Possible role for interleukins as biomarkers for mortality and recurrence in oral cancer.

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Abstract:	Objectives: Salivary and serum levels of interleukin-6 (IL-6) and interleukin-8 (IL-8) have previously been studied in oral cancer with conflicting results. Methods: We designed a controlled study to assess the correlation between pre-treatment salivary and serum levels of IL-6 and IL-8, and all-cause survival and cancer recurrence in oral cancer patients. Results: Fifty-two oral cancer patients and 52 healthy control cases were selected. In univariate analysis, salivary IL-6 and IL-8 seemed to be more expressed in cases ($p<.001$ and $p=.010$ respectively). Multivariate analysis showed that higher pre-treatment saliva IL-6 levels were significantly associated with better survival (HR, 8.62; 95% CI, 1.21-62.50, $p=.031$). Conclusions: To date, this is the largest prospective controlled study that analyses the pre-treatment salivary and serum levels of IL-6 and IL-8 in oral cancer patients, suggesting salivary IL-6 as possible prognostic biomarkers, but further validation on a larger sample is still necessary.
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Response to Reviewers:	Dear editor and reviewer, Ref.: Ms. No. JBM-D-14-00064 Possible role for interleukins in mortality and recurrence in oral cancer. The International Journal of Biological Markers Thank you for your decision and comments regarding the above manuscript. In order to expedite the processing of the revised manuscript, we have attempted to answer the reviewers' comment in a point-by-point manner. The attached highlights the changes in yellow. #Reviewer 1: - As required primary and secondary endpoints have been better described at the beginning of the M&M section. - Some lines about the sample size have been added, as required, when discussing about statistical methods. - As suggested, in the Discussion section the weaknesses and strengths of the study have been discussed; moreover, the potential clinical applications of the achieved results have been better commented. We now hope that our revisions will be acceptable. Yours sincerely, Paolo Giacomo Arduino DDS, MSc (Oral Med) Department of Surgical Sciences University of Turin, Turin, Italy

Possible role for interleukins in mortality and recurrence in oral cancer.

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ABSTRACT

***Objectives*: Salivary and serum levels of interleukin-6 (IL-6) and interleukin-8 (IL-8) have previously been studied in oral cancer with conflicting results.**

***Methods*: We designed a controlled study to assess the correlation between pre-treatment salivary and serum levels of IL-6 and IL-8, and all-cause survival and cancer recurrence in oral cancer patients.**

***Results*: Fifty-two oral cancer patients and 52 healthy control cases were selected. In univariate analysis, salivary IL-6 and IL-8 seemed to be more expressed in cases ($p<.001$ and $p=.010$ respectively). Multivariate analysis showed that higher pre-treatment saliva IL-6 levels were significantly associated with better survival (HR, 8.62; 95% CI, 1.21-62.50, $p=.031$).**

***Conclusions:* To date, this is the largest prospective controlled study that analyses the pre-treatment salivary and serum levels of IL-6 and IL-8 in oral cancer patients, suggesting salivary IL-6 as possible prognostic biomarkers, but further validation on a larger sample is still necessary.**

Key words: Oral cancer, Prognostic markers, Interleukins, Recurrence, and Survival.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most frequent oral cancer, accounting for more than 90% of cases worldwide. Despite substantial improvements in diagnosis and therapy, OSCC still has a low estimated 5-year overall survival rate of 50% even in the United States and Western Europe (1, 2). Knowledge of specific prognostic and predictive factors could be therefore crucial in determining appropriate therapy.

Many different biological and molecular factors have been proposed as prognostic factors in OSCC, but they are far removed from a real impact on routine clinical care (3). Cytokines play an important role in the initiation and maintenance of inflammatory and immune responses as well as intercellular cross-talking (4). Interleukin-6 (IL-6) is a multifunctional cytokine synthesized in response to defined stimuli by a variety of different cells (5). Interleukin-8 (IL-8) belongs to a super family of chemokines that has chemotactic activity for neutrophils, eosinophils, basophils, monocytes, mast cells, dendritic cells, natural killer (NK) cells, and lymphocytes [6]. Salivary and serum levels of IL-6 and IL-8 have been studied in head and neck cancer but with conflicting results, inadequate follow-up and on different types of tumours (7-10).

Therefore, we designed a study to assess the correlation between pre-treatment salivary and serum levels of IL-6 and IL-8, and all-cause survival and cancer recurrence in OSCC patients.

METHODS

This was a prospective cohort study: the primary explanatory variable was pre-treatment salivary and serum IL-6 and IL-8 levels; control variables were

age, sex, smoking, tumour site and stage, and different treatment modalities. The secondary outcome variables were tumour recurrence and all-cause survival.

Patients' selection and control variables

Consecutive Caucasian patients, attending the Oral Medicine Section of the Department of Surgical Sciences, University of Turin, between January and September 2008, were enrolled. Local ethical committee approval was obtained before the trial started and all subjects gave written informed consent.

Newly diagnosed patients with biopsy proven OSCC were approached for participation in this study.

Excluded were those 1) <18 years of age, 2) pregnant or breast feeding, 3) or with mental health issues, 4) with diagnosis of oral lichen planus, 4) or with clinical diagnosis of periodontitis. A group of control subjects, unrelated to the cases, were recruited from the population attending the University Hospital of Turin. All controls presented with no oral lesions.

Demographic information, smoking, alcohol consumption, tumour site, T classification and neck nodes involvement, treatment, outcome and survival rate (at December 31st, 2012) were recorded. Tumour grade (well, moderately or poorly differentiated) (11), was given by two different pathologists. C-reactive protein was also assessed.

Saliva and serum collection

Subjects were asked to refrain from eating, drinking, or oral hygiene for at least one hour prior to unstimulated whole saliva collection; they rinsed out their mouths with tap-water and then waited at least 5 min before expectorating into a 50 cc Falcon tube. The tube was centrifuged at 3500 *rpm* (2600g) for 15 min at 4 °C (Hermle bench centrifuge Z300, Labnet International, Woodbridge, NJ 07095); the supernatant obtained was immediately transferred to a new tube and frozen at -80 °C. Serum samples were collected at the same time, by centrifuging whole venous blood at 3000 *rpm* (1000g) for 10 minutes at 15°C (Hermle bench centrifuge Z300, Labnet International, Woodbridge, NJ 07095). The aliquots were stored at –80°C.

Interleukins

Samples were tested using ThermoScientific/Pierce Biothecnology (Rockford, IL, USA) ELISA kits for recombinant human IL-6 and IL-8. Cytokine protein levels were determined by solid phase quantitative sandwich immunoassay technique. The minimum detectable doses were 1.0 pg/mL for IL-6 and 2.0 pg/mL for IL-8. All testing was performed in duplicate per manufacturer instructions and means were used for data reporting.

Statistical analysis

A study with 52 case patients and 52 control subjects has been planned, in order to detect at least a true difference in the mean response of experimental and control subjects of +-0.52 standard deviation with an 80% power. The Type I error probability associated with this test of the null hypothesis that the population means of the experimental and control groups are equal is 0.05. If

we suppose a Bonferroni setting, the detection of true difference in the mean response is +-1.71 standard deviation.

Means and frequency distributions were examined for all variables. Describing general information, data was reported as means and standard deviation (± SD). Associations between salivary and serum interleukins and control variables were assessed by using analysis of variance. For univariate and multivariate analyses, interleukins were treated as a continuous variable after log transformation [ln(IL + 2)]. Univariate and multivariate Cox proportional hazards models were used to study the relation between salivary and serum IL-6 and IL-8, control variables, and time to recurrence and death. Time to recurrence and death was measured from the pre-treatment blood draw date. Crude and adjusted ORs were computed by using logistic regression analyses to assess the independent effects of control and pre-treatment cases' variables. Statistical analyses were performed using the IBM-SPSS statistics software version 20.

RESULTS

Cases and controls were comparable with regards to age, gender and risk factors.

Fifty-two cases of OSCC were selected, of whom 32 were male and 20 female (mean age 66.19 ± 14.97 years). The follow-up period ranged from 12 to 60 months (median of 39.4 months). During the period considered, 10 (19.2%) patients died because of the original OSCC. There was no recurrence in 29 (55.8%) patients, whilst 13 (25%) developed a recurrence. No second primary tumours were detected.

Control variables and association with pre-treatment levels of interleukins

Table 1 shows the differences between cases and controls in interleukin expression. In univariate analysis, salivary IL-6 and IL-8 seemed to be more expressed in cases (p<.001 and p=.010 respectively) than in controls; similar results were also present in the multivariate analysis, also adding a statistical larger expression of serum IL-8 (p=.006) for pre-treatment cases.

Older persons had higher salivary and serum IL-6 levels than did younger persons, the converse from salivary and serum IL-8 expression, but neither data had statistical significance. Smokers were more likely to have higher salivary IL-6 levels than non-smokers. Those with larger cancers had higher levels of salivary IL-6 (p=.011). There was no association between salivary and serum interleukins and gender, cancer site, node association, serological inflammation data grading or treatment type; there was only a larger expression of salivary IL-6 and salivary IL-8 and lymph node involvement but again without statistical significance.

IL-6, IL-8 and control variables as independent predictors of recurrence and survival

The results of the univariate and multivariate Cox proportional hazards regression models for recurrence and survival are detailed in Table 2. Serum IL-6 was not included in the final model, because the levels were too low to perform the analysis.

Univariate and multivariate analyses did not show that higher pre-treatment salivary or serum levels of interleukins were associated with recurrence.

Analyses showed that patients who underwent radiotherapy remained significantly susceptible to recurrence, even after controlling for the other variables.

Univariate analysis showed that larger cancers, nodal involvement and chemotherapy were significantly negatively associated with survival. Both analyses showed that higher pre-treatment saliva IL-6 levels were significantly associated with survival ($p<.05$). Older age, nodal involvement, cancer site and having chemotherapy were also independently associated with poorer survival in the multivariate analysis, whereas gender was not associated with survival.

DISCUSSION

OSCC is one of the most complex malignancies to control, and only a small improvement has been seen in the survival rate over the last decades; the problem of metastasis, recurrences and difficulties in reconstruction comprise the main obstacles in treatment; therefore, early detection would be a powerful approach in improving quality of life, and outcome and survival rate (12).

The use of saliva as a diagnostic bio-fluid has long been recognised, and it has many advantages over other specimens like blood, exfoliated cells and urine (13). To date, some studies based on salivary and serum cytokine expression profile have endeavoured to provide information relevant to survival rate and recurrence of OSCC, but several critical issues still need to be addressed before these findings can be fully utilized in patient care. One of the biggest problems is that samples have included not only oral cancer but

also oropharyngeal carcinomas - which generally have a different aetiopathogenesis (human papillomavirus) and a better prognosis.

In the present study, we set out to identify whether IL-6 and IL-8 could potentially be useful as biomarkers for *oral* cancers. Interleukin 6 was detected at higher concentrations in the saliva of OSCC cases compared to controls, and IL-8 was detected at higher concentrations both in the saliva and in the serum of patients with OSCC. Moreover, salivary IL-6 levels have been demonstrated to be useful also as independent prognostic factors for OSCC survival rate.

Data confirmed the hypothesis that saliva should be preferred to blood in this type of research, having the potential to serve as non-invasive, widely accessible screening tools that do not rely on the localization of a lesion for diagnosis; collection is also inexpensive and can be performed in any setting. If becoming a routine, saliva, as a diagnostic tool for OSCC, would also comprise a suitable instrument for population screening, monitoring of patients at high risk of recurrences, and subsequently for improving survival rates (12).

Serum measurements of cytokines may reflect the systemic response to tumour development and progression, and may not be simply indicative of local biochemical events at the tumour site. Moreover, several studies have outlined the importance of autocrine IL-6 signalling in different type of carcinoma - such as lung, breast, skin, and in head and neck squamous cell carcinoma cell lines, where findings indicate that endogenous IL-6 could play an important role in the growth of tumour and exert its action by an autocrine growth mechanism. Therefore, it seems that variation of salivary levels of IL-6

and IL-8 could represent disease markers that could possibly reflect accurately tumour status (13-15).

Higher levels of the salivary cytokines (above all IL-6) may have significant usefulness as surrogate biomarkers in the efficiency of chemoprevention therapy (7). Although, the present data is from a relatively small sample size (even if the biggest in oral cancer ever reported), the results are significant enough to merit further investigation.

Determination of the exact mechanisms leading to increased IL-6 and IL-8 secretion in recurrent and advanced disease might also lead to new therapeutic interventions or monitoring.

To the best of our knowledge, this is the largest prospective control study that analyses the pre-treatment salivary and serum levels of IL-6 and IL-8 in OSCC patients, confirming earlier results [16], that have suggested salivary IL-6 and IL-8 as diagnostic biomarkers for OSSC, but further validation on a larger sample is still necessary in a multicentre population study.

Conflict of interest

The authors declare that they have no conflict of interest.

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TABLE I - UNIVARIATE AND MULTIVARIATE ANALYSES OF CONTROL AND PRE-TREATMENT CASES' VARIABLES

	Variable	*β*	*SE*	*OR*	*CI*	*P*
Univariate						
	Saliva IL6	1.83	.483	6.25	2.42-16.11	**<.001**
	Saliva IL8	1.17	.455	3.24	1.33-7.91	**.010**
	Serum IL8	0.77	.445	2.16	0.90-5.17	.083
	Gender	.10	.452	1.11	0.45-2.68	.821
	Smoking	.71	.495	2.04	0.77-5.37	.151
	Age	.69	.450	2.00	0.83-4.83	.123
Multivariate						
	Saliva IL6	1.76	.641	5.83	1.66-20.47	**.006**
	Saliva IL8	1.37	.640	3.95	1.12-13.84	**.032**
	Serum IL8	1.88	.681	6.56	1.73-24.93	**.006**
	Gender	.64	.602	1.89	0.58-6.16	.288
	Smoking	.32	.628	1.38	0.40-4.74	.604
	Age	.54	.606	1.71	0.52-5.62	.375

Legend:
SE: Standard Error
OR: Odds ratio
CI: 95% Confidence Interval

TABLE II - UNIVARIATE AND MULTIVARIATE RATIOS OF IL6 AND IL8 FROM COX PROPORTIONAL HAZARD REGRESSION MODELS FOR RECURRENCE AND DEATH EVENTS

RECURRENCE EVENTS

Variable Name	U.M.R.§			M.M.R.§		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
Log IL6_saliva*	1.77	0.52-5.99	.355	3.94	0.48-32.07	.200
Log IL8_serum*	1.66	0.49-5.55	.412	2.54	0.68-14.70	.162
Log IL8_saliva*	1.98	0.57-6.85	.278	1.52	0.25-5.88	.648
Age (>68 *vs* ≤68)	2.42	0.70-8.33	.160	3.77	0.96-14.70	.056
Gender (female *vs* male)	1.10	0.33-3.66	.869	1.19	0.23-6.10	.827
pT (pT1 *vs* pT2 plus pT4)	2.79	0.82-9.51	.101	1.63	0.19-13.69	.650
N (N0 *vs* N1 plus N2)	1.36	0.41-4.54	.618	1.73	0.44-2.13	.434
G (G2 plus G3 *vs* G1)	1.51	0.40-5.75	.541	1.60	0.25-10.28	.620
Site (others *vs* tongue plus floor)	1.63	0.47-5.63	.440	1.39	0.33-5.89	.650
Surgery (yes *vs* no)	1.46	0.18-11.49	.718	1.71	0.04-76.92	.783
Chemiotherapy (yes *vs* no)	3.14	0.87-11.23	0.78	6.06	0.43-10.75	.349
Radiotherapy (yes *vs* no)	4,83	1.21-19.23	**.026**	5.61	1.33-23.81	**.018**

DEATH EVENTS

Variable Name	U.M.R.§			M.M.R.§		
	Hazard Ratio	95% CI	P	Hazard Ratio	95% CI	P
Log IL6_saliva*	4,83	1.29-29.31	**.026**	8.62	1.21-62.50	**.031**
Log IL8_serum*	1.03	0.26-4.12	.968	1.69	0-13-2.08	.687
Log IL8_saliva*	1.74	0.42-7.30	.448	1.16	0.09-14.70	.909
Age (>68 *vs* ≤ 68)	1.09	0.69-1.69	.130	12.34	1.23-125	**.033**
Gender (male *vs* female)	1.86	0.37-9.24	.446	2.58	0.41-16.02	.307
pT (pT2 plus pT4 *vs* pT1)	1.03	1.26-83.33	**.029**	1.602	0.02-90.68	.819
N (N1 plus N2 *vs* N0)	5.31	1.07-26.31	**.041**	20.05	1.63-245.89	**.019**
G (G2 plus G3 *vs* G1)	3.31	0.40-27.03	.263	3.14	0.17-57.17	.440
Site (others *vs* tongue plus floor)	2.61	0.52-12.94	.240	6.95	1.05-45.93	**.044**
Surgery (no *vs* yes)	1.94	0.30-9.65	0.416	1.85	0.17-20.09	.612
Chemiotherapy (yes *vs* no)	5.81	1.38-24.39	**.016**	8.13	1.38-47.6	**.020**
Radiotherapy (yes *vs* no)	4.83	0.97-2.38	.054	2.17	0.08-58.82	.642

§U.M.R.=univariate model results
§M.M.R.=multivariate model results
* over the mediana *vs* under the mediana

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Signed for and on behalf of the Authors: Paolo G. Arduino

Dear editors,

We are proud to submit to your prestigious journal this brief report detailing salivary and serum levels of interleukin-6 (IL-6) and interleukin-8 (IL-8) in patients with “pure” oral cancer. We designed a prospective controlled study to assess the correlation between pre-treatment salivary and serum levels of IL-6 and IL-8, and all-cause survival and cancer recurrence in oral cancer patients.

Fifty-two oral cancer patients and 52 healthy control cases were selected. In univariate analysis, salivary IL-6 and IL-8 seemed to be more expressed in cases ($p<.001$ and $p=.010$ respectively). Multivariate analysis showed that higher pre-treatment saliva IL-6 levels were significantly associated with better survival (HR, 8.62; 95% CI, 1.21-62.50, $p=.031$). To date, this is the largest prospective controlled study that analyses the pre-treatment salivary and serum levels of IL-6 and IL-8 in oral cancer patients, suggesting salivary IL-6 as possible prognostic biomarkers, but further validation on a larger sample is still necessary.

To the best of our knowledge, similar findings have never been documented.

Regards

Dr. Paolo Giacomo Arduino and co-authors.