

## RESEARCH ARTICLE

# Tissue Expression, Serum and Salivary Levels of IL-10 in Patients with Head and Neck Squamous Cell Carcinoma

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### Abstract

**Background:** Head and neck SCC is a common cancer related to various factors. IL-10, a pleiotropic cytokine produced by macrophages, T-helper-2 cells, and B lymphocytes, is thought to play a potential pathogenetic or therapeutic role in a number of human conditions, such as inflammation, autoimmunity and cancer. The present study was designed to evaluate the relation between tissue expression, serum and salivary levels of IL-10 in head and neck squamous cell carcinomas (HNSCCs) and their correlation with clinicopathologic features. **Materials and Methods:** Samples were collected from 30 patients with HNSCCs and 24 healthy volunteers. IHC analysis was used to examine the tissue expression and ELISA was employed to measure serum and salivary levels. **Results:** Our study showed tissue expression of IL-10 to be significantly higher in patients (P: 0.001), but there was no relation between tissue expression, serum and salivary levels of the marker (P>0.05). Also except for a positive correlation between tissue expression of IL-10 and stage (P: 0.044), there was no relation between this marker and clinicopathologic features. There was no correlation between serum and salivary levels in either patients or controls. **Conclusions:** It seems there is no correlation between level of IL-10 in serum and saliva and this marker in saliva and serum does not reflect tissue expression.

**Key words:** Interleukin-10 - head and neck SCC - serum - saliva

*Asian Pacific J Cancer Prev*, 14 (3), 1681-1685

### Introduction

IL-10 is a human cytokine with a homodimer structure and molecular weight of 37 KDa. Each monomer consists of 160 amino acids with a molecular weight of 18.5 KDa. IL-10. Its gene in humans is located on chromosome 1q31-1q32 (Chandler et al., 2002; Asadullah et al., 2003). Its primary role is to suppress immune activity by blocking pro-inflammatory cytokines synthesis such as TNF- $\alpha$ , TNF- $\gamma$  and IL-1. Various cell populations are able to produce IL-10. In addition to T-cell group (T helper2, T cytotoxic2), monocytes, macrophages and several other cell types such as B cells and keratinocytes may produce IL-10. But the major source of IL-10 production, in studies, is macrophages (Chandler et al., 2002; Asadullah et al., 2003; Sabatet al., 2010). IL-10 is effective on different cell populations, especially circulating and tissue-resident immune cells as well as keratinocytes (Fujieda et al., 1999; Sabat et al., 2010).

It is assumed that IL-10 plays a key role in tumorigenesis and neoplasm metastasis. IL-10 can cause tumor growth by stimulating cancer cells growth and inhibition of apoptosis. Increased serum levels of IL-10 have been

observed in patients with hematopoietic and solid tumors (Toiyama et al., 2010; Liu et al., 2011). IL-10 can inhibit the production of new blood vessels within the tumor, whether directly by influencing the tumor cells or indirectly by affecting infiltrated immune cells. IL-10 can also inhibit angiogenesis by reducing the number of macrophages in the tumor. It has been suggested that IL-10 can inhibit angiogenesis by reducing VEGF secretion in tumor-associated macrophages. Other factors involved in the production of new blood vessels, such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6, will be decreased by IL-10 (Stearns et al., 1999).

Considering dual role of IL-10 and absence of a study on simultaneous occurrence of this marker in tissue, serum and saliva of patients with head and neck SCC, this research is to examine tissue incidence as well as serum and salivary level of this factor.

### Materials and Methods

In this cross-sectional study, 30 patients with head and neck SCC (21 males and 9 females) with a mean age of 12.5 $\pm$ 56.8 years who were referred to Khalili and Chamran

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Hospitals of Shiraz University of Medical Sciences were examined. The control group consisted of 24 healthy individuals (16 male and 8 females), with a mean age of  $54.87 \pm 10.87$  years, who were matched with the patients group in terms of age and sex.

People suffering from any systematic disease, active infection, autoimmune disease, inflammatory disease and periodontal problems were excluded from the study. As for the patients group, those who had a history of radiotherapy or chemotherapy or other cancers were not entered into the study.

H & E slides were examined in patients with SCC and those with sufficient tissue were selected for IHC studies. Clinical information of patients including age, sex, tumor location, tumor size, metastasis stage and grade as well as tobacco consumption habits were extracted from the patients' records. All patients signed an informed consent form to participate in the study.

To prepare serum, 5 cc of blood was drawn from the patients' veins, who were NPO for 12 hours, in the morning before surgery. The blood samples were immediately centrifuged at 3000 rpm for 10 min. The serum was then separated and stored at  $-80^{\circ}\text{C}$  until analysis (Shang et al., 2007).

In order to collect saliva, unstimulated whole saliva samples were collected from the patients, who were NPO for 12 hours, in the morning before surgery.

The patients were asked to refrain from eating, drinking and smoking for thirty minutes. Then, the patients' lips were cleaned and each patient rinsed his/her mouth with plain water. Approximately 5-10 ml saliva was collected from every patient. After centrifugation of the samples ( $g \times 2600$ , 15 min,  $40^{\circ}\text{C}$ ), they were then stored at  $-80^{\circ}\text{C}$  until use (Brooks et al., 2008).

#### Serum and saliva analysis

Concentration of IL-10 (Bender Med Systems GmbH, Germany) was measured using Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's instructions.

#### Immunohistochemical staining

IHC staining was performed by using Envision Labeled Peroxides System (DAKO, Carpinteria, CA, USA). All the samples have been fixed in 10% buffered formalin and have been embedded in paraffin. Sections with  $4\mu$  thickness were prepared, deparaffinized in xylene, rehydrated in graded alcohol and were washed with distilled water. Antigen retrieval was performed by using DAKO cytometry target retrieval solution with  $\text{pH}=9$ , for 20 minutes. Internal peroxidase activity was inhibited by 3%  $\text{H}_2\text{O}_2$ . Tissue sections were then incubated for 30 minutes with the anti-IL-10 (BMS 131/2-UL, BMS 131/2-PL, Bender Med Systems GmbH) at a 1/20 dilution. Normal samples were stained with the same amount of antibody used for staining tumor tissues. Omission of primary antibody was employed as negative control, while tonsil tissue was used as positive control. Brown cytoplasmic staining for IL-10 was considered as positive.

Immunohistochemical results were interpreted by

two pathologists. In cases of disagreement between pathologists in evaluating the staining slides, consensus was reached by joint and simultaneous re-evaluation using multi-headed microscope. The stained slides were initially scanned at low magnification. For the slides showing heterogeneous staining, the regions with higher staining were studied. Randomly five fields were chosen and 500 cells were counted and percentage of staining was calculated. When more than 30% of cells showed cytoplasmic staining, immune reactivity of IL-10 was considered as positive.

#### Statistical methods used in the analysis

After entering the data into the statistical software (SPSS ver.18), the normality of the data was first examined using Kolmogorov-Smirnov test. As the variables under study were not normally distributed, Mann-Witney, Kruskal-Wallis and Chi-Square tests were used in order to compare two groups. Differences were considered significant at  $p < 0.05$ .

## Results

#### Description of variables

The present study was performed on 30 patients with head and neck SCC (21 males and 9 females) with a mean age of  $56.76 \pm 12.46$  years. The minimum and maximum ages were 34 and 85 respectively. Clinicopathologic characteristics of the patients are shown in Table 1.

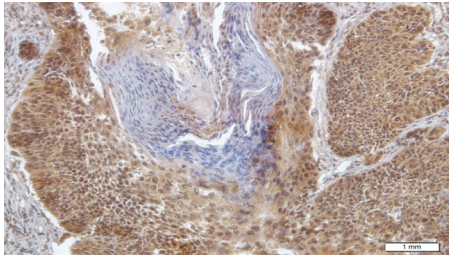
#### Histological study

In examining the incidence and histological staining, no IL-10 staining was observed in normal tissues; whereas IL-10 occurred in 26 patients (86.6%). The results indicated that the tissue expression of IL-10 in cases and controls showed a significant difference ( $P: 0/001$ ), such that it is higher in SCC group than the control (see Figure 1). Also, the expression of IL-10 was observed in inflammatory cells within the tumor stroma (Figure 2).

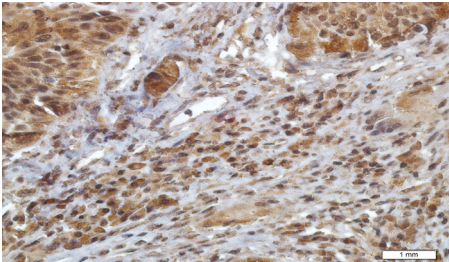
After that, the relationship between IL-10 expression and other variables such as age, sex, grade, M, N, T was examined using Kruskal Wallis test. It was found that there was no significant relationship between IL-10 and

**Table 1. Patient Details**

Age (years)		56.76±12.46
Gender	Male	21 (70.0%)
	Female	9 (30.0%)
Tumor size	T1	12 (40.0%)
	T2	18 (60.0%)
Regional lymph node involvement	N0	27 (90.0%)
	N1	3 (10.0%)
	M0	30 (100.0%)
TNM stage	I	11 (36.7%)
	II	16 (53.3%)
	III	3 (10.0%)
Histological grade	I (well-diff.)	8 (26.7%)
	II (moderately- diff.)	18 (60.0%)
	III (poorly- diff.)	4 (13.3%)
Tumor location	Larynx	22 (73.3%)
	Oral cavity	8 (26.7%)



**Figure 1. Immunohistochemical Staining of IL-10 in HNSCC**



**Figure 2. Positive Staining in Stromal Elements**

other variables. The only significant correlation found, was between IL-10 and the disease stage ( $p: 0.044$ ); such that patients in lower stage disease showed higher chance of positive tissue for IL-10.

#### *Serum and Salivary Levels*

Mean serum level of IL-10 was  $11.69 \pm 7.07$  in patients and  $5.29 \pm 5.1$  in controls. Mean Salivary level of IL-10 was  $11.79 \pm 10.66$  in patients and  $10.01 \pm 6.01$  in controls. No significant difference was found by Mann-Whitney statistical test between controls and patients ( $P: 0.619$ ,  $P: 0.548$ ).

No significant correlation was found by Mann – Whitney statistical test between IL-10 tissue expression rate and its serum and salivary level (Respectively  $P: 0.423$ ,  $P: 0.541$ ). The relationships between serum and salivary levels of IL-10 and other variables such as age, sex, M, N, T, grade and stage were separately examined, and no significant relationship was found between serum or salivary levels and the mentioned variables.

## **Discussion**

AIL-10 is a pleiotropic cytokine which is produced by macrophages, T-helper and B lymphocytes and has the ability to either stimulate or suppress the immune system (Liu et al., 2011).

IL-10 has dual effects on solid tumors which appear as anti-tumor effects or tumor cells evasion of immune system. By preventing tumor antigen from reaching antigen-producing cells, IL-10 causes tumors to escape T-cells (Fujieda et al., 1999).

IL-10 antitumor mechanism is not quite well known yet. Some studies have suggested that IL-10 can cause anti-tumor and anti-metastatic effects by inhibition of angiogenesis. IL-10 can have anti-angiogenesis effects via reducing the production of angiogenic factors, such as VEGF, TNF- $\alpha$ , IL-6 and MMP9, by macrophages associated with tumor. IL-10 is also directly effective on secretion of angiogenic factors from tumor cells (Cervenak

et al., 2000).

The results of this study showed that IL-10 expression was observed in 86.6% of tumor samples; while none of the normal tissues expressed IL-10. The difference between groups of patients and controls in IL-10 expression was statistically significant. The expression of this marker was also observed in mononuclear cells of the tumor stroma.

The results of this study are consistent with the results of the study conducted by Fujieda et al. In their study, the incidence of IL-10 was observed in 82% of oral and pharyngeal SCC tumor tissues and mononuclear cells (Fujieda et al., 1999).

However, in Chandler et al study, 65% of oral SCC tissues showed expression of IL-10, which is lower in comparison with our study, and the expression of IL-10 was not observed in mononuclear inflammatory cells. According to their study, IL-10 did not express even in normal tonsil tissues. They concluded that less occurrence of IL-10 may be due to problems in the process of antigen retrieval or differences in the tendency of primary antibody in binding to antigen (Chandler et al., 2000).

In this study, IL-10 was observed in the cytoplasm of tumor cells. This indicates that head and neck SCC cells are capable of producing IL-10. The expression of IL-10 was not associated with any clinicopathologic factors except 'stage', such that the incidence of IL-10 decreases in higher stages. These findings indicate that decreased expression of IL-10 in tumor destroys anti-tumor mechanisms and causes progression of the disease. In support of this hypothesis, some studies have shown that systematic prescription of IL-10 prevents metastasis in melanoma tumor cells, sarcoma and colorectal cancer (Berman et al., 1996; Zheng et al., 1996).

In head and neck SCC, several cytokines with high concentrations have been detected in patient's serum and plasma (Linkov et al., 2007).

Concerning IL-10 serum level and its role in head and neck tumors, contradictory reports have been presented. According to results of different studies, in patients with advanced head and neck SCC, serum and plasma level of IL-10 increases (Sparano et al., 2004).

However, in other studies, IL-10 has not been detected in serum of the patients with SCC and adenoid cystic carcinoma (Hoffmann et al., 2007).

Also, in the study conducted by Linkov et al. (2007) there had been no difference between IL-10 serum level of patients with head and neck SCC and that of the control group who were smokers (Linkov et al., 2007).

The study of Alhamarneh et al has shown that IL-10 serum level was significantly higher in the advanced stages of the disease (other T3/T4), in patients with Lymph node involvement and in stage III/IV (Alhamarneh et al., 2010).

In our study, serum level of IL-10 in patients with SCC was a little higher than that of patients in control group; which has not a significant difference.

In most studies, the IL-10 increase in serum was detected in advanced stages of disease (Linkove et al., 2007; Alharneh et al., 2010); but in our study, the number of patients who were in the advanced stage of the disease (III and IV Stage, lymph node metastasis and tumor T3/T4) was low. This could explain the low IL-10 serum level.

Another reason for the contradictory results is related to the difference in the location of tumor, which is generally considered as being in the head and neck in this study. While, there are notable differences between tumors in different locations. In addition, difference in methods of measuring IL-10 in serum and plasma, small number of samples in some studies including ours, having radiotherapy or chemotherapy before giving blood sample could be other reasons for the contradictory results.

According to Alhamarneh's study, serum level of IL-10 is reduced four to six weeks after treatment. This finding shows that tumor cells are the major source of IL-10 production. As shown in IHC examination of the tissues in this study as well as Chandler et al (2002), IL-10 expression has been mostly in tumor cells (Alhamarneh et al., 2010; Chandler et al., 2002).

In some studies, other sources have been considered as IL-10 producers including T cells, active macrophages within the tumor, B cells, regulatory B cells and natural killer cells. These can cause an increase in the serum level of IL-10. These cells can remain even after resection of the tumor (Berzofsky et al., 2009; DiLillo et al., 2010).

In order to prove the role of other cells in producing IL-10, other studies are required in which serum levels of IL-10 are measured before and after the treatment.

Saliva is a body fluid which is easily accessible and it can be provided with a non-invasive method. It is now considered as the mirror of the body and it has been used in the diagnosis of systematic diseases and examining tumor markers. Therefore, salivary levels of IL-10 were evaluated. The studies examining the salivary levels of IL-10 in cancer are limited (Teles et al., 2009).

In a study conducted by Nelson et al on cervical cancer, salivary and serum levels of IL-10 were evaluated and it was shown that serum level of IL-10 was higher in patients than controls, but the salivary level showed no significant difference and did not have any relationship with any of the clinicopathologic factors (Nelson et al., 2008).

In the present study, although the salivary levels of IL-10 in patients were a little higher in patients than controls, this difference was not statically significant. It seems that one of the reasons for lack of increase in the level of IL-10 in saliva is 'inhibitory cytokines'. In a study conducted by Wozinak et al, the inhibitory role of parotid saliva and whole saliva on the surfaces of several cytokines was examined. And it was found that whole saliva, compared to parotid saliva, has an inhibitory effect on concentrations of cytokines. In that study, it was suggested that cytokines degradation by mucin-like proteins or large molecules or enzymatic degradation can be among inhibitory mechanisms in saliva (Wozniak et al., 2002).

Therefore, salivary level of IL-10 in parotid saliva can be examined in future studies of this kind. In the present study, most patients were in stage I, II, and it would be better to examine patients with higher-stage diseases in future studies. In this study, there was no significant difference between tissue expression, serum and salivary level of IL-10; that is, serum or salivary levels do not represent tissue level of this marker.

There was an increase in IL-10 expression in SCC tissues; which shows that this marker is associated with

head and neck SCC. No relationship, except between the stage and the tissue expression, was observed between tissue, serum and salivary levels of this marker with clinicopathologic factors.

## Acknowledgements

The authors would like to thank the Vice-Chancellery for Research of Shiraz University of Medical Sciences for providing financial support for this study (Grant#90-5551). This manuscript is relevant to the post graduate thesis of Dr.Marzieh Hamzavi.

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