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10.2478/AMB-2022-0045

REVIEW

IMMUNOREGULATORY MARKERS IN HUMAN SALIVA – A NON-INVASIVE APPROACH IN THE DIAGNOSIS OF ORAL PRECANCEROUS AND CANCEROUS LESIONS

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Abstract. *Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral mucosa. Currently, the diagnosis of oral cancer is based on clinical examination and histopathological analysis. Numerous potential biomarkers have been suggested for oral cancer diagnosis, but the results are contradictory. A great achievement could be identification of biomarkers that indicate the early stage and progression of OSCC. Saliva is an easy to obtain biological fluid containing specific molecules for various pathologies. Some reliable saliva molecules described as oral biomarkers for OSCC are cytokines. A number of studies have shown that higher levels of the proinflammatory and proangiogenic cytokines, like IL-8, IL-6 and TNF-α in saliva could be used for early diagnosis of OSCC. Other studies have shown that the levels of some immunosuppressive cytokines (IL-4, IL-10, IL-13 and IL-1RA) are elevated in saliva in OSCC patients compared to controls. The importance of the immunoregulatory molecules as markers for OSCC is debated. Further studies are needed to identify clinically relevant biomarkers for screening and early detection of oral cancer.*

Key words: *oral squamous cell carcinoma, biomarkers, saliva, cytokines*

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Received: *4 June 2022 –* ***Accepted:*** *13 August 2022*

INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for 90% of all oral malignancies. The 5-year survival of patients diagnosed with OSCC is reported to be approximately 50%, showing low survival rate despite the well-known treatment methods. Most cases of oral cancer are diagnosed at a late stage. The relatively high recurrence rate and the high risk of methastases lead to uncertain prognosis, therefore the early detection by sensitive and specific biomarkers is of paramount importance [1]. In addition, the process of oral carcinogenesis is complex and not yet fully elucidated.

Currently, the diagnosis of oral cancer is based on clinical examination and histopathological analysis, so it may remain undiagnosed until significant interruption of the oral mucosa and clinically detectable lesion are found [2]. Thus the need for an early, rapid and accurate screening and diagnostic method for oral cancer by clinicians is of highly importance. Numerous potential biomarkers for cancer diagnosis have been investigated and suggested, however some discrepancies do not allow their routine clinical application. A great step forward in the diagnosis of oral cancer would be the identification of biomarkers appearing at the early stage of oral cancer.

Specific gene mutations, epigenetic alterations of the gene expression and protein synthesis, as well as the factors of the local environment, are of higly importance for the progression of oral cancer. Some specific genes are known to be of diagnostic significance and related to the prognosis in patients with oral cancer. For this reason, proteomic and genomic techniques for altered gene expression monitoring in oral cancer are being suggested and elaborated. Studies have been conducted in both cancerous tissues and biological fluids (blood serum and plasma, breast secretions, saliva) that may contain altered proteins, DNA, RNA and metabolic products associated with cellular mutations [3, 4].

The key of accurate diagnosis of the cancerous lesions is the application of highly sensitive and specific clinical tests. Inflammatory complications of the oral mucosa caused by odontogenic and non-odontogenic infections are also not uncommon. Such non-neoplastic conditions of the mouth may interfere with the identification of potential biomarkers for OSCC in saliva. Most studies are focusing on searching for potential salivary biomarkers in patients diagnosed with OSCC compared to healthy volunteers, and very few studies are focusing on checking the salivary biomarkers in cancerous patients and patients having an inflammatory disease in the oral cavity.

The key to proper diagnosis is to suggest highly specific and sensitive tests. Inflammatory diseases affecting the oral mucosa, periodontal structures or tooth pulp are not uncommon. These are non-neoplastic pathologies capable of altering the expression of potential salivary diagnostic biomarkers for OSCC. The presence of inflammatory biomarkers in the saliva together with those for neoplasms may compromise the establishment of specific diagnostic and prognostic tests for OSCC [5]. In addition, altered salivary bio-molecules have been identified in other malignancies, i.e. breast cancer and lung cancer [6, 7]. Therefore, the need to validate diagnostic cancerous biomarkers, as well as OSCC-specific panels of markers, is essential for early diagnosis.

DIAGNOSTIC IMPORTANCE OF SALIVA

Many innovative studies focus on the saliva as a biofluid containing specific secretory elements of variable levels as a result of various factors. Our previous reports reveal the diagnostic significance of saliva for some other pathologies, due to the high levels of periodontal disease markers [8], as well as markers for rheumatoid arthrithis [9]. It is expected that the “whole-mouth” saliva, considered a non-invasive type of “liquid biopsy”, to play crucial role for pathogenetic mechanisms identification and diagnosis of the oral cancer. When compared to the blood tests, salivary diagnosis is characterized with the following benefits: easy and non-invasive to obtain, easy to transfer and store. There several tools and kits for saliva collecting and storage – RNAPro, SAL (Oasis Diagnostics); SimplOFy (Oasis Diagnostics); OraGene (DNA Genotek); Salivette (Sarstedt); Saliva DNA Collection Device (Norgen Biotek); SalivaBio (Salimetrics) devices [10]. These technologies provide equivalent or even better results when compared to the passive saliva leakage followed by centrifugation and analysis considered “gold standard” for saliva diagnosis [11].

With the progress of the research and the proteomic analysis, more than 3000 different proteins have been identified in human saliva in the past decades [12]. In wide number pathologies of the oral cavity, i.e. dental caries, gingivitis, periodontal disease, OSCC, cleft lip and palate, Sjögren syndrome, Moebus syndrome, precancerous lesions, un-stimulated whole saliva (USWS) is obtained to identify various proteins. The following techniques are then used: 2D gel electophoresys, MALDI-TOF/TOF, mass spectrometry analysis, liquid chromatography/tandem mass spectrometry, Bradford method, SDS-PAGE, 1D software (BIO-RAD), IEFSDS-PAGE-MALDI-MS/MS, multiplex of Human Luminex and ELISA, immunochystochemistry, etc. New technologies with higher sensitivity for diagnostic purposes are expected in the near future.

SALIVARY CYTOKINES AND IMMUNOREGULATORY MOLECULES IN THE DIAGNOSIS OF ORAL CANCER

Cytokines are suggested promising oral biomarkers for diagnosis of oral cancer. In addition to the role in inflammatory reactions and immune response, the salivary cytokines are known to be linked to the tumor growth and progression, therefore these active mole-

cules are expected to be manifested in oral malignancies. Studies reveal that cytokines capable of mediating the inflammatory process, play a key role in tumor progression [13]. Two groups of cytokines are locally secreted by immune and malignant cells, known as pro-inflammatory and anti-inflammatory [14]. The two groups coordinate the immune response by modulating the cell proliferation and growth (pro-inflammatory cytokines) or by suppressing the functions of malignant cells (anti-inflammatory cytokines) [14].

Studies demonstrate that IL-6 inhibits apoptosis and stimulates uncontrolled cell proliferation, as it inactivates the p53 tumor suppressor gene by hypermethylating its promoter [15]. IL-6 plays a key role in suppressing the immune response, and therefore it inables the prevention of neoplastic alterations. It is known to stimulate angiogenesis and lymphatic vessels growth. When checking the IL-6 concentrations in saliva obtained by patients with OSCC and healthy volunteers, a significantly high mean concentration of 86.5 pg/ml, was observed in the first group, while the healthy patients were negative for the investigated biomarker [16]. Other studies have linked the serum IL-6 concentration to the tumor stage and found the cytokine is significantly decreased following surgical removal of the lesion, radiation therapy and chemotherapy [17].

Other studies reveal that the pro-inflammatory and the pro-angiogenic cytokines like IL-8, IL-6, TNF-β and IL-1β are associated with more aggressive behaviour of OSCC [17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27]. IL-8 plays an important role in stimulating angiogenesis, proliferation and chemotaxis of granulocytes and macrophages, which are some of the components found in the tumour stroma [23]. Elevated IL-6 levels are often associated with cachexia and hypercalcaemia, as these symptoms are particularly common in patients with poor prognosis [23]. These cytokines stimulate tumor growth, therefore the higher the concentration is, the higher metastatic risk as well as the development of larger tumors is expected [18].

A number of studies have confirmed the role of IL-8 and IL-6 in tumorigenesis, although in some ways there is controversy regarding which of the two cytokines is more active. The analysis of 25 samples obtained from patients with OSCC, showing significantly elevated levels of IL-8 confirmed its role in angiogenesis and neoplastic growth progression [19], while another study demonstrates moderate significance of the same marker [22]. Some authors have shown that the levels of IL-6 in saliva are significantly increased in patients diagnosed with OSCC compared with controls, while no difference of the levels of IL-8 between the two groups have been found [20, 27].

The results of the study show a significant increase in IL-8 compared to IL-6, suggesting its key role in the OSCC [23].

Some authors have identified elevated levels of IL-6, IL-8 and TNF in saliva and serum samples of patients diagnosed with oral leukoplakia, oral submucosal fibrosis and lichen planus, suggesting a high potential of these cytokines as biomarkers associated with the oral precancerous lesions [21].

TNF-alpha is another cytokine commonly suggested for analysis in patients with oral lesions. TNF-α stimulates cell proliferation, inhibits apoptosis, and enhances the secretion of proinflammatory cytokines by transcriptional activation of NF-κB [15]. Elevated levels of serum and salivary TNF-α have been observed in patients with OSCC when compared to healthy volunteers and patients with premalignancies. The analysis showed high specificity and sensitivity for salivary TNF-α when differentiating OSCC from premalignant diseases and healthy oral mucosa. Serum and salivary levels of TNF-α also are also dependent on the histologhistopathological and clinical features of the tumor – the markers levels are higher in moderate and poorly differentiated tumors than in well-differentiated, as well as in advanced stage tumors [24].

As previously discussed, the salivary cytokines can be used as biomarkers for the clinical detection of OSCC, as the molecules are associated with the histological subtype and the stage of the tumor. There are significant differences between the saliva levels of TNF-α, IL-1, IL-6, IL-8 in OSCC patients, those having oral premalignant lesions (OPML) and healthy controls. Therefore, it is suggested that these pro-angiogenic inflammatory cytokines in saliva could be used as diagnostic and prognostic tools in OSCC and OPML [15, 25]. Studies confirm levels of salivary cytokines – IL-8, IL-6 and TNF-α as biomarkers important for early diagnosis of oral cancer [2, 26].

Together with the studies revealing the potential of the saliva and serum cytokines (IL-6, IL-8 and TNF-α) as biomarkers for OSCC identification, other authors suggest their role as pro-inflammatory cytokines in pathologies like periodontitis, Sjögren's syndrome, stress, etc. [28, 29, 30, 31, 32]. The results show that despite the importance of IL-6, IL-8 and TNF-α in the pathogenesis of periodontal disease [33], these cytokines are uncertain markers in their diagnosis, as most studies related to saliva diagnosis do not show significant differences in cytokine concentrations in periodontal health and in disease [32]. Reports reveal significant difference between the levels of IL-6,

IL-8 and TNF-α in saliva of OSCC patients and patients with periodontal disease [31, 34, 35, 36]. Further research is needed in the field.

Little is known about the activity of IL-15 as anti-neoplastic agent. IL-15 is an immunostimulatory cytokine with IL-2-like activity. IL-15 has been shown to potentiate T-, B- and NK cells that are directly or indirectly involved in the antitumor response [37]. However, we did not find any studies about this cytokine and its relation to OSCC.

Studies have shown that the levels of the immunosuppressive cytokines IL-4, IL-10, IL-13 and IL-1RA are elevated in the saliva of OSCC patients compared to controls [38]. It is noted that IL-10 and IL-13 levels are significantly elevated, and IL-1RA levels are highest in poorly differentiated OSCC lesions compared to well- and moderately differentiated lesions. Bio-Plex's Luminex Multianalytical Profiling Technology (xMAP) was used in the study. The conclusion says that salivary immunosuppressive cytokines (IL-4, IL-10, IL-13 and IL-1RA) may be considered potential biomarkers of OSCC and can be used for early detection and progression of the tumor [38].

IL-10 is an immunosuppressive and anti-inflammatory cytokine having tumor suppressive and immunomodulatory properties [39]. In addition, Human leukocyte antigen (HLA -G, -E), programmed cell death 1 ligand 1 (PD-L1) and TGF-β are proteins involved in the antitumor immune response. It has been reported that overexpression of these immunomodulatory mediators in oral leukoplakia may interfere with the potential for immune suppression, which is an independent prognostic factor together with the histopathological features and the state of proliferation/apoptosis [40]. The conclusion has been made following investigation the relationship between the expression of the mediators in patients with oral leukoplakia and the potential for malignant transformation [40]. Apart from the epithelial dysplasia stage and the index of proliferation/apoptosis, the expression of HLA-G, -E, PD-L1, IL-10, TGF-β2 and -β3 in tissues is more significant in oral leukoplakia when compared to controls and is similar to the expression in oral squamous cell carcinoma. On the other hand, immunohistochemistry and ELISA tests analysis reveal that the concentrations of sHLA-G, IL-10 and TGF-β in saliva do not appear to be different in oral leukoplakia patients and in healthy volunteers [40]. Another study shows higher expression of IL-10 and HLA-G in tissues samples from oral carcinoma patients in comparison with healthy donors [41]. Determination of the IL-10 concentration in saliva delimitates patients with OSCC from healthy individuals, as a correlation between the tumor stage and the cytokine tissue expression is noticed. According to the same study, IL-10 is a potential salivary biomarker for OSCC, and increased expression of both HLA-G and IL-10 in tumor lesions may protect the neoplastic cells from the immune defense mechanisms [41]. Additional studies of IL-10 in saliva in relation to oral squamous cell carcinoma and oral premalignant lesions would confirm or rule out its importance in the immune response in these processes.

Combinated quantitative assessment of Cyfra 21-1, tissue polypeptide antigen (TPS) and carbohydrate antigens (CA125) in the saliva of patients with squamous cell carcinoma show a similar diagnostic value to those obtained from the serum of the same patients, as these three tumor markers have been significantly increased in the saliva of patients with oral squamous cell carcinoma when compared with healthy individuals [42, 43]. The salivary concentration of CA125 in patients with OSCC was 4.2 times higher than in healthy individuals, and the salivary concentration of TPS in patients with OSCC was 3.9 times higher than in healthy individuals [43]. According to another study, the concentrations of CA125 and TPS in saliva are defined as promising tumor markers for OSCC, as they are significantly elevated in patients with OSCC, both compared to healthy individuals and to patients with non-malignant diseases in the mouth [44]. However, it was also shown that the levels of salivary CA125 and TPS did not correlate with the pathological degree and clinical stage of carcinoma [44]. It has been reported that CA 125 is a biomarker that is not only markedly increased in the saliva of patients with OSCC, but its levels are also dependent on the stage of the tumor [45].

The role of some growth factors and their receptors in activating intracellular signaling pathways of malignancies and affecting the tumor growth is discussed [46]. Brain-derived neurotrophic factor (BDNF) is a member of the family of neurotrophins, which are growth factors involved in the differentiation, proliferation and survival of nerve cells and neurogenesis in the central and peripheral nerve systems. Binding of BDNF to the tropomyosin-related kinase B (TrkB) receptor causes its dimerization through conformational changes and autophosphorylation of tyrosine residues in the intracellular space [47]. In cancer, stimulation of the BDNF/TrkB signaling pathway activates signaling molecules like Akt, STAT3, Src, ERK, and MAPK, leading to increased cell proliferation rate, resistance to apoptosis, invasion, metastasis, and resistance to chemotherapy. Thus, increased BDNF/TrkB expression is associated with poor prognosis [48]. Immunohistochemical analysis showed increased expression of BDNF/TrkB/Akt in OSCC tissues compared to normal mucosal tissues

and oral leukoplakia, as BDNF and p-Akt are considered predictor markers for poor prognosis in OSCC patients [49]. We didn`t find any reports related to BDNF in saliva in association with OSCC diagnosis and prognosis.

Leukemic inhibitory factor (LIF) has been identified in various cancers, but few studies reported its role in the OSCC. LIF regulates the motility of cancer cells by increasing the spread of the tumor to regional lymph nodes. Immunohistochemical analysis of LIF expression in OSCC cell lines showed that patients with lymph node metastases, large tumor size, advanced cancer, and shorter survival showed higher LIF expression. Regulation of LIF by molecules like Inhibin-β-A subunit (INHBA) inhibits the ability of cancer cells for migration in invasion. Therefore, LIF may be a potential therapeutic target for the prevention of metastasis and the spread of cancer [50]. We did not find reports related to this marker in saliva in relation to the manifestation of OSCC, as such a study could establish the importance of LIF as a potential biomarker.

CONCLUSION

In conclusion, we believe that further research is needed to reveal the role of salivary concentrations of cytokines, immunoregulatory molecules and growth factors in the pathogenesis of premalignant and malignant lesions in the oral cavity. Identification of prognostic markers in cancer patients, neoplasms and the parallel with healthy controls may have the potential to define some of the indicators for OSCC screening and early detection. These findings would be important for disease prognosis, quality of life, treatment planning strategies and follow-up.

Acknowledgements: *Project № D-92/04.06.2021, "GRANT-2021" entitled: „Potential for diagnosis of oral premalignant and malignant diseases through identification of cytokines and tumor antigen concentrations in saliva", funded by Medical Science Council, Medical University-Sofia, Bulgaria.*

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