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MESTRADO PROFISSIONAL EM
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**THAMYRES CAMPOS FONSÊCA**

# ANÁLISE IMUNO-HISTOQUÍMICA DAS PROTEÍNAS BRCA1 E ACETIL HISTONA H3 NO CARCINOMA DE CÉLULAS ESCAMOSAS ORAL

Rio de Janeiro

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Dissertação de Mestrado apresentada ao Programa de Pós-Graduação em Odontologia, Mestrado Profissional em Clínica Odontológica, Faculdade de Odontologia, Universidade Federal do Rio de Janeiro como requisito parcial à obtenção do título de Mestre em Clínica Odontológica.

Orientadora: Profª Drª Aline Corrêa Abrahão

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Aprovada em:

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*“A única vida que tem sentido é a que se vive para os demais”*

Albert Einstein

## RESUMO

Fonsêca, Thamyres Campos. Análise imuno-histoquímica das proteínas BRCA1 e Acetil Histona H3 no carcinoma de células escamosas oral. Rio de Janeiro. 2019. Dissertação (Mestrado Profissional em Clínica Odontológica) - Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2019.

O carcinoma de células escamosas da região de cabeça e pescoço é o 4° tipo de câncer mais comum em homens no mundo. A Carcinogênese oral é caracterizada por uma heterogeneidade clínica, patológica e biológica, gerando instabilidade genômica e ocasionando a progressão e desenvolvimento de uma desordem potencialmente maligna. O mecanismo DDR (*DNA damage response*) age como um mecanismo de autodefesa responsável pela manutenção da integridade da cromatina. Sua eficácia é dependente de seu acesso ao local do dano pelas proteínas envolvidas no processo, como as histonas (proteínas que compõem os nucleossomos e que auxiliam no desempacotamento da cromatina). O desequilíbrio da acetilação e deacetilação das histonas em regiões promotoras contribui para a desregulação da expressão gênica e tem sido associado à carcinogênese e à progressão do câncer. O mecanismo DDR é composto por diversas vias de sinalização interconectadas, como as vias do BRCA1. A proteína BRCA1 regula a transcrição, o remodelamento da cromatina, a ubiquitinação e o reparo da quebra de fita dupla de DNA, entre outras funções. Sua identificação tem sido relacionada à quimiorresistência e pior prognóstico, principalmente, em carcinomas de mama e ovário. Assim, o objetivo do estudo foi avaliar a imunoexpressão das proteínas BRCA1 e Acetil Histona H3 em espécimes de biópsia incisional de carcinomas de células escamosas oral (CCEO) em língua móvel, e correlacionar sua expressão com dados epidemiológicos e com a gradação histopatológica dos tumores. Biopsias incisionais de 43 CCEO de língua móvel foram submetidas a análise imuno-histoquímica para os anticorpos BRCA1 e AcH3. As amostram foram microscopicamente subdivididas em bem diferenciado (BD) e pouco diferenciado (PD).

Todos os casos de CCEO apresentaram marcação nuclear positiva para a ACH3 e não houve correlação estatisticamente significativa entre este anticorpo e a gradação histológica ($p$=0.391). Todos os casos foram também positivos para a proteína BRCA1. O grupo PD apresentou uma expressão aberrante do BRCA1, com a maioria dos casos possuindo menos que 10% da marcação nuclear ($p$<0.01). Todos os casos do grupo PD apresentaram marcação citoplasmática ($p$=0.034). O grupo dos BD mostrou uma expressão majoritariamente nuclear com a maioria dos casos expressando mais de 50% das células positivas ($p$<0.01). Nossos resultados mostraram que as proteínas AcH3 e BRCA1 foram encontradas expressas em todos os casos de CCEO. Em geral, casos de CCEO apresentaram-se hipoacetilados. Houve uma diminuição significante na expressão da proteína BRCA1 no grupo PD em comparação ao BD. Além disso, a imunomarcação do BRCA1 nos casos PD foi exclusivamente citoplasmática. Nossos estudos corroboram com os achados de trabalhos anteriores para imuno-histoquímica de ambas proteínas BRCA1 e AcH3 em CCEO. O status hipoacetilado do CCEO evidencia a importância das mudanças epigenéticas na carcinogênese oral e na progressão tumoral como sendo um campo de investigação promissor. A imunoexpressão da proteína BRCA1 mostrou um declínio significativo nos casos PD com a proteína, em sua maioria, sendo encontrada no citoplasma. Apesar do estudo ter usado espécimes de biópsia incisional, nosso BRCA1 reproduziu o mesmo observado nos CCEO e em outras neoplasias, sugerindo a possibilidade de seu uso como um marcador prognóstico.

PALAVRAS CHAVE: HISTONAS, PROTEÍNA BRCA1, CARCINOMA DE CÉLULAS ESCAMOSAS ORAL, EPIGENÉTICA

## ABSTRACT

Fonsêca, Thamyres Campos. Análise imuno-histoquímica das proteínas BRCA1 e Acetil Histona H3 no carcinoma de células escamosas oral. Rio de Janeiro. 2019. Dissertação (Mestrado Profissional em Clínica Odontológica) - Faculdade de Odontologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 2019.

Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck region and is the fourth most prevalent cancer in men worldwide. The oral carcinogenesis is characterized by a clinical, pathological and biological heterogeneity, causing genomic instability with the progression and development of potentially malignant disorders. The DDR *(DNA damage response)* pathway acts as a mechanism of self-defense responsible for the detection of the chromatin integrity. Its efficacy is dependent on its access to the site of damage by the proteins involved in the process, such as the histones (proteins that constitute the nucleosomes and assist the packing of the chromatin). The instability of histone acetylation or deacetylation in promoter regions contribute for the dysregulation of the gene expression and has been associated to the carcinogenesis and cancer progression. The DDR mechanism is composed of several interconnected signaling pathways, such as BRCA1 pathways. The BRCA1 protein regulates transcription, chromatin remodeling and repair of double strand DNA breaks and other functions. Its identification has been related to chemoresistance and poor prognostic, especially in mama and ovarian carcinoma. The aim of the study was to evaluate the immunoexpression profile of BRCA1 and AcH3 proteins in fragments of incisional biopsy oral squamous cell carcinomas of the mobile tongue and correlate with the epidemiological data and histopathological gradation of the tumors. Incisional biopsy samples of 43 oral squamous cell carcinoma of the mobile tongue were submitted to immunohistochemical for AcH3 and BRCA1 antibodies. Samples were microscopically graded in well differentiated and the poor differentiated. All OSCC cases showed AcH3 positive nuclear expression and there was no

correlation between AcH3 and histological grading ($p$=0.391). All the OSCC cases were positive for BRCA-1 expression. The PD group showed an aberrant expression of the BRCA-1 protein, with most of the cases with less than 10% of nuclear staining ($p$<0.01). Moreover, all PD cases showed cytoplasmatic staining ($p$=0.034). The WD cases showed nuclear staining with most of the cases showing more than 50% of positive cells ($p$<0.01). Our results pointed that the AcH3 and BRCA1 protein were found to be expressed in all OSCC. In general, OSCC cases were hypoacetylated. There was a significant decrease in BRCA1 protein expression in the poor differentiated group in comparison to the well differentiated. Also, BRCA1 immunostaining was cytoplasmatic in the poor differentiated cases. Our results corroborate the few previous studies for both AcH3 and BRCA1 protein immunohistochemistry findings in OSCC. The hypoacetylated status of OSCC highlights the importance of epigenetics changes in oral carcinogenesis and tumor progression as a promising field of investigation. The BRCA1 protein imunnoexpression showed a significant decrease in the PD cases and the protein was mostly found into the cytoplasm. Although the present study used biopsy specimens, our BRCA1 results were the same observed not only in OSCC but in other cancers suggesting it as a promising prognostic marker.

KEYWORDS: HISTONES, BRCA1 PROTEIN, ORAL SQUAMOUS CELL CARCINOMA, EPIGENETICS

## LISTA DE ILUSTRAÇÕES

## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| CECP | Carcinoma de Células Escamosas de Cabeça e Pescoço |
| INCA | Instituto Nacional do Câncer |
| CCEO | Carcinoma de Células Escamosas Oral |
| DNA | Ácido Desoxirribonucleico |
| DDR | DNA Damage Response |
| AcH3 | Acetil Histone H3 |
| BRCA1 | Breast Cancer 1 |
| WD | Well differentiated |
| PD | Poor Differentiated |
| OSCC | Oral Squamous Cell Carcinoma |
| WHO | World Health Organization |
| Vetec | Vehicular Technology Conference |
| LSAB | Labelled Streptavidin-Biotin2 System |
| DAB+ | Diaminobenzidine |
| IBM | International Business Machines |
| SPSS | Statistical Package for the Social Sciences |
| HPV | Human Papilloma Virus |
| TNM | Tumor Node Metastasis |

**SUMÁRIO**

## 1. INTRODUÇÃO

O carcinoma de células escamosas da região de cabeça e pescoço (CECP) é o 4° tipo de câncer mais comum em homens no mundo, tendo sido estimados cerca de 500.000 novos casos e aproximadamente 251.000 mortes no ano de 2018 (Ferlay et al., 2018). No Brasil, as estimativas do Instituto Nacional do Câncer (INCA) para o biênio 2018-2019 preveem 634.880 casos novos de câncer no Brasil, sendo 14.700 desses lesões da cavidade oral, onde a região Sudeste é a 4ª com maior incidência. (INCA, 2018).

O carcinoma de células escamosas oral (CCEO) representa cerca de 90% de todos as neoplasias malignas da cavidade oral. Afeta principalmente adultos do sexo masculino (proporção homem:mulher de aproximadamente 2:1), usuários de tabaco e álcool, entre a sexta e oitava décadas de vida (El-Naggar, Chan, Grandis, Takata, & editor, 2017; Ferlay et al., 2018). Apesar dos fatores de risco serem bem reconhecidos, a compreensão dos mecanismos responsáveis pelo estabelecimento e progressão do CCEO (Le, Squarize, & Castilho, 2014), bem como dos mecanismos associados a resistência terapêutica e a identificação de marcadores prognósticos confiáveis ainda não estão bem estabelecidos (Mascolo et al., 2012).

O CCEO é caracterizado por uma heterogeneidade clínica, patológica e biológica (Haddad & Shin, 2008). Acredita-se que sua evolução e a progressão tumoral do CCEO resultem de sucessivas alterações celulares e moleculares controladas por eventos genéticos e epigenéticos que influenciam a expressão gênica (Giudice, Pinto, Nor, Squarize, & Castilho, 2013; Haddad & Shin, 2008), clinicamente representada pela progressão e transformação maligna de uma desordem potencialmente maligna (Haddad & Shin, 2008). Alterações epigenéticas são as mudanças celulares que ocorrem independentemente de modificações na sequência de DNA. A carcinogênese oral é influenciada por alterações epigenéticas, que incluem alterações nas histonas, proteínas diretamente envolvidas no remodelamento da

cromatina e, consequentemente, na regulação da dinâmica de expressão e silenciamento gênico (Webber et al., 2017). A compreensão da biologia molecular do CCEO aumentou significativamente nas últimas décadas, permitindo a elucidação de alterações genéticas e epigenéticas e seus respectivos papéis no desenvolvimento de diferentes tipos de câncer, incluindo o CCEO, e contribuindo para o desenvolvimento e estabelecimento de novas terapias-alvo, de drogas adjuvantes à quimioterapia, e da imunoterapia (Le et al., 2014; Patel et al., 2017).

Os medicamentos quimioterápicos à base de platina ainda são os que representam uma boa eficácia antineoplásica para o CCEO. Mesmo frente à novas terapias-alvo e imunoterapias, seu uso voltou ao cenário quimioterápico para as lesões de cabeça e pescoço, em alguns casos sendo usado como adjuvante. O mecanismo de ação ocorre pela ligação ao DNA e introdução de ligações cruzadas, resultando em dano ao DNA da célula neoplásica. Em consequência, ocorre a parada do ciclo celular e a ativação das vias de resposta ao dano no DNA (DDR – *DNA damage response*), desencadeando a apoptose (Nilsson, Huelsenbeck, & Fritz, 2011). Embora os agentes que causam danos no DNA sejam geralmente efetivos em neoplasias malignas primárias, observa-se a ocorrência de recorrência tumoral associada à quimiorresistência nos pacientes (Davis & Lin, 2011). Os mecanismos de resistência ainda não estão bem estabelecidos, podendo estar relacionados às proteínas das vias de sinalização de resposta DDR. Desse modo, a identificação de marcadores tumorais que contribuam para a predição da resposta ao tratamento quimioterápico com essa classe de medicamentos tem sido alvo de pesquisas nos mais diversos cânceres, incluindo os da região de cabeça e pescoço (Makino et al., 2010).

O mecanismo DDR é composto por diversas vias de sinalização interconectadas e sua eficácia é dependente de seu acesso ao local do dano pelas proteínas envolvidas no processo. Isso requer o desempacotamento da cromatina, que ocorre através de modificações nas histonas

(Helleday, 2008), proteínas que compõe os nucleossomos (subunidades fundamentais da cromatina) e ao redor das quais o DNA se enrola. As histonas são responsáveis pelo empacotamento do DNA e permitem que DNA de grandes dimensões se acomodem no núcleo das células. São conhecidas cinco classes de histonas (H1, H2A, H2B, H3, H4 e H5) e elas podem ser alvo de alterações pós traducionais (geralmente N-terminais), as quais podem afetar a regulação gênica de maneira epigenética, sendo as mais observadas a metilação e a acetilação. A acetilação das histonas, pela ação das acetil transferases, leva ao relaxamento da cromatina, permitindo a transcrição gênica. A reversão desse processo, com consequente empacotamento da cromatina e supressão da transcrição, ocorre através da ação da enzima histona deacetilase ou por metilação. Esse mecanismo é um importante regulador transcricional, possibilitando a transcrição de cromatinas hiperacetiladas, mas cromatinas hipoacetiladas não. Falhas nesse processo levam à instabilidade da cromatina, ao acúmulo do dano ao DNA com a incorporação de mutações, à instabilidade genômica e, por fim, ao desenvolvimento/progressão do câncer. Inibidores das histonas deacetilases têm se mostrado importantes agentes antineoplásicos, demonstrando atividade contra diversos tipos de câncer, bem como efeitos na proliferação celular, apoptose, diferenciação e angiogênese *in vitro* e *in vivo*. (Ferrari & Strubin, 2015; Helleday, 2008; Wu et al., 2011).

A acetilação das histonas H2A e H3 já foi amplamente demonstrada na literatura em diversos tipos de câncer, bem como mutações ou translocações cromossomais nos genes da histona acetil transferase e da histona acetil deacetilase, já foram demonstrados em alguns tipos de leucemias e linfomas, câncer de cólon e câncer gástrico (Costelloe, Fitzgerald, Murphy, Flaus, & Lowndes, 2006; Ferrari & Strubin, 2015; Polo & Jackson, 2011). Webber et al. (2017) demonstraram haver associação entre a hipoacetilação da histona H3 e o pior prognóstico dos pacientes com CCEO. As modificações nas histonas poderiam estar associadas ao desenvolvimento das desordens potencialmente malignas e aos estágios iniciais da

carcinogênese oral, levando à alterações gênicas subsequentes que permitiriam a proliferação e o crescimento tumoral do CCEO (Webber et al., 2017).

O gene *BRCA1* (*breast cancer 1)* é um gene supressor tumoral localizado no cromossoma 17q21, que codifica a proteína BRCA1. Estudos *in vitro* mostraram que células deficientes em BRCA1 ou BRCA2 (*breast cancer 2)* exibem deficiência no reparo da dupla-fita de DNA por recombinação homóloga (Rego, Harney, Mauro, Shen, & Howlett, 2012). Mutações germinativas heterozigóticas no gene *BRCA1* em mulheres levam à um risco elevado de desenvolver câncer de mama e/ou de ovário. Disfunções na proteína BRCA1 são frequentemente observadas no carcinoma de ovário esporádico, e também em outros cânceres, como o de próstata, o colorretal e o melanoma. (Chen et al., 1995; Lee, Jin, Chang, Lin, & Su, 1999). Em conjunto com outras proteínas, a BRCA1 regula a transcrição, o remodelamento da cromatina, a ubiquitinação e o reparo da quebra da dupla fita de DNA induzida por ligações cruzadas, radiação e outros agentes por recombinação homóloga (Lesnock et al., 2013). Pelo fato da proteína BRCA1 poder estar alterada de diversas formas no câncer, existe um grande interesse na investigação de sua disfunção na patogênese e na sensibilidade à quimioterapia, onde estudos prévios sugerem que a baixa expressão do BRCA1 pode aumentar a sobrevida dos pacientes após a terapia com agentes a base de platina (ou baseada no dano de DNA) e reduzir a resposta a outros agentes quimioterápicos no carcinoma de ovário (Lesnock et al., 2013), representando um potencial marcador para escolha do melhor tratamento para o paciente.

Apesar da proteína BRCA1 localizar-se no núcleo celular, Chen et al. (1995) apresentaram um primeiro relato de localização aberrante dessa proteína no citoplasma de células de câncer de mama, sugerindo que tal localização pudesse representar uma anormalidade fundamental para o desenvolvimento do câncer de mama. Posteriormente, essa localização aberrante foi também relatada em outros tumores como os carcinoma de ovário, gástrico e nasofaríngeo (Carser et al., 2011; Chen et al., 2013; Lesnock et al., 2013).

Estudos acerca da expressão do BRCA1 nos cânceres de cabeça e pescoço são escassos. Recentemente, Oliveira-costa et al. (2014) analisaram a expressão da proteína BRCA1 em CCEO, relatando um padrão de expressão tanto nuclear quanto citoplasmática e correlacionaram o padrão aberrante com baixas taxas de sobrevida nos pacientes, sugerindo um potencial papel como marcador biológico.

Apesar de marcadores prognósticos serem amplamente usados para a seleção do tratamento de pacientes com diversas neoplasias malignas como linfomas e câncer de mama, a ausência de marcadores prognósticos identificados e bem estabelecidos nos CECP, incluindo os CCEO, continua a ser um grande desafio e alvo de inúmeras pesquisas (Litman, Gupta, Brosh, & Cantor, 2008; Oliveira-costa et al., 2014). A volta do uso das drogas antineoplásicas a base de platina, em especial a cisplatina no CECP, levaram ao aumento de estudos de seus possíveis mecanismos de resistência tumoral e de identificação de marcadores biológicos de sensibilidade quimioterápica e marcadores prognósticos, que poderiam estar diretamente relacionados ao mecanismo DDR, a fim de apontar genes alvos e novos marcadores biológicos de resistência e susceptibilidade tumoral (Almeida et al., 2014; Lesnock et al., 2013). Isso despertou o interesse na realização no presente estudo, a fim de investigar alterações de proteínas envolvidas nos mecanismos DDR no CCEO, especificamente proteínas já relacionadas à quimiorresistência em outras neoplasias malignas, a fim de contribuir futuramente para a melhoria do tratamento e prognóstico dos pacientes.

## 2. OBJETIVOS

Avaliar a imunoexpressão das proteínas BRCA1 e da AcH3 em espécimes de biópsia incisional de carcinomas de células escamosas oral de língua móvel e correlacionar sua expressão com dados epidemiológicos e com a gradação histopatológica dos tumores.

## 3. ARTIGO

# BRCA1 AND ACETIL HISTONE H3 IMMUNOHISTOCHEMICAL PROFILE IN MOBILE TONGUE SQUAMOUS CELL CARCINOMA

**Running title**: BRCA1 and ACH3 expression in oral squamous cell carcinoma

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**Abstract**

*Objective*

To evaluate the immunoexpression profile of BRCA1 and AcH3 proteins in oral squamous cell carcinomas of the mobile tongue and correlate with the epidemiological data and histopathological gradation of the tumors.

*Material and methods*

Incisional biopsy samples of 43 oral squamous cell carcinoma were submitted to immunohistochemical for AcH3 and BRCA1 antibodies. Samples were microscopically graded in well differentiated (n=21) and the poor differentiated (n=22). Groups were submitted to statistical analysis ($p<0.05$) regarding the percentage of positive cells and categorized into subgroups according to each antibody.

*Results*

All cases showed AcH3 positive nuclear expression and there was no correlation between AcH3 and histological grading ($p=0.391$). All the samples were positive for BRCA-1 expression. The poor differentiated group showed an aberrant expression of BRCA-1, with most of the cases with less than 10% of nuclear staining ($p<0.01$). Moreover, these cases showed cytoplasmatic staining ($p=0.034$). The well differentiated cases showed nuclear staining with most of the cases showing more than 50% of positive cells ($p<0.01$).

*Conclusion*

AcH3 and BRCA1 protein were found to be expressed in all samples. Cases were mostly hypoacetylated. There was a significant decrease in BRCA1 expression in the poor differentiated group with cytoplasmatic immunostaining in these cases. The BRCA1 results suggests its use as a possible prognostic marker.

**Key words:** Oral squamous cell carcinoma, epigenetics, histones, BRCA1 protein

## Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of the head and neck region (Davies & Welch, 2006; Haddad & Shin, 2008; Scully & Bagan, 2009). It is the fourth most prevalent cancer in men worldwide — half million new cases and approximately 251.000 deaths were estimated for 2018 (Ferlay et al., 2018). The National Institute of Cancer (INCA), estimated 634.880 new cases of cancer in Brazil for the same year being 14.700 new cases of OSCC and with the Southeast region as the fourth most incident in Brazil (INCA, 2018).

Regarding all research advances, as well as the identification of reliable biomarkers, the mechanisms involved in OSCC development, progression and resistance of chemotherapy are still unknown (Le et al., 2014). Moreover, there is still a lack of validated markers that could easily be evaluated in OSCC biopsies what would be for treatment planning (Almangush et al., 2017).

Oral carcinogenesis is characterized by a clinical, pathological and biological heterogeneity (Haddad & Shin, 2008). The DDR *(DNA damage response)* pathway acts as a mechanism of self-defense responsible for the detection of DNA damages and chromatin integrity maintenance. The DDR involves histones modifications and its efficacy is dependent on its access to the site of damage, a process that require the unpacking of the chromatin which is guaranteed by the histone modifications (Rego et al., 2012). The DDR mechanism is composed of several interconnected signaling pathways, such as BRCA1 and BRCA2 proteins pathways (Rego et al., 2012).

Histones are proteins that assist in the packaging of DNA and exhibit tails which may undergo post-translational modifications, such as acetylation or deacetylation (Webber et al., 2017). Recently, Webber et al. (2017) evaluated acetyl-histone H3 status in OSCC and found that OSCC were hypoacetylated (increased chromatin condensation) in comparison to oral leukoplakia and normal oral mucosa and it is was associated with poor prognosis.

Breast cancer 1 (BRCA1) is a gene that has multiple roles in cellular functions as to regulate transcription, chromatin remodeling and repair of double strand DNA breaks via homologous recombination (Oliveira-costa et al., 2014; Vora, Shah, Patel, Trivedi, & Choksi, 2003). BRCA1 is a nuclear protein that can show aberrant cytoplasmic localization in breast cancer cells (Chen et al., 1995), ovarian cancer (Russell et al., 2000) and in gastric cancer (Hu, Zhang, Zou, Yang, & Liang, 2010). Despite of the findings that lack of BRCA1 staining suggests that these abnormalities are fundamental to the progression and development of cancer, new

researches have pointed to new combined therapies where the lack or absence of BRCA1 could improve treatment and enhance survival (Lesnock et al., 2013). Only two studies have reported the expression of BRCA1 protein and oral cancer (Oliveira-costa et al., 2014; Vora et al., 2003).

The aim of the present study was to evaluate the immunoexpression profile of BRCA1 and AcH3 proteins of OSCC of the mobile tongue and correlate with the epidemiological data and histopathological gradation of the tumors.

**Material and Methods**

The present study was previously approved by the local Research Ethics Committee of Clementino Fraga Filho University Hospital of Federal University of Rio de Janeiro, Brazil (protocol number nº 2.517.633). Lateral border of mobile tongue OSCC incisional biopsies specimens were retrieved from 2013 to 2018 from the archives of Oral Pathology Laboratory of School of Dentistry of Federal University of Rio de Janeiro, Brazil. Lesions of other oral mucosa sites and oropharynx were excluded. Clinicopathological data of all patients was retrieved from the files of Oral Pathology Laboratory and transcribed for a data sheet for further analysis. Samples were microscopically analyzed by an Oral Pathologist and graded according to WHO grading system (El-Naggar et al., 2017) in well differentiated and poor differentiated (PD) and splitted in two groups.

For immunohistochemistry conduction, 3µm OSCC sections were placed on silanized slides, deparaffinized in xylene and hydrated in descending grades of ethanol. Antigen retrieval was performed in 6% citrate acid and peroxidase blockage in 10% hydrogen peroxide (Vetec Química, Rio de Janeiro, Brazil). Then, the samples were washed in distilled water and immersed in Tris buffer solution pH7,4. Samples were incubated with primary antibodies anti-BRCA1 (C20) (1:1000; sc-642, policlonal rabbit, Santa Cruz Biotechnology Inc., Texas, USA) and anti-AcH3 (Lys9) (1:600; #9671, policlonal rabbit, Cell Signaling Technology, Massachusetts, USA) overnight at 39,2ºF. Sections were then incubated with streptavidin and biotin solutions (Dako LSAB+; Agilent Technologies Inc., California, USA), revealed with diaminobenzidine tetrahydrochloride (DAB+; Agilent Technologies Inc., California, USA) and counterstained Harris Hematoxylin (Vetec Química, Rio de Janeiro, Brazil). A fibrous hyperplasia of the lateral border of mobile tongue was used as positive and negative control in all assays.

Digital images were obtained with the slide scanner ScanScope Aperio (ScanScope Aperio CS2, Leica, Germany) and analyzed with Aperio ImageScope (Aperio ImageScope, Leica,

Germany). In each case, 1000 cells were randomly counted avoiding the inflammatory area, and the results were expressed as the percentage of positive cells. Cases were also scored for AcH3 and when less than 25% tumor cells showed nuclear staining, cases were considered negative (Wasco & Pu, 2008; Webber et al., 2017). BRCA1 immunohistochemistry counting was performed according to the adapted score system for nuclear staining suggested by Lesnock et al. (2013). Slides were scored as 0 if there was no nuclear staining, as 1 if there was scattered staining (<10%), as 2 if 10-50% of cells stained, as 3 if 50-90% of cells were positive and 4 if nearly all (>90%) the nuclei of the cells were positive. Tumors were categorized as having aberrant BRCA1 expression for very low to no staining (≤10% staining, 0 or 1 score) and normal BRCA1 expression for > 10% of BRCA1 staining (2-4 score). BRCA1 staining location (nucleus and cytoplasm) was also analyzed and compared with histological grading and score system.

All data was analyzed with StatTools software (Palisade, Ithaca, NY, USA). Chi-square test and Student`s *t*-test were performed. A *p*-value <0.05 was considered statistically significant in all tests.

**Results**

Forty-three biopsy specimens of patients diagnosed with OSCC of mobile tongue were selected. Clinical and demographic data of the patients are detailed in Table 1. The cohort was represented by 30 men (69.8%) and 13 women (31.2%) with a mean age of 62 years old. Most of the lesions were clinically described as ulcers 21 (48.8%). Information of tobacco and alcohol consumption was present 32 (74.4%) and 21 (48.8%) from patient´s files respectively. Twenty-one OSCC (48.8%) were microscopically classified as WD and 22 (51.2%) as PD tumors (Image 1). Comparison of clinical and demographic data between groups showed no statistical difference ($p>0.05$).

All cases were positive for AcH3. The average percentage of positive cells was 64.83% and no differences was observed between groups (BD 65.93% and PD 63.89%; $p=0.74$). No significant associations with AcH3 and clinical-demographic features were found (Table 3). Using the score, four cases (9%) showed less than 25% of positive cells, three of them were BD (18.6% of cells; 14.3% of BD cases) and only one PD (17.6% of positive cells; 4.5% of PD cases). There was statistical association between acetylated cases (more than 25% positive cells) and the ulcerated lesions ($p<0.01$).

All cases were also positive for BRCA1 (Table 2). Twenty-three cases (53.4%) showed less than 10% of positive cells while 20 cases (46.6%) presented a normal nuclear protein expression (Table 3). Of the aberrant cases, 19 (82.6%) were PD and only 4 (17.4%) were WD, while of the normal expression cases, 17 (85%) were WD and 3 (15%) were PD. This finding was statistically significant ($p<0.01$) as in the PD group most of the case showed aberrant expression and in BD group most of the cases showed a normal expression. Interestingly, 16 (37.2%) cases showed no nuclear staining (score 0), 12 (75%) of them graded as PD (54.5%), and only 12 cases (27.9%) showed more than 50% cells with nuclear staining (score 3), all of them graded as BD (57.1%). None of the cases filled the score 4 group.

Statistical evidence of an interaction was observed between tumor expression of BRCA1 and gender ($p$=0.042), with men having less expression of BRCA1 protein into the nucleus than women (Table 3). It was observed that a statistical association between the lack/lower BRCA1 nuclear expression in PD group and alcohol consumption ($p$ = 0.003) and with ulcerated lesions ($p$=0.041).

The microscopic analysis of BRCA1 staining also disclosed a heterogeneous immunostaining in which was observed cells with only nuclear staining and cells exhibiting cytoplasmatic staining pattern. In this pattern, BRCA1 protein was observed only into the cytoplasmatic or in both nucleus and cytoplasm (Table 4; Figure 2). Only ten (2.3%) cases presented exclusive nuclear staining, all from them graded as WD (47.6% of WD group). All the 22 OSCC graded as PD showed cytoplasmatic staining. This difference of BRCA1 staining pattern between WD and PD groups was statistically significant ($p$=0.034). Lastly, when comparing the staining pattern with the score system, we observed that all the cases with less than 10% of BRCA1 nuclear staining (score 0 and 1) the protein was located into the cytoplasm ($p$<0.01).

**Discussion**

Oral squamous cell carcinoma is the most common cancer in oral cavity that has shown increased incidence worldwide and has aggressive behavior and poor prognosis even at early stages. Although several prognostic markers for OSCC have been published, there is still a lack of validated markers that could easily be evaluated histological sections and contribute to treatment planning (Almangush et al., 2017).

Oral carcinogenesis is a multistep process modulated by genetic and epigenetic events that promote genomic instability and tumor development (Mascolo et al., 2012). Histone modifications modulate a diverse array of biological processes, including gene regulation and

DNA repair and because of this, they have been studied for different types of cancer (J. H. Chen et al., 2013; Le et al., 2014).

In our study we evaluated the expression of AcH3 in cases of well differentiated and poor differentiated OSCC and correlated with clinical-demographic aspects. Ulcers were found to be hyperacetylated in comparison to plaque or spot clinical presentation. The mean percentage of positive AcH3 cells in OSCC (64.8%) agreed with previous works. It has been demonstrated that OSCC tends to be hypoacetylated in comparison to the normal oral mucosa (Giudice et al., 2013; Le et al., 2014; Webber et al., 2017). Histone hyperacetylation is generally correlates with transcriptional activity and with chromatin unpacking (Le et al., 2014; Lydall & Whitehall, 2005). Hypoacetylation of AcH3, which causes the increased of chromatin condensation, is normally correlated to poor prognostic cancers and tumor resistance to chemotherapies (Giudice et al., 2013; Lydall & Whitehall, 2005; Webber et al., 2017). Reduced acetylation levels of histone H3 have been previously reported in lung and esophagus tumors (C. Chen et al., 2011; Song, Kim, Kim, Park, & Jang, 2012). Webber et al. (2017) analyzed AcH3 at the invasive front of OSCC specimens and showed that they were hypoacetylated when compared to oral leucoplakias. However, the authors did not compare AcH3 status to OSCC microscopic grading. To the best of our knowledge, our work is the first to show AcH3 in OSCC and correlate to WHO microscopic grading despite having used incisional biopsies specimens. Our results showed that PD was slightly more hypoacetylated than the WD group, but this difference was not statistically significant ($p>0.05$).

BRCA1 gene has multiple roles in cellular functions including transcription regulation, chromatin remodeling and repair of double strand DNA breaks via homologous recombination (Oliveira-costa et al., 2014; Vora et al., 2003). Although the link between the BRCA1 tumor-suppressor gene and hereditary breast and ovarian cancer is well established, its definition as an independent prognostic marker for survival and efficient therapeutic strategies are still subjects of study (Carser et al., 2011; Lesnock et al., 2013). Russell et al. (2000) reported that in 90% of the cases of sporadic ovarian cancer, the BRCA1 protein expression was reduced or absent. The same finding was further reported by Thrall et al. (2006) and Carser et al. (2011) with advanced stages ovarian cancers; the last one suggested that BRCA1 could be used as a prognostic and predictive biomarker of response to chemotherapy in sporadic ovarian cancer.

Due to the lack of BRCA1 studies in head and neck cancers including OSCC, its role in oral carcinogenesis, tumor progression and prognosis are yet to be established. Only two previous studies reported the expression of BRCA1 protein and OSCC (Oliveira-costa et al., 2014; Vora

et al., 2003). Vora et al. (2003) found a decrease BRCA1 protein staining when compared to leucoplakias. Our study did not compare the oral cancer with any potential malignant disorder but evaluated BRCA1 protein according to OSCC microscopic grading. The PD group results agrees with Vora et al. (2003) results, who also suggested that the complete loss of nuclear BRCA1 expression in OSCC is related with cases of poor prognostic cancers. This lack of nuclear expression could be related to BRCA1 role in cellular functions as tumor suppressor, so as its disruption could deprive the cell of important mechanisms such as stopping cell proliferation, continuing the cell proliferation and developing the cancer (Lee et al., 1999). Most of our OSCC cases with less than 10% of BRCA1 nuclear staining (82.6%) – aberrant expression – were from the PD group while almost all the cases with the nuclear staining were from the WD group. Only WD cases presented more than 50% of cells (score 3) with positive nuclei. Contrary to our results, Oliveira-costa et al. (2014) also reported a cytoplasmatic immunoexpression of BRCA1 protein in OSCC but did not find association between BRCA1 and microscopic grading. However, they associated the higher cytoplasmatic BRCA1 staining as factor of worst disease specific survival in OSCC patients.

The presence of BRCA1 into the cytoplasm was first reported by Chen et al. (1995) breast cancer cells and related to its pathogenesis. After that, other authors reported the same finding in cancer from other sites as cytoplasmatic and nuclear staining in ovarian cancer (Russell et al., 2000) and cytoplasmatic staining in gastric cancer (Hu et al., 2010). Wilson et al. (1999) however, stated that BRCA1 is only found into the nucleus and that cytoplasmic staining is a technical artifact. Our findings corroborate the previous cytoplasm staining finding. All PD cases showed cytoplasm staining (only in the cytoplasm and/or associated to the nucleus). Although some studies correlate this cytoplasmatic staining to prognostic and chemotherapy response, the clinical significance of cytoplasm expression is yet to be elucidated. BRCA1 abnormalities are fundamental to the progression and development of cancer and researches have pointed to new combined therapies where the lack or absence of BRCA1 could improve treatment and enhance survival. Lesnock et al. (2013) showed in their report that reduced BRCA expression in ovarian cancer results in survival improvement and increase sensitivity to cisplatin combined with paclitaxel respectively. Other studies also have been suggesting that loss of BRCA1 function confer enhanced chemosensitivity to platinum-based chemotherapy (Carser et al., 2011; Quinn et al., 2007; Stordal & Davey, 2009). Up to now there are no published studies correlating BRCA1 and chemosensitivity in head and neck cancer, including OSCC.

Finally, our results corroborate the few previous studies for both AcH3 and BRCA1 protein immunohistochemistry findings in OSCC. The hypoacetylated status of OSCC highlights the importance of epigenetics changes in oral carcinogenesis and tumor progression as a promising field of investigation. The BRCA1 protein imunnoexpression showed a significant decrease in the PD cases and the protein was mostly found into the cytoplasm. Although the present study used biopsy specimens, our BRCA1 reproduced the same observed not only in OSCC but in other cancers suggesting a possible use as prognostic marker. Further studies are necessary to the better understanding of the role of BRCA1 and AcH3 and their potential use as prognostic and predictive biomarker in OSCC.

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**Table 1.** Clinicopathological features of oral squamous cell carcinoma patients

| | **WD (n=21)** | **PD (n=22)** | **Total (n=43)** |
|---|---|---|---|
| **Age *(years old)*** | | | |
| Mean | 65 | 58 | 62 |
| Minimum | 33 | 36 | 33 |
| Maximum | 98 | 74 | 98 |
| **Gender** | | | |
| Male | 13 (61.9%) | 17 (77.3%) | 30 (69.8%) |
| Female | 8 (38.1%) | 5 (22.7%) | 13 (30.2%) |
| *Total* | *21 (48.8%)* | *22 (51.2%)* | *43 (100%)* |
| **Ethnicity** | | | |
| White | 8 (38.1%) | 9 (40.9%) | 17 (39.5%) |
| Non-white | 8 (38.1%) | 6 (27.2%) | 14 (32.6%) |
| Not informed* | 5 (23.8%) | 7 (31.8%) | 12 (27.95) |
| **Size (cm)** | | | |
| Mean | 3.48 | 3.33 | 3.4 |
| Minumum | 0.1 | 0.5 | 0.1 |

| | | | |
|---|---|---|---|
| Maximum | 6 | 5 | 6 |
| Not informed* (cases) | 14 | 13 | 27 (63%) |
| **Evolution (months)** | | | |
| Mean | 3.76 | 3.5 | 3.6 |
| Minimum | 0.7 | 1 | 0.7 |
| Maximum | 8 | 12 | 12 |
| Not informed* (cases) | 5 | 8 | 13 (30%) |
| **Clinical aspects** | | | |
| Ulcer | 11(52.4%) | 10 (45.5%) | 21 (48.8%) |
| Spot/plaque/nodule | 5 (23.8%) | 5 (22.7%) | 10 (23.3%) |
| Not informed* | 5 (23.8%) | 7 (31.8%) | 12 (27.9%) |
| **Consistency** | | | |
| Fibroelastic | 3 (14.3%) | 1 (4.5%) | 4 (9.3%) |
| Firm | 11 (52.4%) | 12 (54.5%) | 23 (53.5%) |
| Soft | 0 | 3 (13.6%) | 3 (7%) |
| Not informed* | 7 (33.3%) | 6 (27.3%) | 13 (30.2%) |
| **Tobacco habits** | | | |
| User | 16 (76.2%) | 16 (72.7%) | 32 (74.4%) |
| Non-user | 1 (4.8%) | 1 (4.5%) | 2 (4.7%) |
| Not informed* | 4 (19%) | 5 (22.7%) | 9 (20.9%) |
| **Alcohol Consumption** | | | |
| User | 10 (47.6%) | 11 (50%) | 21 (48.8%) |
| Non-user | 3 (13.6%) | 3 (13.6%) | 6 (14%) |
| Not informed* | 8 (36.4%) | 8 (36.4%) | 16 (37.2%) |

*WD= well differentiated, PD= poor differentiated*
**Data not available or not informed in patient´s records*

**Table 2.** BRCA1 and AcH3 immunohistochemical expression scores in oral squamous cell carcinoma

| | BRCA-1 | | | | | AcH3 | |
|---|---|---|---|---|---|---|---|
| | *Aberrant** | | *Normal*** | | | *Negative* | *Positive* |
| | Score 0 | Score 1 | Score 2 | Score 3 | Score 4 | < 25% | >25% |
| WD (n=21) | 4 (19%) | 0 | 5 (23.8%) | 12 (57.1%) | 0 | 3 (14.3%) | 18 (85.7%) |
| PD (n=22) | 12 (54.5%) | 7 (31.8%) | 3 (13.6%) | 0 | 0 | 1 (4.5%) | 21 (95.4%) |
| *Total (n=43)* | *16 (37.2%)* | *7 (16.27%)* | *8 (18.6%)* | *12 (27.9%)* | *0* | *4 (9.3%)* | *39 (90.7%)* |

*WD= well differentiated, PD= poor differentiated; * Aberrant when <10% of staining; ** Normal when >10% of staining*

**Table 3**. Association of AcH3 and BRCA1 immunoexpression and the clinicopathological parameters.

| Characteristic | Positive (%AcH3 >25%) n=39 | Negative (%AcH3 <25%) n=4 | *p*-value | Normal (%BRCA >10%) n= 20 | Aberrant (%BRCA ≤10%) n= 23 | *p*-value |
|---|---|---|---|---|---|---|
| **Age (years old)** | | | 0.883 | | | 0.45 |
| <= 60 | 18 | 2 | | 10 | 11 | |
| > 60 | 21 | 2 | | 13 | 9 | |
| **Gender** | | | | | | |
| Male | 27 | 3 | 0.81 | 13 | 17 | 0.042* |
| Female | 12 | 1 | | 10 | 3 | |
| **Tobacco habits** | | | | | | |
| User | 28 | 4 | 0.594 | 18 | 14 | 0.222 |
| Non-user | 2 | 0 | | 2 | 0 | |
| **Alcohol Consumption** | | | | | | |
| User | 19 | 2 | 0.432 | 8 | 13 | 0.05 |
| Non-user | 6 | 0 | | 5 | 1 | |
| **Clinical aspects** | | | | | | |
| Ulcer | 26 | 0 | 0.005* | 10 | 16 | 0.374 |
| Spot/plaque/nodule | 8 | 2 | | 6 | 4 | |
| **Histological gradation** | | | | | | |
| Well-differentiated | 18 | 3 | 0.271 | 17 | 4 | <0.01* |
| Poor-differentiated | 21 | 1 | | 3 | 19 | |

**Statistical significance P<0.05 or P<0.01*

**Table 4.** Distribution of immunohistochemical intracellular expression of BRCA1

| | **BRCA1** | | | |
|---|---|---|---|---|
| | Positive | | | Negative |
| | *Nuclear** | *Cytoplasmatic*** | Total | |
| **WD (n=21)** | 11 | 10 | 21 (100%) | 0 (0%) |
| **PD (n=22)** | 0 | 22 | 22 (100%) | 0 (0%) |
| ***Total (n=43)*** | *11* | *32* | *43 (100%)* | *0 (0%)* |

**Exclusive nuclear staining; **Cytoplasm and/or nuclear and cytoplasm staining*

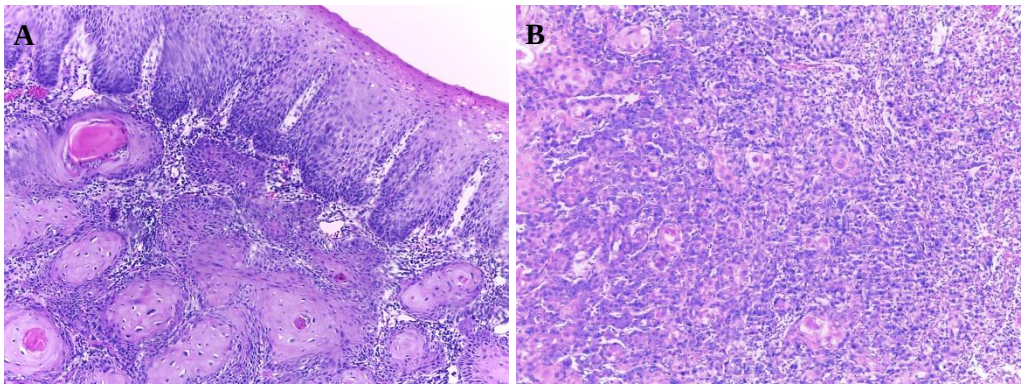


**Figure 1.** Microscopic grades of OSCC. **A.** Well differentiated OSCC. Nests of well differentiated epithelial cells with keratin pearls invading the connective tissue. **B.** Poor differentiated OSCC. Small nests and cords of atypical epithelial cells. Keratin pearls are not observed, and cells are detached inside connective tissue. (HE, 100x)

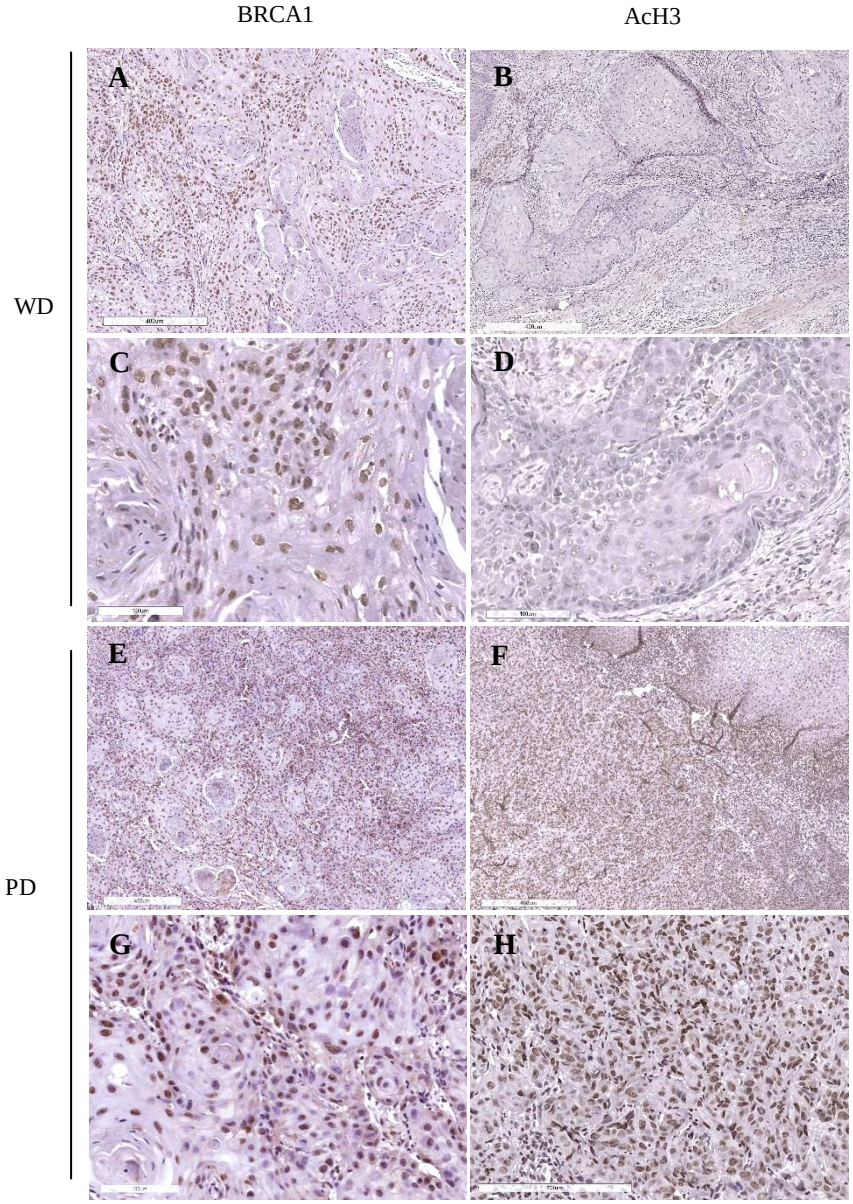


**Figure 2.** Immunohistochemical expression of BRCA1 and AcH3 in mobile tongue OSCC samples. **A-D.** Well differentiated OSCC. *A,C* – BCRA-1 expression mainly into the nucleus. Keratinized cells and mitosis were negative. *B,D* – AcH3 weak nuclear expression. **E-H.** Poor differentiated OSCC. *E,G* – BRCA1 positive cytoplasmatic staining. *D, F* – AcH3 positive immunoexpression.

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## 4. CONCLUSÃO

- O estudo avaliou a expressão da AcH3 no Carcinoma de células escamosas oral de língua móvel, especificamente nos grupos histológicos: bem diferenciados e pouco diferenciados e correlacionou com os aspectos clínico-demográficos dos pacientes. Apesar de não ter sido encontrada nenhuma associação estatisticamente significativa entre os grupos e a AcH3, o grupo pouco diferenciado se mostrou ligeiramente hipoacetilado em relação ao bem diferenciado.
- A avaliação da expressão da proteína BRCA1 relacionada aos grupos histológicos, mostrou que 82% dos casos que apresentavam menos que 10% da marcação nuclear para o BRCA1 eram do grupo dos pouco diferenciados enquanto que quase todos os casos do grupo dos bem diferenciados apresentavam uma expressão do BRCA1 considerada normal. Mostrando assim, uma diminuição da imunoexpressão do BRCA-1 nos casos de CCEO pouco diferenciados.
- Nossos achados apontam para um padrão heterogêneo de marcação imuno-histoquímica do BRCA1 nos Carcinomas de células escamosas oral de língua móvel, com ela podendo ser nuclear, citoplasmática ou nuclear e citoplasmática. Todos os casos de carcinoma pouco diferenciado e todos os casos com expressão imuno-histoquímica do BRCA1 menor que 10%, apresentavam a imunomarcação citoplasmática. A marcação nuclear foi observada na maioria dos casos bem diferenciados.

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## 6. ANEXOS

**ANEXO 1** - PARECER COMITÊ DE ÉTICA EM PESQUISA

UFRJ - HOSPITAL UNIVERSITÁRIO CLEMENTINO FRAGA FILHO DA

### PARECER CONSUBSTANCIADO DO CEP

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** ANÁLISE IMUNOISTOQUÍMICA DAS PROTEÍNAS BRCA-1 E ACETIL HISTONA H3 NO CARCINOMA DE CÉLULAS ESCAMOSAS ORAL

**Pesquisador:** ALINE CORREA ABRAHAO

**Área Temática:**

**Versão:** 1

**CAAE:** 82584018.6.0000.5257

**Instituição Proponente:** UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

**Patrocinador Principal:** UNIVERSIDADE FEDERAL DO RIO DE JANEIRO

**DADOS DO PARECER**

**Número do Parecer:** 2.517.633

**Situação do Parecer:**
Aprovado

**Necessita Apreciação da CONEP:**
Não

RIO DE JANEIRO, 01 de Março de 2018

**Assinado por:**
**Carlos Alberto Guimarães**
**(Coordenador)**

**Endereço:** Rua Prof. Rodolpho Paulo Rocco Nº255, 7º andar, Ala E
**Bairro:** Cidade Universitária **CEP:** 21.941-913
**UF:** RJ **Município:** RIO DE JANEIRO
**Telefone:** (21)3938-2480 **Fax:** (21)3938-2481 **E-mail:** cep@hucff.ufrj.br

# **ANEXO 2 –** NORMAS DA REVISTA CIENTÍFICA ORAL DISEASES



## 5. MANUSCRIPT FORMAT AND STRUCTURE

**5.1. Page Charge**
Articles exceeding 6 published pages, including title page, abstract, references, table/figure legends and tables and figures, are subject to a charge of GBP70 per additional page. As a guide, one published page amounts approximately to 850 words, or two to four small tables/figures. Additional supplementary material (including text and figures), which does not fit within the page limits, can be published online only as supporting information.

**5.2. Format**

**Language**: Authors should write their manuscripts in British English using an easily readable style. Authors whose native language is not English should have a native English speaker read and correct their manuscript. Spelling and phraseology should conform to standard British usage and should be consistent throughout the paper. A list of independent suppliers of editing services can be found at http://authorservices.wiley.com/bauthor/english_language.asp. All services are paid for and arranged by the author, and use of one of these services does not guarantee acceptance or preference for publication.

**Presentation**: Authors should pay special attention to the presentation of their findings so that they may be communicated clearly. The background and hypotheses underlying the study as well as its main conclusions should be clearly explained. Titles and abstracts especially should be written in language that will be readily intelligible to any scientist.

**Technical jargon**: should be avoided as much as possible and clearly explained where its use is unavoidable.

**Abbreviations**: Oral Diseases adheres to the conventions outlined in Units, Symbols and Abbreviations: A Guide for Medical and Scientific Editors and Authors. Non-standard abbreviations must be used three or more times and written out completely in the text when first used.

**5.3. Structure:** All papers submitted to *Oral Diseases* should include:

- Title Page
- Structured Abstract (reviews need not include a structured abstract)
- Main text
- References
- (Figures)
- (Figure Legends)
- (Tables)

**Title Page**: should be part of the manuscript uploaded for review and include:

- A title of no more than 100 characters including spaces
- A running title of no more than 50 characters
- 3-6 keywords

- Complete names and institutions for each author
- Corresponding author's name, address, email address and fax number
- Date of submission (and revision/resubmission)

**Abstract:** is limited to 200 words in length and should contain no abbreviations. The abstract should be included in the manuscript document uploaded for review as well as separately where specified in the submission process. The abstract should convey the essential purpose and message of the paper in an abbreviated form set out under:

- Objective(s),
- Subject(s) (or Materials) and Methods,
- Results,
- Conclusions(s).

**The Main Text of Original Research Articles** should be organised as follows

**Introduction**: should be focused, outlining the historical or logical origins of the study and not summarize the results; exhaustive literature reviews are inappropriate. It should close with the explicit statement of the specific aims of the investigation.

**Materials and Methods** must contain sufficient detail such that, in combination with the references cited, all clinical trials and experiments reported can be fully reproduced. As a condition of publication, authors are required to make materials and methods used freely available to academic researchers for their own use. This includes antibodies and the constructs used to make transgenic animals, although not the animals themselves. Other supporting data sets must be made available on the publication date from the authors directly.

**Results**: should present the observations with minimal reference to earlier literature or to possible interpretations.

**Discussion:** may usually start with a brief summary of the major findings, but repetition of parts of the abstract or of the results sections should be avoided. The section should end with a brief conclusion and a comment on the potential clinical relevance of the findings. Statements and interpretation of the data should be appropriately supported by original references.

**Acknowledgements**: Should be used to provide information on sources of funding for the research, any potential conflict of interest and to acknowledge contributors to the study that do not qualify as authors. All sources of institutional, private and corporate financial support for the work within the manuscript must be fully acknowledged, and any potential grant holders should be listed. Acknowledgements should be brief and should not include thanks to anonymous referees and editors. Where people are acknowledged, a covering letter demonstrating their consent must be provided.

**5.4. References**

References should be prepared according to the *Publication Manual of the American Psychological Association* (6th edition). This means in-text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). For references with three to five authors, all authors should be listed only on the first occurrence of the in-text citation, and in subsequent in-text occurrences only the first author should be listed followed by '*et al.*'. The complete reference list should appear alphabetically by name at the end of the paper.

A sample of the most common entries in reference lists appears below. Please note that a DOI should be provided for all references where available. For more information about APA referencing style, please refer to the APA website. Please note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

**Journal article**

Example of reference with 2 to 7 authors

Beers, S. R., & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. *The American Journal of Psychiatry, 159*, 483–486. doi: 10.1176/appi.ajp.159.3.483

Ramus, F., Rosen, S., Dakin, S. C., Day, B. L., Castellote, J. M., White, S., & Frith, U. (2003). Theories of developmental dyslexia: Insights from a multiple case study of dyslexic adults. *Brain, 126*(4), 841–865. doi: 10.1093/brain/awg076

Example of reference with more than 7 authors

Rutter, M., Caspi, A., Fergusson, D., Horwood, L. J., Goodman, R., Maughan, B., ... Carroll, J. (2004). Sex differences in developmental reading disability: New findings from 4 epidemiological studies. *Journal of the American Medical Association, 291*(16), 2007–2012. doi: 10.1001/jama.291.16.2007

**Book edition**

Bradley-Johnson, S. (1994). *Psychoeducational assessment of students who are visually impaired or blind: Infancy through high school* (2nd ed.). Austin, TX: Pro-ed.

**5.5. Tables, Figures and Figure Legends**

**Figures**: All figures and artwork must be provided in electronic format. Please save vector graphics (e.g. line artwork) in Encapsulated Postscript Format (EPS) and bitmap files (e.g. half-tones) or clinical or in vitro pictures in Tagged Image Format (TIFF).

Detailed information on our digital illustration standards can be found at http://authorservices.wiley.com/bauthor/illustration.asp.

Check your electronic artwork before submitting it: http://authorservices.wiley.com/bauthor/eachecklist.asp.

Unnecessary figures and parts (panels) of figures should be avoided: data presented in small tables or histograms, for instance, can generally be stated briefly in the text instead. Figures should not contain more than one panel unless the parts are logically connected.

Figures divided into parts should be labelled with a lower-case, boldface, roman letter, a, b, and so on, in the same type size as used elsewhere in the figure. Lettering in figures should be in lower-case type, with the first letter capitalized. Units should have a single space between the number and unit, and follow SI nomenclature common to a particular field. Unusual units and abbreviations should be spelled out in full or defined in the legend. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. In general visual cues (on the figures themselves) are preferred to verbal explanations in the legend (e.g. broken line, open red triangles etc).

**Color figures**

Color figures may be published online free of charge; however, the journal charges for publishing figures in colour in print. If the author supplies colour figures at Early View publication, they will be invited to complete a colour charge agreement in RightsLink for Author Services. The author will have the option of paying immediately with a credit or debit card, or they can request an invoice. If the author chooses not to purchase color printing, the figures will be converted to black and white for the print issue of the journal.

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If you would like to send suggestions for artwork related to your manuscript to be considered to appear on the cover of the journal, please follow these general guidelines.

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Bring your research to life by creating a video abstract for your article! Wiley partners with Research Square to offer a service of professionally produced video abstracts. Learn more about video abstracts at www.wileyauthors.com/videoabstracts and purchase on for your article at https://www.researchsquare.com/wiley/ or through your Author Services Dashboard. If you have any questions, please direct them to videoabstracts@wiley.com.