Cytokine 120 (2019) 62–65

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Short communication

# Variations of salivary concentration of cytokines and chemokines in presence of oral squamous cell carcinoma. A case-crossover longitudinal prospective study

Matteo Val*, Girolamo Antonio Sidoti Pinto, Lorenzo Manini, Sergio Gandolfo, Monica Pentenero

*University of Turin, Department of Oncology, Unit of Oral Medicine and Oral Oncology, Italy*

ARTICLE INFO

*Keywords:*
Salivary biomarkers
Cytokines
Chemokines
Oral squamous cell carcinoma

ABSTRACT

Salivary biomarkers represent a promising diagnostic tool. Non-invasive and stress-free sampling results in objective parameters able to overcome the need of clinical expertise required for a correct interpretation of early oral squamous cell carcinoma (OSCC). The present study aims at evaluating potential variations in the salivary concentration of cytokines in presence of OSCC.

Twenty patients who underwent surgical resection of OSCC were enrolled in order to assess the variation of the salivary concentration of cytokines and chemokines in a longitudinal prospective case-crossover setting. The salivary concentration of 27 salivary cytokines/chemokines was assessed before and after surgical resection of OSCC. In cases of relapsing/recurrent disease further assessments were performed.

In presence of OSCC an increased concentration of IL-8($p = 0.004$), IL-6($p = 0.005$), VEGF($p = 0.014$), MIP-1ß($p = 0.033$), IP-10($p = 0.047$), IL-1β($p = 0.049$) was observed; conversely the concentration of IFN-γ($p = 0.036$) and IL-5($P = 0.048$) decreased. In cases with relapsing/recurrent disease IL-5, IL-6 and MIP-1β showed variations consistent with the previously reported results.

Further studies are needed in order to confirm these results and to determine reference values to determine the presence or absence of disease.

## 1. Introduction

Squamous cell carcinoma (SCC) accounts for over 90% of all malignant tumours in the head-neck district. When dealing with oral squamous cell carcinoma (OSCC) the diagnostic path starts from a clinical interpretation of oral lesions in order to identify cases where further biopsy is needed. Nevertheless, the conventional oral examination has several limitations and it depends on human variability and skills. The assessment of salivary biomarkers implies simple, rapid, inexpensive, non-invasive sampling, which can be performed by health professionals without specific skills in oral oncology. Last but not least, saliva sampling is also stress-free for patients. Salivary concentration of cytokines or chemokines including IL-1β, IL-6, IL-8 and VEGF have been reported in the literature as potential biomarker for OSCC [1–4], but comparing different subjects with or without disease could imply potential confounding factors due to inter-subject unknown and unavoidable variations.

The present study aims at evaluating variations in the expression pattern of 27 salivary cytokines and chemokines related to the presence of OSCC.

## 2. Material and methods

### 2.1. Participants

Patients were enrolled at the Oral Medicine and Oral Oncology Unit of the University Hospital San Luigi Gonzaga (Orbassano, Italy) in a longitudinal prospective study with a case-crossover design. Inclusion criteria were a histological diagnosis of OSCC, indication to exclusive surgical treatment and negative history of previous malignancies irrespective of the anatomical site. All patients were recruited at the time of diagnosis, before performing any type of cancer therapy. The following data were collected: age, gender, medical history, systemic diseases, tobacco smoking and alcohol drinking habits. Following the histological diagnosis, patients underwent clinical staging according to the TNM Classification of Malignant Tumours, 7th Edition (2009).

After surgery, patients were excluded from further assessments in case of absence of free surgical margins or pathological staging

* Corresponding author at: Dept. Oncology, Unit of Oral Medicine and Oral Oncology, University of Turin, Regione Gonzole, 10, Orbassano (TO), Italy.
*E-mail address:* matteo.val@outlook.it (M. Val).

https://doi.org/10.1016/j.cyto.2019.04.009
Received 29 December 2018; Received in revised form 12 April 2019; Accepted 13 April 2019
Available online 18 April 2019
1043-4666/ 

requiring adjuvant treatments.

### 2.2. Saliva sampling

Whole non-stimulated (WNS) saliva samples were obtained by draining method. The patients had to avoid to eat and/or brush their teeth one hour before sampling. Saliva was centrifuged for 10 min at 245 g and 1.5 ml of supernatant was immediately frozen (−20 °C) and stored at −80 °C for subsequent analysis.

Salivary sampling was performed at diagnosis before surgery (T0) and 2 months after surgery (T1). Further samplings were performed at occurrence of second tumours or relapse during the follow-up: at the moment of the new diagnosis (T2) and 2 months after the second surgical excision (T3). The onset of OSCC at least 4 months after treatment was considered a second primary consistently with the AJCC Cancer Staging Manual.

In order to evaluate the intra-subject variability, the T0 sample was repeated in 3 different days in 5 patients.

### 2.3. Laboratory analysis

The cytokine analyses were performed using the Bio-Plex® multiplex beads system (Bio-Plex 100 array reader with Bio-Plex Manager software version 6.1, Bio-Rad, Hercules, California), thus allowing the simultaneous assessment of the concentration of 27 cytokines or chemokines (IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, basicFGF, Eotaxine, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF-BB, RANTES, TNF-α and VEGF) in a single sample with small salivary volume. Fluorescently-labelled magnetic microspheres were coupled to specifically capture antibodies and mixed with the samples in which the cytokine concentrations were unknown. Biotinylated detection antibodies and streptavidin R-phycoerythrin were introduced into the mixture, which was then analysed by flow cytometry. Two lasers identified the microsphere type and quantified the amount of the bound antigen. From the Bio-plex Manager software a standard curve is prepared above the concentration range between 0.15 and 3700 pg/ml. The concentration of the molecules was expressed in picograms per millilitre (pg/ml). All measurements were blind performed.

### 2.4. Statistical analysis

All statistical analyses were performed after assessing normality of data (Shapiro-Wilk tests).

The comparison of the salivary concentration of cytokines before and after therapy (T0 versus T1) was assessed through paired data *t*-test or Wilcoxon test. In case of relapse/onset of second primaries the effects of the presence of disease was assessed by comparing concentrations observed at T0 versus T1 and at T1 versus T2. The evaluation of the intra-subject variability was performed using the Friedman test.

Statistical analyses were performed with SPSS software (version 22.0), $p < 0.05$ was considered to indicate a statistically significant difference.

## 3. Results and discussion

Twenty-one (10 males and 11 females) patients with an average age of 65.4 years entered the study and had salivary sampling at T0. Sampling at T1 was performed in 20 patients as one was excluded due to the pathology report showing presence of disease at the resection margins. Table 1 shows clinico-pathological features of enrolled patients. The analysis of repeated samples (3 repeated samples in 5 patients) at T0 did not show any significant intra-subject variation (data not shown).

When comparing T0 and T1 (Table 2), the presence of OSCC was found to drive significantly increased concentration of IL-8 ($p = 0.004$), IL-6 ($p = 0.005$), VEGF ($p = 0.014$), MIP-1ß ($p = 0.033$), IP-10 ($p = 0.047$), IL-1β ($p = 0.049$) and reduced concentration of IFN-γ ($p = 0.036$) and IL-5 ($p = 0.048$). Second primaries were observed in 3 patients and all of them underwent a second surgical excision. When comparing samples at T0, T1, T2 and T3 the concentration of IL-5, IL-6 and MIP-1β showed variations consistent with the significant data above reported. IL-5 concentration increased at T1 after surgery, decreased at onset of the second primary at T2 and increased again at T3 after the second surgery. Conversely both IL-6 and MIP-1β showed an opposite trend.

Although the oral mucosa is easy to be examined, about 60% of patients have diagnosis at late stages (Stages III and IV). Early detection of OSCC is one of the main aim for clinicians trying to improve OSCC survival. This is of utmost importance for high risk patients who are followed-up for the presence of potentially malignant disorders or because of previous primaries. Repetition of incisional biopsies could be a source of stress for patients favouring the loss of compliance at follow-up and could impair the clinical follow-up due to sequelae of multiple biopsy samplings. Repeated tests of highly sensitive biomarkers based on non-invasive sampling would be an important diagnostic aid, free from any potential inter-subject variations.

Saliva is a body fluid currently used for the diagnosis of several diseases and there is increasing literature about its use as a source of biomarkers for OSCC aiming to develop non-invasive diagnostic aids. More than 200 different salivary cytokines and chemokines have been suggested as potential biomarker and significant differential expression in presence of OSCC has been reported for IL-1β, IL-6, IL-8, VEGF-A, TNF-α [5].

Cytokines and chemokines found to be differentially expressed in saliva in presence of OSCC have numerous functions not only related to inflammation (IL-1β) or to immune response (IL-10, TNF-α); they have also been found to be involved in biological pathways connected with tumour progression as cellular growth, differentiation or apoptosis (IL-1, IL-6). Inflammation induced by IL-1β in the microenvironment has been investigated as a potential promoter of tumour invasiveness [6]. Among proinflammatory cytokines, recent evidence suggests that IL-6 has a key role in driving tumour initiation and subsequent growth and metastasis [7]. IL-8 is not only involved in tumour growth and it seems to have an important role in epithelial–mesenchymal transition [8]. VEGF has a well-known regulatory effects on angiogenesis and its expression has been reported to be increased from the normal epithelium through dysplasia, to OSCC [9].

Even if the salivary concentration of such molecules is significantly associated to the presence of OSCC, no single biomolecules meet the requirements to achieve high accuracy in early identification of OSCC, due to the complexity of the human biological system. In the literature no validated cut-off values for the physiological concentrations of salivary cytokines and chemokines have been reported. Of note studies investigating the same molecule report different mean concentration values as associated to OSCC [1,3,4]. The present study was not able to find any significant inter-individual variations neither in presence nor in absence of OSCC, probably due to the low sample size. Anyhow, a case-crossover setting evaluating variations within the same subject, allows to avoid any potential bias related to inter-individual variations and allows the detection of variations most likely related to the presence of disease.

As variations of one single cytokine/chemokine could be induced by different factors, the simultaneous analysis of a panel of molecules has been suggested in order to have more specific results able to differentiate OSCCs, potentially malignant oral disorders and controls [10]. In the present study, the use of a bioplex plate allowed a standardized and simultaneous evaluation of 27 molecules aiming at defining a panel of cytokines and chemokines differentially expressed in presence of disease.

Lee et al.[11] used a similar technology to assess in both saliva and plasma the concentration of 14 molecules in 24 OSCC patients

**Table 1**
Clinico-pathological characteristic of the sample.

| Patient | Age | Gender | Risk habit profile | T stage | Nodal invasion | Histological grade | Site |
|---|---|---|---|---|---|---|---|
| 01 | 95 | F | None | T1 | Negative | Well differentiated | Alveolar ridge |
| 02 | 49 | F | Exclusive smoker | T2 | Negative | Moderately differentiated | Tongue |
| 03 | 49 | M | Exclusive smoker | T1 | Negative | Well differentiated | Buccal mucosa |
| 04 | 57 | M | None | T1 | Negative | Well differentiated | Buccal mucosa |
| 05 | 57 | M | None | T1 | Negative | Well differentiated | Buccal mucosa |
| 06 | 48 | M | None | T2 | Negative | Moderately differentiated | Tongue |
| 07 | 77 | F | None | T1 | Negative | Moderately differentiated | Alveolar ridge |
| 08 | 80 | F | None | T1 | Negative | Well differentiated | Tongue |
| 09 | 60 | F | None | T1 | Negative | Poorly differentiated | Tongue |
| 10 | 67 | F | None | T1 | Negative | Well differentiated | Alveolar ridge |
| 11 | 69 | F | Exclusive smoker | T1 | Negative | Well differentiated | Tongue |
| 12 | 76 | M | Exclusive alcohol drinker | T1 | Negative | Well differentiated | Tongue |
| 13 | 57 | M | Mixed habits | T1 | Negative | Well differentiated | Hard palate |
| 14 | 58 | M | Mixed habits | T4a | Positive | Well differentiated | Floor of the mouth |
| 15 | 60 | M | Mixed habits | T1 | Negative | Well differentiated | Tongue |
| 16 | 87 | F | None | T4a | Positive | Poorly differentiated | Soft palate |
| 17 | 77 | M | None | T2 | Negative | Moderately differentiated | Tongue |
| 18 | 46 | F | None | T1 | Negative | Well differentiated | Tongue |
| 19 | 78 | F | None | T1 | Negative | Well differentiated | Floor of the mouth |
| 20 | 51 | M | None | T2 | Negative | Moderately differentiated | Alveolar ridge |

**Table 2**
Significant variations in salivary Cytokines/Chemokines concentration before and after surgical treatment of OSCC.

| OSCC-associated concentration | | |
|---|---|---|
| Increased | Decreased | Not significant |
| IL-1b (p = 0.049) | IL-5 (p = 0.048) | IL-1ra |
| IL-6 (p = 0.005) | IFN-γ (p = 0.036) | IL-4 |
| IL-8 (p = 0.004) | | IL-7 |
| IP-10 (p = 0.047) | | IL-9 |
| MIP-1b (p = 0.033) | | IL-10 |
| VEGF (p = 0.014) | | IL-12 |
| | | IL-13 |
| | | IL-15 |
| | | IL-17 |
| | | Eotaxin |
| | | FGF |
| | | GCSF |
| | | MCP-1 |
| | | MIP-1a |
| | | PDGFbb |
| | | RANTES |
| | | TNFα |

compared to healthy controls. Saliva showed increased concentration of IL-1β, IL-6, IL-8, MIP-1β, eotaxin and IFN-γ and TNF-α; while in plasma IFN-γ and MIP-1β had a reduced concentration and a significant increase was found only for IP-10. On note, IFN-γ and MIP-1β had significant opposite variations in saliva and in blood.

The present study confirmed the presence of significant increase in the expression of molecules IL-1β (p = 0.0049), IL-6 (p = 0.005), IL-8 (p = 0.004) and VEGF (p = 0.014) in presence of OSCC. Of note, differently from previous studies in the literature, the design of the present study avoids the potential inter-subjects variations which could represent a bias when healthy patients are used as control subjects [1–4].

TNF-α is a quite often investigated molecule, but independent studies reported contrasting results [3,4,12]. Even if the study with the largest sample size (100 patients) was able to find a significantly increased concentration [12], it could be observed that the reported mean value for OSCC patients is not similar to what found in the other studies: ranging about 300 pg/ml versus 35 pg/ml.

Several molecules found to have significantly increased or lowered salivary concentration in the present study have previously been reported in only one study (MIP-1β, IP-10, IFN-γ and IL-5) [11].

Natural killer T cells have a potential role in contrasting tumour development exerting their function through effector molecules among which IFN-γ. The reduced concentration of IFN-γ (p = 0.036) is consistent with the reported negative regulation of NK cell in OSCC tissue [13].

IP-10 has been associated to infectious diseases, immune dysfunction and tumour development. The increased expression of both IP-10 and its receptor CXCR3, has been found to be associated with advanced-stage tumours in malignant melanoma [14]. The present study found a significant increased salivary concentration of IP-10 (p = 0.047) supporting its potential role also in OSCC.

MIP-1β is able to induce an antitumor response attracting both T lymphocytes and NK cells. The increased salivary concentration found in the present study suggests an attempt to counteract tumour grow and is consistent with previous results by Lee [11].

There are no studies in the literature to be contrasted to the present results showing a significantly lower concentration of IL-5 in presence of OSCC (p = 0.048). This is quite surprizing as in other anatomical sites (e.g. lung) IL-5 is usually found to have higher concentration in presence of cancer [15].

In order to rely on salivary cytokines as a marker of the presence of OSCC in a single patient, further evaluations would be needed with sample size large enough to determine reference values indicating presence/absence of disease.

Waiting for such data, a case-crossover approach allows to evaluate the variations within the same subject in the presence/absence of disease, without potential bias related to uncontrollable inter-individual variations, thus highlighting those molecules whose expression is most likely related to the disease.

The present study, despite the small sample size, brings a significant contribution in supporting previous findings showing increased salivary concentrations of IL-6, IL-8, VEGF, IL-1β in patients with OSCC and highlights a potential role of IL-5 never reported before.

## 4. Conflict of interest statements

No competing interests.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2019.04.009.

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