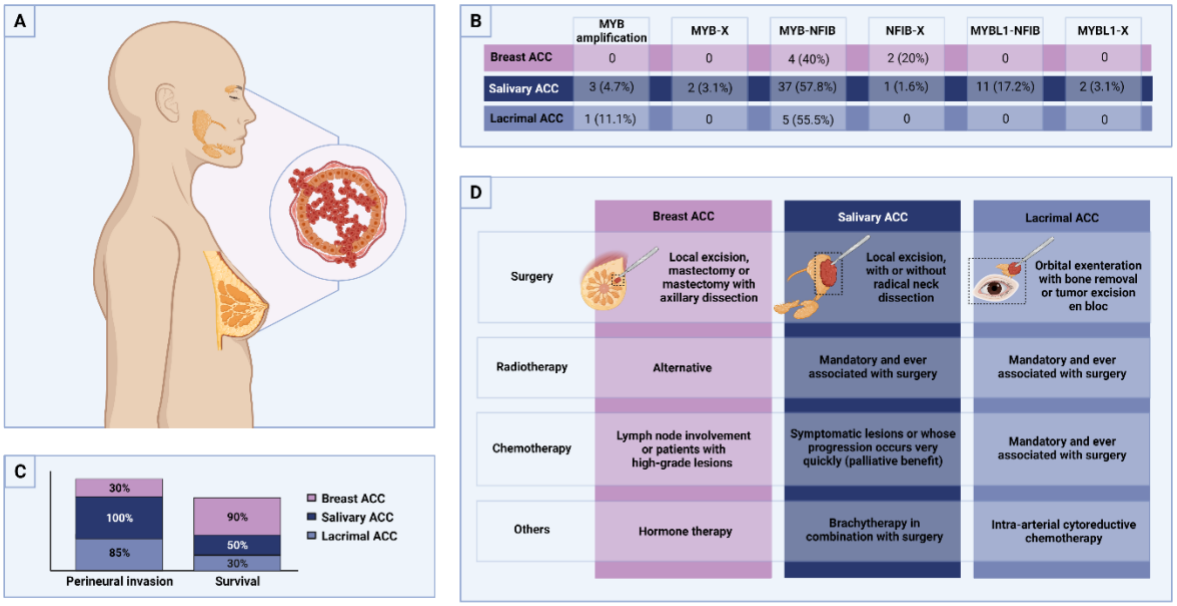

Fig. 2. Adenoid Cystic Carcinoma. A) ACC can develop in the lacrimal, salivary, and mammary glands and is characterized as a biphasic lesion involving epithelial and myoepithelial cells. B) The genomic trademark of ACC is mainly associated with the fusion of MYB and NFIB genes, resulting from translocation t(6; 9). Andreasen et al. (2018) show the genetic spectrum in the three anatomic locations. C) One of the main features of lacrimal and salivary ACC is its invasive behavior that directly affects the prognosis, while breast ACC is less aggressive and is associated with more prolonged overall patient survival. D) Multiple forms of treatment are currently implemented in ACC management. Treatment varies widely accordingly to the site of the lesion. Created with BioRender.com.

2.3 ARTIGO 3: Drug screening and contrasting sensitivities: Adenoid Cystic Carcinoma Cancer Stem Cells and tumor cells differentially react to histone modifier drugs

Drug Screening and Contrasting Sensitivities: Adenoid Cystic Carcinoma Cancer Stem Cells and Tumor Cells Differentially React to Histone Modifier Drugs

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ABSTRACT

Adenoid cystic carcinoma (ACC), a rare malignancy often treated through a multidisciplinary approach, is noted for its slow progression and aggressive behavior. This study focuses on the impact of histone modification drugs (HMD) on ACC tumors and the implications for treatment. By analyzing the effects of HMD on Cancer Stem Cells (CSC) and non-CSC tumor cells, we investigated the inhibitory effect of various histone-modifying compounds on ACC tumor cells. Our dual-pronged approach used reductions in sphere size and increased cell death as indicators of drug efficacy, employing diverse compounds targeting histones. Subsequent validation revealed promising results, with specific drugs showing significant cytotoxicity. Moreover, several as-yet untried in ACC clinical trials showed potential, notably UNC0631, a histone methyltransferase inhibitor, and ITF2357 (Givinostat), an HDAC inhibitor that exhibited significantly high cell-death percentages. Also examined was the specific application of distinct HMD to non-cancer stem cells within ACC tumors. The findings underline the importance of identifying drugs capable of targeting CSC independently from non-CSC tumor cells, as both populations of cells present contrasting sensitivities.

INTRODUCTION

Adenoid cystic carcinoma (ACC) is a rare malignant neoplasm originating mainly in the salivary glands. Known for its aggressive behavior, it has a unique clinical course, characterized by slow growth and a propensity for perineural invasion, that can lead to neurological symptoms upon the involvement of the base of the skull (Cantù, 2021a, 2021b). Despite its generally slow progression, ACC is notorious for its high rate of recurrence and metastasis, usually to distant sites such as the lungs (Alfieri et al., 2017; Emerick et al., 2022b; Sahara et al., 2021). ACC treatment strategies involve a multidisciplinary approach, including surgical resection, radiotherapy, and, in some instances, adjuvant chemotherapy (Alfieri et al., 2017; Almeida et al., 2017; da Silva et al., 2023). ACC's unpredictable and persistent nature highlights the importance of long-term monitoring (Adams et al., 2013).

Epigenetic modifications, which include the modification of histones through acetylation, methylation, phosphorylation, ubiquitylation, or sumoylation, are found deregulated in several tumors and are likely to contribute to the biology of ACC of the salivary glands (Almeida et al., 2017). Histone modifications involve chemical alterations to the proteins around which DNA is wrapped, affecting gene expression without altering the underlying genetic code (Roy et al., 2014). In ACC, these modifications significantly influence tumor development, progression, and response to treatment. For example, histone deacetylase inhibitors (HDACi), a class of drugs with emerging potential for treating ACC, work by blocking the activity of histone deacetylases, enzymes responsible for removing acetyl groups from histones. By inhibiting these enzymes, changes in histones contribute to the maintenance of the stem, self-renewal, and differentiation (Guimarães et al., 2023; Manou et al., 2023).

Cancer stem cells (CSC), also known as tumor-initiating cells, represent a distinct subpopulation within tumors with the capacity for self-renewal and differentiation into various cell types (Almeida et al., 2017; Fujita and Ikeda, 2012). These cells can exhibit a quiescent state, which makes them less susceptible to treatments that target rapidly dividing cells (Adams et al., 2013; Sahara et al., 2021). The concept of CSC challenges the traditional view of cancer by highlighting the hierarchical organization within tumors

(Cojoc et al., 2015). To better understand the population of CSC in a tumor, tumorspheres can be used to mimic the behavior of this subpopulation of cancer cells. Tumorspheres are three-dimensional structures formed by cancer cells under low adhesion culture conditions.

The transition from non-CSC tumor cell culture to tumorspheres can yield invaluable insights into the plasticity of CSC. Gaining a comprehensive understanding of the molecular mechanisms that govern stemness in CSC is crucial for developing targeted therapies. In this study, we aim to investigate the inhibitory effects of histone modification drugs (HMD) on CSC derived from ACC tumors, using both 2D and 3D cell culture techniques, resulting in the expansion of non-CSC tumor cells and the formation of tumorspheres.

MATERIALS AND METHODS

Cell line and culture conditions

The UM-HACC-2A cell line was used as described by Warner et al. (2018) (Warner et al., 2018), which was derived from a non-metastatic adenoid cystic carcinoma (ACC) located at the base of the tongue. Culturing of these cells was achieved in DMEM (Hyclone) supplemented with sodium pyruvate, 10% fetal bovine serum (Hyclone), 200 mM L-glutamine (Gibco), 400 ng/ml hydrocortisone (Sigma-Aldrich), 20 ng/ml human epidermal growth factor (Sigma-Aldrich), 5 µg/ml insulin (Sigma-Aldrich), and 1% antibiotic (Sigma-Aldrich) in 10 cm culture plates. The cells were subjected to incubation in a humidified environment with 5% CO_2 at 37ºC until achieving 70% confluence.

High-throughput drug screening using a histone modification library

The histone modification library was purchased from APExBIO (Boston, MA) and is composed of 157 compounds designed to modify histones. These compounds, initially at a concentration of 10mM dilutions in DMSO, were used at a final concentration of 10µM in the assays. An Opentrons OT-2 automated robot handled all liquid manipulations, from drug dilution to application onto the cells. Post-drug application, changes in cellular viability of non-CSC tumor cells and tumorspheres were analyzed utilizing a high-content imaging system (Molecular Devices ImageXpress Micro 4).

Tumorsphere assay and validation

An automatic liquid handler was used to seed seven thousand UM-HACC-2A cells (4,500 cells per well) into 2 ultra-low attachment round-bottom 96-well plates. The cells were left in a humidified incubator with 5% CO_2 at 37ºC for two days to facilitate sphere formation. Subsequently, epigenetic drugs were administered to the plates at a final concentration of 10µM, and the tumorspheres were observed. After a period of 48 hours, the

tumorspheres were dyed with Propidium Iodide and Hoechst 33342. Image acquisition and sphere quantification were then conducted using ImageXpress (Fig. 1).

Non-CSC tumor cells assay and validation

Ten thousand UM-HACC-2A cells were distributed (5,000 cells per well) into two flat-bottom 96-well plates using a robotic liquid handler. Following a 24-hour incubation period, the cells were exposed to the selected epigenetic drugs at a final concentration of 10µM. On the subsequent day, the cells were dyed with Propidium Iodide and Hoechst 33342, and images were taken and quantified using ImageXpress (Fig. 2).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism v.10 (GraphPad Software, CA, EUA), with data visualization facilitated by Flourish (Canvas, London, UK). In the attempt to analyze drug toxicity index, a non-paired T-test was used. The Shapiro-Wilk was applied to assess data normality when possible. All data resulted in parametric distribution. The results were shown in bar graphs represented with mean and standard deviation. The level of statistical significance was determined by Fisher's exact test at 5% and statistically significant results were marked with asterisks in the following way: * represents $p<0.05$; ** for $p<0.01$; *** when $p<0.001$; **** for $p<0.0001$; and 'ns' symbolizes a non-significant result with $p>0.05$.

RESULTS

Targeting ACC tumorspheres using HMD

Histone modification is a fundamental epigenetic mechanism that involves chemical modifications to histone proteins, influencing the accessibility of genes for transcription. In ACC, aberrant histone modifications contribute to gene expression deregulation, thus influencing tumor behavior. Our research investigated the potential inhibitory impact of 157 unique histone-modifying compounds on ACC tumorspheres. The study was underpinned by a dual-pronged approach, assessing both sphere size and cell death as indicators of drug efficacy in reducing stem cell content and increasing tumor cell death (Fig. 3). The drugs were selected based on their ability to reduce tumorsphere size and increase cell death compared to controls (highlighted in yellow). This study uses a library of compounds that target various epigenetic mechanisms. The targets included HDAC (38% of the drugs selected), bromodomains (28%), histone methyltransferases (14%), histone demethylases (5%), c-RET (5%), EZH2 (5%) and COX (5%). Modulating the epigenetic landscape of cancer cells using epi-drugs result in the interference of aberrant epigenetic remodeling events commonly found in cancer and sensitization of tumor cells to adjuvant chemoterapies (Table 1).

Identification of top HMD capable of targeting ACC tumor stem cells.

The validation process revealed that out of the 11 drugs initially chosen, 8 exhibited non-toxic characteristics towards tumor sphere formation (Fig. 4). However, only 5 out of these 8 drugs showed statistical significance ($p<0.05$). Interestingly, GSK343, a drug that targets specifically EZH2, induced a significant 35.2% of cell death, a metric more than double compared to the control group ($p<0.0001$). In a similar fashion, SGI-1027, a suppressor of histone methyltransferase, accounted for 29% of cell death ($p<0.05$). Another histone methyltransferase inhibitor, UNC0638, was responsible for 24.8% of tumorsphere cell death ($p<0.001$). The HDAC inhibitors, SB939, also identified as Pracinostat, and MS-275 also referred to as Entinostat, recorded cell death figures of 22.33% ($p<0.001$) and 20% ($p<0.05$), respectively. It’s noteworthy, however, that only the

latter two drugs are currently under investigation in clinical trials, yet none of these trials are focused on ACC.

Identification of distinct HMD capable of targeting non-CSC tumor cells.

In order to analyze the impact of epi-drugs on non-CSC tumor cells from ACC, the same histone modification library was employed. Of the 157 compounds tested, 42 exhibited a higher percentage of cell death (indicated in the yellow area) compared to the control group (depicted in the gray area) (Fig. 5). Among the selected drugs, those inhibiting HDAC were the most significant (47%), followed by inhibitors of histone methyltransferase (22%). EZH2 inhibitors accounted for 7%, sirtuin inhibitors for 6%, and inhibitors of histone demethylase, JAK, Pyk2, C-RET, EGFR, and histone acetyltransferase all each accounted for 3%, respectively.

Drug validation and the identification of one histone-modifying drug that significantly affects both, ACC tumorspheres and non-CSC tumor cells.

During the validation of the HMD, 14 drugs were selected, 9 of which were the top drugs capable of inducing toxicity to non-CSC, and 5 have been previously validated for tumorspheres. From a total of 14 validated drugs, half showed statistically significant toxicity against Non-CSC ($p<0.05$) (Fig. 6). The most significant result was the histone methyltransferase inhibitor UNC0631 that resulted in 80.5% of cell death ($p<0.0001$). Following the same inhibition target, LLY-507 and UNC0638 showed 72.6% and 67.5% cell death, respectively ($p<0.05$). ITF2357, commercially known as Givinostat, is an HDAC inhibitor and showed 78% cell death ($p<0.01$). The c-RET inhibitor TG101209 also showed promising results, with 77.67% cell death ($p<0.001$). GSK126, an EZH2 inhibitor, showed 66.83% cell death ($p<0.05$). NSC228155, an EGFR inhibitor, showed 62.5% cell death ($p<0.05$). In this selection, only three drugs (UNC0631, ITF2357, and GSK126) have clinical trial studies, but none focus on ACC. Furthermore, among the drugs tested in the tumorsphere validation, only UNC0638 had expressive results with non-CSC tumor cells (Table 2).

Current clinical trials of identified HMD

From all of the 20 validated HMD, only 8 are undergoing clinical trial studies. The drugs ITF2357 (23 studies), Entinostat (71 studies), PFI 4 (6 studies), and Pracinostat (16 studies), which have been validated in tumor spheres, have their studies focused on different diseases. On the other hand, the drugs validated for non-CSC cells, ITF2357 (23 studies), JNJ-26481585 (6 studies), Panobinostat (151 studies), 4SC-202 (3 studies), and GSK126 (1 study), have studies more focused on immune system diseases. Among these clinical studies, only Entinostat and Panobinostat, both drugs that target HDAC, have studies in head and neck cancer. Despite this, there are no clinical trials with these drugs and ACC (Tables 1 and 2).

DISCUSSION

Adenoid cystic carcinoma (ACC) is a malignancy that notably lacks an effective systemic therapy. Regrettably, there is yet to be a drug approved by the Food and Drug Administration (FDA) specifically dedicated to treating these patients. (Wagner et al., 2023). The lack of drug treatments that enhance patient survival rates can be principally attributed to an insufficient understanding of the pathophysiology surrounding Adenoid Cystic Carcinoma (ACC). For example, one key challenge is the difficulty in establishing a primary cell line culture for use in subsequent studies. Such limitations underscores the complexities faced in making significant strides towards a better understanding of ACC. (Shao et al., 2011; Warner et al., 2018).

Non-CSC tumor cell cultures involve growing cells on a flat surface, resembling a traditional two-dimensional environment. This method is essential for studying the general characteristics of cancer cells, including proliferation, apoptosis, and response to various treatments. On the other hand, tumorspheres, three-dimensional structures formed in non-adherent conditions, better mimic the subpopulation of CSC that are found within the intricate microenvironment found in tumors.

In such environments, CSC, which supposedly drive tumor initiation and progression (Sahara et al., 2021), are more likely to survive under diverse environment including low adhesion conditions and form compact, spherical structures. Tumorspheres derived from cancer cells are characterized by their capacity for self-renewal and differentiation into heterogeneous cell types, like the properties exhibited by CSC in tumors (Franco et al., 2016; Goričan et al., 2020). Researchers use sphere formation assays as a tool to isolate and study CSC in various types of cancer (Goričan et al., 2020; Ishiguro et al., 2017). By using non-CSC tumor cell cultures and tumorspheres, studies have a more comprehensive understanding the biology of ACC This dual approach is essential for revealing the heterogeneity within tumors and deciphering the distinct roles played by CSC in tumor initiation, progression, and resistance to therapy.

As we explore the biology of CSC using various experimental models, such as non-CSC tumor cell cultures and tumorspheres, the influence of histone modifications becomes an important field of study. These chemical alterations to histone proteins,

including acetylation, methylation, and phosphorylation, contribute to the dynamic regulation of chromatin structure. Here, our study explored the impact of 157 histone modifying drugs, and when compared to the normal cell death level of the ACC cell line used, only 11 (7%) drugs obtained a statistically significant result ($p<0.05$) after validation with non-CSC and tumorspheres. Our results also focused on 5 (23.8%) epigenetic targets that modify histones: histone methyltransferase, histone deacetylase (HDAC), EZH2, c-RET, and EGFR inhibitors. We discuss each of them below.

One of the most widely studied histone modification mechanisms is methylation, where methyl groups are added post-translationally to specific lysine or arginine residues in histones. This epigenetic modification plays a fundamental role in regulating gene expression and chromatin structure, influencing cellular processes such as transcription, DNA repair, and replication. Here, UM-HACC-2A cells showed more cell death when tested with histone methyltransferase inhibitors - UNC0631 (80.5% $p<0.0001$), LLY-507 (72.6% $p<0.01$) and UNC0638 (67.5% $p<0.05$) with non-CSC tumor cells and SGI-1027 (29% $p<0.05$) and UNC0638 (24.8% $p<0.001$) with tumorspheres.

UNC0631 and UNC0638 are inhibitors that target the histone lysine methyltransferase G9a, which catalyzes the methylation of histone H3 at lysine 9. By blocking G9a activity, these inhibitors aim to regulate oncogenic modifications, such as cell multiplication, invasion, autophagy, and drug resistance (Ding et al., 2013; Liu et al., 2018; Ma et al., 2020; Wei et al., 2017). In their study with cholangiocarcinoma, Ma et al. (2020) showed that UNC0631 decreased the level of H3K9me2 in human cholangiocarcinoma cells and mice (Ma et al., 2020). Liu et al. (2018) showed in their study with triple-negative breast cancer that UNC0638 also reduced the size and number of the tumorspheres, suggesting that this drug can be used as a therapeutic option against metastatic cancers (Liu et al., 2018).

LLY-507 is a selective histone lysine methyltransferase SMYD2 inhibitor responsible for the monomethylation of histone H4 on lysine 20 (Nguyen et al., 2015; Zhang et al., 2020). The inhibition of SMYD2 by LLY-507 has an anti-inflammatory effect on mouse cells and can potentially inhibit cell proliferation in non-small cell lung cancer (Munawwar et al., 2023). SGI-1027, on the other hand, is an inhibitor that targets DNA methyltransferases (DNMTs) and histone methyltransferases. By inhibiting DNMTs, SGI-

1027 contributes to DNA demethylation, while its effects on histone methyltransferases help regulate chromatin structure (Datta et al., 2009; García-Domínguez et al., 2013; Sun et al., 2018). Sun et al. (2018) demonstrated that SGI-1027 causes cell apoptosis to inhibit the proliferation of human hepatocellular carcinoma cells (Sun et al., 2018), but there is no evidence of molecular mechanisms of SGI-1027 in ACC management.

A different type of histone methyltransferase grouped as EZH2 (Enhancer of Zeste Homolog 2), also emerged in the validation of non-CSC tumor cells and tumorspheres. It catalyzes the addition of methyl groups to histone H3 on lysine 27 (H3K27), leading to the formation of a repressive chromatin state, and was described as a stemness target (Xu et al., 2019; Yu et al., 2017; Zhou et al., 2020). Here, two epigenetic drugs presented statistical results with UM-HACC-2A cells: GSK126 showed 66.83% ($p<0.05$) cell death in non-CSC cells, while GSK343 showed 35.2% ($p<0.0001$) cell death in tumorspheres. GSK126 has recently been studied as a regulator of antigen presentation in head and neck cancer, and it has been shown that combining an EZH2 inhibitor and an anti-PD-1 can decrease tumor growth (Zhou et al., 2020). On the other hand, GSK343, when applied to glioma cells, reduces the size and number of tumorspheres 5 days after treatment, suggesting that it suppresses tumor proliferation and invasion (Yu et al., 2017).

The second inhibition target that showed the most results in this study was histone deacetylase. They represent a compound class that targets the enzymes that remove acetyl groups from histone proteins. By inhibiting HDACs, they alter chromatin structure and gene expression. ITF2357/Givinostat (78% $p<0.01$) was the HDAC inhibitor that showed potential in therapy against non-CSC cells, while SB939/Pracinostat (22.33% $p<0.001$) and MS-275/Entinostat (20% $p<0.05$) showed significant results in validating ACC tumorspheres.

ITF2357/Givinostat, primarily targeting HDAC1, HDAC2, and HDAC6 (Chifotides et al., 2020), has demonstrated anti-cancer effects in preclinical and early clinical studies (Rambaldi et al., 2021; Viviani et al., 2008). Its modulation of signaling pathways involves regulating genes related to cell cycle control, apoptosis, and immune response. Givinostat has been heavily studied as a treatment for polycythemia vera, and phase I and II studies have shown a promising clinical-hematological response (Chifotides et al., 2020; Rambaldi et al., 2021).

SB939/Pracinostat inhibits HDACs class I, II, and IV, affecting cell cycle arrest, apoptosis, and differentiation-related signaling pathways (Chen et al., 2020). In a study of human colorectal cancer using mouse models, this drug has already been described as having around twice the potency of Vorinostat (Novotny-Diermayr et al., 2010). A phase I multicenter trial with Pracinostat alone and with Azacitidine showed that Pracinostat was well tolerated in patients with advanced hematological malignancies (Abaza et al., 2017). Chen et al. (2020) showed that Pracinostat had promising results against breast cancer, inhibiting tumor proliferation, migration, and invasion and presenting a more substantial anti-metastatic effect than Vorinostat in vitro and in vivo (Chen et al., 2020).

MS-275/Entinostat, capable of inhibiting HDAC1 and 3, has been investigated in various cancers, influencing signaling pathways associated with cell cycle progression, apoptosis, and immune response (Solta et al., 2023; Trapani et al., 2017). A phase II study with Entinostat and Pembrolizumab, a PD-1 inhibitor in patients with uveal melanoma, showed durable responses, remaining the importance of combining epigenetic and immunotherapy to induce tumor regression (Ny et al., 2021).

While Givinostat, Pracinostat, and Entinostat have shown promise in hematological malignancies, their specific relation to ACC is not documented. Only two clinical trials of HDACi address patients with ACC. A phase II showed stable disease in 27 of 30 patients using Vorinostat, suggesting that this drug can be included in future studies with ACC (Goncalves et al., 2017). A phase I with Vorinostat included not only ACC but a few advanced cancers and showed antitumor activity. Although these studies with Vorinostat have shown significant results, our study did not select this drug for validation. Vorinostat showed 41% cell death in non-CSC cells, while it showed 38% cell death in tumorspheres.

Another important HMD in our study, c-RET inhibitors, has emerged as a targeted therapeutic approach to block the aberrant signaling pathways associated with RET alterations (cancer-associated mutations in the rearrangement during transfection) (Salvatore et al., 2021). TG101209 is one such c-RET inhibitor that showed promise in our study with non-CSC cells, with 77.67% ($p<0.001$) cell death. TG101209 is a small molecule that inhibits JAK2 and exerts its effects on c-RET to stop the uncontrolled cell

proliferation and survival characteristics of cancer cells with RET mutations (Wang et al., 2009). Sun et al. (2011), in a study with TG101209 in mouse xenografts, presented a decrease in tumor proliferation and increased apoptosis in lung cancer models (Sun et al., 2011). This drug was developed less than 20 years ago and has no registered clinical trial despite being linked to cancer research (Pardanani et al., 2007).

Finally, NSC228155 was described as an EGFR-activating drug and showed 62.5% ($p<0.05$) of dead cells in the validation with non-CSC cells. However, the scant literature on this drug and its relationship with EGFR limited us to discussing it, and, in the only article in which it appears, it is a coadjuvant in the treatment of colorectal cancer (Chen et al., 2022). We found that EGFR activation increased DNA methyltransferase (DNMT) activity acutely. It seems to be an indirect activator of DNMT through the continuous activations of EGFR (Samudio-Ruiz and Hudson, 2012).

One of the most significant difficulties in finding therapies that can treat ACC is that, as we don't have a standard drug, there are no ethical limitations to new single-arm studies (Wagner et al., 2023). In addition, some studies include ACC with other solid tumors that have a different clinical course and prognosis, and consequently, the treatment ends up being ineffective in treating this lesion (Kelly et al., 2005). To kick-start the treatment of rare tumors, such as ACC, the US established the "Orphan Drug Act" in 1983, giving priority and exclusivity to these tumors (United States of America, 1983). Today there are four FDA-approved drugs that have benefited from this law: Dovitinib (pan-tyrosine kinase inhibitor) (2013), Para-toluenesulfonamide (carbonic anhydrase inhibitor) (2017), AL101 (gamma-secretase inhibitor) (2019) and Apatinib (tyrosine kinase inhibitor) (2021). None of them were present in this study.

The National Comprehensive Cancer Network (NCCN) recommends various chemotherapy drugs for the treatment of metastatic or unresectable salivary gland tumors, such as ACC. These drugs include paclitaxel alone or in combination with carboplatin, cisplatin in combination with vinorelbine, cisplatin in combination with doxorubicin and cyclophosphamide, or carboplatin in combination with gemcitabine (NCCN Guidelines, 2023). In addition, other drugs such as lenvatinib (Tchekmedyian et al., 2019), axitinib alone or with avelumab (Ferrarotto et al., 2023), and sorafenib (Thomson et al., 2015) are also suggested in the guidelines (NCCN Guidelines, 2023).

CONCLUSION

Although these targeted therapies are still in the early stages of investigation, exploring drugs that act against ACC by modulating histone modifications represents a promising future in searching for more effective and personalized treatment strategies for individuals facing ACC. After completing this study, it was evident that CSC and non-CSC react differently to various HMDs. This finding suggests that personalized drug therapies for solid tumors, specifically those originating from salivary glands, may need to target both cancer cell populations.

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TABLES

Table 1. HMDs selected for validation in tumorspheres with their target, percentage of cell death and information on clinical trials and FDA approval.

Name	Target	Cell death (%)	Number of clinical trials (CT)	Most studied disease (CT)	HNSCC (CT)	ACC (CT)	FDA
UNC 0631	Histone Methyltransferase	5%	0	-	0	0	No
UNC0638	Histone Methyltransferase	10%	0	-	0	0	No
ITF2357 (Givinostat)	HDAC	41%	23	Musculoskeletal diseases	0	0	No
2,4-Pyridinedicarboxylic Acid	Histone Demethylases	41%	0	-	0	0	No
Entinostat (MS-275,SNDX-275)	HDAC	41%	71	Breast cancer	2	0	No
TG101209	c-RET	42%	0	-	0	0	No
PFI 4	Bromodomain	42%	6	Drinking behavior	0	0	No
GSK343	EZH2	42%	0	-	0	0	No
NCH 51	HDAC	50%	0	-	0	0	No
Pracinostat (SB939)	HDAC	50%	16	Bone Marrow and hematologic diseases	0	0	No
SGI-1027	Histone Methyltransferase	64%	0	-	0	0	No

Table 2. HMDs selected for validation in non-CSC cells with their target, percentage of cell death and information on clinical trials and FDA approval.

Name	Target	Cell death (%)	Number of clinical trials (CT)	Most studied disease (CT)	HNSCC (CT)	ACC (CT)	FDA
UNC 0631	Histone Methyltransferase	58%	0	-	0	0	No
2,4-Pyridinedicarboxylic Acid	Histone Demethylases	58%	0	-	0	0	No
UNC0638	Histone Methyltransferase	68%	0	-	0	0	No
TG101209	c-RET	69%	0	-	0	0	No
ITF2357 (Givinostat)	HDAC	81%	23	Musculoskeletal diseases	0	0	No
JNJ-26481585	HDAC	82%	6	Immune system diseases	0	0	No
Tenovin-6	Sirtuin	83%	0	-	0	0	No
LAQ824 (NVP-LAQ824, Dacinostat)	HDAC	84%	0	-	0	0	No
Panobinostat (LBH589)	HDAC	84%	151	Immune system diseases	4	0	Yes
LLY-507	Histone Methyltransferase	84%	0	-	0	0	No
Nexturastat A	HDAC	85%	0	-	0	0	No
NSC228155	EGFR	89%	0	-	0	0	No
4SC-202	HDAC	90%	3	Neuroendocrine tumors	0	0	No
GSK126	EZH2	90%	1	Immune system diseases	0	0	No

FIGURES

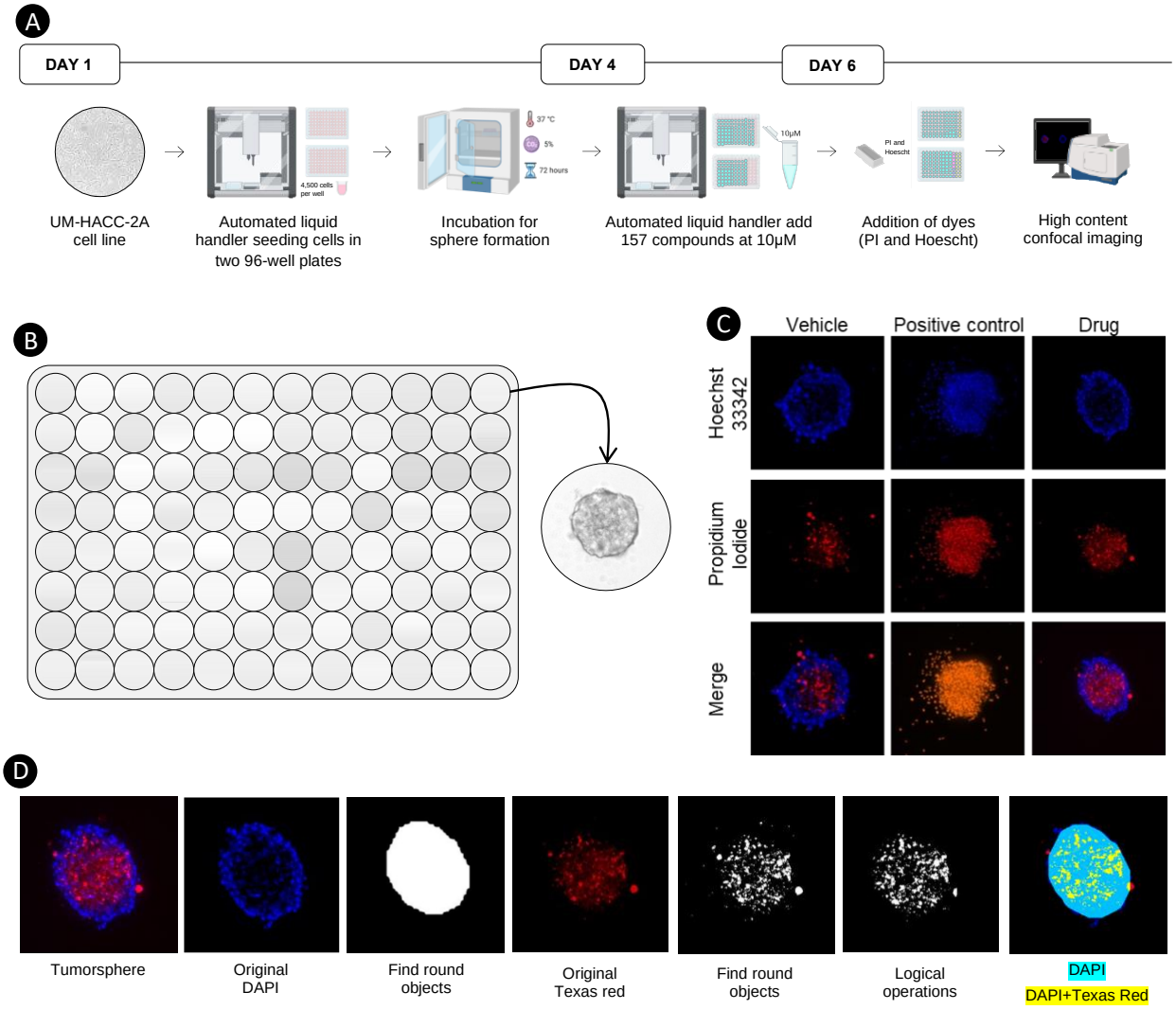

Figure 1. Tumorspheres drug screening. (A) Diagram showing the stages of the drug screening experiment, from day 1, with the addition of the cells to the 96-well plate; day 4, with the addition of the HMDs; day 6, with the addition of the dyes and acquisition of the images for analysis. (B) Representation of a 96-well plate with the formation of a sphere in each well. (C) Immunofluorescent panel representing three UM-HACC-2A tumorspheres: the first column on the left shows an untreated sphere (vehicle), where the first image is stained only with Hoescht 33342 which delimits the nucleus of the cells; the second image stained with Propidium iodide to delimit dead cells; and the third image with the sphere delimited by live and dead cells. The middle column shows a sphere with

the addition of the positive control H202, which targets cell death - note that all the cells are stained red. The third column shows a sphere treated with a drug, and more cell death can be seen compared to the first image in the third row. (D) Steps of the mask created to recognize both tumorsphere size and number of dead cells using the high-throughput system analysis software algorithms.

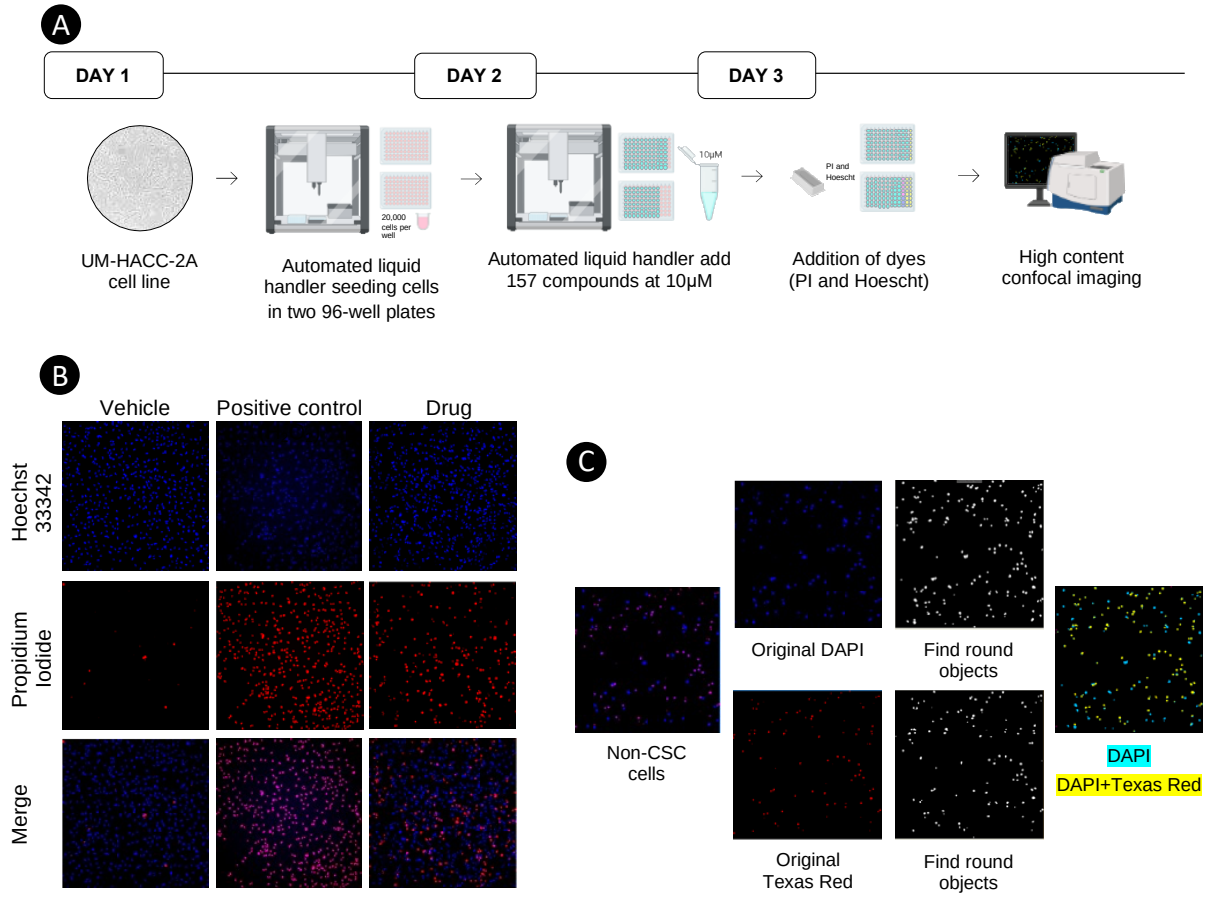

Figure 2. Non-CSC drug screening. (A) Diagram showing the stages of the drug screening experiment, from day 1, with the addition of the cells to the 96-well plate; day 2, with the addition of the HMDs; day 3, with the addition of the dyes and acquisition of the images for analysis. (B) Immunofluorescent panel showing three images containing non-CSC UM-HACC-2A cells: the first column shows untreated cells (vehicle), the second shows cells with the addition of the positive control H202 and the third shows treated cells. The first row shows Hoechst 33342 staining for cell nuclei and the second row Propidium Iodide staining for dead cells. Note that the amount of cell death is much greater in the treated cells than in the control cells (vehicle). (C) Steps of the mask created to recognize number of dead cells using the high-throughput system analysis software algorithms.

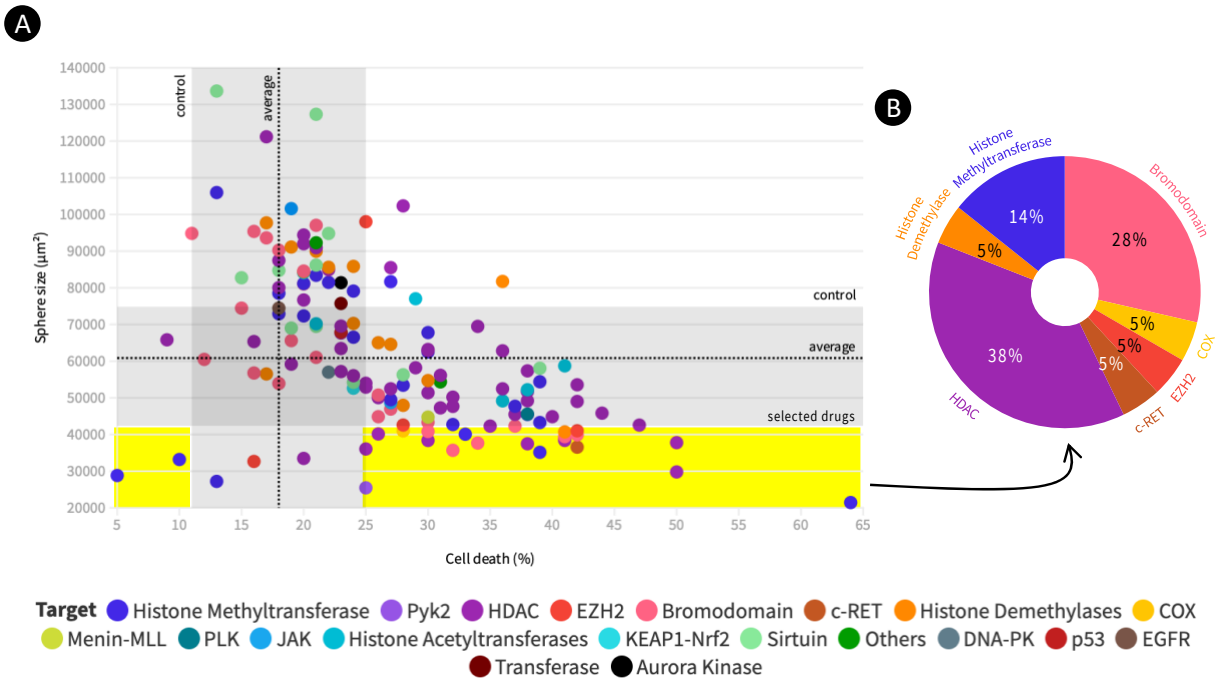

Figure 3. Panel (A) shows a scatter plot about a library of 157 HMD, where each drug is represented by a colored dot, the X-axis refers to the size of the tumorspheres when the respective drugs are added, and the Y-axis represents their relationship with cell death in UM-HACC-2A cells. The rectangles in gray represent the drug control zone, divided by a dashed line showing the average of this zone. The yellow rectangles are labeled to indicate the drugs that obtained the best results when compared to the control. Panel (B) shows a plot divided according to the therapeutic targets of the drugs present in the yellow rectangles of panel (A).

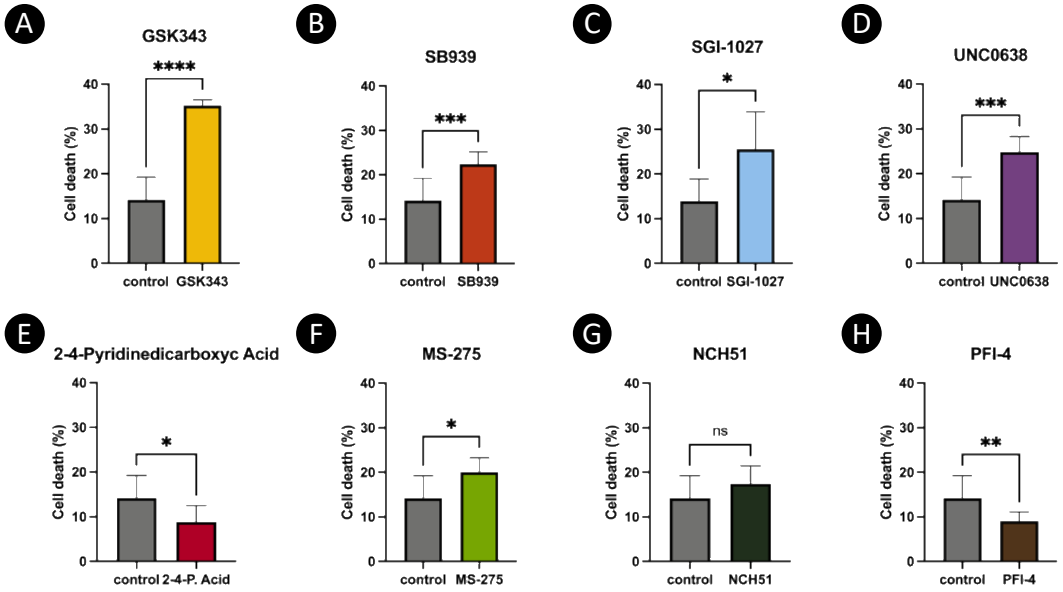

Figure 4. Each plot shows a drug result after validation with UM-HACC-2A in relation to cell death of tumorspheres. Only five drugs showed a statistically significant result: (A) GSK343 (35.2% p<0.0001); (B) SB939 (22.33% p<0.001); (C) SGI-1027 (29% (p<0.05); (D) UNC0638 (24.8% p<0.001) and (F) MS-275 (20% p<0.05). (*p<0.05; **p<0.01; *** p<0.001; ****p<0.0001; ns: p>0.05).

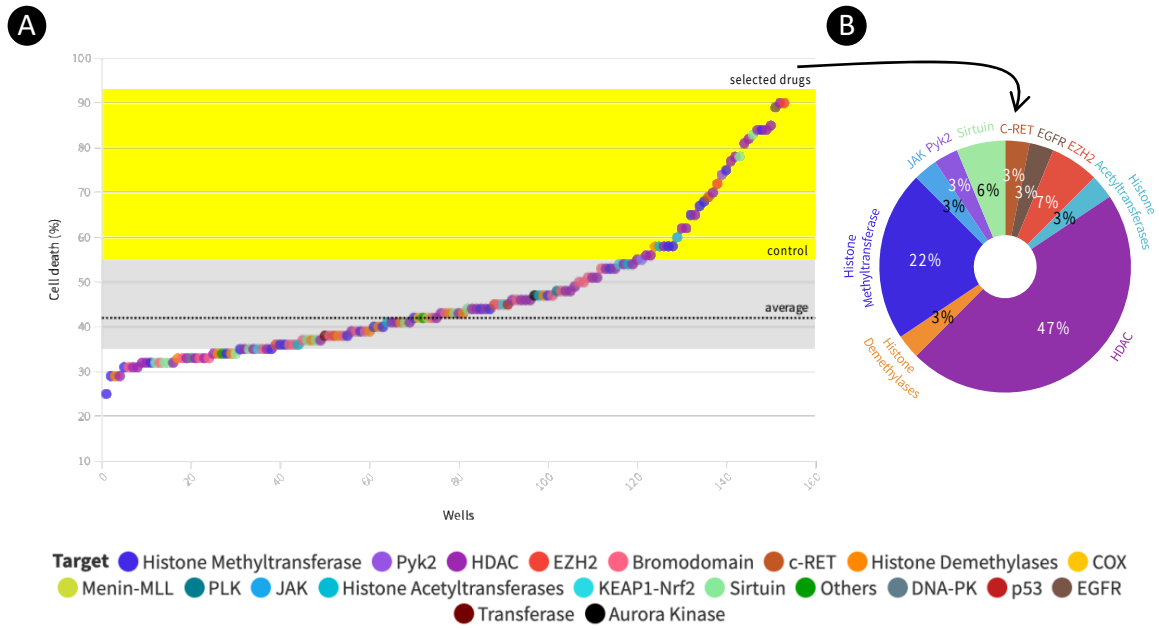

Figure 5. Panel (A) shows a scatter plot in ascending order where each colored dot represents drug after treating non-CSC UM-HACC-2A cells, the X-axis represents the level of cell death and the Y-axis each well of the 96-well plates. The gray rectangle is the control zone, and the dashed line is the mean of the control zone. The yellow rectangle represents the drugs that showed more cell death than the control. Panel (B) shows a graph divided by therapeutic targets for the drugs selected in the yellow rectangle.

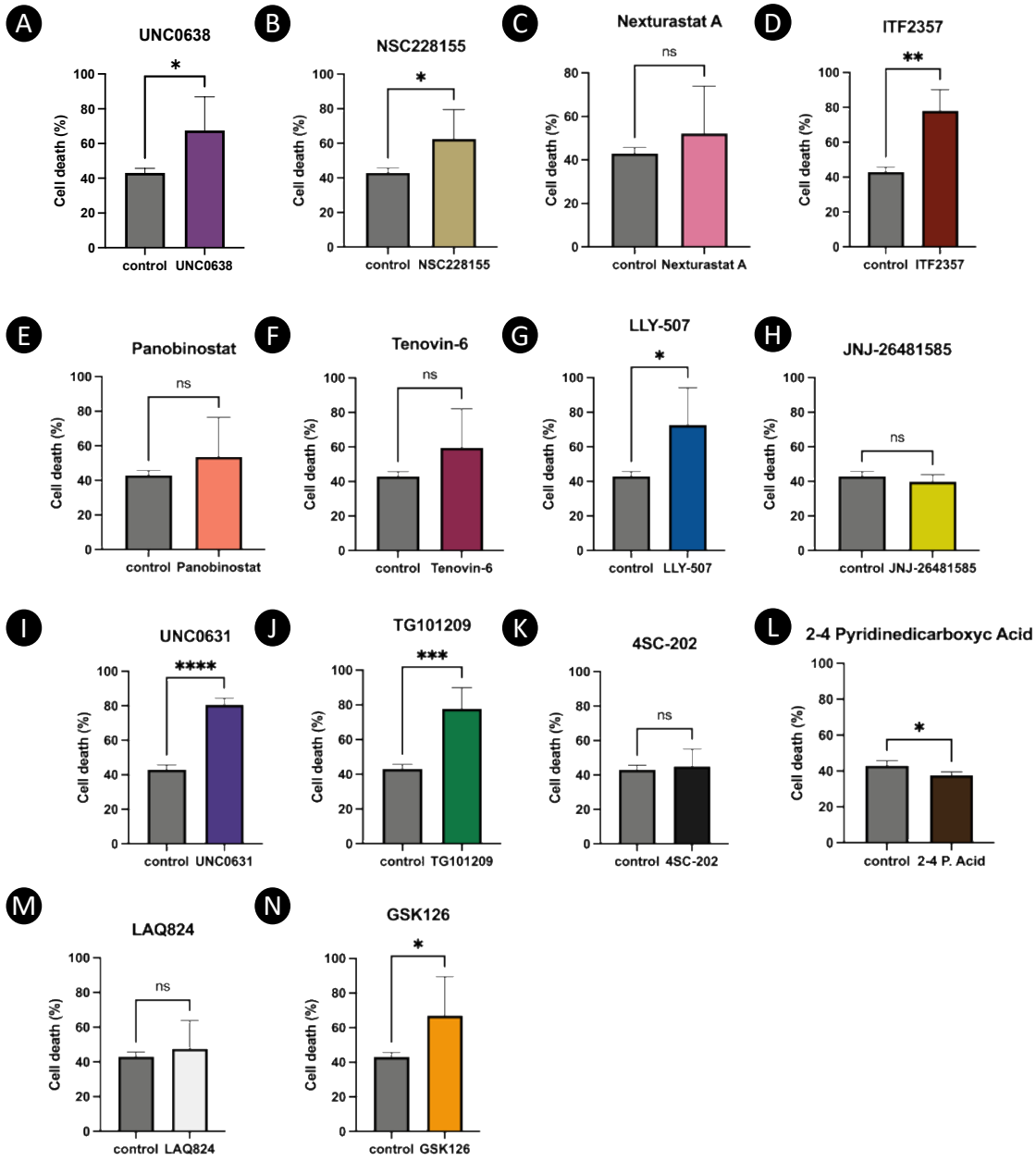

Figure 6. Each plot shows a drug result after validation with UM-HACC-2A in relation to cell death of non-CSC tumor cells. Only seven drugs showed a statistically significant result: (A) UNC0638 (67.5% $p<0.05$); (B) NSC228155 (62.5% $p<0.05$); (D) ITF2357 (78% $p<0.01$); (G) LLY-507 (72.6% $p<0.05$); (I) UNC0631 (80.5% $p<0.0001$); (J) TG101209 (77.67% $p<0.001$) and (N) GSK126 (66.83% $p<0.05$). (*$p<0.05$; **$p<0.01$; *** $p<0.001$; ****$p<0.0001$; ns: $p>0.05$).

3 DISCUSSÃO

O carcinoma adenoide cístico apresenta um curso clínico prolongado e sombrio nas glândulas salivares e lacrimais, enquanto na mama tende a ser indolente. A base genética do CAC é consistente em todos os sítios de acometimento, mas não explica as diferenças dependentes do local no comportamento clínico (Andreasen et al., 2018). O CAC é tratado principalmente com ressecção cirúrgica e radioterapia adjuvante, mas surgem desafios devido à sua propensão à invasão perineural e à extensão intracraniana. Cada vez mais, estratégias terapêuticas estão sendo exploradas para o tratamento de recorrências e metástases, enfatizando um prognóstico mais eficaz por meio da utilização de tecnologia de imagem avançada, terapias combinadas e o estudo aprofundado da biologia molecular (Ellington et al., 2012; Jiang et al., 2019; Laurie et al., 2011).

A construção desse trabalho foi baseada inteiramente nos problemas enfrentados pelos pacientes no tratamento de lesões de CAC. Primeiro, a carta ao editor “Spotlights on rare cancer” (Emerick et al., 2022a) nos guiou quanto a definição desse grupo de câncer e ilustrou como países da Europa e os Estados Unidos lidam com esse problema de saúde pública. O incentivo às pesquisas, as diretrizes dos órgãos de saúde e a condução do tratamento baseado em evidências mostram o porquê que essas regiões apresentam taxas muito melhores de sobrevida e mortalidade. Além disso, o artigo chama atenção e incentiva o leitor a mudar o foco quando se trata de alvo terapêutico, antes baseado prioritariamente no tipo histológico do tumor, convidando a olhar também para o perfil molecular das lesões. Dessa forma, tumores que apresentarem alterações genéticas e/ou epigenéticas similares podem receber terapias semelhantes.

Diante desses achados, nosso segundo passo foi reunir informações quanto ao perfil clínico, genético, de prognóstico e tratamento de lesões de CAC. A revisão “Adenoid Cystic Carcinoma from the salivary and lacrimal glands and the breast: Different clinical outcomes to the same tumor” (Emerick et al., 2022b) abordou três sítios de acometimento de CAC e destacou como o sítio anatômico pode influenciar completamente na sobrevida do paciente. As lesões de mama, em sua maioria, tendem a ser mais brandas e com curso clínico mais favorável, enquanto as lesões de glândula

salivar e lacrimal apresentam prognóstico sombrio com maiores chance de invasão perineural e metástases.

O terceiro artigo nos levou, então, a detalhar o papel de drogas epigenéticas em lesões de CAC salivar. A pesquisa, que ainda não foi publicada, teve como objetivo analisar o efeito inibitório de 157 drogas modificadoras de histonas nas CSC de CAC usando células aderidas e esferas tumorais. Nessa pesquisa, o ensaio de formação de esferas foi usado como uma ferramenta para isolar e estudar CSC em CAC. A capacidade das células cancerígenas de formar esferas tumorais é considerada um indicativo de suas propriedades semelhantes às das células-tronco. O uso de tecnologia de alto rendimento é um diferencial neste estudo pioneiro, que destacou 11 possíveis drogas com diferentes alvos que atuam na inibição de histonas. Após a conclusão desta pesquisa, ficou evidente que as CSC e as células aderidas de CAC reagem de maneira diferente a essas drogas.

Mudanças epigenéticas são descritas como qualquer mecanismo baseado na cromatina capaz de regular a expressão gênica independente de alterações na sequência de DNA. A administração de medicamentos que interfiram nas histonas já vem sido estudado há alguns anos pelo nosso grupo (Almeida et al., 2017; Giudice et al., 2013; Guimarães et al., 2023; Le et al., 2014). O uso de medicamentos de outros estudos bem-sucedidos continua sendo um norte para o tratamento de tumores sólidos como CAC (Wagner et al., 2023). As descobertas presentes nessa tese sugerem que as terapias personalizadas de medicamentos para tumores sólidos, como CAC, possivelmente requerem o desenvolvimento de uma combinação de drogas capazes de atingir ambas as populações de células cancerígenas.

4 CONCLUSÃO

As estratégias de tratamento para cânceres raros continuam sendo desafiadoras. O manejo clínico, o diagnóstico tardio e o não entendimento do curso da doença contribuem para um prognóstico ruim. O CAC apresenta-se como um tumor complexo, mas o incentivo a pesquisas que envolvem esse grupo de lesões tem feito a diferença, como vimos nos artigos descritos aqui.

A carcinogênese ocorre de forma complexa em diversas etapas por meio de muitas mutações e translocações, além de alterações epigenéticas como metilação do DNA, densidade de histonas, modificações pós-traducionais e mecanismos baseados no RNA. Atualmente, está bem estabelecido que a epigenética representa um papel crucial para o desenvolvimento do tumor. Porém, os mecanismos epigenéticos envolvidos no desenvolvimento e progressão dos tumores das glândulas salivares permanecem pouco compreendidos, incluindo os que envolvem CAC. Assim, se tornam necessários novos estudos com foco em cânceres raros, que abordem uso da epigenética como um campo promissor para entender melhor o comportamento desses tumores, além de buscar novas terapias-alvo.

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ANEXOS

ANEXO 1 – COMPROVANTE DE PUBLICAÇÃO DO ARTIGO 2.1

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ORAL DISEASES WILEY

LETTER TO THE EDITOR

Spotlight on rare cancers

In the United States, rare cancers have been defined as diseases with an incidence of fewer than 15 cases per 100,000 people per year. Combined, they affect about 40,000 people yearly and represent a quarter of all cancers in the United States. (DeSantis et al., 2017; Greenlee et al., 2010). The European Surveillance of Rare Cancers project (RARECARE) created a database used to classify rare cancers based on an incidence of fewer than 6 people per 100,000 per year (Casali & Trama, 2020). Similar to the U.S. statistics, close to 25% of all cancers in Europe are diagnosed as rare, presenting a lower survival rate of 49% compared with 63% for non-rare cancers (Gatta et al., 2017).

Managing rare cancers can be challenging. The lack of basic information on the etiopathogenesis, alongside the suboptimal genetic characterization and limited availability of research tools (e.g., animal models, cell lines, and tumor sample sizes), imposes serious challenges to advancing knowledge on rare cancers. Difficulties in recruiting patients for clinical trials are also a limitation, and when combined with other obstacles related to the study of rare cancers, many funding organizations choose to fund and focus on more prevalent diseases. Fortunately, research funding for rare cancers has recently gained significant traction, with increased funding opportunities from the National Institutes of Health and the Department of Defense. Moving to include rare cancers into the portfolio of major funding agencies is partially due to the push to classify tumors not by their histological morphology but by considering their molecular signature. As a result, rare cancers are not-so-rare, as previously suggested (Komatsubara & Carvajal, 2016). Lesions such as glandular secretory carcinoma and infantile fibrosarcoma, which have distinct histological features, could receive the same molecularly targeted therapy since both have NTRK fusion (Solomon et al., 2019). Advances in therapeutic strategies for a specific rare cancer, such as the adenoid cystic carcinoma (ACC) from the salivary glands (Ferrarotto et al., 2021), are likely to translate into effective therapy for ACC tumors from other organs, like the ones from the breast and lacrimal glands. Both tumors present distinct disease progression and survival rates from the salivary gland ACC and are, to date, vastly understudied due to their rarity.

It is of great importance to support the research of rare cancers. Support should come in different flavors, from federal agencies creating and expanding programs targeting rare tumors to non-profit foundations that are capable of mobilizing patients, communities, and researchers towards the common goal of finding a cure to neglected tumors. Successful stories of foundations include the Adenoid Cystic Carcinoma Research Foundation (https://accrf.org/), which has changed the research landscape on ACC tumors by incentivizing collaboration among researchers. Foundations can play a crucial role in centralizing databases and support the development of core services to increase access to animal models and other research tools. Scientific publishers also play an important role in rare cancer research by creating special issues on rare tumors and continue to make available meaningful genomic information to the research community.

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AUTHOR CONTRIBUTIONS

Carolina Emerick: Conceptualization; data curation; formal analysis; methodology; project administration; writing – original draft; writing – review and editing. **Fernanda Viviane Mariano:** Conceptualization; writing – review and editing. **Cristiane H Squarize:** Conceptualization; writing – review and editing. **Rogerio M Castilho:** Conceptualization; data curation; formal analysis; methodology; project administration; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST

None declared.

PEER REVIEW

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ANEXO 2 – AUTORIZAÇÃO DA EDITORA PARA A UTILIZAÇÃO DO ARTIGO 2.1

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Adenoid Cystic Carcinoma from the salivary and lacrimal glands and the breast: Different clinical outcomes to the same tumor

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ABSTRACT

Adenoid cystic carcinoma (ACC) is a biphasic malignant lesion that can develop at various anatomical sites. Salivary and lacrimal ACC lesions have a high risk of local invasion, metastasis, and poor prognosis. In more distant organs, such as the breast, ACC is a rarer and less aggressive lesion. One of the major predictors of mortality of ACC is perineural invasion, which can be seen in 30 % of breast lesions, 85% of salivary lesions, and almost 100 % of lacrimal gland tumors. The biological differences between these three ACC tumors are still poorly understood. We focused on the current understanding of the genetic variations observed on ACC tumors and prognostic differences associated with distinct anatomical sites. A special effort was made to present the currently available therapies alongside the emerging strategies under development.

1. Introduction

Adenoid Cystic Carcinoma (ACC) is a biphasic malignant tumor comprised of a similar number of epithelial and myoepithelial cells presented in 3 distinct histological patterns, tubular, cribriform, or solid (Ferrarotto et al., 2021; Gatta et al., 2020) (Fig. 1). Most commonly found in the salivary gland, ACC presents a high risk for local invasion and metastatic dissemination, resulting in poor prognosis with a 10-year disease-free survival between 29 % and 50 % of all patients. With similar histological and behavioral characteristics, ACC from the lacrimal gland presents a 10-year survival rate of approximately 30 %. In tissues outside of the head and neck anatomical area, such as the breast, ACC is rare, less aggressive, and presents a 10-year survival rate of 90 % of all patients. Further, ACC from the mammary gland also presents a triple-negative subtype for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2) (Andreasen et al., 2017; Andreasen et al., 2018; Rafizadeh et al., 2022; Liu et al., 2021; Ji et al., 2022). Although locally distinct, ACC from the salivary and lacrimal glands and the breast share genetic similarities. Yet, current therapeutic strategies differ from the anatomical location of the tumor. Here, we will discuss the unique aspects of ACC from each of these sites, focusing on impacts on the clinical outcome for patients.

A key point that draws attention to these tumors is the complexity of clinical management. Salivary and lacrimal ACC present high rates of perineural invasion and an increased propensity for intracranial invasion, both associated with slow and indolent growth. Interestingly, the same does not occur with breast ACC, which, although it presents a similar progression rate, its metastatic potential and perineural invasion are significantly reduced (Andreasen et al., 2018; Thompson et al., 2011). As for the histological pattern found in all three anatomical sites, cribiform is the most common histological pattern for all 3 ACC anatomical localizations (Fig. 1A and B). The solid pattern is known to be the most aggressive as it presents with myoepithelial dedifferentiation (Fig. 1D) (Zhang et al., 2020).

The genomic trademark of ACC is the fusion of the *MYB* and *NFIB* genes or, less often, *MYBL1* and *NFIB*, both resulting from translocation t (6; 9) (Andreasen et al., 2018). ACC is characterized by genomic stability, with few mutations and relatively small changes in the number of

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ANEXO 4 – AUTORIZAÇÃO DA EDITORA PARA A UTILIZAÇÃO DO ARTIGO 2.2

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