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MAIN ARTICLE

Serum levels of interleukins 4 and 10 in head and neck squamous cell carcinoma

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Abstract

Objective: There is currently controversy over the association between serum interleukin-4 and -10 levels and head and neck squamous cell carcinoma in patients of different ethnicity. This study aimed to investigate serum levels of these cytokines in Iranian patients with this pathology, and to analyse correlations with tumour location and tumour stage at diagnosis.

Design: Serum cytokines levels were quantified using commercial enzyme-linked immunosorbent assays.

Subjects: Study groups comprised 93 untreated patients and 53 healthy donors.

Results: Serum interleukin-4 levels were significantly increased in patients compared with controls ($p < 0.000$), but were not significantly associated with tumour stage. Serum interleukin-10 levels were not raised in patients, nor associated with tumour stage.

Conclusion: Serum levels of interleukin-4, but not -10, were increased in Iranian head and neck squamous cell carcinoma patients. These data do not support an association of these cytokines with tumour progression; this is consistent with previous findings.

Key words: Carcinoma, Squamous Cell; Interleukin-4; Interleukin-10; Head And Neck Neoplasms; Immunosuppression

Introduction

Head and neck squamous cell carcinoma (SCC) is the sixth most common malignancy world-wide. It originates from the mucosal surfaces of the upper aerodigestive tract, including the oral cavity, pharynx and larynx. Despite improvements in diagnosis, surgery and chemotherapy, the five-year survival of head and neck SCC patients has not increased significantly in recent decades.[1]

Cytokines comprise various soluble factors. They have been associated with the initiation, progression, prognosis and therapeutic response of malignant processes.[2–4] Although a variety of cells can produce cytokines, including cancer cells, a major source is the different subsets of T helper lymphocytes. The T helper cells are classified into two distinct functional subsets, the Th1 and Th2 cells. They are differentiated from naïve T helper lymphocytes by distinct stimuli. The Th1 subset is characterised by the secretion of Th1 cytokines, such as interferon-γ, which activate macrophages, cytotoxic T lymphocytes and natural killer cells – a cell population responsible for direct execution of cancer cells. In contrast, the Th2 subset produces Th2 cytokines, such as interleukin (IL) 4 and IL-10, which support tumour growth and progression partly through inhibition of Th1 cytokines.[2,3] Dysregulation of the Th1/Th2 cytokine ratio, with a predominance of Th2 cytokines, has been shown to favour the development of head and neck SCC.[5–10]

Interleukins 4 and 10 are hallmark Th2 cytokines. Interleukin-4 is mainly produced by Th2 cells, and also by mast cells and basophils.[2] Its production by cancer cells has been observed only in lymphoma and pancreatic cancer.[11] Interleukin-10 is produced by a variety of immune cells (e.g. Th2 cells) and non-immune cells (e.g. cancer cells).[3] Both these cytokines reduce Th1 cytokine production, enhance antibody production by B cells and aggravate Th2-mediated disease (e.g. allergic disorders). In particular, IL-4 is the signature cytokine for the induction of naïve T cell differentiation into Th2 subset cells, while IL-10 blocks the expression of co-stimulatory molecules for T helper cell activation (e.g. cluster of differentiation 80 and 86 glycoproteins, and major histocompatibility complex class II) and co-stimulatory molecules for cytotoxic T cell activation (e.g. major histocompatibility complex class I). Interleukins 4 and 10 can also promote tumourigenesis via several nonimmunological

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mechanisms, such as protection of cancer cells from apoptosis.[2,3]

Interleukin-4 and -10 'gene knockout' animal models display significant suppression of tumour growth, suggesting that these cytokines play a role in tumour growth *in vivo*. Increased IL-4 and IL-10 production is usually seen in cancer patients' peripheral blood lymphocytes, tumour infiltrating lymphocytes and/or sera.[2,3,8–10] A limited number of studies have investigated IL-4 and IL-10 serum levels in head and neck SCC patients, with sometimes controversial results.[5–10]

Despite evidence that IL-4 and IL-10 act to support tumour growth, some researchers have described anti-tumour activities for these cytokines. Evidence suggests that the nature of cytokines' effect on tumours – whether promoting or suppressing – depends upon their dose, administration route (i.e. local or systemic), and whether they are expressed early or late in the tumour development process.[2,3] For example, in mice with inoculated tumours, early IL-4 expression suppresses tumour growth but late IL-4 expression does not.[2] It has been suggested that IL-4 suppresses tumours *in vivo* by inhibiting angiogenesis.[2]

It is generally accepted that data from several studies, and from patients of various ethnic backgrounds, is needed in order to adequately investigate the importance of any factor involved in disease aetiopathogenesis. We have previously studied pretreatment serum IL-6 levels in Iranian head and neck SCC patients.[12] In keeping with results from other populations,[8,13] we found that serum IL-6 levels were associated with head and neck SCC progression.[12]

The association between serum IL-4 and IL-10 levels and head and neck SCC is controversial.[5–10] There has been no previous investigation of the levels of these cytokines in Iranian head and neck SCC patients.

Therefore, the present study assessed pretreatment serum levels of IL-4 and IL-10 in Iranian head and neck SCC patients, compared with levels in healthy control individuals. We also evaluated the correlation between serum levels of these two cytokines and tumour location and stage at diagnosis.

Materials and methods

Subjects

The study was approved by the ethics committee of the Shiraz University of Medical Sciences. All participants were informed that blood samples would be used for serum analysis for research projects, and their consent was obtained.

The study assessed 93 unrelated patients (61 men and 32 women; mean age ± standard deviation (SD), 61.8 ± 15.4 years) and 53 unrelated healthy control subjects (34 men and 19 women; mean age ± SD, 60.7 ± 11.9 years).

The patients were admitted to Khalili Hospital, Shiraz, Iran. The diagnosis of SCC was confirmed histopathologically. Disease stage was determined according to the tumour-node-metastasis classification.[14]

Control subjects were staff members of Shiraz University of Medical Sciences, or blood donors attending the Shiraz Blood Transfusion Center. They were apparently healthy, with no history of malignant, metabolic or autoimmune diseases.

All participants were questioned and physically examined to detect any symptoms or signs of infection, such as cough, fever, or throat or ear inflammation. Routine laboratory tests (e.g. full blood count) were also performed for patients and the blood donor controls. None of the participants had any evidence of acute infection within the past month.

Interleukin-4 and -10 assays

Blood samples were taken from the 93 newly diagnosed patients and 53 healthy controls. Collected serum samples were aliquoted and stored at −70°C until use.

Serum levels of these cytokines were determined using commercial enzyme-linked immunosorbent assay kits (eBioscience, Vienna, Austria), according to the manufacturer's protocols. Briefly, standards (diluted serially in assay buffer), serum samples (diluted 1:2 in assay buffer) and blanks (comprising 100 µl assay buffer) were added in triplicate, at a volume of 100 µl per well. Standard dilutions ranged from 500.0 to 7.8 pg/ml. Biotin-conjugate anti-human interleukin-4 (IL-4) monoclonal antibody was added to all wells and incubated for 2 hours at room temperature, followed by washing. Streptavidin horse radish peroxidase was then pipetted into all wells and incubated for 1 hour at room temperature. Wells were then washed three times with wash buffer, and tetramethyl-benzidine substrate solution was added. The plates were incubated for approximately 10 minutes. The reaction was stopped by phosphoric acid.

The microplate reader (Anthos 2020; Anthos Labtec, Salzburg, Austria) was blanked and the colour intensity measured at 450 nm. The minimum detectable limits were 1.3 pg/ml for the IL-4 assay kit and 1.0 pg/ml for the IL-10 kit. Concentrations below the detection limits were reported as undetectable.

Statistical analysis

Levels of IL-4 and IL-10 were not normally distributed in head and neck SCC patients, according to the Kolmogorov–Smirnov test. Therefore, non-parametric tests (i.e. the Mann–Whitney U test or the Kruskal–Wallis test) were used to calculate differences in the levels of these cytokines, comparing cases versus controls, and to assess their associations with clinical disease characteristics.

Data were analysed using the Statistical Package for the Social Sciences version 11.5.0 software program (SPSS Inc, Chicago, Illinois, USA). Findings were

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considered statistically significant at a p value of less than 0.05.

Results

Table I shows the mean and median serum levels of interleukin (IL) 4 and IL-10 in the 93 untreated head and neck SCC patients and 53 healthy controls. The median serum IL-4 level was significantly higher in patients than controls ($p < 0.000$). However, there was no significant difference in IL-10 level, comparing patients and controls.

Table II shows the associations between median IL-4 and IL-10 serum levels, tumour stage and tumour location. There was no significant association between serum IL-10 levels and tumour stage or location. There was a trend towards increased serum IL-4 level with more advanced tumour stage, but this did not reach statistical significance.

Tumours were located in the oral cavity in 34 patients (36.5 per cent), in the pharynx in 14 (15.1 per cent) and in the larynx in 45 (48.4 per cent). Tumour location was not significantly associated with either IL-4 or IL-10 serum level.

Discussion

Serum cytokines have attracted much attention in the field of cancer research, as potential diagnostic markers, prognostic indicators and guides for therapy.[8,12,13] Several publications have provided data relevant to the potential feasibility, or otherwise, of cytokines as cancer biomarkers. Several studies, including reasonable numbers of patients, have demonstrated that serum levels of the inflammatory cytokine interleukin (IL) 6 has potential as a biomarker for head and neck SCC diagnosis and progression.[8,12,13] However, the associations between head and neck SCC and other cytokines have not been studied as extensively.

Cytokines can function as tumour-promoting factors (e.g. Th2 cytokines) or tumour-suppressing factors (e.g. Th1 cytokines).[2,3] Increased expression of Th2 cytokines has been observed in the lymphocyte cytokine profiles, tumour tissues and sera of head and neck SCC patients.[5–10] Evidence from serum analysis supports a partial Th2 cytokine bias and a decline in cytokine interrelationships, as head and neck SCC progresses.[8,9]

Two important Th2 cytokines are IL-4 and IL-10. A limited number of studies have investigated the association between serum IL-4 and IL-10 levels and head and neck SCC, and their results have been controversial.[5–10] For example, Lathers *et al.*[8] found significantly increased IL-4 and IL-10 serum levels in 101 head and neck SCC patients, compared with 40 controls; in contrast, Hoffmann *et al.* found no significant increase in IL-4 and IL-10 serum levels in 20 head and neck SCC patients, compared with 20 controls.[10] In the present study, we measured serum levels of IL-4 and IL-10 in 93 head and neck SCC patients and 53 healthy controls, and observed significantly increased

TABLE I
IL-4 AND IL-10 SERUM LEVELS IN HNSCC PATIENTS AND CONTROLS

IL level (pg/ml)	Patients*	Controls†	p‡
IL-4			
– Mean ± SD	7.9 ± 10.4	3.1 ± 1.4	<0.000
– Med (range)	6.3 (1.5–87.0)	2.9 (UD** to 6.8)	
IL-10			
– Mean ± SD	2.3 ± 2.0	1.9 ± 0.3	0.34
– Med (range)	1.7 (UD§ to 14.8)	2.0 (1.0–2.6)	

*n=93; †n = 53. ‡Mann–Whitney U test, patients *vs* controls. Enzyme-linked immunosorbent assay sensitivities: **1.3 pg/ml for interleukin (IL) 4; §1 pg/ml for IL-10. HNSCC = head and neck squamous cell carcinoma; SD = standard deviation; med = median; UD = undetectable

TABLE II
IL-4 AND IL-10 SERUM LEVELS, BY TUMOUR SITE AND STAGE*

Parameter	Pts (n (%))	IL-4 (pg/ml)				IL-10 (pg/ml)			
		Med	Percentile 5th	Percentile 95th	p†	Med	Percentile 5th	Percentile 95th	p†
Tumour site					0.84				0.49
– Oral cavity	34 (36.5)	6.4	1.7	15.3		2.0	1.1	5.9	
– Pharynx	14 (15.1)	6.4	3.0	8.7		1.6	1.1	7.9	
– Larynx	45 (48.4)	6.3	2.8	49.2		1.7	UD‡	9.1	
Tumour stage**					0.26				0.47
– $T_{1/2/3}$	64 (69.6)	6.1	2.1	16.7		1.7	1.1	7.5	
– T_4	28 (30.4)	7.1	3.0	25.3		2.0	UD‡	10.3	

*In 93 patients with head and neck squamous cell carcinoma. †Mann–Whitney U test or Kruskal–Wallis test, as appropriate. ‡Enzyme-linked immunosorbent assay sensitivity for interleukin (IL) 10 = 1 pg/ml. **Sum of patient numbers does not equal the true total as data is missing for one patient. Pts = patients; med = median; UD = undetectable; T = tumour stage

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serum IL-4 levels, but not IL-10 levels, in the cancer patients compared with the controls.

Of the nine cytokines studied by Lathers *et al.*[8] in head and neck SCC patients, IL-4 showed the most prominent change (i.e. a 47-fold increase), compared with controls. Aberrant results and loss of serum cytokine interrelationships have been reported in head and neck SCC patients.[8] However, simultaneous analyses of serum IL-4 and IL-10 levels in Western head and neck SCC patients have generally shown either a clear association, or lack of association, with head and neck SCC.[8–10]

- **Head and neck squamous cell carcinoma (SCC) is commonly associated with increased Th2 cytokine levels**
- **This study investigated serum interleukin (IL) 4 and -10 levels in Iranian head and neck SCC patients**
- **Serum levels of IL-4, but not IL-10, were increased in patients compared with controls**
- **Serum IL-4 and -10 levels were not associated with tumour stage, as previously reported**

In our study, the observed association between head and neck SCC and serum IL-4 levels, but not IL-10 levels, may have been due to an extreme increase in IL-4 levels in the head and neck SCC patients, compared with controls.[8] We also note that our control subjects had raised serum IL-4 levels, compared with control values reported by Western studies (Table I).[8–10] Our control subjects' serum IL-4 levels were close to those reported by researchers working in India,[15] a country closer to Iran (compared with Western European and the Americas). This may indicate shared environmental or genetic factors, resulting in elevated serum IL-4 levels in normal individuals of both countries.

Evidence from animal models indicates that IL-4 and IL-10 are involved in tumour progression.[2,3] For example, in animal models, neutralisation of IL-4 (using an IL-4-specific monoclonal antibody) decreased the metastatic ability of a highly metastatic melanoma cell line, whereas systemic IL-4 administration increased the metastatic ability of a low-metastasis melanoma variant.[2]

However, in human head and neck SCC cases, there is consensus that differences in cytokine levels may not represent the extent of clinical disease, but merely reflect the presence of tumour, IL-6 being the exception.[8,9] We have previously observed that serum IL-6 levels increase as head and neck SCC progresses.[12]

In the present study, we examined the association between tumour stage and serum IL-4 and IL-10 levels, and found no significant association.

We also investigated the association between tumour location and serum IL-4 and IL-10 levels. Jebreel *et al.*[5] found that IL-10 detectability varied according to the tumour (primary) site, being more commonly observed in hypopharyngeal and laryngeal tumours.[5] However, we found no significant association between either IL-4 or IL-10 serum levels and tumour location.

Conclusion

Our data, obtained from a reasonable number of patients, reveal that pretreatment serum levels of IL-4, but not IL-10, are increased in Iranian head and neck SCC patients, compared with healthy controls. In keeping with most previous studies, we found that serum levels of these cytokines were not affected by tumour stage.

Our study represents the first attempt to analyse serum levels of these two Th2 cytokines in Iranian head and neck SCC patients. Our findings further support the impracticality of using serum IL-4 and IL-10 levels as markers for disease progression in head and neck SCC patients.

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