UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

WENDEL FERREIRA COSTA

VALIDATION OF AN ARTIFICIAL INTELLIGENCE TOOL FOR DETERMINING THE AMPLIFICATION OF HER2 GENE IN BREAST AND SALIVARY GLAND CARCINOMAS

VALIDAÇÃO DE UMA FERRAMENTA DE INTELIGÊNCIA ARTIFICIAL PARA DETERMINAÇÃO DA AMPLIFICAÇÃO DO GENE HER2 EM CARCINOMAS DE MAMA E DE GLÂNDULAS SALIVARES

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

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Orientador: Prof. Dr. Ciro Dantas Soares
Coorientador: Prof. Dr. Oslei Paes de Almeida

Este trabalho corresponde à versão final da dissertação defendida pelo aluno Wendel Ferreira Costa e orientada pelo Prof. Dr. Ciro Dantas Soares.

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RESUMO

A despeito do avanço significativo no desenvolvimento de aplicações fundamentadas em aprendizado de máquina (do inglês, *machine learning*) e sistemas automatizados de inteligência artificial (IA) para análise de imagens microscópicas patológicas, que foram implementadas com sucesso em uma extensa variedade de aplicações, a sua incorporação no campo da citogenética não foi ainda extensivamente estudada e validade. O objetivo central dessa dissertação consistiu na realização de um estudo de validação com o intuito de discernir o potencial emprego de uma metodologia de Análise Automatizada de Imagens (AAI) na avaliação da amplificação do gene Receptor 2 do Fator de Crescimento Epidérmico Humano (*HER2*) em espécimes de carcinoma ductal invasivo da mama e das glândulas salivares. Para isso, seções provenientes análises de Hibridização Fluorescente In Situ (FISH) e Imunoistoquímica (IHC) foram selecionadas – carcinomas ductais invasivos de mama (CDIM) e carcinomas do ducto salivar (CDS), atendendo aos seguintes critérios: (1) todos os casos com escore HER2 de 2+ por IHC, apresentando expressão heterogênea; (2) material disponível para a realização da análise por FISH. A amplificação do gene *HER2* foi avaliada mediante duas metodologias: avaliação visual e quantificação automatizada por meio do sistema de Imagem Espectral Aplicada (ASI), que computou a média dos sinais vermelhos e verdes detectados por FISH. O estudo compreendeu um total de 45 pacientes, dos quais 30 com CDIM e 15 com CDS. A utilização de instrumentos de pareamento tecidual (do inglês, *tissue matching*) para determinar as áreas focais nas secções correspondentes de IHC e FISH revelou-se de importância crucial. A concordância na categorização da razão *HER2*:CEP17 (amplificado versus não amplificado) entre a ferramenta de Inteligência Artificial e o consenso de especialistas alcançou um coeficiente kappa ponderado quadrático de 0,932 (Intervalo de Confiança de 95%: 0,800–1,000) para CDIM, e de 0,706 (Intervalo de Confiança de 95%: 0,343–1,000) para CDS, denotando uma concordância de boa a excelente, principalmente nos tumores mamários. Este estudo de validação validou a aplicação de ferramentas de pareamento tecidual para intensificar a detecção da amplificação do gene *HER2*, enfatizando o potencial da análise computacional em patologia. Os achados robustecem o crescente compêndio de evidências que advogam pela integração da IA como uma ferramenta auxiliar na patologia.

Palavras-chave: Citogenética; Genes erbB-2; Carcinoma ductal; Inteligência artificial; Glândulas salivares.

ABSTRACT

Despite the burgeoning development of deep learning-based applications for the analysis of pathological microscopic images, which have been efficiently implemented across a diverse array of applications, their integration into the cytogenetics domain has not been extensively realized. The primary aim of this study was to perform a validation study to determine the potential use of Automated Image Analysis (AIA) methodology for evaluating Human Epidermal growth factor receptor 2 (*HER2*) gene amplification in breast and salivary gland ductal carcinoma specimens, employing fluorescent in situ hybridization (FISH) and immunohistochemical (IHC) analyses. We selected sections of breast (n=30) and salivary invasive ductal carcinomas (n=15), considering the following criteria: (1) all cases with a HER2 2+ score by IHC, with heterogeneous expression, and (2) available material for FISH analysis. *HER2* gene amplification was evaluated using two approaches: visual assessment and automated quantification using the Applied Spectral Imaging (ASI) system, which calculates the mean of the red and green signals detected by FISH. The study included 45 patients: 30 with breast carcinoma (BC) and 15 with salivary duct carcinoma (SDC). The utilization of 'tissue matching' tools for determining hotspot areas in the corresponding IHC and FISH sections proved to be pivotal. The agreement on *HER2*:CEP17 ratio categorization (amplified vs. non-amplified) between the AI tool and expert consensus yielded a quadratic weighted kappa of 0.932 (95% Confidence Interval [CI]: 0.800–1.000) for BC and 0.706 (95% CI: 0.343–1.000) for SDC, indicating good-to-excellent concordance, predominantly in breast tumors. This validation study advocates the application of tissue-matching tools to enhance the detection of *HER2* gene amplification, thus underscoring the potential of computational analysis in pathology. These findings fortify the burgeoning corpus of evidence advocating the integration of AI in clinical diagnostics, paving the way for extended applications across diverse molecular markers and neoplastic entities, and signaling the advent of an epoch of precision medicine.

Key Words: Cytogenetics; Genes erbB-2; Carcinoma, ductal; Artificial intelligence; Salivary glands.

SUMÁRIO

1 INTRODUÇÃO

Nos últimos anos, a pesquisa no campo da oncologia tem sido notavelmente influenciada pela identificação de marcadores moleculares críticos para a compreensão dos mecanismos patogênicos subjacentes às diversas neoplasias, e por conseguinte, no desenvolvimento de terapias alvo-específicas (Naito, Urasaki, 2018; Sarhangi et al., 2022; Wahida et al., 2023). Nesse contexto, desde o advento das terapias-alvo direcionadas a tumores com amplificação no gene ERBB2 (também conhecido como HER2 – Human Epidermal growth factor Receptor 2), a técnica de hibridização *in situ* por fluorescência (FISH) tem sido indicada como a mais confiável e reprodutível para o diagnóstico dessa alteração, cuja amplificação está associada a um subconjunto de tumores caracterizados por maior agressividade (Jacquemier et al., 2013; Eswarachary et al., 2017; Figueroa-Magalhães et al., 2014; Huang et al., 2022).

A avaliação da amplificação do gene HER2, geralmente realizada através da Hibridização Fluorescente in Situ (FISH), constitui uma ferramenta molecular fundamental, especialmente no estudo do carcinoma ductal invasivo de mama (CDIM). Estudos têm expandido essa análise para outras neoplasias, incluindo o Carcinoma Ductal Salivar, ou carcinoma do ducto salivar (CDS). Devido à raridade dos CDS e à heterogeneidade desses tumores, as evidências sobre o uso do trastuzumabe nesse contexto ainda são bastante limitadas e carecem de evidências científicas robustas (Krishnamurthy et al., 2013; Takahashi et al., 2019; Wu, Quan, Han, 2019; Uijen et al., 2022; Boey et al., 2023).

O CDS, apesar de ser uma neoplasia relativamente rara, apresenta características semelhantes com o CDIM em vários aspectos histológicos e moleculares (Hosal et al., 2003; Simpson, 2013; Rooper, Gagan, Bishop, 2022).

Esta similaridade estabelece uma base importante para investigações comparativas, incluindo, mas não se limitando ao estudo da amplificação do gene HER2 por meio de estudos moleculares nestas neoplasias (Jalaly et al., 2018; Dalin et al., 2016; Balatti et al., 2019). Uma análise comparativa rigorosa sobre a prevalência desta amplificação no CDS e no carcinoma de mama pode enriquecer o entendimento molecular destas doenças, sugerindo possíveis estratégias terapêuticas convergentes.

Alguns estudos exploratórios tiveram como objetivo avaliar a expressão do receptor HER2/neu em carcinoma do ducto salivar para determinar se o trastuzumabe poderia ser uma opção terapêutica viável (Krishnamurthy et al., 2013; Takahashi et al., 2019; Wu, Quan, Han, 2019; Uijen et al., 2022; Boey et al., 2023). Dentre eles, podemos citar alguns resultados promissores, tais como os obtidos por Aoyama et al. (2023), que representa um avanço significativo para a compreensão das implicações da terapia direcionada ao HER2 em modelos de xenotransplantes e organoides derivados de CDS positivo para HER2. Este estudo fornece uma base fundamental para futuras investigações clínicas e ressalta a eficácia potencial da terapia direcionada ao HER2, sinalizando possibilidades para o manejo clínico do CDS.

Outro avanço significativo observado na área de Patologia são as ferramentas baseadas em aprendizagem de máquina, ou métodos automatizados para processamento de imagens histológicas. Dentre elas, podemos citar diversas ferramentas de inteligência artificial que têm sido amplamente utilizadas em patologia para auxiliar no trabalho de mensuração de áreas, contagem de células positivas para determinado marcador de imuno-histoquímica, dentre inúmeras outras aplicações (Wang et al., 2019; Acs,

Rantalainen, Hartman, 2020; Pallua et al., 2020; Sultan et al., 2020). No entanto, seu uso em citogenética ainda é pouco explorado.

Portanto, a análise comparativa da amplificação do gene *HER2* por FISH em CDS e CDIM pode fornecer insights substanciais sobre o perfil molecular destas doenças. O estudo deste tema visa esclarecer a prevalência da amplificação do gene *HER2*, fomentando um caminho para o desenvolvimento de estratégias terapêuticas direcionadas, com o potencial de melhorar o prognóstico e a qualidade de vida dos indivíduos afetados por esses tumores malignos agressivas.

2 ARTIGO

AUTOMATED IMAGE ANALYSIS OF FLUORESCENCE *IN SITU* HYBRIDIZATION IMPROVES THE DETECTION OF *HER2* GENE AMPLIFICATION IN BREAST AND SALIVARY CARCINOMAS: A VALIDATION STUDY

Artigo científico submetido ao periódico *Histopathology*

(Anexo 2)

Running title: *HER2* gene amplification.

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ABSTRACT

Aims: To validate an Automated Image Analysis method for assessing *HER2* gene amplification in breast and salivary gland ductal carcinomas using fluorescent *in situ* hybridization (FISH) and immunohistochemistry (IHC) slides.

Methods: It was included 45 patients in this study, with 30 diagnosed with invasive breast carcinomas (IBC) and 15 with salivary duct carcinoma (SDC). It was selected sections of invasive ductal carcinomas from the breast and salivary glands based on the following criteria: (1) Presence of HER2 2+ score by IHC with heterogeneous expression, and (2) availability of sufficient material for FISH analysis. The evaluation of *HER2* gene amplification was performed by visual inspection and an automated approach using the applied spectral imaging system. The automated method quantified the average of red and green dots in FISH images. To account for tumor heterogeneity, a 'tissue matching' tool was employed to identify corresponding hotspot areas in IHC and FISH sections.

Results: The 'tissue matching' tool proved valuable in identifying hotspot areas. For IBC, the quadratic weighted kappa between the AI tool and consultant consensus was 0.932 (95% CI: 0.800 to 1.000). For SDC, the kappa was 0.706 (95% CI: 0.343 to 1.000) when determining *HER2* gene amplification status based on the HER2:CEP17 ratio (amplified or not amplified).

Conclusion: Our study demonstrates that the automated method exhibits good-to-excellent agreement with the conventional approach for determining *HER2* gene amplification, particularly in breast tumors. We suggest considering the integration of tissue matching tools to enhance the accuracy of *HER2* gene amplification detection in future research.

Keywords: *HER2* gene amplification, invasive breast carcinoma, salivary duct carcinoma, fluorescent *in situ* hybridization, immunohistochemistry.

INTRODUCTION

The Human Epidermal Growth Factor Receptor 2 (HER2), denoted as ERBB2, represents a firmly established oncogenic entity situated on chromosome 17. Its pivotal involvement in oncogenesis is notably prominent, especially within the domain of breast cancer research.[1] The consistent observation of HER2 protein expression has consistently revealed a compelling correlation with the progression and unfavorable clinical trajectory of breast carcinoma.[1,2] Nevertheless, the precise role and implications of HER2 in the context of malignant neoplasms originating from the salivary glands have persisted as an intriguing and enigmatic subject, thereby beckoning for comprehensive scientific exploration.[3-6]

The advent of targeted therapeutic interventions, exemplified by trastuzumab (Herceptin™), has heralded a transformative era in research initiatives dedicated to unraveling the intricate patterns of *HER2* gene amplification across a spectrum of tumor types.[3,4] These investigative endeavors have predominantly centered on the evaluation of *HER2* amplification within prevalent malignancies, notably breast and gastric cancers. However, it is regrettable that the scope of research pertaining to salivary gland tumors within this context has remained conspicuously limited, thereby underscoring a notable lacuna in our comprehension of the molecular mechanisms governing these less-frequented neoplastic entities.[3-6]

Fluorescent in situ hybridization (FISH) has solidified its status as the quintessential method for delineating *HER2* gene amplification, renowned for its precision and specificity in this domain. However, it is incumbent upon us to recognize that the evaluation of *HER2* status via FISH is accompanied by considerable financial implications and is vulnerable to an array of pitfalls, spanning the spectrum from the preanalytical to the postanalytical phases of the diagnostic continuum.[7] The preeminent challenge that looms over the process of *HER2* status determination, exacerbated by the inherent tumor heterogeneity, lies in the intricate task of pinpointing the precise regions warranting analysis to ascertain the presence or absence of *HER2* amplification with unerring accuracy. This inherent complexity underscores a pivotal facet of the diagnostic conundrum.[8]

Despite witnessing a surge in development across various domains of pathological microscopic image analysis, deep learning-based applications remain an underexplored frontier when it comes to their integration into cytogenetic investigations.[7,8] Although these sophisticated methods have demonstrated notable success in diverse areas, their utilization within the purview of cytogenetics, particularly concerning the assessment of *HER2* amplification, remains notably limited.[8]

Salivary duct carcinoma (SDC) occupies a distinct niche within the tumor classification framework as established by the World Health Organization (WHO), thereby underscoring its unique pathological and molecular attributes.[9] Intriguingly, both breast and SDCs manifest convergent morphological, pathological, and molecular characteristics, chief among them being *HER2* gene amplification, thus necessitating a deeper investigation.[10] While the role of *HER2* amplification in breast carcinomas has undergone extensive scrutiny, its analogous role within the context of SDCs remains a relatively underexplored frontier within the scientific landscape. Consequently, there exists a compelling and immediate need for comprehensive research endeavors directed towards unraveling the intricacies of *HER2* amplification in the salivary counterparts.[9,10]

To bridge these critical knowledge gaps, our study harnesses a state-of-the-art automatic detection AI tool. The primary aim is to discern the HER2 amplification status in FISH images derived from both breast and salivary carcinomas. Our research endeavors to substantially elevate the precision and accuracy of diagnostic procedures, with the ultimate goal of enhancing our capacity to ascertain the *HER2* status in these malignancies. The integration of AI within this diagnostic framework harbors the potential to herald transformative advancements, thereby facilitating improved clinical decision-making and more refined therapeutic stratification.

METHODS

Case Selection and Clinicopathological Features

The surgical pathology archives of three distinct institutions, namely Getulio Sales Diagnósticos (Natal, RN, Brazil), Centro Clínico de Cabeza y Cuello (Guatemala City, Guatemala), and Piracicaba Dental School (Piracicaba, SP, Brazil), were meticulously examined in pursuit of invasive breast carcinomas

(IBC) and SDC. This extensive search encompassed the diagnostic period spanning from 2000 to 2020, aligning with the latest World Health Organization (WHO) classification guidelines.[9,10]

The case selection process adhered to two fundamental inclusion criteria, thereby underpinning the robustness and relevance of the selected cohort. The initial criterion mandated the inclusion of purely invasive carcinomas characterized by heterogeneous HER2 expression, with a conclusive score of 2+ (indicative of equivocal overexpression). The second criterion pertained to the availability of suitable tissue specimens amenable to FISH analysis.

This study was approved by the Piracicaba Dental School research ethics committee (61565722.8.1001.5418).

HER2 *immunohistochemistry and evaluation*

Sections of 4-µm from the FFPE tissues were submitted to IHC staining using pre-diluted VENTANA anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody, the most widely adopted and reliable HER2-IHC. All reactions were performed in a fully automated Ventana Benchmark XT™ platform. The reactions were analyzed manually in optical microscope and the whole-slide image (WSI) with the tool HiPath™ of the GenASIS™ software (Applied Spectral Imaging – ASI, Carlsbad, CA, USA), v. 8.3.0.59918. Initially, the cases were classified for HER2 IHC expression following the criteria of the HER2 Testing in Breast Cancer - 2023 Guideline Update.[11] Only cases with score 2+ (equivocal overexpression, with weak to moderate complete membrane staining in more than 10% of tumor cells) were considered to this study. The software classified the tumor cells into four categories (0 – no expression; 1+ – weak; 2+ – moderate, and 3+ – high HER2 expression), and the visual score was achieved by the analysis of an experienced pathologist, using the same microscope.

HER2 Fluorescent in-situ hybridization (FISH)

FISH reactions were done in the automated platform Thermobrite Elite™ (Leica Biosystems, Buffalo Grove, IL) using a FISH dual-color probe Kreatech™ ERBB2 (17q12) / SE 17 (ERBB2 (17q12) direct-labeled with PlatinumBright™550 and SE 17 specific FISH probe gene region, direct-labeled with PlatinumBright™495). Briefly, four-micron thick formalin-fixed paraffin-embedded (FFPE) sections were immersed in pretreatment Solution A (Sodium Citrate 0,01M, LK-110C) for 15 minutes. After this step, the sections were incubated with

2 × SSC and subsequent pretreatment using pretreatment solution B (Kreatech, catalog # LK-100C). The enzymatic digestion process was done with Proteinase K (Sigma Aldrich) during 5 minutes. After that, the sections were processed for denaturation (75°C for 5 min) and hybridization (37°C for 16h) and also counterstained with DAPI.

HER2 Fluorescent in-situ hybridization (FISH): image analysis

Scoring for both methods was performed on a minimal 120 nuclei. Amplification was defined when a HER2 (red)/CEP17 (green) signals ratio ≥2.0 was found or ≥6.0 red signals or clusters were detected, also following the ASCO/CAP recommendations to determine HER2 amplification.[11] Manual scoring was performed by 2 pathologists blinded to AI tool results. We used a microscope (Manual, Olympus BX43), vendor provided (Applied Spectral Imaging - Carlsbad, CA) and two Image Analysis software (GenASIs HiPath™ and PathFusion™ Imaging platform for use with HER2 FISH slides). First of all, the IHC were scanned and areas with high and low expression of HER2 were matched to FISH analysis. The process of FISH analysis is fully automated after determining the exact areas required by the operator.

Statistical analysis

Cohen's kappa coefficient was used to calculate concordance between IA tool for determining HER2 amplification and visual method using the Statistical Package for the Social Sciences (SPSS, Version 25) software.

RESULTS

Clinicopathological features

The patient cohort for this study comprised two distinct subgroups: 30 individuals diagnosed with breast duct carcinomas (with a mean age of 47 ± 15.09 standard deviation (SD) years) and 15 individuals with SDC (with a median age of 58 ± 14.05 SD years). A comprehensive repository of detailed demographic and clinical information is accessible in Table 1.

Microscopic analysis of IBC unveiled the presence of invasive tumor cells that exhibited a spectrum of morphological configurations. These cells were arranged in cords, clusters, and trabeculae, infiltrating the fibrovascular stroma. Remarkably, all cases were designated as "no special type." Furthermore, among the cases, 12 displayed the concurrent presence of intraductal carcinoma. An in-

depth evaluation of the nuclear score was executed, culminating in the classification of 21 out of 30 cases as grade 2, 9 out of 30 as grade 3, with none falling under the category of grade 1.

The SDCs showed neoplastic cells arranged in a cribriform pattern that featured the presence of comedonecrosis. Furthermore, a notable prevalence of nuclear pleomorphism and perineural invasion was discernible. In-situ components were identified in nine instances. The preponderance of these tumors earned classification as high-grade carcinomas, with seven out of fifteen cases classified as grade 2, six out of fifteen as grade 3, and two out of fifteen designated as grade 1.

HER2 *protein expression and analysis*

Our selection criteria were characterized by stringency, with a specific focus on cases manifesting a score of 2+ for HER2 protein expression, indicative of equivocal overexpression. This classification relied on the observation of weak to moderate complete membrane staining, encompassing more than 10% of tumor cells. It is noteworthy that these criteria closely align with the stipulations delineated in the "Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer: ASCO-CAP Guideline Update."

Upon subjecting the tumors to automated analysis, a predominant pattern of heterogeneity emerged in terms of HER2 protein expression profiles. Within the confines of these tumors, discrete regions exhibited distinctive HER2 immunohistochemistry (IHC) expression patterns, a visual representation of which can be gleaned from Figure 1. In view of this conspicuous heterogeneity, our investigative approach accorded primacy to the selection of areas that exhibited both high and moderate HER2 IHC expression for subsequent tissue matching analyses.

HER2 FISH: automated and manual scoring

The evaluation of HER-2/neu gene status followed immunohistochemistry (IHC) analysis, involving a meticulous matching process with the corresponding areas displaying heightened HER2 expression. Our approach identified HER2 amplification in 13 cases (43.3%) of IBC and 6 cases (40.0%) of SDCs when utilizing the AI tool. Manual analysis, in comparison, classified 12 cases (40.0%) of BC and 4 cases (26.7%) of SDC as exhibiting HER2 amplification.

Upon a comprehensive review of the entire slide sets to establish a general consensus, both groups of cases indicated that the results obtained through the AI tool were the most congruent. This led to the reclassification of one BC case and two SDC cases as HER2 amplified, compared to the initial assessments. Consequently, when considering the initial results, the concordance rate, calculated using the quadratic weighted kappa, revealed values of 0.932 (95% CI: 0.800 to 1.000) for BC and 0.706 (95% CI: 0.343 to 1.000) for SDC when comparing the AI tool and manual analysis. Discordant cases were infrequent, encompassing one IBC (3.3%) and two SDCs (6.6%). These discordant cases, however, were uniformly identified as amplified by the AI tool but classified as non-amplified upon visual analysis, underscoring the challenges associated with delineating in-situ and invasive components within FISH slides.

The summarized results regarding HER-2/neu status in IBC and SDC, as ascertained through FISH, are presented in Table 2. Additionally, the strategic utilization of the tissue matching tool, as illustrated in Figure 2, has emerged as a highly valuable approach for precisely delineating areas characterized by elevated HER2 Immunohistochemistry (IHC) expression. This process facilitates the analysis of corresponding FISH regions and enables the differentiation of in-situ and invasive components. This methodological integration has proven pivotal in achieving precise and insightful assessments of HER2 amplification status within the study specimens.

DISCUSSION

The determination of HER2 gene status via FISH represents a pivotal step that serves as a critical decision point guiding the administration of targeted therapeutic interventions, most notably trastuzumab (Herceptin™).[1-3] While HER2 gene amplification has been exhaustively investigated in the context of breast and gastric carcinomas, its precise role in salivary duct carcinoma (SDC) remains a subject of ongoing debate and scrutiny within the scientific community.[4,5,12] The present study endeavors to address this knowledge gap by conducting a comprehensive evaluation of both invasive breast carcinoma (IBC) and SDC. These two neoplastic entities, characterized by architectural and molecular similarities, were scrutinized in cases displaying equivocal IHC expression of HER2 protein, designated as a score of 2+. The study further

encompasses the validation of an Artificial Intelligence (AI) tool for determining HER2 status in these tumors.

A primary challenge encountered in the analysis of FISH slides revolves around the precise identification of areas corresponding to heightened IHC expression of HER2.[8] Consequently, the past few years have witnessed a burgeoning interest in the utilization of AI tools to address this challenge effectively. Notably, a spectrum of AI-driven solutions has emerged, focused on automating and enhancing various aspects of pathology routines. These innovations encompass improvements in scanning, visualization, and analysis of not only IHC but also conventional histology and FISH specimens.[13,14] The adoption of these tools has yielded tangible benefits, such as the attainment of more accurate results through the analysis of thousands of cells, expedited and streamlined workflow processes, and the establishment of secure archival systems to improve data retrieval for subsequent deep learning initiatives.[15]

Our innovative approach in this study centered on the validation of an automated AI tool designed to facilitate the precise alignment of areas within IHC and FISH slides. Additionally, the AI tool was employed to determine the HER2:CEP17 ratio, a pivotal metric in HER2 gene status assessment. Our findings underscore the utility of the tissue matching tool, which proved to be highly valuable in accurately delineating regions characterized by heightened IHC expression of HER2 and aligning them with corresponding areas in the FISH sections. Furthermore, our methodology encompassed a comprehensive two-step automated workflow devised for the quantification of HER2 and CEP17 signals. This approach yielded exceptionally favorable rates of concordance when compared to manual analysis.

In our study, an important observation was the presence of a heterogeneous pattern in HER2 expression within certain breast and salivary carcinomas. This heterogeneity encompassed areas displaying both amplification and non-amplified HER2 expression profiles. This phenomenon can be attributed to the concurrent presence of in-situ carcinoma alongside frankly invasive carcinoma components within the same specimens. It is imperative for pathologists to exercise diligence in distinguishing the in-situ component, which frequently exhibits robust HER2 expression (often denoted as a 3+ score) and HER2 gene amplification. Conversely, when determining the HER2 gene status,

it is essential to exclusively consider the invasive regions, disregarding the in-situ component. The tissue matching tool employed in our study played a pivotal role in achieving precise concordance, enabling the targeted visualization of these invasive areas. Our findings, in part, align with the observations made by Di Palma et al.12 in their study conducted in 2012. Their research also indicated a heightened HER2 expression within the in-situ component when compared to the frankly invasive regions, supporting the importance of discerning and excluding in-situ areas when evaluating HER2 gene status.

In our study, we encountered a limited number of discordant cases, comprising one IBC and two SDCs. Subsequent reevaluation revealed that the results obtained through the utilization of the AI tool were notably more reliable. This disparity in reliability was primarily attributable to the inherent challenges associated with visual analysis, particularly in discerning precise areas exhibiting heightened Immunohistochemistry (IHC) HER2 expression. Indeed, the intricate task of distinguishing between the in-situ and frankly invasive components within the carcinomas in the context of Fluorescent In Situ Hybridization (FISH) slides emerged as a predominant hurdle in our diagnostic process. The AI tool, with its capacity for enhanced precision and objectivity, demonstrated its potential to mitigate these challenges and yield more dependable results.

Collectively, these findings offer compelling evidence for the potential utility of the AI tool in the determination of HER2 gene status in both breast and salivary gland carcinomas. This validation study serves as the inaugural step toward the prospective integration of the AI tool into the routine practices of cytogenetics. It is vital to acknowledge, however, that our study does bear certain limitations. We must acknowledge its status as a hypothesis-generating observational study, underscoring the necessity for subsequent investigations with larger sample sizes to formally validate our initial findings. Moreover, the retrospective and multi-institutional nature of our study inherently hinders the precise estimation of the direct impact of HER2 amplification on the clinical outcomes of patients grappling with SDCs. Consequently, we underscore the urgent need for further dedicated research endeavors in this pertinent research domain to obtain more nuanced insights.

In summary, our study culminated in the successful validation of an AI tool specifically designed for the analysis of HER2 status in both salivary and breast

ductal carcinomas. As we conclude, it is imperative to emphasize the potential transformative role of future research in this domain, envisioning a future where pathologists can leverage AI-driven tools to expedite and enhance the reliability of FISH analysis, ultimately benefiting patient care and clinical decision-making.

Acknowledgements

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Author contributions

Conceptualization: CDS, AOS, PAV, OPA, FPF.

Case contribution: AOS, OPA, PAV, JCHG.

Methodology: LLS, WFC, JESA, TGFJ.

Software: CDS, AOS, TGFJ.

Resources: FPF, PAV, OPA, AOS, CDS.

Data curation: JESA, TGFJ.

Writing - original draft preparation: WFC, LLS.

Writing - review and editing: CDS, PAV, FPF, JCHG.

Supervision: CDS, AOS, OPA.

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TABLES

Table 1. Clinical and Pathologic Features of breast and salivary duct carcinomas included in this study.

Breast carcinomas		Salivary duct carcinomas	
Features	**N/Total (%)**	**Features**	**N/Total (%)**
Age, mean (SD) y	47 (15.09)	Age, mean (SD) y	58 (14.05)
Sex		Sex	
Male	0/30 (0.0%)	Male	11/15 (73.3%)
Female	30/30 (100.0%)	Female	4/15 (26.7%)
Nuclear score		Nuclear score	
1	0/30 (0.0%)	1	2 (13.3%)
2	21/30 (70.0%)	2	7 (46.7%)
3	9/30 (30.0%)	3	6 (40.0%)
In-situ component		In-situ component	
Yes	12/30 (40.0%)	Yes	9/15 (60.0%)
No	18/30 (60.0%)	No	6/15 (40.0%)
Histological subtype		Histological subtype	
No special type	27/30 (90.1%)	Sarcomatoid	4/15 (26.6%)
Oncocytic	2/30 (6.6%)	Micropapillary	3/15 (20.0%)
Lipid-rich	1/30 (3.3%)	Basal-like	2/15 (13.3%)
Glycogen-rich	0/30 (0.0%)	Oncocytic	6/15 (40.1%)

Table 2. Classification of breast carcinomas and salivary duct carcinomas (SDCs) according to the HER2 gene status by IA tool automated analysis and visual method.

Groups	IA tool		Visual	
Breast carcinomas	**n**	**%**	**n**	**%**
Non-amplified	17	56.7	18	60.0
HER2 gene amplified	13	43.3	12	40.0
Salivary duct carcinomas	**n**	**%**	**n**	**%**
Non-amplified	9	60.0	11	73.3
HER2 gene amplified	6	40.0	4	26.7

FIGURES

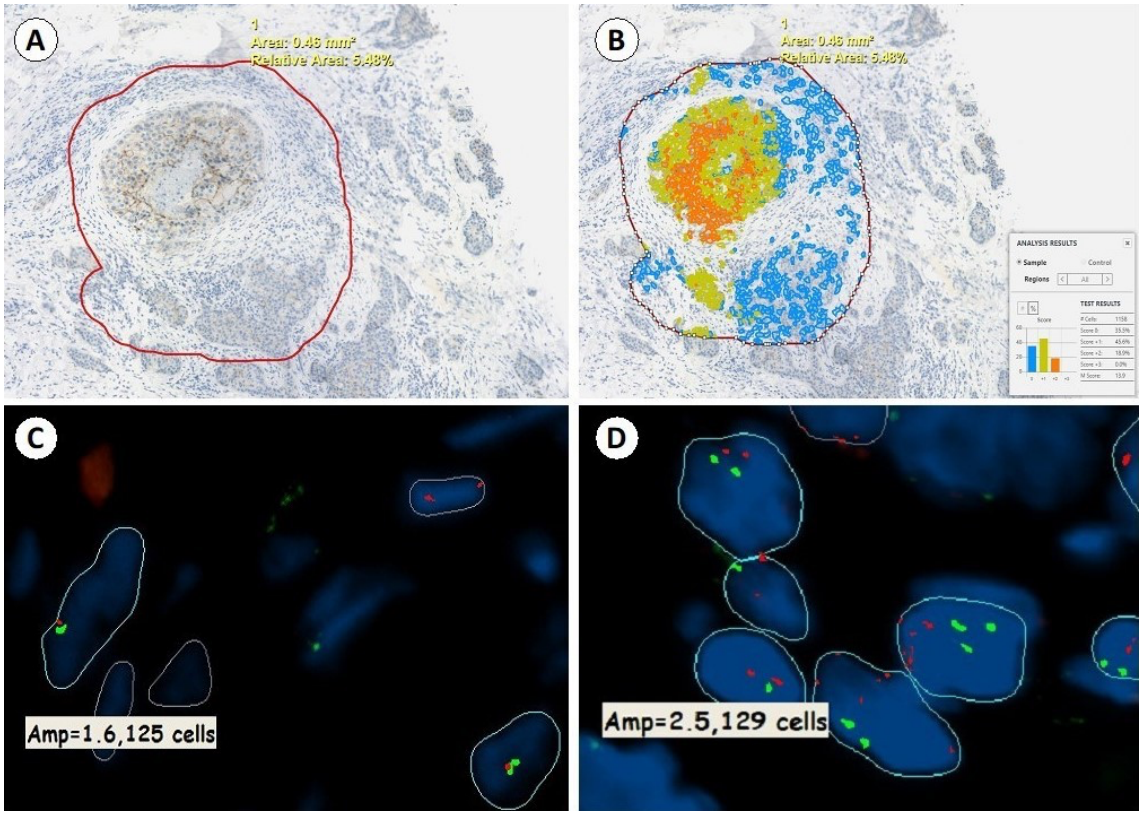

Figure 1. Complete workflow automated analysis of HER2 IHC expression (A, B), tissue matching and FISH analysis (C, D). Salivary duct carcinoma without amplification of HER-2/neu gene in the invasive component (C, HER2:CEP17 ratio=1.6) and low amplification in the in-situ component (D, HER2:CEP17

ratio=2.5). Note the mention to the number of cells and the result to these specific areas.

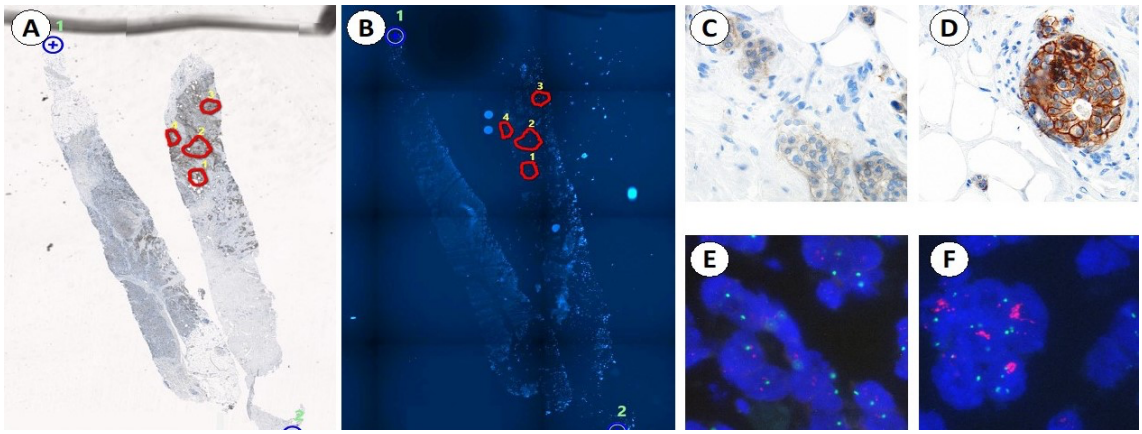

Figure 2. Complete workflow automated analysis of HER2 IHC expression in four distinct areas (A, B), tissue matching and FISH analysis. Areas 1, 2 and 3 had predominantly invasive areas, whereas area 4 had predominantly in-situ carcinoma. In this breast carcinoma, the invasive component shows no HER2 amplification (C, E) and the in-situ component shows HER2 amplification (D, F).

3 CONCLUSÃO

Como principais conclusões do presente estudo, podemos citar que:

- A validação do método automatizado baseado em Inteligência Artificial para determinar o status da amplificação do gene *HER2* em carcinomas ductais de glândulas salivares e de mama foi bem-sucedida.
- A ferramenta de pareamento (*tissue matching*) possui uma excelente capacidade de aumentar a precisão e a objetividade, gerando resultados mais confiáveis para análise das áreas francamente invasivas e intraductais, sobretudo nos carcinomas de mama.
- É essencial ressaltar, ao finalizarmos este estudo, o papel preponderante que as futuras pesquisas poderão desempenhar nesse campo, antevendo um cenário em que os patologistas possam se valer de dispositivos aprimorados por IA para otimizar e incrementar a precisão da análise por FISH, sendo uma ferramenta interessante para uso na prática de citodiagnóstico.

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ANEXOS

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Pesquisador: CIRO DANTAS SOARES

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Versão: 2

CAAE: 61565722.8.1001.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.614.313

Apresentação do Projeto:

O parecer inicial é elaborado com base na transcrição editada do conteúdo do registro do protocolo na Plataforma Brasil e dos arquivos anexados à Plataforma Brasil. Os pareceres de retorno, emendas e notificações são elaborados a partir do último parecer e dos dados e arquivos da última versão apresentada. A EQUIPE DE PESQUISA citada na capa do projeto de pesquisa inclui CIRO DANTAS SOARES (Cirurgião Dentista, Docente do PPG em Estomatopatologia da FOP-UNICAMP, Pesquisador responsável), ERICLENE FARIAS DE OLIVEIRA (Cirugião-Dentista, Mestrando no PPG em Estomatopatologia da FOP-UNICAMP), WENDEL FERREIRA COSTA (Médico Oncologista, Mestrando em Estomatopatologia da FOP-UNICAMP), OSLEI PAES DE ALMEIDA (Cirurgião-Dentista, Professor Titular da Área de Patologia da FOP-UNICAMP), o que é confirmado na declaração dos pesquisadores e na PB.

DELINEAMENTO DA PESQUISA: Trata-se de estudo laboratorial, comparativo, observacional, retrospectivo, com base em arquivos, que envolverá amostras emblocadas em parafina de 190 indivíduos adultos, diagnosticados com carcinomas de glândulas salivares ou carcinomas ductais

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Continuação do Parecer: 5.614.313

Projeto Detalhado / Brochura Investigador	1_Projeto_HER2_MestDout.pdf	15/08/2022 05:50:01	CIRO DANTAS SOARES	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	5TCLE_HER2_MestDout.pdf	15/08/2022 05:49:41	CIRO DANTAS SOARES	Aceito
Declaração de Pesquisadores	declaracao_pesquisadores.pdf	13/08/2022 00:46:35	CIRO DANTAS SOARES	Aceito
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Solicitação Assinada pelo Pesquisador Responsável	SOLICITACAO_URGENCIA_CEP_FOP UNICAMP.pdf	13/08/2022 00:31:02	CIRO DANTAS SOARES	Aceito
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Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Anexo 3 – Comprovante de submissão de artigo científico

Histopathology <no-reply@atyponrex.com>
para mim

inglês › português Traduzir mensagem

Dear Ciro Soares,

Your manuscript "Automated image analysis of fluorescence in situ hybridization improves the detection of HER2 gene amplification in breast and salivary carcinomas: a validation study" has been successfully submitted and is being delivered to the Editorial Office of *Histopathology* for consideration.

You will receive a follow-up email with further instructions from the journal editorial office, typically within one business day. That message will confirm that the editorial office has received your submission and will provide your manuscript ID.

Thank you for submitting your manuscript to *Histopathology*.

Sincerely,
The Editorial Staff at Histopathology