



Serum concentrations of interleukin-8, vascular endothelial growth factor, and epidermal growth factor receptor in patients with squamous cell cancer of the head and neck

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Received 23 April 2004; accepted 9 June 2004

KEYWORDS

Head and Neck cancer;
IL-8;
VEGF;
EGFR

Summary Squamous cell cancer of the head and neck (SCCHN) is associated with production of pro-inflammatory and pro-angiogenic cytokines. We hypothesized that cytokine serum levels will correlate with tumor volume and aggressiveness. We investigated interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) in SCCHN.

The patient population consisted of normal and irradiated controls: patients with newly diagnosed SCCHN, and patients with recurrent or metastatic disease. Pretreatment sera were studied by ELISA.

Serum IL-8 levels, as opposed to VEGF or EGFR, were consistently elevated in patients with recurrent or metastatic disease. The differences in mean serum IL-8, compared to controls, were significant ($p=0.02$).

Serum levels of IL-8 are consistently elevated in patients with recurrent or metastatic SCCHN and elevated levels may correlate with advanced or aggressive disease. Further, more intensive, study of IL-8 as a biomarker in SCCHN is warranted.

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Introduction

Head and neck cancers represent approximately 3% of all malignancies in the United States, with approximately 90% being SCCHN.¹ Though

improvements in management of head and neck cancers have occurred over the past 30 years, increases in long-term survival have been minimal.² Advances in the biomedical sciences have allowed clinicians and researchers to look at the malignancy at the molecular level in an effort to define better prognostic indicators and to develop more precise, targeted therapies. In vitro analyses of cell lines derived from squamous cell cancers of the head and neck and in situ studies of the local head and neck tumor microenvironment have demonstrated that head and neck tumors express a number of autocrine and paracrine growth factors and cytokines.^{3–5} These growth factors and cytokines are thought to alter host inflammatory, angiogenic, and immune processes to the benefit of the tumor.³ As critical factors in tumor growth and survival, inhibition of the activity of these cytokines provides a possible therapeutic intervention. Among the cytokines that have been identified as being produced by head and neck tumors are interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF).^{4–6}

In addition to established research findings indicating expression of IL-8 and VEGF in the local microenvironment of tumors, studies have also investigated the circulating levels of these cytokines in patients with various malignancies. Serum IL-8 levels were found to be inconsistently elevated and hence a possible prognostic marker in malignant melanoma, soft tissue sarcoma, B-cell chronic lymphocytic leukemia, and hepatocellular carcinoma.^{7–10} Similarly, serum VEGF has been found to be elevated in patients with colorectal cancer, breast cancer, lung cancer, and malignant melanoma.^{10–12} Additionally, elevated serum VEGF levels have been correlated with advanced stage and formation of distant metastasis in colorectal cancer.¹²

The mechanisms enabling the production of these cytokines in tumors and in particular SCCHN involve a complex series of cell signaling events. Activation of the epidermal growth factor receptor (EGFR) may play a vital role in initiating cell signaling pathways that promote tumor growth and survival, including the expression of pro-inflammatory and pro-angiogenic cytokines. EGFR, a tyrosine kinase receptor found on the cell surface of both normal and malignant cells, has been found to be highly expressed on SCCHN tumor cells.¹³ We hypothesized that cytokine serum levels will correlate with tumor volume and aggressiveness, and investigated interleukin-8 (IL-8), vascular endothelial growth factor (VEGF), and epidermal growth factor receptor (EGFR) in SCCHN.

Methods and materials

Subjects were seen and evaluated in the Head and Neck Oncology Program at the Dana-Farber Cancer Institute. Subjects signed an IRB approved informed consent prior to participation in this study. Normal controls were healthy men and women aged 45–65 with no history of chronic systemic disease or malignancies. Patients could not have underlying significant morbidity or active medical problems such as congestive heart failure, active infection, autoimmune disease, hepatitis, HIV, abnormal renal function, active alcohol addiction, or active drug addiction. Patients with newly diagnosed disease were included only if adequate TNM staging was available and were followed to complete staging. Patients with recurrent disease were defined as having local regionally recurrent disease or metastatic disease. Patients with metastatic disease at the time of initial diagnosis were placed in the recurrent disease group rather than newly diagnosed Stage III/IV disease. Patients with local/regional recurrent disease who had prior radiotherapy were required to be more than 6 months from completing radiotherapy. Radiation control patients were required to be at least 2 years from completion of radiotherapy and were followed for an additional 6 months to ensure that there were no recurrences in the time frame. All patients and controls were not allowed to be taking any steroid or NSAID, with the exception of acetaminophen, within one week of sample collection.

Patients and controls underwent phlebotomy of venous blood. Blood was allowed to clot for a minimum of 0.5 h and a maximum of 2 h and then centrifuged at 1200g for 10 min. Serum was aspirated and aliquoted into 4 × 0.5 ml aliquots in vials and stored at –80 °C until use. Samples were freshly thawed prior to use.

Serum levels IL-8, VEGF, and EGFR were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits following the manufacturers' instructions. All samples were run in duplicate, and concentration values were averaged. Patient information for each assay displaying the number of patients analyzed and demographic data is shown in Table 1. Serum IL-8 concentrations were determined by a quantitative sandwich ELISA commercial kit (BD Biosciences Pharmingen, San Diego, CA). Circulating levels of VEGF in patient sera were analyzed using two different commercial ELISA kits. One kit utilized a sandwich ELISA (Quantikine Human VEGF Immunoassay, R&D Systems, Minneapolis, MN) to measure circulating

Table 1 Demographics of patients studied

Patient group	Total # patients	Male/female	Age range	Mean age
<i>IL-8 assay</i>				
Newly diagnosed disease	15	14/1	29–73	51
Stage I/II	3	3/0	29–48	41
Stage III/IV	12	11/1	29–73	55
Recurrent disease	22	20/2	45–65	55
Local/regional recurrent disease	6	4/2	51–65	56
Metastatic disease	16	16/0	45–63	55
Radiated control group	7	6/1	34–76	55
Normal healthy controls	7	5/2	–	all >50
<i>S-VEGF assay</i>				
Newly diagnosed disease	3	3/0	29–48	41
Stage I/II	2	2/0	29–48	39
Stage III/IV	1	1/0	46	46
Recurrent disease	16	14/2	47–64	56
Local/regional recurrent disease	4	2/2	54–61	58
Metastatic disease	12	12/0	47–64	55
Normal healthy controls	5	3/2	–	all >50
<i>Total VEGF assay</i>				
Newly diagnosed disease	4	4/0	29–71	50
Stage I/II	2	2/0	29–48	39
Stage III/IV	2	2/0	41–61	51
Recurrent disease	13	11/2	47–62	55
Local/regional recurrent disease	3	1/2	54–60	57
Metastatic disease	10	10/0	47–62	55
Normal healthy controls	6	5/1	–	all >50
<i>EGFR assay</i>				
Newly diagnosed disease	3	3/0	29–48	41
Stage I/II	2	2/0	29–48	39
Stage III/IV	1	1/0	46	46
Recurrent disease	12	10/2	47–62	55
Local/regional recurrent Disease	4	2/2	54–61	58
Metastatic disease	8	8/0	47–62	53
Normal healthy controls	5	4/1	–	all >50

levels of VEGF165 (S-VEGF), a blood soluble and predominant isoform of VEGF. A second kit utilized a competitive ELISA (Neogen Corporation, Lexington, KY) to measure circulating levels of all isoforms of VEGF (total VEGF), including receptor-bound VEGF. Serum EGFR concentrations were determined by a commercial sandwich ELISA kit (Oncogene Research Products, San Diego, CA) that measured all forms of circulating EGFR, including activated EGFR (Figs. 1–3).

Serum levels of IL-8, VEGF, and EGFR in patient samples were statistically analyzed in comparison

to normal controls using *t*-tests. All *t*-tests were two tailed and regarded significant if $p < 0.05$. Microsoft Excel was used to carry out the analysis.

Results

Interleukin-8

The results of the circulating levels of IL-8 in the various patient categories are shown in Table 2.

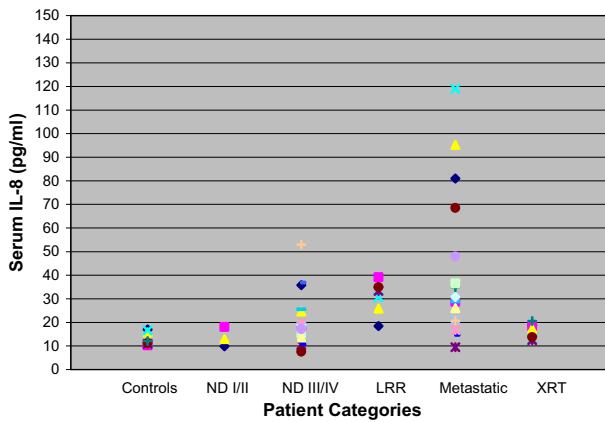


Figure 1 Serum IL-8 levels in patients with SCCHN. Individual data points for each of the patient groups are represented. Legend: ND I/II, new disease stage I/II patients; ND III/IV, new disease stage III/IV patients; LRR, local/regional recurrent disease patients; Metastatic, metastatic disease patients; XRT, radiated control group.

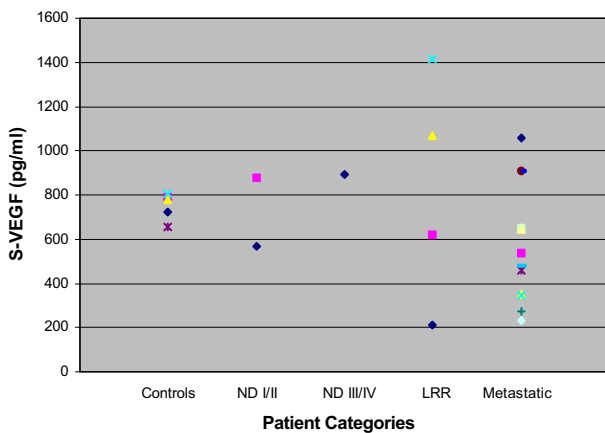


Figure 2 Serum S-VEGF levels in patients with SCCHN. Individual data points for each of the patient groups are represented. Legend: ND I/II, new disease stage I/II patients; ND III/IV, new disease stage III/IV patients; LRR, local/regional recurrent disease patients; Metastatic, metastatic disease patients.

The difference in mean serum IL-8 between normal controls and patients with newly diagnosed disease was not found to be statistically significant regardless of stage. Similarly, no statistically significant difference in serum IL-8 concentration was seen between normal healthy controls and patients in the radiated control group. In contrast, patients with metastatic disease and local/regional recurrent disease were found to have mean serum IL-8 concentrations that had a statistically significant difference ($p = 0.03$ and 0.001 respectively) from

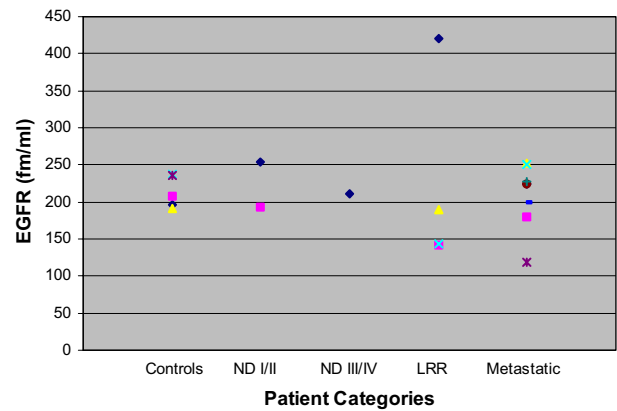


Figure 3 Serum EGFR levels in patients with SCCHN. Individual data points for each of the patient groups are represented. Legend: ND I/II, new disease stage I/II patients; ND III/IV, new disease stage III/IV patients; LRR, local/regional recurrent disease patients; Metastatic, metastatic disease patients.

that of the normal controls. The overall combined recurrent disease group was also found to have a mean serum IL-8 concentration that was significantly different from that of the normal controls ($p = 0.02$).

Another measure of comparing patients with head and neck cancers to normal controls is to define the number of patients in each category that had IL-8 levels above the upper limit of normal. In this study, the upper limit of serum IL-8 levels in the normal control group was 18 pg/ml. Using this value, it is shown in Table 1 that serum IL-8 levels exceeded 18 pg/ml in 3 out of 15 patients (20%) with newly diagnosed disease (0 of 3 with Stage I/II disease, 3 of 12 with Stage III/IV). In comparison 19 of 22 patients (86%) with recurrent disease had levels exceeding the upper limit of the normal controls. Further subdivision shows that 81% of patients (13 of 16) with metastatic disease and 100% of patients (6 of 6) with local/regional recurrent disease exceeded the 18 pg/ml level.

VEGF

Table 3 displays the results of circulating serum levels of S-VEGF in the various patient groups. Statistical analysis of the S-VEGF data demonstrated no statistically significant difference between normal controls and each of the other patient categories.

Using 810 pg/ml as the upper limit of the normal controls, Table 3 shows that 1 of 2 patients with Stage I/II disease and the lone patient with Stage III/IV disease exceeded the 810 pg/ml level. 31% of patients with recurrent disease (5 of 16) had serum VEGF levels that exceeded the upper limit of

Table 2 Serum interleukin-8 levels in patients with SCCHN

Group	# of patients	IL-8 ± SD (pg/ml)	p value	Range	>18 pg/ml
Normal controls	6	13.4 ± 2.9		10.3–17.0	0/6
Newly diagnosed	20	19.9 ± 10.9	0.14	7.6–52.9	8/20
Stage I/II disease	3	13.6 ± 4.2	0.94	9.4–18.1	0/3
Stage III/IV disease	17	21.0 ± 11.5	0.1	7.6–52.9	8/17
Recurrent disease	22	40.2 ± 28.7	0.02	9.5–119.0	19/22
Metastatic disease	16	44.0 ± 32.3	0.02	9.5–119.0	13/16
Local regional recurrence	6	28.7 ± 6.7	0.0001	18.4–35.0	6/6
Radiated control group	7	16.3 ± 3.2	0.1	12.4–20.6	1/7

Table 3 Serum S-VEGF levels in patients with SCCHN

Group	# of patients	VEGF ± SD (pg/ml)	p value	Range	>850 pg/ml
Normal controls	5	749 ± 60.8		656–803	0/5
Newly diagnosed	3	779 ± 181.6	0.84	569–893	2/3
Stage I/II disease	2	722 ± 215.7	n/a	569–874	1/2
Stage III/IV disease	1	893 ± 0.0	n/a	—	1/1
Recurrent disease	16	635 ± 348	0.48	210–1412	5/16
Metastatic disease	12	570 ± 281	0.22	232–1056	3/12
Local regional recurrence	4	827 ± 523	0.75	210–1412	2/4

normal. Further examining the patients with recurrent disease revealed that 25% (3 of 12) of patients with metastatic disease and 50% (1 of 2) of patients with local/regional recurrent disease exceeded the 810 pg/ml level.

Assays measuring serum levels of total VEGF were conducted on two different occasions one month apart. Of the total 17 patients studied (6 controls, 2 Stage I/II, 2 Stage III/IV, 3 local/regional recurrent disease, and 10 metastatic disease), six patient samples were run on both occasions. These replicate studies were performed using vials of patient serum aliquoted from the same tube of

blood; hence serum total VEGF levels should have been nearly identical in the replicates of these six patients. However, circulating total VEGF serum levels were 3–5 times higher when measured on the second occasion compared to the first. Based on the great variance demonstrated in these six patients, the entire results of the total VEGF ELISA cannot be viewed as reliable.

EGFR

Table 4 displays the results for circulating EGFR levels in the various patient groups. No statistically

Table 4 Serum EGFR in patients with SCCHN

Group	# of patients	EGFR ± SD (fm/ml)	p value	Range	>240 fm/ml
Normal controls	5	213.9 ± 21.5		192–238	0/5
Newly diagnosed	3	219.7 ± 31.2	0.76	193–254	1/3
Stage I/II disease	2	223.7 ± 42.9	n/a	193–254	1/2
Stage III/IV disease	1	211.6 ± 0.0	n/a	—	0/1
Recurrent disease	12	214 ± 77.9	0.99	119–420	3/12
Metastatic disease	8	209 ± 43.7	0.84	119–252	2/8
Local regional recurrence	4	223 ± 132	0.87	141–420	1/4

significant difference was demonstrated in controls versus each of the patient groups with respect to circulating levels of EGFR in sera.

The upper limit of serum EGFR in normal controls was 240 fm/ml. 33% of newly diagnosed patients (1 of 3) had serum levels exceeding this upper limit of normal. Only 25% (2 of 8) of patients with metastatic disease and 25% (1 of 4) of patients with local/regional recurrent disease had serum levels above 240 fm/ml.

Discussion

Prior studies looking at serum levels of IL-8 in patients with head and neck cancers have been limited. Chen³ and colleagues demonstrated in 11 patients (1 Stage II, 10 Stage III/IV) with head and neck cancers that serum levels of IL-8 were elevated compared to normal healthy controls. In that study, serum IL-8 levels in patients ranged from 0.0 to 194.5 pg/ml with the highest IL-8 concentration found in the lone patient in the study with metastatic disease. The same study showed a weak direct relationship between serum IL-8 concentration and tumor burden.³

This study was unique in that it analyzed serum IL-8 levels in a diverse group of patients with head and neck cancers including those with newly diagnosed disease, local/regional recurrent disease, and metastatic disease and included normal control and disease-free irradiated control populations. In our sample group, patients with recurrent disease had concentrations of IL-8 that were consistently elevated compared to normal controls. 81% of patients with metastatic disease and 100% of patients with local/regional recurrent disease had serum IL-8 levels that exceeded the upper limit of IL-8 concentrations in our healthy control group. The results of the radiated group, whose IL-8 levels were similar to the control subjects, demonstrated that these elevations in serum IL-8 in the recurrent disease patients could not be explained by prior radiotherapy. However, the increase in serum IL-8 in recurrent disease patients may be explained by increased tumor production in recurrent disease, increased volume of disease, increased local inflammation, or more permeable stroma.

These initial survey results of serum IL-8 levels in head and neck patients foretell the potential benefit of measuring serum IL-8 levels in the clinical setting. Measurement of serum IL-8 at the time of diagnosis may screen out patients who are more likely to have aggressive or metastatic disease.

Given the elevation of serum IL-8 in patients with recurrent disease, longitudinal studies through the course of treatment may demonstrate that IL-8 has early predictive value for disease progression or regression. An increase in serum IL-8 in the years following curative treatment may also be indicative of the onset of recurrent disease.

The elevation of serum IL-8 levels in patients with recurrent disease also suggests that IL-8 plays a mechanistic role in the pathogenesis of head and neck cancer progression. Therapeutic intervention aimed at IL-8 might be of value. Kawano⁶ and colleagues conducted both in vivo and in vitro studies of the inhibitory effects of an IL-8 antibody and an angiogenesis inhibitor TNP470, a synthetic fumagillin, on a head and neck cancer cell line known to produce IL-8 (KB cell line). In their study, proliferation of KB cells in culture was inhibited by both IL-8 antibody and TNP470 individually and synergistically when combined. Combination IL-8 antibody and TNP470 reduced tumor volume considerably when administered intratumorally in mice.⁶ Clinical trials determining the impact of IL-8 antibody in human subjects with head and neck tumors have not been conducted.

Circulating serum levels of S-VEGF in head and neck cancers have been reported in a number of studies.¹⁴ Shang¹⁵ and colleagues reported in a study looking specifically at serum S-VEGF levels in patients with oral squamous cell carcinomas that patients with oral squamous cell cancers (9 Stage I/II, 22 Stage III/IV, no metastatic disease) had a mean serum S-VEGF level that was significantly elevated compared to 10 healthy controls. Additionally, the study found that mean S-VEGF concentrations were directly correlated to disease stage and presence of nodal metastasis.¹⁵ The study did not report the number or percentage of head and neck patients in each category that exceeded the upper limit of serum S-VEGF of the normal controls. Therefore, it is not possible to conclude that serum S-VEGF levels are consistently elevated in patients with oral squamous cell carcinomas compared to normal controls based on the results presented.

The results of the present study with respect to circulating levels of VEGF were inconsistent and inconclusive. The variance in results from the same patient's sera could be explained by factors related to serum collection and the thawing process. Jelkmann¹⁶ described one of the potential pitfalls associated with measuring circulating levels of VEGF being that platelets and leukocytes release VEGF during the clotting process. Collection of sera intrinsically requires blood clotting and therefore VEGF levels in sera will measure both the VEGF

found in the systemic circulation and that released by platelets and leukocytes during clotting. Studies have also shown that VEGF levels in serum fluctuate with the duration of clotting and temperature.¹⁶ A better method of measuring systemic circulating levels of VEGF would be to measure plasma levels.

Serum EGFR concentrations were also studied. Based on the current results in this report, there does not appear to be any difference in circulating serum levels of EGFR between healthy controls and each of the patient groups. While the activation of EGFR on the cell surface of head and neck tumors has been demonstrated to lead to tumor growth, EGFR is not necessarily expressed in supernormal levels or shed into the systemic circulation of patients with SCCHN.

In conclusion, this study represents a pilot study of IL-8, VEGF, and EGFR in the sera of patients with SCCHN. Serum IL-8 concentrations were consistently elevated in patients with local/regional recurrent or metastatic disease and a small fraction of patients with newly diagnosed, localized disease compared to controls. IL-8 may therefore be considered a potential prognostic marker in SCCHN. Determination of the mechanisms leading to increased IL-8 secretion in recurrent and advanced disease might lead to new therapeutic interventions or monitoring. In contrast, serum levels of S-VEGF and EGFR were not demonstrated to be elevated in our patient populations regardless of stage or aggressiveness of disease. Larger patient populations with broader representation of disease sites within the oral mucosa and stage, as well as prospective studies of selected treated patient populations, will be needed to confirm the results of this study with respect to IL-8.

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