ORIGINAL ARTICLE

ABERRANT CYTOKINE EXPRESSION IN SERUM OF PATIENTS WITH ADENOID CYSTIC CARCINOMA AND SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Abstract: *Background.* Squamous cell carcinoma (SCC) and adenoid cystic carcinoma (ACC) represent 2 clinically important subtypes of head and neck cancer. Our objective was to characterize and compare cytokine profiles in the systemic circulation of patients with SCC and ACC.

Methods. Multiplex analysis of 10 different cytokines (interleukin [IL]-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, granulocyte-macrophage colony-stimulating factor [GM-CSF], interferon [IFN]-γ, and tumor necrosis factor [TNF]-α) in the serum of patients with SCC ($n = 20$) and ACC ($n = 20$) and healthy controls ($n = 20$) was performed using the Luminex fluorescent-bead technology.

Results. Patients with SCC as well as patients with ACC showed an altered cytokine profile compared with healthy individuals. In patients with SCC, significantly elevated serum levels of the proinflammatory cytokines, IL-6 and IL-8, were observed. In patients with ACC, IL-8 serum levels were significantly elevated, and IL-6 serum levels were only increased in a subset of patients.

Conclusions. A similar serum cytokine profile, with the predominance of proinflammatory cytokines, was observed in patients with SCC and ACC. The newly defined cytokine profile in ACC patients may form the basis for future investigations to explore the role of cytokines in ACC tumor progression and their potential value as predictive biomarkers. *Head Neck* **29:** 472–478, 2007

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Head and neck carcinomas are among the most common types of human cancer, with an annual incidence of more than 500,000 cases worldwide.[1] Head and neck carcinomas include tumors with different histologic phenotypes and distinct clinical characteristics. Squamous cell carcinomas (SCC) of the upper aerodigestive tract mucosa represent the most common histologic subtype of head and neck cancer.[1,2] Adenoid cystic carcinoma (ACC) is a rare malignant epithelial tumor arising from salivary glands.[3–5] In the last decade, there has been only a limited improvement in the survival of patients with head and neck cancer, despite advances in surgery, chemotherapy, and

radiation therapy.[3,6,7] In several studies, we and others have identified and characterized a profound immune suppression in patients with SCC.[8,9] However, it is unknown whether ACC patients have similar alterations in immune responses. Furthermore, no biomarkers allowing for prediction of disease progression, recurrence, or therapeutic responses have been described for ACC.

Cytokines are soluble, low-molecular-weight proteins secreted by immune cells, which mediate immune and inflammatory responses. Various tumor types have been shown to produce cytokines, which often act in an autocrine fashion and are thought to alter host inflammatory, angiogenic, and immune processes for the benefit of the tumor.[10,11] Head and neck SCC cell lines in vitro as well as SCC tumor tissues in vivo express a number of cytokines, including interleukin (IL)-1α,[10] IL-6,[12] IL-8[13–15], and granulocyte-macrophage colony-stimulating factor (GM-CSF).[10,16] Moreover, several cytokines have been detected in higher concentrations in the serum and saliva of SCC patients, suggesting that these proteins may serve as biomarkers in this disease.[10,17–19] In contrast to head and neck SCC, the cytokine production of ACC cells and the serum cytokine profiles of patients with ACC have not yet been characterized.

In the present study, serum cytokine concentrations of patients with head and neck SCC as well as patients with ACC were measured using the Luminex fluorescent-bead technology. In both patient groups, aberrant serum cytokine profiles with the predominance of proinflammatory cytokines were observed, suggesting that cytokines might serve as potential biomarkers and may contribute to tumor progression in both head and neck cancer entities.

MATERIALS AND METHODS

Study Population. Peripheral blood samples were collected from white patients with SCC, ($n = 20$) and ACC, ($n = 13$), as well as from age-matched normal controls ($n = 20$) at the Department of Otorhinolaryngology, University Hospital Düsseldorf, Germany. Moreover, peripheral blood samples from white patients with ACC ($n = 7$) were collected at the Department of Otorhinolaryngology, University of Pittsburgh Medical Center, PA. The diagnosis was confirmed clinically and histopathologically for each patient, and clinical as well as pathologic T status and nodal status data were collected. At the time of blood sample collection, all patients had active disease, before any oncologic therapy. Patients did not have underlying significant morbidity or active medical problems such as congestive heart failure, active infection, systemic inflammatory or autoimmune disease, hepatitis or human immunodeficiency virus infection, or abnormal renal function. Healthy controls did not have a history of chronic systemic disease or malignancies and were not on medications. All patients were followed routinely after treatment. The study was approved by the local ethic committees, and informed consent was secured from each patient prior to sample acquisition. Serum from whole blood samples was obtained by centrifugation and stored at -80°C until analyzed. Serum aliquots were assayed blind to diagnosis.

Cytokine Analysis. The levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were measured in the serum samples using immunobead-based multiplex assays. Panels of capture antibody-coated beads and labeled detection antibodies were purchased from Biosource, Camarillo, CA. The reagents were pre-tested and qualified by the manufacturer to ensure the absence of cross-reactivity among antibody-coated beads. The assay sensitivity varied from 5 to 15 pg/mL, depending on the analyte. Comparisons between the ten-plex assay and enzyme-linked immunosorbent assays (ELISAs) for the individual cytokines were performed by the laboratory to confirm specificity and were found to be satisfactory. The assays were performed in the Immunologic Monitoring Laboratory at the University of Pittsburgh Cancer Institute using the Bio-Plex System (the instrument and software for data analysis) purchased from Bio-Rad, Hercules, CA.

Statistical Analysis. The Kruskal-Wallis test was used to compare the age of the study groups. Serum levels of individual cytokines between groups were compared by the nonparametric Mann-Whitney U test. Correlations between individual cytokine levels as well as between cytokine levels and the time to recurrence were analyzed by the nonparametric Spearman's correlation test. To assess the influence of cytokine levels on the time to recurrence, logistic regression models were fitted to estimate the probability of having an early recurrence, as a function of serum cytokine levels. We used a binary outcome of early recurrence (within 36 months after therapy in ACC patients, and within 48 months after therapy in SCC patients) or late/no recurrence (later than 36 months after

Table 1. Demographics of study subjects.

Group	No. of patients	Age, y Median (range)	Sex		T status		N status	
			Female	Male	T1–T2	T3–T4	N0	N1–N3
ACC*	20	65 (32–88)	11	9	8	10	16	2
SCC	20	64 (36–84)	4	16	13	7	12	8
Normal controls	20	60 (30–82)	4	16	NA	NA	NA	NA

Abbreviations: ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma; NA, not applicable.
**For 2 of 20 patients with ACC, T and N classification was not available.*

therapy, or no recurrence in ACC patients, and later than 48 months after therapy, or no recurrence in SCC patients). Statistical analyses were performed using the SPSS 12.0 software. A value of $p < .05$ was considered to be statistically significant.

RESULTS

Serum concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, and TNF-α were measured by Luminex fluorescent-bead technology in the serum of patients with ACC ($n = 20$), patients with SCC ($n = 20$), and normal controls ($n = 20$). Demographics of the study subjects are shown in Table 1. Among the 3 subject groups, there were no significant age differences (Table 1 and Figure 1).

Cytokine levels measured in the serum are displayed in Table 2. Multiplex analysis of serum cytokine levels demonstrated that both ACC and SCC patients showed altered cytokine profiles when compared with those of healthy individuals, with a significant increase in levels of proinflammatory cytokines. T helper 1 (Th1)-type (IFN-γ) and T helper 2 (Th2)-type (IL-4, IL-5 and IL-10) cytokines, as well as a hematopoiesis-regulating cytokine, GM-CSF, were either detected at a very low level or were below the detection limit in normal controls as well as ACC and SCC patients. IL-2 was present at variable levels in the serum of head and neck cancer patients as well as healthy controls.

Among the proinflammatory cytokines, higher levels of IL-6 and IL-8 but not of IL-1β or TNF-α were detected in the sera of SCC patients as compared with normal controls (Table 2). Comparisons of the individual cytokine levels between groups revealed that the serum levels of IL-6 were significantly elevated in SCC patients as compared with healthy controls ($p < .05$; Table 2 and Figure 2A). IL-8, a chemokine that has been associated with different steps of tumor progression, was either undetectable or detected at a low level in the serum of healthy controls (Table 2 and Figure 2B). However, in the serum of SCC patients, IL-8 levels were significantly elevated ($p < .05$; Table 2 and Figure 2B), in accordance with previous studies.[17,20,21] Interestingly, in SCC patients, a positive correlation was observed between the serum levels of IL-6 and IL-8 ($r = .51$, $p < .05$, Spearman's correlation).

Elevated IL-6 levels could also be observed in a subset of patients with ACC (Table 2 and Figure 2A), although the difference in IL-6 levels between ACC patients and controls was not statistically significant. Moreover, a significant increase of serum IL-8 levels could also be observed in patients with ACC ($p < .01$; Table 2 and Figure 2B). Notably, in 6 of 20 ACC patients, IL-8 levels exceeded the highest level observed in the SCC group (9.33 pg/mL, Table 2). In ACC patients, no statistically significant correlation could be detected between the levels of IL-6 and IL-8. Although TNF-α serum levels in ACC patients did not differ significantly from those measured in healthy controls and SCC patients on average, a subset of ACC patients had high levels of TNF-α (Table 2). Interestingly, patients with high TNF-α levels also had high levels of IL-6 and/or IL-8 in the serum, and statistical analysis revealed a positive correlation between

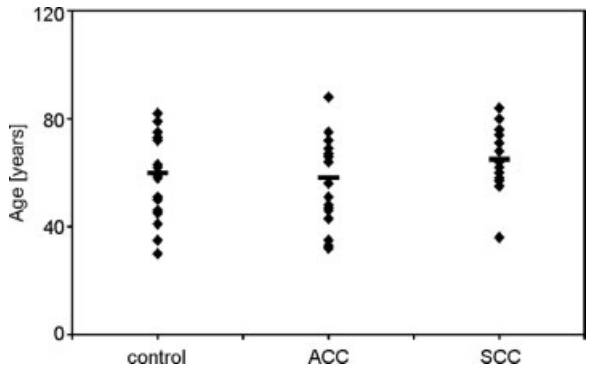

FIGURE 1. Age distribution of the study groups consisting of healthy controls ($n = 20$), patients with adenoid cystic carcinoma (ACC, $n = 20$), and patients with squamous cell carcinoma (SCC, $n = 20$).

Table 2. Serum cytokine levels in head and neck cancer patients and in healthy controls.

Cytokine	Mean ± SE, pg/mL	Range, pg/mL
IL-1β		
Normal controls	7.3 ± 3.6	0.0–52.5
ACC	1.0 ± 1.0	0.0–20.5
SCC	38.6 ± 36.9	0.0–739.0
IL-2		
Normal controls	50.7 ± 14.4	0.0–167.8
ACC	39.2 ± 15.7	0.0–207.9
SCC	49.5 ± 16.0	0.0–188.6
IL-4		
Normal controls	0.0 ± 0.0	0.0–0.0
ACC	0.0 ± 0.0	0.0–0.0
SCC	0.0 ± 0.0	0.0–0.0
IL-5		
Normal controls	0.0 ± 0.0	0.0–0.0
ACC	0.0 ± 0.0	0.0–0.0
SCC	0.0 ± 0.0	0.0–0.0
IL-6		
Normal controls	1.0 ± 0.5	0.0–6.2
ACC	3.3 ± 1.4	0.0–24.0
SCC	3.8 ± 1.2*	0.0–19.9
IL-8		
Normal controls	0.5 ± 0.3	0.0–5.6
ACC	19.3 ± 8.4†	0.0–120.7
SCC	2.5 ± 0.8*	0.0–9.33
IL-10		
Normal controls	0.0 ± 0.0	0.0–0.0
ACC	0.0 ± 0.0	0.0–0.0
SCC	0.0 ± 0.0	0.0–0.0
GM-CSF		
Normal controls	3.9 ± 2.7	0.0–43.3
ACC	0.0 ± 0.0	0.0–0.0
SCC	0.0 ± 0.0	0.0–0.0
IFN-γ		
Normal controls	0.0 ± 0.0	0.0–0.0
ACC	1.5 ± 1.5	0.0–30.8
SCC	0.4 ± 0.4	0.0–7.86
TNF-α		
Normal controls	5.8 ± 5.8	0.0–115.8
ACC	29.4 ± 27.0	0.0–541.2
SCC	0.7 ± 0.7	0.0–13.5

Abbreviations: SE, standard error of the mean; ACC, adenoid cystic carcinoma; SCC, squamous cell carcinoma; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; TNF, tumor necrosis factor.
**$p < .05$ compared with normal controls (Mann–Whitney U test).*
†$p < .01$ compared with normal controls (Mann–Whitney U test).

the levels of TNF-α and IL-6 ($r = .47$, $p < .05$; Spearman's correlation) as well as TNF-α and IL-8 ($r = .56$, $p < .05$; Spearman's correlation).

To analyze the possible association between serum cytokine levels and disease stage, cytokine levels were compared in patients with different pathologic T and N status. Analysis of IL-6 and IL-8 levels in SCC patients with different T classifications demonstrated that both IL-6 and IL-8 levels were higher in patients with higher T status (IL-6: 2.7 ± 4.7 pg/mL in patients with T1/T2 classification, 7.6 ± 7.6 pg/mL in patients with T3/T4 classification; and IL-8: 1.8 ± 2.7 pg/mL in patients with T1/T2 classification, 3.3 ± 3.9 pg/mL in patients with T3/T4 classification), although statistical analysis did not indicate significant differences between these groups. Neither IL-6 nor IL-8 showed a significant association with N classification in SCC patients.

Interestingly, in ACC patients, IL-8 levels were higher in patients with T1/2 classification (40.4 ± 54.0 pg/mL) than in those with T3/4 classification (6.4 ± 8.1 pg/mL), although this difference was not statistically significant. Serum levels of IL-6 did not show any difference by T classification in ACC patients.

Next, we analyzed the association of cytokine levels and time to recurrence in those cases in which recurrence was observed during the follow-up time. No significant correlation was observed between the levels of IL-6 or IL-8 and the time to recurrence in SCC or ACC patients.

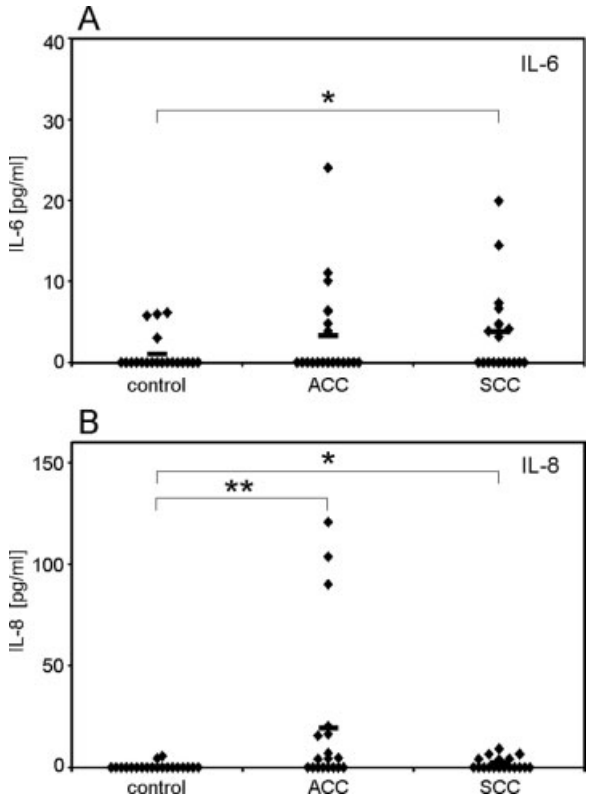

FIGURE 2. Serum levels of interleukin (IL)-6 (**A**) and IL-8 (**B**) in healthy controls ($n = 20$) as well as in patients with adenoid cystic carcinoma (ACC, $n = 20$) and squamous cell carcinoma (SCC, $n = 20$), determined by Luminex fluorescent-bead technology. Serum cytokine levels in individual patients as well as means are shown. *$p < .05$, **$p < .01$, Mann–Whitney U test.

To estimate the probability of developing early recurrence depending on the cytokine serum levels, we used logistic regression models. No significant influence of IL-6 or IL-8 or the combination of these 2 cytokines on the time to recurrence in ACC or SCC patients could be found.

DISCUSSION

In head and neck cancer, altered immune, inflammatory, and angiogenesis responses are observed, and many of these responses have been associated with a poor clinical outcome.[8,10] The local microenvironment of these tumors is rich in immune cells, such as T lymphocytes, dendritic cells, B cells, natural killer (NK) cells, macrophages, eosinophils, and the soluble factors these cells produce. These factors, including inflammatory cytokines and chemokines, are likely to have an effect on the promotion/progression of the tumors arising in this microenvironment.[22] In addition to the soluble factors produced by nonmalignant cells in the microenvironment, tumor cells also produce a number of growth factors and cytokines.[15,23]

Here, we show that ACC as well as SCC patients show an altered serum cytokine profile compared with healthy controls. Although these 2 tumor types arise from different cell types, ACC and SCC patients showed a similar, predominantly proinflammatory cytokine profile. Elevated levels of serum IL-6 and IL-8 were detected in both patient groups as compared with control subjects. Moreover, a positive correlation was observed between the serum levels of the proinflammatory cytokines IL-6 and IL-8 in SCC patients, and between TNF-α and IL-6 as well as TNF-α and IL-8 serum levels in ACC patients, suggesting that these cytokines are part of an inflammatory profile reflecting the immune dysregulation in head and neck cancer patients.

IL-8/*CXCL8*, a proinflammatory chemokine and a potent chemotactic factor for neutrophils, has been shown to be upregulated in several human malignancies.[24–26] In general, IL-8 production is linked with tumor vascularization, metastatic phenotype, tumor growth, and overall poor prognosis.[24,27–30] In head and neck SCC, tumor cells have been shown to express both IL-8 and its receptors, IL8RA/*CXCR1* and IL-8RB/*CXCR2*,[15,20,31,32] and it has been proposed that IL-8 may act both in a paracrine and autocrine manner. IL-8, interacting with its receptor on SCC cells, can induce tumor cell migration and invasion,[15] and through its receptors on endothelial cells, it contributes to angiogenesis.[33] Our results showing elevated IL-8 levels in SCC patients are in accordance with previous studies demonstrating elevated serum concentrations of IL-8 in head and neck SCC patients.[10,17,21] Here we show for the first time that serum levels of IL-8 are significantly elevated in ACC patients, suggesting that this chemokine may play an important role in the pathogenesis of ACC. Further studies involving in situ detection of cytokines in ACC tumors are needed to determine whether tumor cells or cells in the microenvironment produce this chemokine, whether IL-8 may serve as a biomarker in ACC patients, and what role it plays in ACC tumor progression. Cell lines derived from ACC tumors do not express the IL-8 receptors, IL8RA/*CXCR1* and IL-8RB/*CXCR2,*[32] suggesting that IL-8 may play a role in angiogenesis rather than tumor invasion and migration in ACC patients.

Serum levels of the proinflammatory cytokine IL-6 were significantly elevated in SCC patients and showed elevated levels also in a subset of ACC patients. IL-6 is produced by T cells, monocytes, and fibroblasts, but it has also been shown to be secreted by tumor cells and act as an autocrine growth factor for various carcinomas.[34–36] Head and neck SCC cells have been shown to express both IL-6 and its receptors.[12,37] Our results showing elevated serum levels of IL-6 in SCC patients are in accordance with previous studies.[38–40] Current evidence suggests that IL-6 can promote tumor cell proliferation in several tumor cell lines including head and neck SCC,[12,34] and patients with higher IL-6 serum levels have been shown in patients with more advanced tumor.[38] However, high IL-6 transcript levels in oral SCC have been associated with better survival,[41] indicating that the association of IL-6 and SCC tumor progression is not straightforward. In our study, IL-6 serum levels were also elevated in a subset of ACC patients. Although there was no correlation between IL-6 levels and T or N classification, there might be other, yet unexplored factors associated with elevated IL-6 levels in this subgroup. In ACC patients, IL-6 is likely to be produced by tumor-surrounding cells in an environment similar to that of SCC; however, it is also conceivable that tumor cells may produce this cytokine. The cellular source and the factors associated with elevated IL-6 levels in ACC patients as well as the presence of the IL-6 receptor on ACC cells enabling this cytokine to act as paracrine or autocrine factor on tumor cells, still needs to be determined.

In a subset of ACC patients with high serum levels of IL-6 and IL-8, high levels of TNF-α were

also detected. However, on average, the serum level of this cytokine in ACC patients did not differ significantly from healthy controls. Further studies are required to identify the cell types producing TNF-α in ACC patients and its biological role in ACC tumor progression.

The observation that only a subset of head and neck cancer patients had high levels of inflammatory cytokines suggests that serum levels of inflammatory cytokines are associated with disease-associated factors such as disease stage. The presence of proinflammatory cytokines and inflammation in the microenvironment of the tumor has been shown to promote tumor growth and invasion,[22] suggesting that patients with high levels of inflammatory mediators may represent a subgroup with more advanced disease or worse prognosis. Our results showing a positive association of IL-6 and IL-8 serum levels with T classification, although not significant, indeed suggest that the serum levels of these cytokines are associated with disease status in SCC patients. In accordance with our results, previous studies have demonstrated altered cytokine profiles in the peripheral circulation of SCC patients[17,19,21] and suggested that cytokines might be used as biomarkers for disease status or therapy response. Interestingly, in ACC patients, high IL-8 levels were observed in patients with lower T classifications, a pattern distinct from that observed in SCC patients. This inverse relationship between IL-8 levels and T status suggests that IL-8 may be produced at higher levels at early stages of tumorigenesis and that IL-8 serum levels may be useful as biomarkers for early-stage ACC rather than for advanced disease.

No significant association was observed between serum cytokine levels and the time to recurrence; hence, our results do not support the utility of serum cytokines for prediction of early recurrence. However, these observations need to be confirmed on larger patient cohorts.

Further studies on larger patient populations will also be needed to evaluate the association of serum cytokine levels with disease stage and the potential use of these cytokines as biomarkers in different types of head and neck cancer. It also has to be noted that serum cytokine profiles might be influenced by inflammatory processes independent of head and neck cancer; therefore it might be necessary to combine serum cytokine profiles with other markers independent of inflammation, for more sensitive and specific prediction of recurrence or response to therapy.

Collectively, our analysis indicates that 2 different subtypes of head and neck cancer, ACC and SCC, show an altered serum cytokine profile as compared with healthy subjects. Both tumor types were characterized by a predominant elevation of proinflammatory cytokines. This study is the first to characterize the serum cytokine profile in ACC, which may be the basis of future investigations to explore the role of cytokines in ACC tumor progression. Future studies clarifying the role of cytokines and chemokines secreted by tumor cells and surrounding immune cells and the effect of these mediators on local immune response may lead to novel therapeutic approaches in head and neck cancer and may identify potential predictive biomarkers.

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