Early Detection of Head and Neck Cancer: Development of a Novel Screening Tool Using Multiplexed Immunobead-Based Biomarker Profiling

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Abstract

Squamous cell carcinoma of the head and neck (SCCHN) is an aggressive disease that has been linked to altered immune, inflammatory, and angiogenesis responses. A better understanding of these aberrant responses might improve early detection and prognosis of SCCHN and provide novel therapeutic targets. Previous studies examined the role of multiplexed serum biomarkers in small cohorts or SCCHN sera. We hypothesized that an expanded panel comprised of multiple cytokines, chemokines, growth factors, and other tumor markers, which individually may show some promising correlation with disease status, might provide higher diagnostic power if used in combination. Thus, we evaluated a novel multianalyte LabMAP profiling technology that allows simultaneous measurement of multiple serum biomarkers. Concentrations of 60 cytokines, growth factors, and tumor antigens were measured in the sera of 116 SCCHN patients before treatment (active disease group), 103 patients who were successfully treated (no evidence of disease group), and 117 smoker controls without evidence of cancer. The multimarker panel offering the highest diagnostic power was comprised of 25 biomarkers, including epidermal growth factor, epidermal growth factor receptor, interleukin (IL)-8, tissue plasminogen activator inhibitor-1, a-fetoprotein, matrix metalloproteinase-2, matrix metalloproteinase-3, IFN-α, IFN-γ, IFN-inducible protein-10, regulated on activation, normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein-1α, IL-7, IL-17, IL-1 receptor-α, IL-2 receptor, granulocyte colony-stimulating factor, mesothelin, insulin-like growth factor binding protein 1, E-selectin, cytokeratin-19, vascular cell adhesion molecule, and cancer antigen-125. Statistical analysis using an ADE algorithm resulted in a sensitivity of 84.5%, specificity of 98%, and 92% of patients in the active disease group correctly classified from a cross-validation serum set. The data presented show that simultaneous testing using a multiplexed panel of serum biomarkers may present a promising new approach for the early detection of head and neck cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(1):102–7)

Introduction

Squamous cell carcinoma of the head and neck (SCCHN) occurs in 50,000 new cases annually in the United States, resulting in >13,000 deaths each year (1). Epidemiologic data suggest that the most important risk factors for the development of SCCHN are tobacco and alcohol consumption (1); however, increasing evidence suggests that viruses (EBV and human papillomavirus) also play an important part in the development of SCCHN subsets (2, 3). No effective screening procedure is available to diagnose SCCHN at the earliest stages when it is still highly curable (4). In particular, because no established biomarker exists for SCCHN, tumor surveillance after treatment of the initial primary malignancy is often ineffective at identifying recurrent disease. High-risk individuals, including chronic smokers and those previously treated for SCCHN, would benefit greatly from such a test (4). In particular, the latter group is at high risk for recurrent disease (30-40% relapse rates within 2-3 years after treatment) as well as for second primary cancers of the upper aerodigestive tract, which occur at rates of 3% to 4% annually for life (5).

In previous studies, individual serologic inflammatory, angiogenesis, and tumor growth factors have been correlated with disease status (6-11). Indeed, despite the growing number of published research indicating the complex interactions between these inflammatory and angiogenic biomarkers in the development of SCCHN, existing research studies have nearly exclusively concentrated on one or a few markers in studies comprising relatively small sample sizes, and thus with limited generalized applicability to a heterogeneous SCCHN population. Additionally, existing studies rarely investigated the roles of less common biomarkers such as hormones, proteases, and adhesion molecules. Whereas we have previously published that a 10-plex panel of cytokines and chemokines had reasonable (~85%) diagnostic accuracy in a small cohort of SCCHN patients (8), we hypothesized that a broader panel of analytes and a much larger set of specimens would lend greater clinical usefulness. Thus, we used a novel multianalyte LabMAP profiling technology (Luminex Corp., Austin, TX), which allows simultaneous measurement of multiple biomarkers in serum of SCCHN patients and controls (12). Our work related to chemokines macrophage inflammatory protein (MIP)-3β and secondary lymphoid-tissue chemokine and chemokine receptor 7–related tumor metastasis and survival highlights the significance of these studies (13, 14). SCCHN populations and others at risk for development of this...
Patients and Methods

Patient Population. The study population comprised patients treated at the Division of Head and Neck Surgery, University of Pittsburgh Department of Otolaryngology. The SCCHN groups included carcinomas of the oral cavity, pharynx, and larynx. Because squamous cell carcinoma accounts for the majority of head and neck cancers, tumors of other histopathologic types, such as salivary and thyroid cancers, were not included in this study. Data were obtained from serum samples of 116 samples from patients with active, untreated SCCHN; 103 samples from patients with successfully treated tumors with no evidence of disease (NED) for at least 3 years; and 117 samples from age-, sex-, and smoking status–matched controls. The demographics of the study populations are shown in Table 1.

Chronic tobacco smokers without evidence of SCCHN were recruited from the same regional population, through the University of Pittsburgh Lung Cancer Specialized Program of Research Excellence, and were matched individually based on age and gender to the active SCCHN cases. The high-risk smoker population was chosen to eliminate confounding serum-proteomic patterns attributable to carcinogen exposure with a high causal relationship to SCCHN. De-identified serum samples from patients with head and neck cancer and control groups were obtained from the Division of Head and Neck Oncologic Surgery in the Department of Otolaryngology/Head and Neck Surgery. Written informed consent was obtained from each subject after approval by the University of Pittsburgh Institutional Review Board.

Collection and Storage of Blood Serum. Ten milliliters of peripheral blood were drawn from subjects using standardized phlebotomy procedures and allowed to clot. Handling and processing was similar for all three groups of patients. Samples were obtained from patients diagnosed with head and neck cancer before surgery and before administration of anesthesia. Blood samples were collected without anticoagulant into red top vacutainers and allowed to coagulate for 20 to 30 min at room temperature. Sera were separated by centrifugation and all specimens were immediately aliquoted, frozen, and stored in a dedicated –80°C freezer. No more than one freeze-thaw cycle was allowed for each sample.

Multiplex Serum Analysis. The LabMAP technology (Luminex) combines the principle of a sandwich immunoassay with the fluorescent-bead-based technology, allowing individual and multiplexed analysis of up to 100 different analytes in a single microtiter well. The LabMAP serum assays were done in 96-well microplate format according to the protocol by Biosource International (Camarillo, CA). A filter-bottom, 96-well microplate (Millipore, Billerica, MA) was blocked for 10 min with PBS/bovine serum albumin. The differentially dyed polystyrene microspheres served as a solid support for sandwich immunoassay. In this study, a panel of 60 serologic markers including cytokines, chemokines, growth and angiogenic factors, cancer antigen α-fetoprotein (AFP), vascular cell adhesion molecule (VCAM), and other markers was analyzed in sera of active SCCHN patients before treatment, SCCHN patients 3 years after successful treatment, and healthy controls with positive smoking status.

Detailed description of multiplexed analysis done by our group, along with the steps of development of LabMAP assays for cancer antigen (CA)-125, has been described in our previous publications. The biomarkers investigated in this study included cytokines and receptors, chemokines, growth factors, their receptors and binding proteins, proteases, cancer antigens, adhesion molecules, and other biomarkers. Table 1 provides a complete list of biomarkers analyzed in this study.

Sources of Bead-Based Immunoassays. xMAP bead-based technology (Luminex) allows for multiplexed analysis of several analytes in one sample. Forty-six bead-based xMAP immunoassays for most known ovarian cancer serum biomarkers were used in this study. Multiplexed bead-based immunoassays for cytokines were purchased from Biosource International; assays for matrix metalloproteinase (MMP)-2 and MMP-3 were from R&D Systems (Minneapolis, MN); multiple assays for soluble intercellular cell adhesion molecule (sICAM-1), soluble VCAM (sVCAM)-1, sE-selectin, tissue plasminogen activator inhibitor-1 (tPAI-1), myeloperoxidase (MPO), macrophage migration inhibitory factor (MIF), soluble Fas, and soluble Fas ligand (FasL) for prolactin, FSH, GH, TSH, LH, and ACTH were obtained from Linco Research (St. Charles, MO). Assays for CA-125, epidermal growth factor (EGF) receptor (EGFR), v-erb-b2 erythroblastic leukemia viral oncogene homologue 2 (ErbB2), carcinoembryonic antigen (CEA), CA 15-3, CA 19-9, insulin-like growth factor binding protein 1 (IGFBP-1), human kallikrein-8, Cyfra 21-1, and mesothelin; CA 72-4, AFP, human chorionic gonadotropin β, and human kallikrein-10; soluble mesothelin-related protein (SMRP); and human epididymis protein 4 (HE4) were developed in Luminex Core Facility of University of Pittsburgh Cancer Institute according to the protocol by Luminex. Antibody pairs for kallikreins were kindly provided by Dr. Diamandis; antibody pairs against HE4 and SMRP were a kind gift of Fujeribio Diagnostics (Malverna, PA). All antibodies are available for nominal purchase from Dr. Anna Lokshin. Overall, five different multiplexed panels and two individual bead-based assays (HE4 and SMRP) were used. The interassay variability within the replicates was in the range of 3.5% to 7%. Intraassay variability was between 11% and 15%. Each assay was further validated in comparison with appropriate ELISA and has shown 89% to 98% correlation. Recovery from serum was assessed and was determined to range from 80% to 110%.

Statistical Analysis of Data. Descriptive statistics and graphical displays (i.e., dot plots) for serum concentrations of 60 biomarkers for each disease state were calculated using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The Wilcoxon test was used (Fig. 1) as an initial screen for estimation of significance, and the median and Kruskal-Wallis tests were used with similar results. Spearman’s (nonparametric) rank correlation was also calculated to quantify the relationships between each pair of markers. Data sets consisting of 116 active SCCHN samples and 117 healthy smoker controls were randomly split into the training and test sets at

Table 1. Demographics of study subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>Males, n (%)</th>
<th>Median age (y)</th>
<th>Median age (y)</th>
<th>Range age (y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy smokers</td>
<td>117</td>
<td>79 (67.5)</td>
<td>60.0</td>
<td>58.5</td>
<td>37-86</td>
</tr>
<tr>
<td>Active SCCHN</td>
<td>116</td>
<td>79 (68.1)</td>
<td>59.9</td>
<td>58.5</td>
<td>37-86</td>
</tr>
<tr>
<td>NED &gt;3 y</td>
<td>103</td>
<td>79 (76.7)</td>
<td>62.6</td>
<td>64</td>
<td>39-82</td>
</tr>
</tbody>
</table>
55:45 ratio; then the training set was used to build the classification model and the test set was used to determine the total number of classification errors as well as the specificity/sensitivity. Because of the out-of-range values observed with markers IFN-γ and interleukin (IL)-17, the actual concentration status and their contribution to the classification results are doubtful. The described procedure was repeated 20 times for each algorithm and the average error rate and the cumulative specificity/sensitivity dependency were calculated. This set was used in a new series of 55:45 cross validation tests that were repeated 100 times for each subset of projections corresponding to the increasing number of markers.

The ADEPT (ADE+PT) Algorithm. A novel approach to the multivariate two-class events classification of sparse data in a multidimensional space has been developed based on classification in multiple k-dimensional projections with subsequent combining of the classification scores obtained from these projections to form a final classifier. The discrimination within a single k-dimensional projection was done using a kernel based probability density estimator with adoptive bandwidth (ADE) by creating a separate density probability estimations for both classification events and then generating the logit score reflecting the probability of a given data point to fall into one of the two event classes. The resulting score was obtained as a sum of scores over all selected projections. The optimal set of projections was obtained by using the projection pursuit technique (PT) applied to the simulation set, which was comprised of series of the training subsets created by repetitive random subsampling of the original data set and adding the white noise to each data point as well as a scale noise to the whole training subset in the form of linear transform with random coefficients to reflect the scale desynchronization between successive xMAP runs. The projections were chosen in such a way that when combined together they maximized the discrimination rate simultaneously for all training sets.

Results

LabMAP-Based Analysis of Serum Concentrations of Cytokines, Growth Factors, and Other Biomarkers in SCCHN Patients. As listed in Table 2, concentrations of 60 different serum markers belonging to different functional groups (i.e., cytokines/chemokines, growth factors, or tumor antigens) were evaluated in multiplexed assays using LabMAP technology. Using this approach, we interrogated serum samples from three separate clinical groups: active, previously untreated SCCHN patients (n = 116); patients who had undergone successful treatment for SCCHN (NED; n = 103); and age- and gender-matched, tobacco-smoking cancer-free controls (n = 117).

Serum concentrations of several biomarkers, IGFBP-1, EGFR, tPAI-1, hepatocyte growth factor (HGF), IL-2 receptor (IL-2R), MMP-3, MIP-3β, IL-6, IL-8, IL-1β, CEA, and ErbB2, were significantly different between the active SCCHN and...
Table 2. Biomarkers studied in the multiplex panels

<table>
<thead>
<tr>
<th>Biological groups</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines/chemokines</td>
<td>IL-6, IL-8, TNF-α, IL-12p40, IL-2R, IL-1β, IL-2, IL-4, IL-5, IFN-γ (IFN-γ), IL-13, IL-15, IFN-α, IL-1α, IL-7, IL-9, IL-11, IL-15, IL-17, IL-18, IL-19α, IL-22, TNF-R1, TNF-R2, EOTAXIN, MIP-1α, MIP-1β, IP-10, MIG, RANTES</td>
</tr>
<tr>
<td>Growth/angiogenic factors</td>
<td>EGF, VEGF, FGF-β, G-CSF, HGF, GM-CSF, ErbB2, EGFR, IGFBP-1</td>
</tr>
<tr>
<td>Proteins</td>
<td>Kallikrein-8, kallikrein-10, MMP-2, MMP-3</td>
</tr>
<tr>
<td>Cancer antigens</td>
<td>CA-153, CEA, CA-19-9, CA-125, AFP, CA-72-4</td>
</tr>
<tr>
<td>Adhesion molecules</td>
<td>sE-selectin, sVCAM, sICAM</td>
</tr>
<tr>
<td>Other markers</td>
<td>Cytokeratin-19, β-hCG, MPO, mesothelin IgY</td>
</tr>
</tbody>
</table>

NED groups. On the other hand, MMP-2, MPO, monokine induced by γ-interferon (MIG), fibroblast growth factor-β (FGF-β), tumor necrosis factor (TNF-α), TF receptor (TFN-R)-I, CA-125, sE-selectin, IL-1Rα, MIP-3α, granulocyte colony-stimulating factor (G-CSF), sCAM, vascular endothelial growth factor (VEGF), monocytectactic protein-1 (MCP-1), AFP, TNF-RII, IL-15, CA-19-9, IL-5, mesothelin IgY, IL-2, regulated on activation, normal T-cell expressed and secreted (RANTES), sVCAM, FasL, IL-13, etoaxin, IFN-γ, cytokertatin-19, MIP-1β, death receptor (DR)-5, CA-153, IL-7, IFN-α, Fas, IL-1α, granulocyte macrphage colony stimulating factor (GM-CSF), kallikrein-10, β human chorionic gonadotropin (β-hCG), MIF, IL-12p40, IFN-inducible protein-10 (IP-10), kallikrein-8, IL-10, and MIP-1α did not differ between the active SCCHN and NED groups. EGF, ErbB2, IL-6, AFP, MMP-3, IL-β, CEA, and IL-2R were lower in patients with active SCCHN as compared with the NED group. IL-8, IGFBP-1, HGF, and tPAI-1 were significantly higher in the active cancer group as compared with the NED patients.

Medians of 19 biomarkers were shown to be statistically different between the active SCCHN and healthy control groups: EGF, IL-8, EGFR, IGFBP-1, MPO, MMP-2, ErbB2, AFP, sVCAM, IL-1Rα, IL-2R, kallikrein-8, MMP-3, IL-6, MIG, RANTES, MIF, HGF, and tPAI-1. Median serum concentrations of EGF, IL-8, IGFBP-1, MPO, IL-2R, MIG, MIF, IL-1Rα, and HGF were higher in those with active SCCHN versus health control group. Median serum concentration of EGFR, MMP-2, ErbB2, IL-6, kallikrein-8, sVCAM, MMP-3, and tPAI-1 were lower in cancer group versus control group (P < 0.05-P < 0.0001). Measurable concentrations of the biomarkers IL-1α, MIP-3α, IL-4, sCAM, Fas, MCP-1, hCG, TNF-α, CA-125, IL-15, Fas-L, TNF-RII, cytokertatin-19, IL-5, CA-19-9, CEA, mesothelin IgY, G-CSF, IP-10, kallikrein-10, TNF-R1, IL-1β, EOTAXIN, IFN-α, IL-12p40, IL-17, MIP-1α, VEGF, DR5, IL-10, FGF-β, MIP-1β, CA-153, IFN-γ, IL-2, sE-selectin, GM-CSF, MIP-3β, and IL-7 did not differ between the cancer and control groups (data not shown).

**Statistical Analysis of Individual Serum Cytokines as Head and Neck Cancer Biomarkers.** As shown in Fig. 1, certain analytes were found to be significantly different between each of the clinical groups in the study. Based on our individual analyses, however, none of the single biomarkers used in this study had high enough sensitivity and specificity to be used alone as a screening test for SCCHN. Thus, we sought to combine the biomarkers using recursive partitioning for discriminative, predictive power in a cross-validation set of serum samples.

**Multiplexed Analysis of Serum Biomarkers for Predictive Profiles of Head and Neck Cancer Biomarkers.** To identify a multimarker combination with the best performance for discrimination of patients with SCCHN from healthy smokers, the ADEPT algorithm was applied as described above. The results for sensitivity at 98% specificity, the total number of correctly identified samples, as well as variation of these variables among the 200 subsets, are presented in Fig. 2 A and B. As shown in Fig. 2, the efficiency of classification reaches its near maximum at about 23 biomarkers.

The multimarker panel offering the highest diagnostic power was comprised of 25 biomarkers including EGF, EGFR, IL-8, tPAI-1, AFP, MMP-2, IFN-γ, IL-1Rα, mesothelin IgY, MMP-3, IP-10, RANTES, MIP-1α, IL-7, IGFBP-1, sE-selectin, G-CSF, cytokertatin 19, sVCAM, IL-2R, IFN-α, and CA-125. The ADE algorithm used for our statistical analysis offered 84.5% sensitivity at 98% specificity with 92% patients correctly classified as shown by receiver-operator characteristic curve presented in Fig. 2. Analysis of correlations between individual markers of the entire panel of 60 biomarkers using Spearman rank correlation method revealed that some markers were significantly correlated to each other. In the active SCCHN group, the strongest correlations of 0.6 to 0.7 were found between etoaxin and MCP-1, IL-5 and DR5, and IL-1β and IL-15. In the NED group, there were correlations between IL-2R and MIG, IL-1Rα and MIP-1α, and IL-7 and IL-1β. These

**Figure 2.** Statistical analysis of predictive capability of serum biomarker profiles. For each number of markers, the random split cross-validation test was run 100 times and then the upper 2.5%, upper 25%, median, and corresponding lower 25% and 2.5% population boundaries were found, both for sensitivity at 98% specificity (A) and for the average percentage of correctly identified samples (B). C, receiver-operator characteristic (ROC) curve.
results show the ability to discriminate clinically distinct subgroups of sera from a cross-validation set with a highly accurate algorithm.

Discussion

Head and neck cancer can be treated effectively with high cure rates if detected at an early stage; but, unfortunately, these patients are usually discovered at a point of advanced disease. Our group has previously shown that multiplexed analysis of a cytokine biomarker panel could be used for the development of a screening test for SCCHN (8). In this expanded study, we used LabMAP technology to show its ability to discriminate SCCHN patients from control populations, analyzing 60 cytokines, chemokines, growth and angiogenic factors, cancer antigen AFP, adhesion molecule VCAM, and other markers in the sera of patients diagnosed with SCCHN before surgery. These data were compared with serum from patients successfully treated for SCCHN 3 years previously and also with a noncancer group of smoker controls (free of upper aerodigestive tract malignancy at the time of blood draw). Our highly accurate, discriminatory algorithm holds promise for application to screening those individuals at high risk for development of recurrent or second primary malignancy of the head and neck.

We were reassured that the results of our study supported previous research suggesting that several biomarkers, individually, including EGF, EGFR, MMP-3 (15), and others may play an important role in the early detection of SCCHN. However, no single analyte proved capable of sufficient classification accuracy to permit its widespread use for association with SCCHN. After performing a multimarker head-based immunoassay screening, we confirmed that the following biomarkers are expressed differently in SCCHN patients versus healthy smoker controls: elevated EGF (16) and EGFR (17), elevated AFP, elevated MMP-2 (18), and IL-1Rα (19). To our knowledge, we were the first group to report that sVCAM has prognostic value for SCCHN. Although statistical analysis indicated that the correlation of each of the above markers with head and neck cancer was modestly present when evaluated alone, a combined biomarker panel showed very strong association with malignant disease and suggested potential usefulness for early diagnosis of head and neck cancer.

Despite the potential application of our findings related to multiplexed analysis of serum biomarkers, it is essential that our data corroborate other published reports. Indeed, individual cytokines, inflammatory, and angiogenic factor tumor markers have been correlated with disease status, including IL-1, IL-6, IL-8, GM-CSF, and VEGF; growth-regulated oncogene 1 (GRO-1); and HGF (20-24). Individually, IL-1 and IL-6 were found to promote survival and proliferation of SCC cells, and IL-8, GRO-1, VEGF, and HGF have been shown to contribute to angiogenesis, tumorigenesis, and metastasis. Furthermore, median serum concentrations of IL-6, IL-8, HGF, VEGF, and GRO-1 were found to be increased in patients with SCCHN (6). 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 (7, 8).

Future studies in this area should concentrate on examining the longitudinal changes in serum concentrations of these biomarkers and investigating their associations with treatment response, relapse, complications, and survival. There need to be more studies examining the roles of biomarkers in smoking versus nonsmoking SCCHN patients. We also believe that integration of such a promising serum-based profile into currently useful screening techniques is essential to validating a potential role. These studies must weigh whether any incremental predictive power is found over current radio-

graphic and clinical screening practices in high-risk individuals. The inflammatory cytokine IL-6 has been linked to poor health outcomes in older adults (25), supporting the idea that systemic chronic inflammation is related to all-cause mortality risk in older persons. Further exploration of biomarkers implicated in the development of SCCHN has a great potential to improve treatment modalities not only for cancer patients but also for all patients presenting other conditions. Biomarkers such as EGF, EGFR, IL-8, TpAI-1, AFP, MMP-2, and others identified in our panel merit further investigation as markers that could be used for the further exploration of the screening test for head and neck cancer.

Increasing our understanding of the role of biomarkers in the etiology and the course of SCCHN has a great potential in facilitating the development of new treatment modalities for this challenging disease. In conclusion, we show here that analysis of multiple serum biomarkers using a novel LabMAP technology is a promising approach for the development of a diagnostic assay for patients with SCCHN or those who are at risk for development of this disease.

In this study, it was interesting to observe that several markers were correlated with each other in the active SCCHN group. These correlations were observed between eotaxin and MCP-1, IL-5 and DR5, and IL-1β and IL-15 in cancer group. Similarly correlations between some of the biomarkers were also discovered in the NED group. Several of the markers we have studies were described in the literature as useful for the identification of several types of cancers, including lung cancer and breast cancer. However, their role in the development of head and neck cancer has rarely been described in the literature, and further work must be done in vitro using human specimens to validate the significance of our findings.

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References

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