Salivary biomarkers related to potentially malignant disorders and oral carcinomas: Brief update

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ABSTRACT
Early diagnosis of oral cancer can improve the quality of life and increase the survival rate of patients. Saliva is a biological fluid of non-invasive access and easy collection, in which it is possible to detect proteins that play an important role in the discrimination of diseases. The presence of salivary protein biomarkers, such as cytokines (IL-6, IL-8, TNF-α)), may aid in the early diagnosis of malignant changes and may be an indicator of the presence of potentially malignant disorders and oral carcinoma. However, due to the variability of the methodologies of the different studies, the absence of standardization of saliva collection, the lack of consensus on the concentration of biomarkers, and also the absence of multicenter studies with larger samples, the reality of clinical use of these biomarkers for early diagnosis of oral cancer is still for the near future.

Keywords: Saliva, Oral Neoplasms, Tumor Biomarkers, Salivary Proteins, Peptides.

1 INTRODUCTION

1.1 ORAL CANCER

Cancer is the leading cause of death worldwide and was responsible for approximately 9.6 million deaths in the year 2018, with 1.2 million deaths in the Americas. This mortality in the Americas is predicted to increase to 2.1 million by the year 2030. A total of 70% of deaths occurred in low- and middle-income countries, such as Brazil (PAHO, 2020).

For the triennium 2023-2025, the National Cancer Institute (INCA) estimates that there will be 704,000 new cases of cancer in Brazil. Of these, a total of 15,100 new cases of oral cavity cancer are estimated, 10,900 in men and 4200 in women, with an estimated incidence of 10.30 cases per 100,000 men and 3.83 per 100,000 women. Disregarding non-melanoma skin tumors, oral cavity cancer ranks eighth among the most frequent types (INCA, 2022a). In 2020, in Brazil, there were 6,192 deaths from oral cavity cancer, corresponding to a risk of death of 2.92 per 100,000 inhabitants, with this risk being higher in men (4.60 per 100,000) than in women (1.32 per 100,000) (INCA, 2020; BRAZIL, 2022).

Cancer mortality can be reduced if cases are detected early through screening and early diagnosis (PAHO, 2020).

Concerning carcinoma of the oral cavity, most of the time, the patient presents for treatment late, with the disease in its advanced stage (SOARES et al., 2019), and a consequent decrease in the survival rate (HU et al, 2008; LEE et al, 2011). Between 2004-2015, more than 60% of malignant neoplasms of the mouth diagnosed and treated in Brazil were in the advanced stage (stage IV) (SOARES et al., 2019). In addition, the treatment of these patients can still be disfiguring and debilitating (GILLENWATER et al., 1998).

Despite improvements in therapeutic and diagnostic modalities, the prognosis of patients with oral carcinomas remains poor (KHALILI, 2008). This worse prognosis is related to late diagnosis, with a five-year survival rate of only 40% to 50% (SCHAAIJ-VISSER et al., 2010; PFAFFE et al., 2011).

In this context, it is important to make an early diagnosis of oral cancer to improve the quality of life and survival rate of patients. In clinical practice, early diagnosis, conventionally performed, requires the performance of accurate clinical examination and biopsy for histopathological evaluation of the suspected site to determine its malignant potential (GILLENWATER et al., 1998). However, several factors contribute to the difficulty of effective development of tools for early detection of oral cancer, such as inexperienced professionals who do not recognize changes indicative of dysplasia and neoplasia (BOUQUOT et al., 2010), the distinction between inflammatory changes, which are much more prevalent, of potentially malignant disorders (INCA, 2022b) and reluctance to perform biopsy.

Epithelial dysplasia is traditionally divided into three categories: mild, moderate, and severe. Mild dysplasia is restricted to architectural changes accompanied by cellular atypia located in the basal layer without extending beyond the lower third of the epithelium. Moderate dysplasia demonstrates the proliferation of atypical cells that extend to the middle third of the epithelium. Severe dysplasia comprises more than 2/3 of the epithelium and may have architectural and cytological changes throughout the epithelium, from the basal layer to the upper third of it. In this case, it can already be considered carcinoma *in situ*. The classification should be based on the area most severely involved, even if it represents only a small portion of the tissue (BOUQUOT et al., 2006). The evaluation of dysplasia can be an important indicator of the malignant potential of a lesion (BARNES et al., 2005).

Among the potentially malignant disorders, we can mention leukoplakia, erythroplasia and actinic cheilitis (NEVILLE, DAY, 2002; INCA, 2022b). Leukoplakia represents 80% of oral lesions with the potential for malignant transformation, affecting mainly white men over 40 years of age (PETTI, 2003). Leukoplastic lesions located on the tongue, oral floor and lip often present dysplastic or malignant changes (NEVILLE, DAY, 2002). Erythroplasia is an alteration of the mucosa with a reddish aspect, with great chances of having dysplastic alteration. Actinic cheilitis is a diffuse alteration located in the lower labial semi-mucosa, which results from excessive or long-term exposure to the

ultraviolet component of solar radiation. The lesions rarely occur in people under 45 years of age and have a strong predilection for the male gender. They initially present as atrophy of the border of the labial semi-mucosa and, as the lesions progress, rough and scaly areas develop on the more dry portions of the lip. With progression, chronic ulcers can develop and last for a few months or progress to squamous cell carcinoma (NEVILLE et al., 2016).

Early diagnosis is the key to decreasing morbidity and mortality associated with the early stages of squamous cell carcinoma and potentially malignant disorders. In Western countries, such as Brazil, leukoplakia, leukoplakia, erythroplakia and cheilitis/actinic keratosis are the most frequent entities and are classically associated with the risk of malignant transformation. (INCA, 2022b). The latter may remain clinically undetectable for years until they progress to the surface. If the change is detected early, the treatment is less aggressive and leaves the patient with a better quality of life after treatment.

Therefore, accurate, objective and non-invasive diagnostic methods are necessary as auxiliary means for detecting suspicious lesions, such as diagnosis through saliva.

2 SALIVARY DIAGNOSIS

Saliva is an important fluid for detecting physiological and pathological changes in the human body (SHAH et al., 2011). Due to the ease of access to the mouth, its obtainment is an easy, inexpensive and non-invasive procedure having been used as a diagnostic tool (CASTAGNOLA et al., 2011; KAKARADDI et al., 2012; MELGUIZO-RODRÍGUEZ, 2020) for oral cancer (WONG, 2006; Zimmermann et al., 2007; LEE; WONG, 2009). This oral fluid can be taken as the mirror of the body, being the perfect medium to be explored in the analysis of health and disease (LEE et al., 2009).

The molecular composition of saliva can reflect the physiological state of the body, including emotional, endocrine, immunological, metabolic, and nutritional variations, and provides a source for monitoring oral and systemic health (ZIMMERMANN et al., 2007; SPIELMANN, WONG, 2011), besides being a marker of infectious or neoplastic diseases (ZIMMERMANN et al., 2007).

Salivary diagnosis could enable clinicians to monitor diseases frequently and easily, which could impact medical research and future therapies. Saliva can help detect lung cancer, pancreatic cancer, breast cancer, and type II diabetes. However, for each disease, there is a need for scientific validation, as well as confirming its superiority over other fluids (LEE, WONG, 2009). Also, saliva can be used to detect the risk of caries, periodontitis, salivary gland diseases and systemic disorders such as hepatitis, HIV and HCV (LEE et al., 2009; CASTAGNOLA et al., 2011).

The direct contact between saliva and altered mucosa makes salivary biomarkers an attractive alternative to blood. The DNA, RNA, and protein molecules of the altered cells can then be conveniently obtained by this fluid, reflecting physiological and pathological stages (LEE, WONG, 2009; SHAH et al., 2011). Oral epithelial cells can routinely be detected in saliva and oral

mouthwashes, making cytological and molecular analyses such as oral cancer monitoring quite attractive. Saliva analysis could monitor the presence of genetically altered oral epithelial cells (GILLENWATER et al., 2006), and also function as a fluid for the detection of biomarkers.

The term biomarker refers to measurable and quantifiable biological parameters that can serve as indicators for physiological measures, as well as parameters of pathological processes, environmental exposures, disease diagnoses and prognosis, and pharmacological response to a therapeutic intervention. Saliva is the only biological fluid that contains components found in serum, offering advantages over the latter. The correlation between plasma and saliva precedes the fact of plasma extravasation through intra- and extracellular pathways, including gingival crevicular fluid, produced by the epithelial cells of the gingival sulcus. Saliva and plasma, therefore, share essential proteins necessary for the maintenance of their physiological functions as body fluids and proteins derived from cellular debris are in close contact with these fluids (SPIELMANN; WONG, 2011). Depending on the size and ionic charge, some molecules penetrate from the blood to saliva via passive diffusion, such as hormones, via filtration and/or active transport, such as IgA, turning saliva into a diagnostic medium (PFAFFE et al., 2011).

Saliva contains hundreds of smaller proteins or peptides that are present in low concentrations but play an important role in discriminating diseases. In addition to proteomes, salivary transcriptomic technology advances the diagnostic potential of saliva for medical applications (LEE, WONG, 2009; LEE et al., 2011; SPIELMANN; WONG, 2011).

The proteome is the analysis of the portion of the genome that is expressed, being the protein complement of the latter. Analysis of the salivary proteome in the course of an alteration can detect early-stage morbidity and monitor disease progression (LEE, WONG, 2009). The amino acid sequence of proteins provides a link between proteins and coding genes via genetic code, and mainly a link between cellular physiology and genetics (SPIELMANN; WONG, 2011). The salivary transcriptome is messenger RNA (mRNA) molecules that cells use to carry instructions provided by DNA for subsequent protein production (LEE, WONG, 2009).

Proteomic constituents are the first choice for salivary analysis (ZIMMERMANN et al., 2007, BARBOSA et al., 2012). The importance of analyzing proteins directly lies in the fact that they perform physiological and pathological functions in the cells. In addition, they are subject to post-transcriptional modifications. The transcription rate, measured through mRNA, does not always have a direct relationship with the biological activity of the protein, its function or quantity (BISCH, 2004).

The collection of unstimulated total saliva is often used for the diagnosis of systemic diseases because it can be readily collected and, more importantly, contains serum constituents (PFAFFE et al., 2011). When using saliva to detect biomarkers, the salivary method has sufficient specificity and

sensitivity, and the collection can be done without the need for prior training (BRINKMANN et al., 2010).

Although biomarkers are in lower concentrations in saliva compared to serum, there are currently sensitive methods capable of detecting them. Also, concerning serum, the possibility of contamination of the professional in the manipulation is greatly reduced. And as the collection is simple, it reduces anxiety, in addition to allowing more frequent monitoring without generating discomfort (LEE; WONG, 2009; SPIELMANN; WONG, 2011).

Although proteomic constituents are the first choice for saliva analysis, genomic targets have emerged as informative and discriminatory biomarkers. The future for salivary diagnosis consists of combining biomarker panels used as *screening* tools to improve diagnostic accuracy and specificity. A biomarker alone is not sufficient as a reliable source to aid in the investigation of the pathogenesis of a given disease. The combined use of biomarkers should provide additional and powerful diagnostic information (PFAFFE et al., 2011).

According to Shah et al. (2011), salivary screening may be the best choice as the primary test for oral cancer screening. The systematic analysis of genomic and proteomic biomarkers facilitates the identification of sensitive and specific parameters for oral cancer and should help identify high-risk patients, in addition to indicating better treatment modalities, improving the survival of these patients.

The use of saliva to monitor the patient's health and disease status is highly desirable for health promotion and health research. It is also possible to evaluate the concentration of drugs, hormones, antibodies and molecules in saliva (PUNYANI; Shatwane, 2012).

As a clinical tool, saliva has numerous advantages over serum, including ease of collection, storage and ease of obtaining in sufficient quantities for analysis at low cost (WONG, 2006). This is particularly relevant in developing countries, where many health and disease risks remain poorly defined and receive inappropriate treatment. In addition, little information about the disease is available to guide the population in health decisions (LEE et al., 2009).

The ease of saliva collection, as well as the fact that it offers less risk of contamination to the operator when handling it, represent important aspects to support the choice of saliva as a means of diagnosis. In addition, it is a reliable method when compared to other scientifically proven methods, such as blood and urine, with validated applicability (MOURA et al., 2007).

According to Loo et al. (2010), approximately 73% of salivary proteins are not present in plasma, providing unique opportunities to discover exclusively salivary proteomic markers. Regarding immunoglobulins, there is relative linearity in the distribution of these proteins between saliva and plasma, suggesting that antibodies can be found concomitantly in plasma and saliva, with salivary concentration being linear and reflecting serum concentration.

It was verified that high levels of Interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) were found in saliva in patients with leukoplakia, concerning patients without oral alterations. IL-6 inactivates the tumor suppressor gene P53, hypermethylating its promoter region, resulting in suppression of apoptosis and uncontrolled cell proliferation. TNF-α activates the transcription factor NFκB, which stimulates cell proliferation and blocks apoptosis, in addition to increasing the secretion of pro-inflammatory cytokines (BRAILO et al., 2006).

Sato et al. (2010) verified an increase in the concentration of IL-6 in the saliva of patients with oral carcinoma, when compared to controls.

IL-8 is a pro-angiogenic and pro-inflammatory mediator, which is significantly increased in the saliva of patients with oral carcinoma when compared to control patients (PUNYANI; Shatwane, 2012).

Osman et al. (2012), in a literature review, reported the need for new large-scale studies for the validation of salivary cytokines (IL-6 and IL-8, mainly) as biomarkers for the diagnosis of oral carcinomas, taking into account that their expression was also altered in periodontal disease.

When assessing the salivary levels of pro-angiogenic and pro-inflammatory TNF-α, IL-1, IL-6 and IL-8 in patients with potentially malignant disorders and squamous cell carcinomas, Rhodus et al. (2005) observed significant differences between groups and patients without lesions. Such cytokines were significantly elevated in patients with potentially malignant disorders with patients without lesions and significantly increased in patients with oral carcinomas, when compared to patients without lesions.

Brailo et al. (2012) observed that IL-6 was significantly increased in the saliva of patients with oral cancer, when compared to patients with leukoplakia and control patients. However, the authors did not observe significant differences in IL-6 levels among patients with leukoplakia when compared to control patients. In addition, they observed that there were no differences in salivary levels of TNF-α when comparing the three groups. The authors noted that the increase in pro-inflammatory cytokines in saliva reflects the development of oral cancer from leukoplakia, but was reluctant to state whether the increase in IL-6 and IL1β occurred before the carcinoma became clinically evident or whether it could be used to monitor the transformation of leukoplakia into carcinoma.

Importantly, many salivary biomarkers are not specific to a particular disease and these can be used to aid the diagnosis of various pathologies. Because of this, it is important to consider the different biomarkers that are affected in each disease for a more specific diagnosis and prognosis (MELGUIZO-RODRÍGUEZ, 2020). According to a simple literature review by these authors, information on the usefulness of biomarkers in oral leukoplakia has not yet been conclusive, and more research is needed to explore new biomolecules that are candidates for this purpose.

Bugshan and Farooq (2020) reported in their review that, for the diagnosis of oral cancer, biopsy should be performed, but salivary biomarkers could also be used as aids in the early diagnosis of the same. According to Rusling et al. (2010), the diagnosis and detection of cancer using salivary protein biomarkers should require accurate detection of panels with approximately 4 to 10 biomarkers for each type of cancer.

Second Li et al. (2020), reliable proteomic biomarkers should be not only highly related to disease but also have stability and be collected from saliva in a standardized way. In addition, the sample should be processed immediately so that there are no changes, and protease inhibitors and refrigeration of the sample should also be used. In addition, protein biomarkers require extensive validation by cohort research before they can be used clinically so that, shortly, they present sufficient specificity to be applied in early-stage cancer screenings and thus greatly improve the quality of life of patients.

Chiamulera et al. (2021) conducted a systematic review and meta-analysis, evaluating 28 case-control studies that used cytokines as cancer biomarkers. The studies described 10 different cytokines, with interleukins (IL-6 and IL-8) being the most studied. According to the meta-analysis, the authors found that salivary levels of some cytokines were different between healthy patients and those with oral carcinoma and potentially malignant disorders. This fact would indicate that these cytokines could represent potential biomarkers for these alterations. However, the salivary levels of these cytokines varied greatly between studies, requiring better standardization in the evaluation by the ELISA technique (*Enzyme Linked Immunosorbent Assay*), to be able to transpose the results into clinical practice. According to the authors, IL-6, IL-8 and TNFα could be considered as biomarkers of oral carcinoma, since their concentration was significantly higher in patients with carcinoma when compared to healthy patients. But levels of IL-6, IL-8 were also shown to be increased in potentially malignant disorders. However, carcinomas were significantly increased than in potentially malignant conditions, and it is important to determine the level of this difference between these two conditions, although in both were more increased than in healthy patients. According to the authors, these two biomarkers are the most promising as reliable diagnostic tools, since none of the other biomarkers showed significant differences in their concentration in oral cancer and potentially malignant disorders. Meta-analysis with twenty-three studies showed that salivary levels of IL-8, IL-6, TNF-α, IL-1β and IL-1α were significantly higher in patients with oral carcinomas compared to healthy patients. Salivary IL-8 and IL-6 levels were statistically higher in patients with carcinoma compared to patients with potentially malignant lesions. In the comparison between healthy patients and patients with potentially malignant disorders, the authors observed an increase in salivary levels of IL-6 in the latter. To use these biomarkers in clinical practice, standardization of saliva collection and cytokine dosage is necessary, as well as further studies with larger samples and multicenter studies.

In the systematic review conducted by Ferrari et al. (2021), using 27 observational studies (cross-sectional and longitudinal), which used the ELIZA technique to identify cytokines, the authors identified that cytokines IL-6, IL-8, TNF-α were in higher concentrations in the saliva of patients with oral carcinoma, when compared to healthy controls, and can be considered biomarkers and serve as a basis for the development of rapid tests for early diagnosis of oral cancer. They also found that the levels of these cytokines were higher in patients with carcinoma when compared to those with potentially malignant disorders. For the authors, there should be an appropriate combination of biomarkers in longitudinal studies to avoid confounding factors, such as patients' behavioral habits and periodontitis. Further studies would also be needed to confirm the reliability of salivary screening tests, with an increase in the sample size and multicenter studies, with a combination of several cytokines, and standardized quantification of the same, using reliable and quality reagents, in addition to paired control patients, according to their periodontal health.

Shaw et al. (2022), in their systematic review and meta-analysis using 13 studies with a total of 1048 patients, found that most of the eight salivary biomarkers (mRNA, miRNA, DUSP100, s100P, IL-8, IL-1B, TNF-a and MMP-9) evaluated in the studies showed good diagnostic accuracy. The authors reported that the detection of mRNA and micro-RNA had a sensitivity of 91% and specificity of 90% when they were detected by the PCR (polymerase chain reaction) technique. IL-8 had 54% and 74% for ELIZA, and 89% and 90% for CRP, of sensitivity and specificity, respectively. According to the authors, the early detection of oral squamous cell carcinoma was best achieved by mRNA and micro-RNA screening by PCR, and these biomarkers can potentially be used for early diagnosis of oral squamous cell carcinoma.

3 FINAL CONSIDERATIONS

It is possible to detect malignant changes with the aid of salivary tests to verify protein biomarkers, such as IL-6, IL-8 and TNF-α. However, these saliva tests should be standardized, using reliable reagents, combination of several biomarkers and standardized quantification of them. In addition, multicenter studies are also necessary before the validation of these markers for their use in clinical practice.

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