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Immunohistochemical investigations on the expression of programmed cell death ligand 1, human leukocyte antigens G and E, and granzyme B in intraoral mucoepidermoid carcinoma

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ABSTRACT

Objective: To identify the expression of nonclassical human leukocyte antigen G and E (HLA-G and -E), programmed cell death ligand-1 (PD-L1) and granzyme B (GB) in intraoral mucoepidermoid carcinomas (MECs), and to assess whether such expressions are related to metastasis, survival, staging, tumor grade and number of GB-positive cells.

Design: For this cross-sectional study, samples of MEC (n = 30) were selected and classified as low-grade (LG), intermediate-grade (IG) or high-grade (HG), according to the WHO grading system. HLA-G, -E and PD-L1 were identified by immunohistochemistry and quantified as the proportion of positive neoplastic cells. The density of GB+ cells was also evaluated. The Kruskal-Wallis test was used with a 5% significance level.

Results: Expressions of HLA-G, -E and PD-L1 were identified in the majority of epidermoid, intermediate and clear cells, but not in the mucous cells of the MECs. The quantitative analysis of the total percentage of positive neoplastic cells showed overexpression of this set of proteins in all MEC samples. The expression of these proteins and histological grading were positively correlated [HLA-G (LG = 79% positive cells, IG = 96%, HG = 99%; $p = 0.0004$), HLA-E (LG = 70%, IG = 96%, HG = 99%; $p < 0.0001$) and PD-L1 (LG = 34%, IG = 79%, HG = 80%; $p = 0.01$)]. No relationship was observed between the immunosuppressive proteins and other clinicopathological parameters. Low GB density was found in all MEC samples.

Conclusions: The augmented expression of HLA-G, -E and PD-L1 in the intraoral MEC might suggest a role of these molecules in the scape of neoplastic cells from immunosurveillance.

1. Introduction

Mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland tumor and presents a high incidence in the minor salivary glands of the palate (Byrd, Spector, Carey, Bradford, & McHugh, 2013; Fonseca et al., 2012; Ito, Ito, Vargas, de Almeida, & Lopes, 2005; Spiro, 1986; Shigeishi et al., 2015; Wang et al., 2012). Intraoral MEC is a locally-invasive, heterogeneous tumor composed of varying proportions of mucous, epidermoid, intermediate, columnar and clear cells (Auclair, Goode, & Ellis, 1992; Barnes, Eveson, Reichart, & Sindransky, 2005, Chap. 5; Byrd et al., 2013). They are classified by the World Health Organization (WHO) into low (LG), intermediate (IG) and high grade (HG) malignancies (Auclair et al., 1992; Barnes et al., 2005; Byrd et al., 2013). High-grade MEC has been associated with higher rates of

Abbreviations: CTL, cytotoxic T lymphocytes; GB, granzyme B; HAJ/ACCG, Goiás Combat Cancer Association's Araújo Jorge Hospital; HG, high grade; HLA, human leukocyte antigen; IG, intermediate grade; LG, low grade; MEC, mucoepidermoid carcinoma; NK, Natural Killer; OSCC, oral squamous cell carcinoma; PAS-D, Periodic acid Schiff with diastase; PD-L1, programmed cell death 1 ligand 1; TNM, extent of primary tumor/regional lymph node metastasis/distant metastasis; UFG, Federal University of Goiás; WHO, World Health Organization

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metastasis, and consequently, with worse clinical prognosis (Auclair et al., 1992; Chen, Roman, Sosa, & Judson, 2014).

An effective host immune response, both locally and in the circulatory system, is pivotal to the tumor in containing progression, local invasion and tumor metastasis (Abbas, Lichtman, & Pillai, 2015; Balkwill & Mantovani, 2001; Liotta & Kohn, 2001). The main immune cells involved in antitumor immunity are antigen-presenting cells (macrophages and dendritic cells), cytotoxic T lymphocytes (CTL) and Natural Killer (NK) cells (Abbas et al., 2015; Balkwill & Mantovani, 2001). The CTL and NK cells work through the release of cytotoxic granules such as perforin and granzyme B (GB) (Afonina, Cullen, & Martin, 2010; Trapani et al., 1998). Perforin is a protein that creates pores in the membrane of the altered cells which act as channels for the influx of GB. The proteolytic nature of GB then brings about the apoptotic death of the neoplastic cell (Afonina et al., 2010; Logue & Martin, 2008).

Most cancers develop strategies of immunosuppression and escape from the host defense system (Borrego, Ulbrecht, Weiss, Coligan, & Brooks, 1998; Braud et al., 1998; Carosella, Moreau, Lemaoult, & Rouas-Freiss, 2008; Carosella, Favier, Rouas-Freiss, Moreau, & Lemaoult, 2008; Cho, Yoon, Lee, Hong, & Hong, 2011; Hamanishi et al., 2007; Homet Moreno & Ribas, 2015; Rouas-Freiss, Moreau, Menier, & Carosella, 2003; Rouas-Freiss, Moreau, Ferrone, & Carosella, 2005; Rouas-Freiss, Moreau, Menier, LeMaoult, & Carosella, 2007; Zou & Chen, 2008). There is surmounting evidence for important counter-regulatory roles of the nonclassical human leukocyte antigen-G and -E (HLA-G and -E) in host response during cancer progression (Borrego et al., 1998; Braud et al., 1998; Rouas-Freiss et al., 2007). Similarly, the co-stimulatory or inhibitory molecule of the B7 family, termed programmed cell death 1 ligand 1 (PD-L1, B7-H1 or CD274), also exhibits this property (Hamanishi et al., 2007; Homet Moreno & Ribas, 2015; Zou & Chen, 2008). The HLAs and PD-L1 proteins bind to inhibitory receptors present in the main cells (e.g., NK cells, CTLs and Antigen Presenting Cells) involved in the development of an effective antitumor immune response, thereby negatively regulating the capacity effector of these cells (Borrego et al., 1998; Braud et al., 1998; Carosella, Moreau et al., 2008; Chen et al., 2004; Dong et al., 2004; Hamanishi et al., 2007; Homet Moreno & Ribas, 2015; Korman, Peggs, & Allison, 2006; Rouas-Freiss et al., 2003; Rouas-Freiss et al., 2005; Rouas-Freiss et al., 2007; Zou & Chen, 2008).

Augmented expression of HLA-G, -E or PD-L1 was identified in oral squamous cell carcinoma (OSCC) (Cho et al., 2011; Fregonezi et al., 2012; Gonçalves et al., 2014; Gonçalves et al., 2015; Lin et al., 2015; Straub et al., 2016; Tsushima et al., 2006; Yu et al., 2015) and associated with a poor clinical prognosis (metastasis and shorter survival) (Gonçalves et al., 2014; Lin et al., 2015; Straub et al., 2016). In salivary gland carcinomas the PD-L1 was associated with poor disease free survival (Mukaigawa et al., 2016). This is the first to investigate this set of immunosuppressive mediators in intraoral MEC. The study of HLA-G, -E and PD-L1 in malignancies is relevant because these proteins are potential therapeutic targets that contribute to the control of neoplastic progression (Bell, 2016; Borghaei et al., 2015; Carosella, Ploussard, LeMaoult, & Desgrandchamps, 2015; Homet Moreno & Ribas, 2015; Lin & Yan, 2015; Pai, Zandberg, & Strome, 2016; Postow et al., 2015; Ravindranath, Terasaki, Pham, & Jucaud, 2015; Robert et al., 2015; Straub et al., 2016; Tsushima et al., 2006; Yu et al., 2015). Consistently, the pembrolizumab and nivolumab (target of the PD1/PD-L1) have provided promising clinical results in the treatment of metastatic melanoma (Homet Moreno & Ribas, 2015; Postow et al., 2015; Robert et al., 2015) and squamous cell carcinoma of the head and neck (Bell, 2016; Pai et al., 2016). Herein, we investigated the expression of HLA-G, -E and PD-L1 in intraoral MEC, and assessed the relationship between the expression of these proteins with clinical and microscopic parameters.

2. Material and methods

2.1. Collection and selection of samples

This study was approved by the Research Ethics Committees at the Federal University of Goiás (UFG) and the Goiás Combat Cancer Association's (ACCG), Araújo Jorge Hospital (HAJ) (N° 1.460.804). For this cross-sectional study, the medical records at HAJ/ACCG were consulted and specimens selected from patients affected by intraoral MEC. The patients had been diagnosed and treated between 1992 and 2013 at the Head and Neck Division. After selection, the blocks of surgical specimens and slides were obtained and evaluated by consulting the Anatomopathology Service files at HAJ/ACCG.

The inclusion criteria demanded that samples be taken from patients with minor salivary gland MEC of the oral cavity (intraoral MEC), diagnosed and treated at HAJ/ACCG, with a minimum follow-up of 24 months (global survival). Their records needed to contain the following data: clinical TNM staging, information about the survival or death of the patient and information on the presence of metastasis and tumor location. Exclusion criteria were: defects in slides and blocks; insufficient for analysis, MECs from other locations; cases without clinical follow-up or with incomplete records; cases which had been subjected to previous treatments (chemotherapy, radiotherapy, etc.) and recurrences.

Thus, after considering the inclusion and exclusion criteria, 30 samples of MEC were selected. Initially the number of high-grade MEC was very low (only 5 cases), but in the course of the study (considering the tendency for higher expression of HLA-G, HLA-E and PD-L1 in high-grade MEC and the possible association of these molecules with histological grading), we performed an active search in Cancer Hospital (HAJ/ACCG) of intraoral MEC of the high-grade. Then, more 4 recent cases of the high-grade MEC were included in the present investigation. The objective was to obtain a higher number of cases in this group (high-grade MEC group) that would allow an adequate statistical analysis of the data.

Healthy oral mucosa samples with minor salivary gland (n = 10) were selected from the store of slides and blocks of the Dental School Pathology Laboratory at the Federal University of Goiás (FO/UFG) for analyses of GB+ cell density, inflammatory infiltrate intensity, HLA-G, HLA-E and PD-L1.

2.2. Histopathologic grading of samples

The material selected was sectioned by microtome (Leica 2165 model RM Microsystems, Inc., Bannockburn, IL, USA), with each block yielding consecutive 5 μm sections, with one placed on a histological slide and stained using the Hematoxylin and Eosin (HE) method.

A second serial slice was subjected to the histochemistry technique of the Periodic Acid-Schiff with Diastase (PAS-D) to detect neutral glycoconjugates. The slides were deparaffinized and hydrated, then human saliva was pipetted onto the slide for 20 min in a humid chamber. After washing in distilled water, the slides were oxidized in 0.5% periodic acid for 15 min, washed and incubated with Schiff reagent for 30 min, washed again and finally counter-stained for 5 min with Harriśs hematoxylin.

The sections stained with HE and PAS-D were used for the microscopic characterization of the samples, based on WHO classification (Barnes et al., 2005). Then, for tumor grading classification of the MECs, the following histopathologic parameters were assessed: cystic components < 20% = 2 points, neural invasion = 2 points, presence of necrosis = 3 points, four or more mitoses/ten high power fields = 3 points and anaplasia = 4 points. MEC samples with the point score between 0 and 4 were classified as low-grade, 5–6 points as intermediate-grade and 7 or more points as high-grade (Auclair et al., 1992; Barnes et al., 2005; Coca-Pelaz et al., 2015). The predominant cell type in the tumor (mucous, intermediate or epidermoid cells) (Auclair et al.,