Immunohistochemical Analysis of Phosphotyrosine Signal Transducer and Activator of Transcription 3 and Epidermal Growth Factor Receptor Autocrine Signaling Pathways in Head and Neck Cancers and Metastatic Lymph Nodes

Raja R. Seethala,1,2 William E. Gooding,3 Phoebe N. Handler,1 Bobby Collins,5 Qing Zhang,1,2 Jill M. Siegfried,4 and Jennifer R. Grandis1,2

Abstract

Purpose: To determine the effect of tyrosine-phosphorylated signal transducer and activator of transcription 3 (pSTAT3) immunoexpression on survival in two independent cohorts of patients with squamous cell carcinoma of the head and neck (SCCHN) and to evaluate pSTAT3, transforming growth factor-α (TGF-α), epidermal growth factor receptor (EGFR), and gastrin-releasing peptide receptor (GRPR) expression in matched tumor and lymph node metastases in one of these cohorts.

Experimental Technique: Immunostaining for pSTAT3, TGF-α, EGFR, and GRPR was done in two SCCHN cohorts (cohort 1, 61 tumors; cohort 2, 69 paired primary tumors and lymph node metastases). Semiquantitative scores derived from the product of staining intensity (scale 0-3) score and percentage of positive tumor cells were correlated with clinical outcome.

Results: Immunoeexpression of pSTAT3 did not correlate with clinical outcome in either cohort (cohort 1, P = 0.914; cohort 2, P = 0.312). In cohort 2, TGF-α and EGFR expression in the primary tumors showed some association with decreased disease-free survival (P = 0.0306 and P = 0.0985, respectively). Both pSTAT3 and EGFR showed a correlation of expression between tumor and matched lymph node metastasis (P < 0.0001 and P = 0.0046, respectively). In addition, the expression of EGFR and GRPR in the primary tumors correlated with TGF-α expression in paired nodal metastases (P = 0.0043 and P = 0.0268, respectively). In the nodal metastases, TGF-α expression correlated with EGFR expression (P = 0.0069). In primary tumors, GRPR expression correlated with TGF-α and EGFR expression (P = 0.0378 and P = 0.0026, respectively).

Conclusions: These findings support an autocrine signaling pathway involving TGF-α, EGFR, and pSTAT3 in metastatic SCCHN as well as transactivation of EGFR by GRPR via TGF-α, but fails to identify an independent prognostic role for pSTAT3 immunoexpression.

Squamous cell carcinoma of the head and neck (SCCHN) is the most common histologic subtype of malignancies that arise in the mucosa of the upper aerodigestive tract. Approximately 40% of individuals with SCCHN have metastatic disease to the cervical lymph nodes at the time of diagnosis (1). SCCHN survival is strongly correlated with the stage of disease at diagnosis, with the nodal status (N stage) as the most important component because survival drops precipitously in patients with positive nodes.

Pathologic features that also contribute to clinical outcome include positive margins, extracapsular spread of lymph node metastases, perineural invasion, and possibly, tumor grade/pattern of invasion (2–4). Although these histologic variables have prognostic value, they are not consistently predictive of biological behavior with perhaps the exceptions of positive surgical margins and extracapsular spread. Hence, an understanding of tumorigenesis and progression at a molecular level may yield individual markers that predict biological behavior (biomarkers), particularly response to therapy and clinical outcome. Much attention has been directed to the study of these biomarkers because in addition to their potential to predict survival, they may also support the design of targeted therapies. Generally, biomarkers consist of proteins (or phosphoproteins) that are aberrantly and selectively expressed in tumor. Clinically applicable biomarkers are typically assessed by routine immunohistochemical methods on formalin-fixed paraffin-embedded tissue.

The epidermal growth factor receptor (EGFR) has been extensively studied as a prognostic biomarker in many epithelial malignancies including SCCHN. Increased expression of EGFR in SCCHN tumors has been associated with decreased.
survival in several cohorts (5, 6). In both of these earlier studies, EGFR was assessed by quantitative image analysis of the immunohistochemical staining. Using this method, which requires both specialized equipment and dedicated pathologic expertise, levels of transforming growth factor-α (TGF-α) and EGFR were highly correlated with each other, and expression of both ligand and growth factor receptor in the primary tumors were shown to be independent of the nodal staging (5). Several EGFR targeting strategies have been developed and the monoclonal antibody cetuximab was approved by the Food and Drug Administration in 2006 for use in patients with SCCHN based on the increased survival of patients treated with this compound in combination with radiation (7). In at least one trial, the level of EGFR in the tumor was associated with clinical response to cetuximab when combined with cisplatin (8).

Despite the ubiquitous overexpression of EGFR in SCCHN, the response rate to EGFR targeting when administered as monotherapy is generally <10% (9). Although the mechanisms of resistance to EGFR targeting are incompletely understood, it is plausible that persistent activation of downstream signaling pathways could be a contributing factor. Signal transducers and activators of transcription (STAT) proteins, including STAT3, can be activated by cell surface tyrosine kinase receptors, such as EGFR. In SCCHN, STAT3 is recruited to specific tyrosine residues in the EGFR cytoplasmic domain leading to transcriptional activation (10). Studies from our laboratory and others have shown antitumor effects when targeting STAT3 in SCCHN preclinical models (11–13).

The expression of phosphorytrosine STAT3 (pSTAT3), an active form of STAT3, was reportedly associated with decreased survival in two separate cohorts (14, 15). In both of these studies, pSTAT3 was the only biomarker examined by immunohistochemistry in the SCCHN tumors. We undertook the present study to test the hypothesis that EGFR autocrine signaling leading to STAT3 activation is a poor prognostic marker in patients with SCCHN. This hypothesis was based on the observation that STAT3 activation mediates tumor cell growth, survival, and invasion. In addition, we previously reported that gastrin-releasing peptide receptor (GRPR) mRNA levels in the primary head and neck cancer were associated with decreased survival (16). Further investigation showed a role for TGF-α in the transactivation of EGFR by GRPR in SCCHN (17, 18). In the present study, two cohorts were examined for the expression of pSTAT3, including a cohort previously examined for the prognostic significance of EGFR (5), and a second cohort with advanced disease and metastasis to cervical lymph nodes with extracapsular spread that was treated on an adjuvant chemoradiation protocol (19). An additional goal was to assess any potential interplay between pSTAT3, EGFR, GRPR, and TGF-α–mediated signaling pathways in clinical tumor samples. Hence, the immunoenexpressions of these markers were assessed in both primary tumors and lymph node metastases of the second cohort. The expression levels of these markers were assessed for any interrelationship and also correlated with various outcome measures.

### Materials and Methods

**Patients and tissue samples.** Cohort 1 consisted of patients with SCCHN who underwent primary surgical resection with curative intent during the period from February 1990 to December 1993 at the University of Pittsburgh, as described previously (5). Tumors from 91 patients were previously stained for TGF-α and EGFR, and expression levels measured using computerized image analysis. Tissue was still available in 61 samples from this cohort for pSTAT3 immunohistochemical staining. Clinical follow-up was available for all patients until January 2005. Pertinent patient information was abstracted from the computerized head and neck tumor registry.

Cohort 2 consisted of SCCHN patients who were found to have extracapsular spread of cervical metastases upon lymph node dissection, which is a known poor prognostic feature. All patients underwent surgical resection with curative intent between August 1982 and May 1992 and had negative surgical margins of the primary carcinoma. Postoperative irradiation consisted of 50 to 60 Gy for 5 to 6 weeks followed by methotrexate and 5-fluorouracil on an outpatient basis on days 1 and 8 every 21 days as described previously (19). Of the 131 subjects who received surgery, irradiation, and chemotherapy, we identified 69 tissue blocks (paired tumor and metastatic lymph nodes) that were deemed to be suitable for immunohistochemical staining for GRPR, TGF-α, EGFR, and pSTAT3. Patient characteristics are presented in Table 1. Clinical follow-up was available for all patients until June 2004. Pertinent patient information was abstracted from the computerized head and neck tumor registry. Tissues from both cohorts were examined under the auspices of an Institutional Review Board–approved protocol.

**Histologic examination and immunohistochemistry.** The original diagnoses of squamous cell carcinoma were verified by two pathologists (B. Collins and R. Seethala) by review of either original H&E-stained slides of tumor or recuts. For immunohistochemical staining, 4-μm sections were cut from formalin-fixed paraffin-embedded tissue blocks and deparaffinized and rehydrated using successive washes of xylenes followed by ethanol. Heat-induced epitope retrieval was done on the sections in a microwave oven (medium power for 10 min) using citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 3% hydrogen peroxide. Sections were incubated in Universal Protein Blocker (Shandon Lipshaw) for 20 min at room temperature. For TGF-α staining, a saponin wash pretreatment was done as well. All slides were examined using a standard bright-field microscope (Olympus BX51). All photomicrographs were taken at 100×.
magnification using an attached digital camera (SPOT insight 4MP; Diagnostic Instruments).

The sections were then incubated with anti-pSTAT3 (1:50 dilution, Tyr 705; Cell Signaling Technology), GRPR (1:100 dilution; Alpha Diagnostics International), EGFR (1:50 dilution, H11; Dako), and/or TGF-α (1:50 dilution, Ab2; Oncogene Science) for 1 h at room temperature. Detection of and chromogen application to the antibody-antigen complex was achieved by use of a standard biotin-avidin complex kit (Vector Laboratories) and by use of the 3,3′-diaminobenzidine kit (Vector Laboratories).

For EGFR, both cytoplasmic and membranous staining were considered positive, whereas for GRPR and TGF-α, cytoplasmic staining was considered positive. For pSTAT3, only nuclear staining was considered positive. Tumor sections were subjectively scored from 0 to 3+ (none, weak, moderate, strong) based on the intensity of the stain, with strong reactivity defined as at least equivalent to the staining intensity of positive control tissues included on a given staining run and/or known internal positive controls. In addition, the percentage of tumor cells that expressed the target protein was estimated. A composite score was generated by multiplying the intensity score by the percentage of cells that were stained producing scores ranging from 0 to 300.

**Statistical analysis.** Disease-free survival was computed from the date of surgery until the first instance of disease recurrence or a second primary tumor. Patients were censored on the last date they were known to be disease-free or the date of death from a cause other than cancer of the head and neck. Analysis of disease-free survival was conducted with Cox proportional hazards regression. Tumor and/or known internal positive controls. In addition, the percentage of intensity of positive control tissues included on a given staining run was considered positive. Tumor sections were subjectively scored from 0 to 3+ (none, weak, moderate, strong) based on the intensity of the expression in SCCHN, we analyzed the expression of pSTAT3 in cohort 1, which was a subset of the same primary tumors we had previously assessed for TGF-α and EGFR expression (5). In contrast to the strong association between TGF-α and EGFR and their effect on disease-specific and overall survival, pSTAT3 levels in the 61 tumors did not correlate with clinical outcome ($P = 0.914$; Table 2).

To verify that the lack of association between pSTAT3 expression and SCCHN survival was not restricted to a single cohort, we also measure pSTAT3 expression in 69 tumors obtained from a second cohort of patients (cohort 2). These individuals were all found to have extracapsular spreading on pathologic analysis of their therapeutic neck dissections and were treated with radiation and chemotherapy in the adjuvant setting (19). Similar to the observations in the first cohort, pSTAT3 expression levels in the tumor were not associated with clinical outcome ($P = 0.252$). The pattern of biomarker expression is often an important consideration in biomarker analysis. It has been noted that pSTAT3 immunoeexpression may be localized to the tumor edge or border in lung carcinomas (23). When we examined our cohort for pattern of expression, we found this edge distribution in only 10 cases. Similar to pSTAT3 immunoeexpression overall, the pattern of staining did not have an effect on clinical outcome ($P = 0.655$).

However, it should be noted that the staining was done on archived surgical specimens and not processed under a protocol optimized for evaluation of phosphoproteins. Fixation time on such a cohort would depend on multiple factors including

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**Abbreviations:** T, tumor; LN, lymph node.
*25th and 75th percentiles of immunohistochemical score which is defined as staining intensity (0, 1, 2, or 3) times percentage of cells stained × 100.
1 Risk of disease recurrence or new primary attributable to an increase in score from the 25th to the 75th percentile. A hazard ratio of 1.0 indicates no influence on disease-free survival.
2 Raw P values were adjusted using the step-down Bonferroni method.

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laboratory workload variation, specimen size, specimen complexity, and gross prosector proficiency. It is thus likely that this variation could contribute to a modulation of pSTAT3 reactivity as reported previously (24, 25).

**STAT3-mediated TGF-α/EGFR signaling is detected in metastatic cervical lymph nodes.** The expression of GRPR, TGF-α, EGFR, and/or pSTAT3 has not been previously examined in lymph node metastases from patients with cancer. Because the subjects in cohort 2 all had cervical metastases with extracapsular spreading, we had the opportunity to examine the expression of these signaling proteins in the lymph nodes and determine their potential correlation with clinical and pathologic variables. However, GRPR, TGF-α, EGFR, or pSTAT3 immunoexpression levels in the nodal metastases were not significantly associated with sex, age 65 years or greater, tumor site, tumor stage, or tumor grade. The primary tumors were also stained for TGF-α and EGFR to allow for the evaluation of the association between expression levels in the tumor and levels in the metastatic lymph node. Although we did not observe as strong an association between TGF-α or EGFR tumor expression levels and survival compared with our earlier study of cohort 1 in which we used quantitative image analysis to assess immunohistochemical staining, TGF-α or EGFR were moderately associated with decreased disease-specific survival in cohort 2 (unadjusted \( P = 0.0306 \) and \( P = 0.0985 \), respectively). Similar to our previous observations, tumor levels of TGF-α and EGFR were highly correlated, underscoring the role of this autocrine EGFR ligand in SCCHN \( (P = 0.0002; \text{Fig. 1}) \). Others have reported an interaction between nuclear EGFR and STAT3 in breast cancers (26). Using the Dako anti-EGFR monoclonal antibody, we did not detect any nuclear EGFR immunoreactivity.

EGFR signaling has been reported to contribute to SCCHN invasion and metastasis (27, 28). If metastatic deposits of tumor cells are derived from EGFR-overexpressing cells in the primary tumor, then it might be expected that expression levels in the two sites may be correlated (Fig. 2). Indeed, we found that expression of EGFR in the primary tumor correlated with EGFR levels in the lymph node metastases \( (P = 0.0046; \text{Table 3}) \). Similarly, pSTAT3 expression levels in the tumor were correlated with pSTAT3 levels in the paired lymph node metastases \( (P < 0.0001) \). In contrast, TGF-α levels in the tumor did not correlate with levels in the lymph nodes \( (P = 0.4612) \). We previously reported that TGF-α stimulates pSTAT3 expression in SCCHN cell lines. In this study, however, we found that within the nodal metastases, TGF-α expression did not correlate with pSTAT3 expression \( (P = 0.4263) \). We also previously noted that stimulation of GRPR induces EGFR activation via cleavage and secretion of TGF-α with subsequent cell proliferation and invasion (17, 18). The correlation between TGF-α \( (P = 0.0378) \) and EGFR \( (P = 0.0026) \) levels in the primary tumor with GRPR levels provides indirect evidence of this transactivation pathway.

**Discussion**

Identification of a biomarker as a therapeutic target is generally based on (a) increased expression of the protein in tumors compared with normal tissue, (b) antitumor effects observed in preclinical models using strategies that target the protein, and (c) correlation of expression levels of the protein in the tumor with decreased survival. These criteria have generally been fulfilled for EGFR in SCCHN as shown by the recent finding of a survival advantage of an EGFR-targeted therapy (cetuximab) and Food and Drug Administration approval of this agent for SCCHN treatment in combination with radiation therapy (7). Tyrosine-phosphorylated STAT3 is overexpressed in SCCHN tumors and targeting STAT3 has consistently shown antitumor efficacy in SCCHN preclinical models (12, 29–31). Although two prior reports found that pSTAT3 expression in the primary tumor was associated with decreased survival, we failed to identify pSTAT3 as a prognostic factor in two retrospective cohorts of patients with SCCHN (14, 15). The potential reasons for these discrepant findings include the relatively homogeneous cohort of tongue/oral cancers studied in the earlier report and the use of different staining protocols and interpretation criteria to detect pSTAT3 expression. The lack of prognostic significance of pSTAT3 expression in primary or metastatic SCCHN tumors suggests that other factors contribute to tumor progression and patient survival in this malignancy.

The analysis of signaling in human tumors has revealed the importance of cross-talk between pathways. Activation of EGFR by G protein–coupled receptors including GRPR leads to the phosphorylation of EGFR and downstream targets including mitogen-activated protein kinase and Src (18, 32). Primarily using cell line models, we and others have shown that activation of EGFR by G protein–coupled receptor ligands induces tumor cell proliferation and invasion (33, 34). We analyzed GRPR expression in the samples that were also assessed for TGF-α and EGFR. Indeed, a correlation between expression levels of these proteins in the tumor with GRPR supports an interaction between signaling pathways in human cancers.
STAT3 can be activated by upstream receptor and non-receptor tyrosine kinases as well as cytokines. In SCCHN, stimulation of EGFR, Src family kinases, as well as interleukin 6/gp130 have all been reported to lead to the activation of STAT3 (5, 35, 36). STAT3 is activated by both tyrosine and serine phosphorylation. Although most studies have focused on the phosphorylation of tyrosine 705, emerging evidence supports the signaling capacity of phosphoserine STAT3 and unphosphorylated STAT3 (37, 38). Therefore, in the absence of measurements of phosphoserine STAT3 and total STAT3, one can only conclude that phosphotyrosine STAT3 levels alone do not contribute to disease-free survival in SCCHN. The lack of correlation between pSTAT3 levels in the tumor and tumor levels of TGF-α or EGFR could be attributed to the contribution of interleukin 6 and/or Src to STAT3 activation in this malignancy.

Although the EGFR signaling axis has not been previously evaluated in lymph node metastases, it is not surprising that the expression levels of EGFR and pSTAT3 in the tumor correlated with levels in the paired lymph node metastases representing a preservation of tumorigenic phenotype with regards to these biomarkers. However, expression of TGF-α in the metastatic lymph nodes did not correlate with TGF-α levels in the primary tumors. Interestingly though, TGF-α expression in lymph node metastases correlated with EGFR expression, and nearly correlated with GRPR in the primary tumor. Additionally, within the lymph node metastases, TGF-α correlated with EGFR and GRPR levels. It is therefore possible that TGF-α levels are

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**Table 3.** Spearman rank correlation coefficients

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<tr>
<td></td>
<td>Stat3</td>
<td>EGFR</td>
</tr>
<tr>
<td>Tumor</td>
<td>-0.11</td>
<td>-0.17</td>
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<tr>
<td>EGFR</td>
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<td>0.23</td>
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<tr>
<td>TGF-α</td>
<td>-0.26</td>
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<tr>
<td>GRPR</td>
<td>-0.26</td>
<td>0.37</td>
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NOTE: Significant correlations with expected 5% false discovery rates are shown in boldface.
induced by these signaling pathways in nodal metastases rather than simply reflecting the expression level intrinsic to a particular primary tumor.

The correlations between signaling proteins in tumors and metastatic lymph nodes suggests that analysis of pathways might be more biologically relevant than a restricted assessment of a single protein. In the absence of a single genetic event that is critical to tumorigenesis (such as the translocations found in various hematopoietic and soft tissue neoplasms), any one epigenetically altered protein is unlikely to contribute to tumor progression by itself. The "panel" approach to immunohistochemical biomarker analysis allows for the evaluation of multiple components of a signaling axis using methodology available to most pathology immunohistochemistry laboratories. Whereas even a single marker such as EGFR may yield prognostically vital information, as we have shown, a "pathway analysis" approach will also reveal interactions between components which may be important mechanistically, and perhaps ultimately, therapeutically.

Although demonstrating several clinical and biological correlations, the results of the present study also underscore the limitations in routine immunohistochemical analysis for the purpose of biomarker validation even if two well characterized cohorts are used. When biomarkers are tested in human cancers, variability in antibody selection, assay conditions, tissue quality, interpretation methodology, and difference in cohort type are all contributing factors to interstudy variability, as indicated by our pSTAT3 findings in comparison with the two prior reports (14, 15). Even well studied markers such as EGFR are subject to the same preanalytic, analytic, and postanalytic variabilities. Our previous findings in cohort 1 (5) showed that quantitative image analysis of immunohistochemical staining for TGF-α and EGFR showed a more significant prognostic association than that seen using semiquantitative light microscopic analysis in cohort 2. This provides indirect evidence that automated, quantitative, high-throughput techniques might be necessary to standardize the assessments of biomarker expression. Such an endeavor is not feasible in a community hospital and probably might be challenging even in the academic medical center without a dedicated pathology core facility. It must be noted, however, that a head-to-head comparison of qualitative or semiquantitative immunohistochemical analysis of EGFR in SCCHN by light microscopy has not been made to quantitative immunohistochemical analysis by imaging software.

There are no fixation, staining, or interpretation guidelines, and no gold standards of benchmarks for EGFR immunohistochemistry, let alone other biomarkers in HNSCC. Furthermore, the precise method to measure EGFR (EGFR or phosphorylated EGFR, immunohistochemistry, EGFR mutation, or EGFR fluorescence in situ hybridization) in SCCHN has not been determined. Several studies advocate the assessment of EGFR amplification by fluorescent in situ hybridization for prognosis (39, 40). Ultimately, if EGFR (and other biomarkers) are used for prognosis and therapeutic management, the approach to evaluation should, to some extent, be modeled after the breast cancer biomarker Her-2/Neu. The literature shows that overexpression of Her-2/Neu by 3+ immunohistochemical staining and/or fluorescent in situ hybridization confers a poor prognosis, and that adjuvant therapy with trastuzumab, which targets Her-2/Neu, confers a survival advantage in patients with breast cancer (41). Recent College of American Pathologists/American Society of Clinical Oncology recommendations for Her-2/Neu testing have reviewed the major adjuvant trastuzumab trials, quality assurance results of the major governing quality assurance organizations in the international pathology community, and the various assays for Her-2/Neu overexpression to yield standard guidelines for testing, interpretation, and proficiency evaluation with respect to benchmark performance (42). The establishment of a similar set of standards for EGFR and other biomarkers requires a comparable volume of well-designed studies supporting the utility of staining as a prognosticator and comparative analysis of different methodologies.

References

13. Gao SP, Bromberg JF. Touched and moved by Stat3: epigenetically altered protein is unlikely to contribute to tumor progression by itself. The "panel" approach to immunohistochemical biomarker analysis allows for the evaluation of multiple components of a signaling axis using methodology available to most pathology immunohistochemistry laboratories. Whereas even a single marker such as EGFR may yield prognostically vital information, as we have shown, a "pathway analysis" approach will also reveal interactions between components which may be important mechanistically, and perhaps ultimately, therapeutically.

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Correction: Article on Immunohistochemical Analysis of Phosphotyrosine STAT3 and EGFR Autocrine Signaling Pathways

The article on immunohistochemical analysis of phosphotyrosine STAT3 and EGFR autocrine signaling pathways by Seethala and associates beginning on page 1303 of the March 1 issue of Clinical Cancer Research was a research article, not a review article.

Seethala RR, Gooding WE, Handler PN, et al. Immunohistochemical analysis of phosphotyrosine signal transducer and activator of transcription 3 and epidermal growth factor receptor autocrine signaling pathways in head and neck cancers and metastatic lymph nodes.
Immunohistochemical Analysis of Phosphotyrosine Signal Transducer and Activator of Transcription 3 and Epidermal Growth Factor Receptor Autocrine Signaling Pathways in Head and Neck Cancers and Metastatic Lymph Nodes

Raja R. Seethala, William E. Gooding, Phoebe N. Handler, et al.


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