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Immunosuppressive mediators of oral squamous cell carcinoma in tumour samples and saliva

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ABSTRACT

The goal of this study was to compare the salivary concentrations of IL-10, TGF-β1 and soluble HLA-G (sHLA-G) in patients with oral squamous cell carcinoma (OSCC) to those in healthy individuals (control group), and to correlate the expression of these mediators in saliva with that in the tumour microenvironment. Neoplastic tissue and saliva samples from patients with OSCC ($n = 22$) were analysed by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) respectively. We detected high expression of IL-10 and HLA-G in the tumour microenvironment when compared to healthy oral mucosa samples. Determination of IL-10 salivary concentration enabled us to distinguish patients with OSCC from healthy individuals ($P = 0.038$), which showed correlation with tissue expression of this cytokine. HLA-G salivary release was similar in both groups ($P = 0.17$) and no correlation with tumour expression was observed. TGF-β1 expression was low or absent in tumours, and salivary concentration was similar between groups. Our results suggest that of the three markers analysed, IL-10 is a potential salivary biomarker. Furthermore, the elevated expression of HLA-G and IL-10 in tumour sites could favour the escape of tumour cells from immune defense mechanisms.

1. Introduction

Saliva is a body fluid has proven very useful for the early diagnosis, monitoring and treatment of diseases [1–3]. It is rapidly and easily collected, and has had intimate contact with oral neoplastic lesions [1–5]. As such, salivary biomolecules have been put forward as promising markers for ovarian cancer [6], breast cancer [7], oral squamous cell carcinoma (OSCC) [1,5,8] and other neoplasms [9]. However, no salivary molecule has so far met all requirements to be a disease marker or prognostic factor for OSCC. Therefore, several biomarkers are still candidates for future clinical use [5].

Of the prognostic markers being investigated, those involved in tumour evasion mechanisms such as interleukin-10 (IL-10), transforming growth factor beta (TGF-β) and human leukocyte antigen G (HLA-G) are of particular interest [10,11,23]. IL-10 and TGF-β are immunosuppressive and anti-inflammatory cytokines involved in tumour evasion [10,11]. IL-10 regulates the differentiation of regulatory T (T reg) cells [12]. IL-10 also contribute to the tumoural escape inhibit the functioning of macrophages and dendritic cells, which are presenting antigen cells (APCs) essential for the presentation of the tumour antigen to the cytotoxic T lymphocytes (CTLs) [13–15]. IL-10 could also be active in tumour evasion through the generation of a phenotype of neoplastic cells resistant to the action of CTLs. This comes about because of a low expression or inhibition of MHC class I molecules on the cell surface. In turn, this effect could possibly be due to a reduction in the expression of the transporters associated with antigen processing (TAP-1 and TAP-2),

Abbreviations: OSCC, oral squamous cell carcinoma; IL-10, interleukin-10; TGF-β, transforming growth factor beta; HLA-G, human leukocyte antigen G; sHLA-G, soluble HLA-G; NKs, natural killer cells; CTLs, cytotoxic T lymphocytes; APCs, antigen-presenting cells; WHO, World Health Organisation; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay.

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which leads to poor translocation of peptides into the endoplasmic reticulum, which then brings about this reduction in the expression of MHC class I [13–15].

TGF-β also contributes to the growth and differentiation of such neoplastic cells, as well as to the process of angiogenesis [16]. The main sources of these cytokines are macrophages, T and B lymphocytes and tumour cells [17–19]. Previous studies have demonstrated that high expression of IL-10 predicts a poor prognosis in OSCC [20], colon cancer [18] and oesophageal carcinoma [17]. Similarly, high expression of TGF-β1 has been associated with a poorer prognosis, represented by greater tumour proliferation and invasion, higher blood vessel density (angiogenesis) and lower survival in patients with gastric cancer [16], lung adenocarcinoma [19] and OSCC [21].

The HLA-G is a non-classical class I HLA which differs from other classical HLA-I molecules, basically because of its characteristics, such as limited polymorphic gene and biological properties which give immune tolerance [22,23]. This molecule is expressed in the foetal–placental interface, on the surface of the trophoblast, with the aim of protecting the fetus against the mother's immune response [22,23]. After birth, the expression of the HLA-G molecule is restricted to immune privileged tissues, such as the thymus [24], cornea [25], pancreas [26] and erythroid and endothelial precursor cells [27]. HLA-G can be expressed as seven isoforms, being four membrane-bound (HLA-G1–4) and three soluble isoforms (sHLA-G; HLA-G5–7), owing to alternative splicing of the primary transcript [23,28–30]. Another soluble form of HLA-G (sHLA-G1) can be released through proteolytic cleavage of the HLA-G1 protein present in the cellular membrane, as a shedding form of HLA-G1 [25]. This molecule is reported to inhibit the activity of natural killer cells (NKs), CTLs and APCs, which are the main cells involved in the development of an effective cytotoxic anti-tumour immune response [31–33]. Such inhibition occurs through a direct interaction with the inhibitory receptors KIR2DL4 (CD158d), immunoglobulin-like transcript 2 (ILT2) (CD85j) (LILRB1) and ILT4 (CD85d) (LILRB2), which are expressed by NKs and CTLs [31–33]. Use of HLA-G has therefore been suggested as a possible strategy by tumour cells in certain neoplasms [33–36], including OSCC [34] to resist or escape host immunosurveillance [33–36].

Although the inhibitory and immunosuppressive properties of the above-mentioned molecules have already been described, no studies have evaluated the presence of all these markers in the saliva of patients with OSCC. The aim of this study was to compare the salivary concentrations of IL-10, TGF-β1 and soluble HLA-G (sHLA-G) in patients with OSCC to concentrations in healthy individuals (control group), and relate these salivary mediators to expression of these factors in the primary tumour.

2. Materials and methods

2.1. Patient selection

This study was conducted between 2008 and 2012, and was approved by the Research Ethics Committees at the Federal Universities of Minas Gerais (UFMG) (087/2007) and Goiás (UFG) (032/2011). A signed informed consent form was obtained from all participants. A tumour sample and saliva from the 22 patients with a histopathological diagnosis of OSCC were included. The control groups consisted of: (1) saliva from 23 healthy individuals and (2) healthy oral mucosa tissue samples obtained from biopsies of 10 healthy patients with pigmentation in the oral cavity mucosa (tongue, floor of the mouth and gums). A microscopic examination of these tissues (control group) showed oral mucosa without any histopathological changes.

Patients and controls who presented signs of significant morbidity or active health problems such as autoimmune disease, HIV infection, abnormal renal function, congestive heart failure, active infection and hepatitis were excluded from the study. Medical records provided data on age, gender, ethnicity, tobacco and alcohol consumption (yes or no), tumour location, histological grading, T stage and metastasis (yes or no).

2.2. Tumour samples

Specimens obtained by incisional biopsy were fixed in 10% buffered formalin (pH 7.4) and paraffin embedded. Microscopic features were assessed by analysis of 3-μm sections of each sample stained with haematoxylin and eosin. OSCC samples were graded according to the WHO (World Health Organisation) tumour classification [37]. The T and N stages of oral cavity carcinomas were graded according to the American Joint Committee on Cancer/International Union Against Cancer classification [38].

2.3. Immunohistochemistry (IHC)

Immunohistochemistry was performed as previously described [34,39]. For retrieval of the IL-10, TGF-β1 and HLA-G antigen, sections were immersed in a citrate buffer (pH 6.0), both for 25 min. The slides were incubated with the following primary antibodies: anti-human TGF-β1 (Santa Cruz Biotechnology – sc130348, CA, USA, diluted 1:50), anti-human HLA-G (clone MEM-G2, Exbio, Prague, Czech Republic, diluted 1:100) and anti-human IL10 (Abcam Inc. – ab34843, Cambridge, MA, EUA, diluted 1:300). The sections containing anti-IL-10 and anti-HLA-G antibodies were incubated with the Starr Trek Universal HRP Detection System (Biocare Medical, CA, USA), while those containing anti-TGF-β1 were incubated with an LSAB Kit (K0690, DAKO, Carpinteria, CA). Trophoblast samples were used as positive controls for HLA-G detection. Smooth muscle walls of arterioles and immune-inflammatory cells of the OSCC samples were used as internal positive controls for TGF-β1 and IL-10, respectively. Negative controls were obtained by omitting the primary antibody, which was replaced with phosphate-buffered saline (PBS; 0.4 mM NaCl, 10 mM $NaPO_4$) containing 1% bovine serum albumin (BSA) and by non-immune rabbit (X0902, DAKO) or mouse (X501-1, DAKO) serum.

The evaluation of the IL-10, TGF-β1 and HLA-G proteins was undertaken in all OSCC samples in both neoplastic cells and cells of the inflammatory infiltrate. A semiquantitative scoring system was performed from previous studies [34,40,41]. The percentage of positive tumour cells was scored as: 0, no tumour cells stained; 1, <25% of cells stained; and 2, ⩾25% of cells stained. Staining intensity was scored as: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining; according the intensity of positive control (inflammatory cells to IL-10, smooth muscle walls of arterioles to TGF-β1 and trophoblast to HLA-G). The immunoreactive score (IRS) was calculated by multiplying the percentage of positive cells (scored 0–2) by the staining intensity (scored 0–3). IL-10, TGF-β1 and HLA-G expression in tumours with IRS = 0 was considered absent, IRS ⩽ 2 was considered low expression, whereas tumours with IRS > 2 were considered to have high expression. All sections were analysed blind by two examiners using a light microscope with high-power fields (400×).

2.4. Saliva collection

After diagnosis of the oral lesions, saliva collection from the major and minor saliva glands was performed as previously described [42]. Participants were instructed to refrain from eating, drinking, smoking, or carrying out oral hygiene procedures for at least one hour before the collection. A cotton wool swab (Salivette®, Sarstedt AG & Co., Nümbrecht, Oberbergischer Kreis, Germany) was inserted into the patient's mouth for 5 min to