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ANÁLISE DA EXPRESSÃO IMUNOISTOQUÍMICA DA PROTEÍNA GLUCOSE-REGULATED PROTEIN 78 (GRP78) NA CARCINOGÊNESE BUCAL

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Trabalho de Conclusão de Curso apresentado ao Curso de Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do título de Cirurgiã-Dentista.

Orientadora: Profa. Dra. Fernanda Visioli

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RESUMO

BARBOSA, Nathalia Mocellin**. Análise da expressão imunoistoquímica da proteína Glucose-Regulated Protein 78 (GRP78) na carcinogênese bucal**. 2015. 36 f. Trabalho de Conclusão de Curso (Graduação em Odontologia) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

O processo de desenvolvimento e progressão do câncer de cabeça e pescoço não está bem esclarecido na literatura. O desafio atual é a busca por biomarcadores que, a partir de sua expressão, possam auxiliar no diagnóstico precoce e acompanhamento clínico da doença, aumentando as taxas de sobrevida dos pacientes com carcinoma espinocelular. Estresse crônico é altamente induzido no microambiente tumoral prejudicando a função do retículo endoplasmático resultando na expressão de GRP78, uma importante chaperona da via celular conhecida como *Unfolded Protein Response*, que ativa uma resposta de pró-sobrevivência e aumenta a resistência das células tumorais à apoptose. Alta expressão de GRP78 está associada a um comportamento tumoral mais agressivo e menor sobrevida em diversos tipos de câncer. Entretanto, o papel da GRP78 como indicador de prognóstico em pacientes com câncer de cabeça e pescoço ainda permanece indeterminado. O objetivo deste trabalho foi verificar se a expressão imunoistoquímica de GRP78 pode ser utilizada para comparar diferentes leucoplasias bucais e prever o potencial de transformação maligna destas lesões, além de verificar seu potencial como indicador de prognóstico no câncer de cabeça e pescoço. A amostra consistiu em 55 casos de carcinoma espinocelular, 57 casos de leucoplasia e 7 amostras de mucosa bucal normal. Nas lâminas histológicas dos tumores, foram capturadas imagens de cinco campos de três diferentes sítios: epitélio adjacente, centro do tumor, e fronte de invasão tumoral. Nas leucoplasias, toda a extensão de tecido epitelial visível foi capturada. Foi realizada a técnica imunoistoquímica para detecção de GRP78. Três examinadores cegos e calibrados (ICC = 0,83) calcularam a porcentagem da marcação imunoistoquímica multiplicando a quantidade de células coradas pela intensidade da coloração. A expressão de GRP78 foi correlacionada com as características clínico-patológicas da doença e o prognóstico do paciente, tanto em leucoplasias quanto em carcinoma espinocelular. Após cinco anos de acompanhamento, observamos que há um aumento significativo da expressão de GRP78 nas células tumorais em comparação às leucoplasias e à mucosa bucal normal. Os parâmetros clínico-patológicos avaliados e o prognóstico do paciente não interferiram de forma significativa na expressão de GRP78. Foi verificada uma diferença estatisticamente significativa da expressão de GRP78 no centro do tumor em pacientes com metástase regional quando comparados aos pacientes sem metástase regional (p = 0,048). Concluímos que a expressão de GRP78 não está correlacionada com a evolução da doença, e, portanto, não deve ser considerada um biomarcador de prognóstico em pacientes com leucoplasia e câncer de cabeça e pescoço.

Palavras-chave: Carcinogênese. Neoplasias bucais. Carcinoma de células escamosas. Leucoplasia. Imuno-histoquímica. GRP78.

ABSTRACT

BARBOSA, Nathalia Mocellin**. Immunohistochemical expression analysis of Glucose-Regulated Protein 78 (GRP78) in oral carcinogenesis**. 2015. 36 p. Final Paper (Graduation in Dentistry) – Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2015.

The development and progression of head and neck cancer are unclear in the literature. The current challenge is the search for biomarkers that can assist in the early diagnosis and clinical management of the disease, increasing the survival rates of patients with squamous cell carcinoma. Chronic stress is highly induced in the tumor microenvironment impairing the endoplasmic reticulum (ER) normal function. ER stress results in GRP78 expression, a major chaperone of the cellular pathway known as Unfolded Protein Response, which activates a pro-survival response and increase tumor cells resistance to apoptosis. Overexpression of GRP78 is associated with aggressiveness and lower survival in several types of cancer. However, the role of GRP78 as a prognostic indicator in patients with head and neck cancer remains undetermined. The objective of this study was to determine if the GRP78 immunohistochemical expression can be used to compare different oral leukoplakia lesions and to predict the malignant potential of them, and to investigate its potential as a prognostic indicator in head and neck cancer. The sample consisted in 55 cases of squamous cell carcinoma, 57 cases of leukoplakia and 7 samples of normal oral mucosa. In histological slides of tumors, five fields of three different sites were captured: normal epithelium adjacent to the tumor, center of the tumor and invasive front of the tumor. In leukoplakia, the entire visible length of epithelial tissue has been captured. Immunohistochemistry was performed for GRP78 detection. Three blinded and calibrated pathologists (ICC = 0.83) calculated the percentage of immunohistochemical staining multiplying the amount of stained cells by the intensity of staining. GRP78 expression was correlated with clinicopathological features of the disease and patient's prognosis. After five years of follow-up, we observed that there is a significant increase of GRP78 expression in tumor cells compared to leukoplakia and normal oral mucosa. Clinicopathological parameters evaluated and patient's prognosis did not interfere significantly in GRP78 expression. A statistically significant difference of GRP78 expression in the center of the tumor in patients with regional metastasis was observed when compared to patients without regional metastasis ($p = 0.048$). We conclude that GRP78 expression does not correlate with disease progression, and, therefore, should not be considered a prognostic biomarker in patients with leukoplakia and head and neck cancer.

Keywords: Carcinogenesis. Mouth neoplasms. Carcinoma, squamous cell. Leukoplakia. Immunohistochemistry. GRP78.

SUMÁRIO

1 ANTECEDENTES E JUSTIFICATIVA

Os tumores de cavidade oral e faringe ocupam a sexta posição das neoplasias mais prevalentes na população mundial (PARKIN et al., 2005). No Brasil, estima-se 11.280 novos casos de câncer da cavidade oral em homens e 4.010 em mulheres em 2014, sendo o quinto tipo mais comum de câncer em homens brasileiros (Instituto Nacional do Câncer, 2014). O carcinoma espinocelular representa 95% de todas as neoplasias malignas que acometem a cavidade bucal (NEVILLE; DAY, 2002).

Apesar dos inúmeros esforços realizados no sentido de prevenir, diagnosticar precocemente e buscar novos protocolos de tratamento, o prognóstico desta doença pouco tem se modificado nas últimas décadas (NEVILLE; DAY, 2002; VAN DER WAAL, 2013) sendo mantidas taxas de sobrevida em torno de 50% em 5 anos (ANTUNES et al., 2001; BIAZEVIC et al., 2006). Em função disso, o câncer de boca requer a implementação de estratégias de combate mais eficazes. Em todo o mundo, cerca de 50% dos pacientes com câncer de boca são diagnosticados já com a doença avançada. A redução dos fatores de risco tais como álcool e fumo pode ser uma ferramenta eficaz para reduzir a morbidade e mortalidade (VAN DER WAAL, 2013).

A etiologia do câncer bucal é multifatorial. Os fatores etiológicos associados a essa patogenia podem ser intrínsecos - condições sistêmicas e hereditariedade - ou extrínsecos – exposição ao tabaco, ao álcool e à radiação ultravioleta (no caso específico do câncer de lábio inferior). Diversos estudos mostram que o câncer bucal surge como resultado do acúmulo de eventos mutagênicos, decorrentes principalmente do efeito do tabaco e do álcool (LA VECCHIA et al., 1997). O dano genético causado pela exposição a diferentes agentes mutagênicos pode causar o comprometimento de diversos processos regulatórios celulares, resultando em aumento da proliferação, inibição de processos apoptóticos e potencial para a invasão de tecidos adjacentes (HANAHAN; WEINBERG, 2011).

A carcinogênese na cavidade oral é um processo de múltiplas etapas, com alterações progressivas no genoma celular, portanto, o desenvolvimento do câncer na mucosa bucal pode ser precedido por uma lesão potencialmente maligna que consiste em um tecido alterado em que o câncer ocorre mais frequentemente em comparação a

sua contraparte normal (MAO, 1997; REIBEL, 2003). Dentre essas lesões, a leucoplasia é a mais frequente. De acordo com Carrard et al. (2010), a prevalência de leucoplasia na população da região metropolitana de Porto Alegre é de 1,01%. Quando nos deparamos com uma leucoplasia, é necessário realizar uma biópsia para permitir o diagnóstico histopatológico, uma vez que estas lesões podem ter inúmeras alterações epiteliais (KRAMER et al., 1978). Os distúrbios epiteliais presentes em leucoplasias são classificados de acordo com suas características morfológicas em hiperplasia epitelial, hiperceratose (hiperortoceratose ou hiperparaceratose), acantose e displasia epitelial (WARNAKULASURIYA et al., 2008). No diagnóstico histopatológico de uma leucoplasia essas alterações epiteliais podem estar presentes isoladamente ou em conjunto (WALDRON; SHAFER, 1975).

Estudos anteriores revelam uma taxa de transformação maligna dessas lesões entre 8,9 e 17,5% (BOUQUOT; WHITAKER, 1994; LIND, 1987; SILVERMAN; GORSKY; LOZADA, 1984). Atualmente, estima-se que a taxa anual de transformação maligna de leucoplasia é de cerca de 1% (VAN DER WAAL, 2009). Quando evidência histológica de displasia epitelial está presente, o risco pode aumentar para até 36%, assim considera-se que a presença de displasia epitelial é o fator preditor mais importante de transformação maligna das lesões potencialmente malignas (LUMERMAN; FREEDMAN; KERPEL, 1995; KRAMER et al., 1978; SILVERMAN; GORSKY; LOZADA, 1984). No entanto, todas as leucoplasias devem ser consideradas suspeitas, pois lesões pequenas e aparentemente inocentes também podem apresentar displasia epitelial ou até mesmo carcinoma espinocelular (NEVILLE; TERRY, 2002; SILVERMAN, 1968; WALDRON; SHAFER, 1975).

Apesar das leucoplasias com displasia epitelial apresentarem maior risco de transformação maligna, alguns carcinomas espinocelulares de boca se desenvolvem a partir de leucoplasias sem displasia epitelial (REIBEL, 2003). Silverman, Gorsky e Lozada (1984) verificaram que 36% das leucoplasias com displasia epitelial sofreram transformação maligna, entretanto, no mesmo período de acompanhamento, 15% das leucoplasias sem displasia epitelial também sofreram transformação maligna.

Apesar do enorme progresso no campo da biologia celular e molecular, o prognóstico dessa doença pouco se modificou nas últimas décadas. Não há um

marcador que permita de forma confiável prever a transformação maligna de uma leucoplasia nem auxiliar no prognóstico e acompanhamento de pacientes com carcinoma espinocelular bucal. A identificação de indivíduos com maior risco de desenvolvimento para o câncer bucal tem fundamental importância para a adoção de medidas eficazes que favoreçam o diagnóstico precoce dessa neoplasia, além de possibilitar a utilização de medidas preventivas, como o abandono do uso de substâncias carcinogênicas, aumentando as taxas de sobrevida destes pacientes.

Recentemente, a relação dos componentes da via celular conhecida como *Unfolded Protein Response (UPR)* com o fenótipo do câncer está sob investigação. O retículo endoplasmático (RE) é constituído por uma rede complexa de membranas e tem um papel fundamental no funcionamento normal da célula. Para realizar o enovelamento tridimensional de proteínas, o RE apresenta altos níveis de proteínas chaperonas que auxiliam no dobramento correto das proteínas nascentes (SAIBIL, 2008) e encaminham a proteína à destruição, caso não seja possível atingir a configuração terciária correta (FENTON; HORWICH, 2003).

Estresse crônico no retículo endoplasmático refere-se a qualquer distúrbio que altere a função de dobramento de proteínas. O RE é altamente sensível a estresses que podem reduzir a sua capacidade de dobramento, o que resulta no acúmulo e na agregação de proteínas mal-dobradas. Agregação de proteínas é tóxica para a célula e muitas condições patológicas estão associadas ao estresse no RE, sendo reconhecidas como um fator importante no desenvolvimento e estabelecimento do câncer (LI; ZHAN; LI, 2011; SZEGEZDI et al., 2006).

Para combater o estresse do RE, as células desenvolveram o mecanismo da UPR, um sistema complexo mediado por três sensores transmembrana do RE: *Inositol Requiring Enzyme 1α (IRE1α), Pancreatic ER eIF2α Kinase (PERK)* e *Activating Transcription Factor 6 (ATF6)*. O domínio intraluminal destas proteínas está ligado à proteína chaperona do RE G*lucose-Regulated Protein 78 (GRP78)* que se desconecta destas proteínas sensores para atuar como chaperona ativando a UPR. A UPR é um mecanismo associado a uma resposta de pró-sobrevivência que irá aumentar a capacidade de dobramento de proteínas reduzindo o acúmulo de proteínas mal-dobradas, e assim, irá restaurar a função do RE. Entretanto, se a agregação de

proteínas persistir e o estresse não puder ser eliminado, a sinalização muda de pró-sobrevivência para pró-apoptótica, levando à morte celular (HEALY et al., 2009).

Estresse do RE e UPR são altamente induzidos no microambiente tumoral, pois as células tumorais se multiplicam em alta velocidade, crescem mais rápido do que seu suprimento sanguíneo e possuem um aumento do metabolismo de glicose, resultando em pobre oxigenação da massa tumoral, falta de nutrientes e mudanças no pH extracelular (LEE, 2007; VAUPEL; KALLINOWSKI; OKUNIEFF, 1989; VISIOLI et al., 2014). Embora a expressão de GRP78 seja mantida a um nível basal na maioria dos tecidos e órgãos adultos, ela é fortemente induzida em tumores (DONG et al., 2004; LI; LEE; LEE, 2006). Evidências mostram que níveis elevados de GRP78 estão correlacionados com agressividade, recorrência e menor sobrevida em pacientes com alguns tipos de câncer (AL-RAWASHDEH et al., 2010; LEE et al., 2006; LEE, 2007; URAMOTO et al., 2005; WANG et al., 2005; ZHENG et al., 2008). Em uma variedade de tumores malignos, o aumento da expressão de GRP78 confere resistência a agentes quimioterápicos e o silenciamento de GRP78 sensibiliza as células tumorais a estes agentes terapêuticos (DONG et al., 2008; LEE et al., 2006; LEE, 2007). Lin et al. (2010) verificaram uma correlação positiva entre a expressão tecidual de GRP78 e o potencial maligno de lesões orais. Assim, a proteína GRP78 é induzida tanto por estresses fisiológicos quanto condições patológicas capazes de desequilibrar a homeostase do retículo endoplasmático celular, com o objetivo de proteger os tecidos e órgãos contra danos, assumindo um papel importante na sobrevivência de células estressadas como se caracterizam as células tumorais.

O objetivo geral deste trabalho é verificar se a expressão de GRP78 pode ser utilizada para auxiliar na comparação de leucoplasias bucais com diferentes alterações epiteliais, além de permitir prever o potencial de transformação maligna destas lesões, bem como auxiliar na determinação do seu potencial como preditor prognóstico em pacientes com carcinoma espinocelular de cabeça e pescoço. Os objetivos específicos são analisar a marcação imunoistoquímica de GRP78 em carcinomas espinocelulares bucais em três diferentes sítios: fronte de invasão tumoral, centro do tumor e tecido epitelial adjacente ao tumor, correlacionando-a com os parâmetros clínico-patológicos da doença e a evolução do paciente, e também avaliar a imunomarcação de GRP78

em leucoplasias bucais em toda a extensão das lesões e correlacioná-la com os parâmetros clínico-patológicos da doença e a ocorrência de transformação maligna.

2 ARTIGO CIENTÍFICO

Immunohistochemical expression analysis of GRP78 (glucose-regulated protein 78) in head and neck carcinogenesis

Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology

ABSTRACT

Background: This study was designed to evaluate if GRP78 immunohistochemical expression can be used to compare and to predict the potential of malignant transformation of leukoplakia lesions and to investigate its potential as a prognostic biomarker in head and neck cancer.

Materials and Methods: A total of 119 samples, 55 cases of SCC, 57 cases of leukoplakia and 7 samples of normal oral mucosa were collected. GRP78 expression was detected by immunohistochemistry. Five fields of three different sites were captured in histological slides of tumors (x200): normal epithelium adjacent to the tumor, center of the tumor and invasive front of the tumor. In leukoplakia, the entire visible length of epithelial tissue was captured. Three blinded and calibrated pathologists (ICC = 0.83) calculated the percentage of immunohistochemical staining. GRP78 expression was correlated with clinicopathological features of the disease and patient's prognosis.

Results: There is a significant increase of GRP78 expression in tumor cells compared to leukoplakia and normal oral mucosa. Clinicopathological parameters evaluated and patient's prognosis did not interfere significantly in GRP78 expression. A statistically significant higher GRP78 expression in the center of the tumor in patients with lymph node metastasis was observed when compared to patients without lymph node metastasis ($p = 0.048$).

Conclusions: GRP78 expression does not correlate with disease progression, and, therefore, should not be considered a prognostic biomarker in patients with leukoplakia and head and neck cancer.

Keywords: Carcinogenesis. Mouth Neoplasms. Carcinoma, Squamous Cell. Leukoplakia. Immunohistochemistry. GRP78.

INTRODUCTION

The tumors of oral cavity and pharynx occupy the sixth position of the most prevalent malignancies in the world population (PARKIN et al., 2005). Among them, squamous cell carcinoma (SCC) accounts for 95% of all malignant tumors that affect the oral cavity (NEVILLE; DAY, 2002). Despite several efforts in the prevention, diagnosis and new treatment protocols, the prognosis of this disease has little improved in the last years, with survival rates of approximately 50% in 5 years (ANTUNES et al., 2001; BIAZEVIC et al., 2006). Oral cancer can be preceded by a potentially malignant lesion, and among them, leukoplakia is the most frequent (DELIBASI et al., 2003; JAHANBANI, 2003; SCHEIFELE; REICHART; DIETRICH, 2003).

To date, there is no appropriate biomarker that help us to identify individuals at high risk of developing malignancies and even to assist in the monitoring of individuals with head and neck cancer. Therefore, the adoption of a reliable biomarker is fundamentally important to introduce preventive measures to avoid malignant transformation, promote early diagnosis and to improve clinical follow-up over time, increasing the survival rates of these patients (AMAGASA; YAMASHIRO; ISHIKAWA, 2006).

The carcinogenesis process results in the formation of a tumor microenvironment featured by low levels of glucose, severe hypoxia, acidosis and ischemia. These pathological environmental changes as well as the accumulation of gene mutations results in protein changes that causes endoplasmic reticulum stress (ERS) (HEALY et al., 2009; RUTKOWSKI; KAUFMAN, 2007), affecting the protein folding and injuring the normal cell function. The accumulation of misfolded proteins increases GRP78/BiP (glucose-regulated protein 78/immunoglobulin heavy-chain binding protein) expression, a chaperone protein of endoplasmic reticulum (ER) which aids the correct proteins folding (KAUFMAN, 2002). It activates a series of signaling cascades known as Unfolded Protein Response" (UPR) associated with a pro-survival response that will increase the folding capacity by reducing the accumulation of misfolded proteins. Evidence shows that activation of the UPR also confers an increase of cancer cells survival, more resistance to immune system and to chemotherapeutic agents (LEE,

2007; ROLLER, 2013). It is an essential mechanism for cancer cells to survive the harsh tumor microenvironment (LI, ZHAN, LI, 2011).

Previous research consider that GRP78 is a protein induced by tumor microenvironment and its high levels are correlated with aggressiveness, recurrence and lower survival in patients with breast cancer (LEE et al., 2006; LEE et al., 2011), liver (AL-RAWASHDEH et al., 2010), prostate (DANESHMAND et al., 2007), kidney (FU et al., 2010) and stomach (ZHENG et al., 2008), and there are conflicting results regarding lung cancer (KIM et al., 2012; URAMOTO et al., 2005; WANG et al., 2005). In regards to head and neck cancer, the literature is restricted; a previous study has found that the GRP78 expression in oral cancer is associated with poor tumor staging (XIA et al., 2014). Lin et al. (2010) found a positive correlation between GRP78 tissue expression and the potential of malignant transformation of oral precancerous lesions suggesting that this protein participates in the initial stage of the oral carcinogenesis process.

Therefore, we investigated if the evaluation of the immunohistochemical GRP78 expression can aid in the differentiation and comparison of leukoplakia with different epithelial changes, as well as its may help as a predictor of prognosis of head and neck squamous cell carcinoma (HNSCC). In our study, the objective was to evaluate the immunohistochemical staining of GRP78 protein in HNSCC correlated with disease progression and prognosis. In potentially malignant oral lesions, we evaluate the correlation between GRP78 immunostaining with clinical and microscopical changes observed in leukoplakia, appearance of new lesions and malignant transformation.

MATERIALS AND METHODS

Sample

In this retrospective study, SCC sample is derived from the Head and Neck Surgery Service of Hospital de Clínicas de Porto Alegre (Porto Alegre, RS, Brazil) and leukoplakia sample was derived from Oral Pathology Department of School of Dentistry of Federal University of Rio Grande do Sul (Porto Alegre, RS, Brazil). The sample consists of 55 samples of patients with histopathological diagnosis of SCC and 57

lesions with clinical diagnosis of leukoplakia. In addition, 7 samples of normal oral mucosa derived from frenectomy surgery were used for comparison. To characterize the sample, all clinicopathologic information was obtained from the database of biopsy or surgical records. This study was approved by the local Ethics Committee (Federal University of Rio Grande do Sul, number. 237 008).

Immunohistochemistry

All tissue samples were fixed in formalin and embedded in paraffin. We performed 3μm sections for each sample, which were deparaffinized in xylene and rehydrated in alcohol. For blocking endogenous peroxidase, the sections were immersed in methanol with 0.3% hydrogen peroxide. Antigen retrieval was performed with pH 6.0 citrate buffer solution (Dako Corporation, Carpinteria, CA, USA) in a pressure cooker at 115°C for 3:30 minutes. Then, the slides were incubated at 30°C overnight with the primary antibody, antiGRP78 antibody (Clone C50B12, Monoclonal Rabbit Anti-human Cell Signalling Technology, Danvers, MA, USA) was used at 1: 200 dilution. For immunohistochemical detection, it was used EnVision+® System (Dako Corporation, Carpinteria, CA, USA) for 2 hours at 30°C. Slides were counter-stained with Harris hematoxylin. The negative control consisted in primary antibody omission. For antibody positive control, it was used an oral squamous cell carcinoma (OSCC) sample that after previous test showed confirmed positive immunohistochemical staining for this protein.

Immunohistochemistry analysis

For each section of SCC five microscopic fields were captured at 200x magnification of three different sites of the surgical tissue: the normal epithelium adjacent to the tumor, which consists of a tumor-free area of non-neoplastic tissue; the center of the tumor with cancer cells located in the central region of the lesion discarding areas of necrosis; and the invasive front of the tumor, determined by the last six layers of cancer cells in contact with the adjacent connective tissue. These areas were chosen randomly using Qcapture® software. For oral leukoplakia samples, we

captured at 200x magnification all visible fields of the epithelial tissue of each lesion and the average was taken across all of them.

Three blinded and calibrated pathologists (ICC = 0.83) evaluated semi-quantitatively the percentage of stained cells in each field, which were considered positive when they showed brown color. Furthermore, the average percentage of staining cells were subsequently classified into five categories as previously described (LIM et al., 2005; XING et al., 2006): score 0 (negative); score 1 (1-25% positive cells); 2 (25-50% positive cells); 3 (50-75% positive cells); 4 (75-100% positive cells). Staining intensity was categorized into the average percentage of cells with 0, 1, 2 and 3, denoting negative, weak, moderate and strong staining, respectively. The final score was obtained by multiplying the intensity score by the percentage of positive cells score.

Statistical analysis

The data of this study were computed using the SPSS computer program (Statistical Package for Social Sciences) for statistical analysis. After the data distribution analysis, Chi-square Test, Fisher's Exact Test and Kruskal Wallis Test were used to test the correlation of GRP78 immunohistochemical expression with disease progression and SCC patient's prognosis. $P<0.05$ was considered statistically significant. For SCC samples, good prognosis was considered as the patient being alive without new lesions and bad prognosis was related to the patient alive with recurrence or metastasis, or who died due to tumor. For leukoplakia lesions, the evolution was considered good if the injury does not relapsed after an excisional biopsy, if it remained unchanged after an incisional biopsy and if the clinical aspect improved and/or decreased in size; the prognosis was considered bad if the injury worsened its clinical aspect and/or increased in size, if there was recurrence, if it emerged new lesions or if there was malignant transformation. Based on this final score, the cut point was 8 for OSCC sample and 6 for leukoplakia lesions, considering the previous findings of increased GRP78 expression with the higher grade of malignancy of the lesions (LIN et al., 2010).

RESULTS

Clinical characteristics of HNSCC and leukoplakia samples of the study and evaluation of histological grade

A total of 55 patients with squamous cell carcinoma (46 men and 9 women with an average age of 57 years) was recruited. The demographic profile of the patients is summarized in Table 1. Most of the patients were males (84%), over 60 years of age (53%). The tumor sites included tongue (n = 12; 20%), palate (n = 9, 16%), oropharynx (n = 16; 29%), and others intra-oral sites (n = 19; 35%). Regarding prevalence of carcinogenic habits, 81% were current smokers (n = 45) and 74% were current consumer of alcohol (n = 41). T stage was found for 64% to be T1-T2, most of the tumors was well differentiated (n = 25; 45%) without lymph node metastasis (n = 36; 65%). The mean duration of follow-up for patients with HNSCC was 5 years, and after this time, about 55% of patients were alive without recurrence.

Table 1 - Demographic profile of SCC sample

Characteristics	Patients (n = 55) No. (%)
Gender	
Male	46 (84)
Female	9 (16)
Age	
<60y	26 (47)
≥60y	29 (53)
Sites	
Tongue	11 (20)
Palate	9 (16)
Oropharynx	16 (29)
Others	19 (35)
Smoking status	
Current	45 (81)
Formant	8 (15)
Never	2 (4)
Alcohol intake	
Current	41 (74)
Former	4 (7)
Never	10 (19)
LN metastasis	
Present	19 (35)
Absent	36 (65)
Prognosis	
Good	30 (55)
Bad	23 (42)

Other 57 patients with leukoplakia (35 men and 22 women with an average age of 56.7 years) were also examined. The demographic profile of the patients is summarized in Table 2. The majority was composed of males (61%) over 60 years of age (54%). The lesions sites included tongue (n = 14; 25%), palate (n = 18; 32%), oropharynx (n = 1; 2%), and others intra-oral sites (n = 24; 41%). Regarding prevalence of carcinogenic habits, 46% were current smokers (n = 26) and 39% were current consumer of alcohol (n = 22). The most prevalent clinical type of leukoplakia was non-homogeneous (n = 27; 47%) without epithelial dysplastic changes (n = 32; 56%). The mean duration of follow-up in patients with leukoplakia was 4,3 years, and for the most of them, the course of the disease has worsened (n = 22; 39%).

Table 2 - Demographic profile of leukoplakia sample

Characteristics	Patients (n = 57) No. (%)
Gender	
Male	35 (61)
Female	22 (39)
Age	
<60y	21 (37)
≥60y	31 (54)
Sites	
Tongue	14 (25)
Palate	18 (32)
Oropharynx	1 (2)
Others	24 (41)
Smoking status	
Current	26 (46)
Former	3 (4)
Never	5 (9)
Alcohol intake	
Current	22 (39)
Former	4 (7)
Never	7 (12)
Dysplastic changes	
Yes	25 (44)
No	32 (56)
Clinical type	
Homogeneous	7 (13)
Non-homogeneous	27 (47)
Prognosis	
Good	10 (18)
Bad	22 (39)

Increased GRP78 immunostaining in head and neck cancer in comparison with potentially malignant lesions and normal tissue

The GRP78 protein was detected in the cells cytoplasm. High expression of GRP78 occured in both potentially malignant and malignant lesions. The comparison among groups revealed an increase of GRP78 expression in cancer tissues, in both center of the tumor and the invasive front of the tumor, in relation to leukoplakia, to the adjacent epithelial tissue of the tumor and to normal oral mucosa samples (Figure 1).

Immunohistochemical staining of GRP78 in these tissue sections samples are shown in Figure 2.

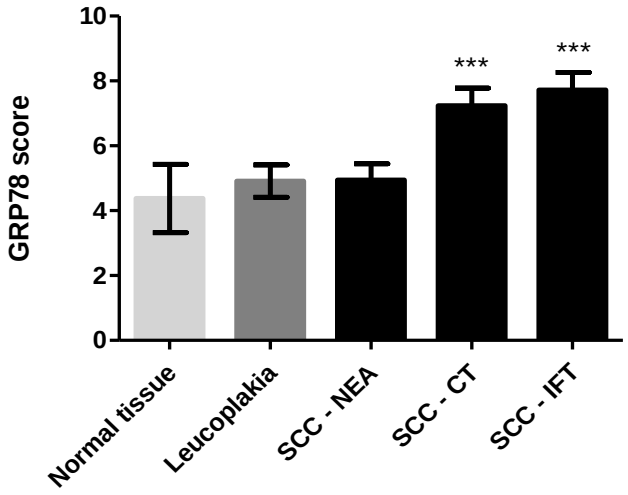

Figure 1 - Comparison of GRP78 immunoexpression along the carcinogenesis process. SCC: Squamous Cell Carcinoma; NEA: Normal Epithelium Adjacent to the tumor; CT: Center of The Tumor; IFT: Invasive Front of the Tumor. Kruskal Wallis Test, *** p<0,001.

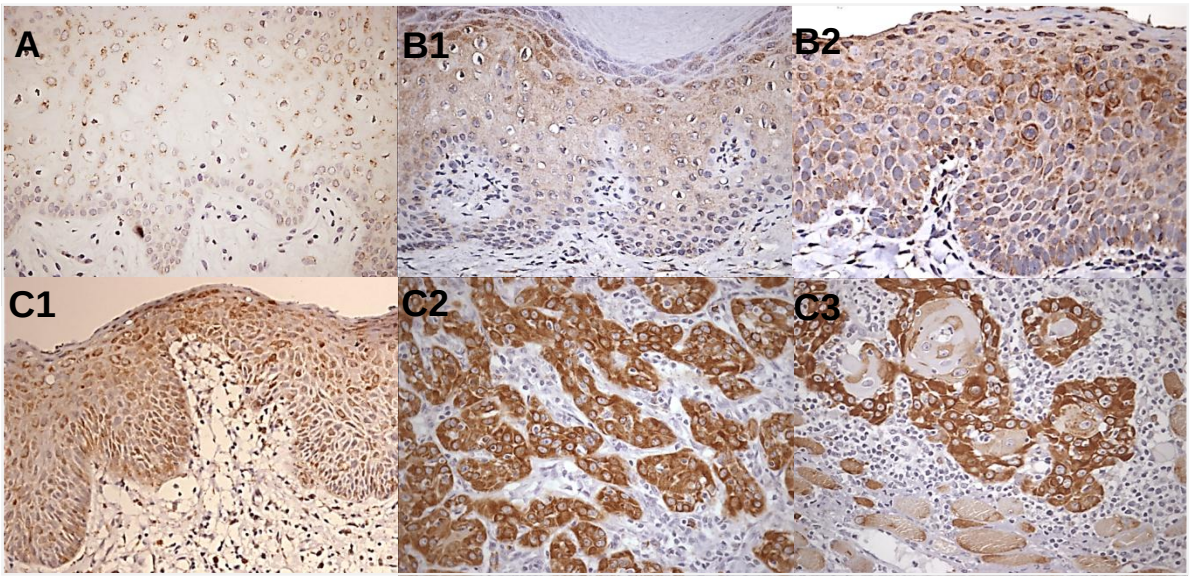

Figure 2 - Immunohistochemical staining (x400) of GRP78 expression in the tissue samples. A: normal oral mucosa; B1: leukoplakia without epithelial dysplasia; B2: leukoplakia with dysplastic changes; C1: normal epithelium adjacent to SCC; C2: center of the tumor; C3: invasive front of the tumor.

Correlation of GRP78 expression with head and neck cancer clinicopathological characteristics

To investigate the role of this protein in head and neck carcinogenesis, we evaluated some important clinical and microscopic features that describe the aggressiveness of the tumor, in addition to the comparison of this protein expression with the survival rates of the patients. We observed that regardless of the site of the tumor tissue, the clinical characteristics as tumor location, tumor size, tumor differentiation and prognosis did not significantly correlate with the immunostaining of GRP78. However, tumors with lymph node metastasis presented a statistically significant higher GRP78 expression in the center of the tumor when compared with tumors without LN metastasis (p = 0,048; Table 3).

Table 3 - Correlation of GRP78 expression in HNSCC with the clinicopathological parameters. *p<0,05, Chi-square Test and Fisher's Exact Test.

Site		**Normal epithelium adjacent**		**Center of the tumor**		**Invasive front of the tumor**	
Score		**<8%**	**≥8%**	**<8%**	**≥8%**	**<8%**	**≥8%**
Location	Mouth	25 (69%)	11 (31%)	17 (46%)	20 (54%)	14 (38%)	23 (62%)
	Oropharynx	11 (73%)	4 (27%)	6 (38%)	10 (62%)	5 (31%)	11 (69%)
	p	1,0		0,569		0,646	
T stage	T1 - T2	23 (70%)	10 (30%)	16 (47%)	18 (53%)	12 (36%)	21 (64%)
	T3 - T4	14 (74%)	5 (26%)	13 (65%)	7 (35%)	13 (65%)	7 (35%)
	p	0,759		0,386		0,92	
LN metastasis	Present	11 (69%)	5 (31%)	5 (26%)	14 (74%)	7 (37%)	12 (63%)
	Absent	26 (72%)	10 (28%)	20 (54%)	17 (46%)	12 (35%)	22 (65%)
	p	1,0		0,048*		0,91	
Tumor differentation	Well	18 (78%)	5 (22%)	9 (41%)	13 (59%)	8 (36%)	14 (64%)
	Moderate/ Poor	17 (68%)	8 (32%)	10 (40%)	15 (60%)	8 (32%)	17 (68%)
	p	0,313		0,835		0,947	
Prognosis	Good	18 (69%)	8 (31%)	11 (41%)	16 (59%)	11 (42%)	15 (58%)
	Bad	13 (76%)	4 (24%)	9 (50%)	9 (50%)	7 (39%)	11 (61%)
	p	0,734		0,54		0,82	

Correlation of GRP78 expression with leukoplakia clinicopathological characteristics

GRP78 expression in leukoplakia lesions was lower than HNSCC. When correlated to the use of carcinogenic substances, patients were classified as follows: *current* for smokers or alcoholics who smoked/drank at the time of data collection; *former* for patients who have quit smoking for more than ten years; and *never* for people who never smoked or drank. The GRP78 staining showed no significant correlation with the use of carcinogenic substances, the epithelial microscopic diagnosis of the lesions and patient's prognosis (Table 4).

Table 4. Correlation of GRP78 expression in leukoplakia with the clinicopathological parameters. Chi-square Test and Fisher's Exact Test.

		Epithelium tissue	
Score		**<6%**	**≥6%**
Clinical type	Homogeneous	4 (57%)	3 (43%)
	Non-homogeneous	18 (67%)	9 (33%)
	P	0,384	
Dysplastic changes	Present	18 (72%)	7 (28%)
	Absent	15 (48%)	17 (52%)
	P	0,666	
Alcohol intake	Yes	13 (59%)	9 (41%)
	No	5 (45%)	6 (55%)
	P	0,529	
Smoking status	Yes	13 (52%)	12 (48%)
	No	4 (57%)	3 (42%)
	P	1,0	
Prognosis	Good	5 (50%)	5 (50%)
	Bad	12 (60%)	8 (40%)
	p	0,602	

DISCUSSION

It is necessary to found reliable biomarkers to help us to improve the therapeutic approach and the follow-up of patients with head and neck cancer. GRP78 is a stress-induced ER chaperone and glucose-dependent protein (LEE, 2001) necessary to embryonic cells growth and development (LEE et al., 2006). Genetic damage and development of pathologic conditions in tumors cells result in ER stress inducing increase of GRP78 expression, which is fundamental to cancer cells survival. This induction is responsible for immunoresistance of the cancer cells against cell death and therefore to chemotherapy and others drugs therapy (VISIOLI et al., 2014).

It is important to understand if GRP78 expression is part of the biological mechanisms responsible for microscopic and clinical aspect of the disease and what is it influence on the course and worsening of lesions and consequently the relation with patient's prognosis. In our study it was clear the increase of GRP78 in cancer cells in comparison to potentially malignant and normal tissues, however, this increase was not correlated with patient's prognosis, although an association with lymph node metastasis was observed. Some studies showed the existence of a positive correlation between the clinicopathological aspects of the disease and GRP78 staining in a variety of malignant tumors. High levels of GRP78 were associated with adverse clinical and pathological features, such as tumor progression and proliferation, poor tumor clinical staging (PIRKO, 2007), lymph node metastasis, recurrence and lower survival rates in patients with high-grade breast cancer, prostate cancer, colon cancer and gastric cancer (DANESHMAND et al., 2007; LEE, 2007; XING et al., 2006; ZHENG, 2008). In the other hand, it was also showed that early tumor stage and longer survival are significantly correlated with high levels of GRP78 in esophageal and neuroblastic tumor cells (LANGER et al., 2008), showing that GRP78 expression can vary considerably among different types of tumors. Kim et al. (2012) showed that GRP78 expression was positively associated with favorable clinicopathological parameters and longer survival in lung cancer patients. Currently, the relation among development and progression of malignant lesions and GRP78 biomarker staining is controversy and needs to be assessed for each type of tumor.

The role of GRP78 in head and neck cancer is still unclear in the literature. Only two studies have recently investigated GRP78 protein in OSCC and have tried to explain the role of this protein in OSCC. Both papers analyzed OSCC samples to evaluate the GRP78 immunohistochemical expression and its influence on the clinical and pathological parameters of the disease. Xia et al. (2014) correlated GRP78 expression with oral cancer behavior. The evaluated criteria of tumor size (>3.1cm), advanced T stage (T3-T4), lymph node metastasis, advanced pathologic stage and poor histological grade were closely related with increase of GRP78 rates. However, Huang et al. (2010) noted that advanced tumor stage (T3-T4) showed weak intensity of GRP78 staining, suggesting that high-grade cells probably developed a mechanism to overcome the effects of ER stress as well as increased expression of GRP78. Our results showed no association with overall prognosis, only with lymph node metastasis. A closer comparison among our and their studies reveals some differences on GRP78 immunohistochemical quantification criteria. Xia et al. (2014) multiplied the score for the intensity of staining by the score for the number of positive cells to obtain the final score like we performed in our study, however next they classified the results as ≤1 being negative and >1 as positive, differing from our methodology. Considering this way of categorization, our results are in contrast with Xia et al. (2014), who found positive expression in only 58.7% of the OSCC sample, while 97% of our samples were positive. In normal oral mucosa, Xia et al. (2014) didn't observe any GRP78 expression, whereas in our study 100% of samples from normal oral tissue showed GRP78 expression. Considering that GRP78 is constitutively expressed, the absence of staining observed in most of Xia et al. (2014) samples may be due to low sensitivity of the antibody used for them. Moreover, GRP78 is also expressed by normal samples. According to Huang et al. (2010), GRP78 was moderately expressed in six of eight normal oral mucosa samples while the others two showed weak expression, demonstrating that the control group also presents basal levels of GRP78.

Few studies have investigated the role of GRP78 in tumor metastasis. Cancer metastasis refers to the ability to cells leave the primary tumor, travel through the circulation and form a secondary tumor. In head and neck squamous cell carcinoma, the lymphatic system is the preferred dissemination route (LEBER; EFFERTH, 2009).

GRP78 expression was significantly higher in highly metastatic tumors in comparison to non-metastatic tumors in esophageal, liver, gastric and oral cancer (SU et al., 2010; XIA et al., 2014; ZHANG; ZHANG, 2010; ZHAO et al., 2015; ZHENG et al., 2008). We showed statistically significant increase of GRP78 expression in the center of the tumor in patients with LN metastasis. One important factor that explains this positive association is that GRP78 regulates the activity of matrix metalloproteinases (MMPs) which are the most common enzymes involved in invasion process in head and neck cancer and have been previously correlated with LN metastasis (LOTFI et al., 2015). MMPs are enzymes involved in proteolysis and degradation of the main components of the basement membrane and extracellular matrix, helping to mediate the migration and invasion and thus facilitating tumor metastasis (LI et al., 2009; ZUCKER; VACIRCA, 2004).

With the progress of cell biology techniques, the field cancerization concept evolved. Molecular changes in adjacent non-tumor epithelium results in formation of a changed field, justifying the considerable GRP78 expression found in this site, being similar to the GRP78 expression in leukoplakia sample. This indicates the presence of epithelial cells with changes already underway with high cancerous potential even in cancer-free zones (HILDEBRAND et al., 2014). Higher expression of GRP78 occurred in head and neck cancer when compared to potentially malignant lesions. This observation is probably due to the fact that malignant tumor tissue is characterized by higher number of cellular changes and genetic mutations, being composed of large clutter, cellular atypia and high protein synthesis in order to improve tumor proliferation and progression. This tumorigenesis process results in higher ER stress compared to the stress caused by epithelial changes observed in leukoplakia lesions, which already present some initial changes such as increased epithelial cells proliferation and genetic mutations, however in a much lesser amount than cancer. Lin et al. (2010) also showed increase of GRP78 tissue expression in oral cancer in comparison to premalignant lesions. They studied leukoplakia, erythroplakia and verrucous lesions, wherein the leukoplakia group of lesions showed the lower number of positive cases. Only 14% of leukoplakias were positive for GRP78 staining, what is in contrast to ours findings that all leukoplakias were GRP78 positive. The early steps of oncogenesis consist in

subsequent mutagenic genetic events and cellular changes that facilitate oral cavity malignant tumors growth and progression. About 30% of these tumors develop from precancerous epithelial lesions, and the most common potentially malignant disorder is leukoplakia. There are no considerable scientific bases that discuss the role of GRP78 in potentially malignant disorders. To the best of our knowledge this is the first study to correlated GRP78 with leukoplakia features and disease evolution. We verified that GRP78 overexpression is not correlated with clinical and microscopic epithelial changes, as well as oral carcinogenic habits and poor prognosis, which included malignant transformation.

In conclusion, we observed that many critical cellular pathways are activated during the carcinogenesis process resulting in overexpression of GRP78 in HNSCC compared with leukoplakia and normal tissue. In addition, our study showed that high levels of GRP78 are involved with migration and invasion ability favoring the development of regional metastasis. However, this protein is not sensitive enough to detect a difference in prognosis in patients with head and neck cancer or to predict the malignant transformation of leukoplakia. With the sample evaluated and the follow-up period proposed, GRP78 protein should not be used as a prognostic biomarker in patients with potentially malignant lesions and head and neck cancer, in contrast to the data found in the literature for other types of cancer. The role of GRP78 in head and neck cancer regional metastasis should be further investigated.

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3 CONSIDERAÇÕES FINAIS

O comportamento biológico do carcinoma espinocelular de cabeça e pescoço ainda é incerto, e representa um campo de pesquisa bastante promissor na busca por maiores esclarecimentos sobre este tema. Embora obtidos avanços no diagnóstico e, principalmente, na terapêutica, seu prognóstico continua sendo desfavorável e as taxas de mortalidade têm-se permanecido inalteradas nas últimas décadas, ao contrário de outros tipos de câncer como de mama, próstata e útero que apresentaram um declínio na sua mortalidade. Portanto, a identificação precoce do câncer de cabeça e pescoço é a conduta mais favorável para reduzir a mortalidade e a morbidade produzidas, contribuindo para um melhor manejo clínico de nossos pacientes (SCOTT, 2006). Para isto, é fundamental investir no estudo de biomarcadores que possam auxiliar o cirurgião-dentista a detectar precocemente essas lesões, a identificar indivíduos com maior predileção para desenvolver lesões malignas e a conduzir o acompanhamento clínico de pacientes com câncer.

Este trabalho se propôs a aprofundar o estudo sobre o mecanismo de atuação da GRP78 e seu impacto no curso clínico da doença. Com a amostra avaliada e o período de acompanhamento proposto, os resultados obtidos neste trabalho demonstram que a proteína GRP78 é altamente expressa em tumores malignos de cabeça e pescoço, porém, não é significativa o suficiente para determinar maior probabilidade de desenvolvimento de lesões malignas nem mesmo detectar diferenças de prognóstico, não estando correlacionada com a evolução do câncer.

É indispensável a realização de futuros estudos longitudinais com ampliação das amostras e do tempo de acompanhamento dos pacientes, tendo em vista que o período para que ocorra a transformação maligna das lesões é variável e pode ser muito longo. Desta forma, seria possível obter resultados mais conclusivos a cerca do impacto da expressão de GRP78 no processo de surgimento de câncer na cavidade bucal.

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ANEXO - PARECER CONSUBSTANCIADO DO COMITÊ DE ÉTICA EM PESQUISA

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL / PRÓ-REITORIA DE PESQUISA -

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ANÁLISE DA EXPRESSÃO IMUNOISTOQUÍMICA DA PROTEÍNA GRP78 (GLUCOSE REGULATED PROTEIN 78) NA CARCINOGÊNESE BUCAL

Pesquisador: Fernanda Visioli

Área Temática:

Versão: 2

CAAE: 12706013.5.0000.5347

Instituição Proponente: Universidade Federal do Rio Grande do Sul

Patrocinador Principal: Faculdade de Odontologia

DADOS DO PARECER

Número do Parecer: 237.008

Data da Relatoria: 04/04/2013

Apresentação do Projeto:

O projeto encontra-se completo. Contempla aspectos importantes para o desenvolvimento de métodos de diagnóstico de processos carcinogênicos bucais.

Objetivo da Pesquisa:

Avaliar a EXPRESSÃO IMUNOISTOQUÍMICA DA PROTEÍNA GRP78 (GLUCOSE REGULATED PROTEIN 78) NA CARCINOGÊNESE BUCAL

Avaliação dos Riscos e Benefícios:

Adequadamente apresentados. Ressalta-se que as solicitações constantes no parecer anterior, em relação aos procedimentos frente a um diagnóstico diferente daquele anteriormente realizado, foram conduzidas. Neste sentido, os autores acrescentaram que o "novo"diagnóstico, caso exista, será encaminhado para o profissional que solicitou o exame anterior. Caberá a este profissional, de acordo com os autores, informar ou não ao paciente sobre o diagnóstico.

Comentários e Considerações sobre a Pesquisa:

O projeto encontra-se adequadamente descrito, abordando um tema de importância frente à alta prevalência de câncer bucal observada na população brasileira.

Considerações sobre os Termos de apresentação obrigatória:

Os termos foram apresentados e estão completos.

Endereço: Av. Paulo Gama, 110 - 2º andar do Prédio da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

Recomendações:

Sugere-se aprovação.

Conclusões ou Pendências e Lista de Inadequações:

Não existem pendências.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Encaminhe-se.

PORTO ALEGRE, 04 de Abril de 2013

Assinador por:
José Artur Bogo Chies
(Coordenador)