STAT1 Activation in Squamous Cell Cancer of the Oral Cavity
A Potential Predictive Marker of Response to Adjuvant Chemotherapy

Klaus Laimer, MD1
Gilbert Spizzo, MD2,3
Peter Obrist, MD4
Guenther Gastl, MD2,3
Thomas Brunhuber, MD4
Georg Schäfer, MD4
Burghard Norer, MD1
Michael Rasse, MD1
Michael C. Haffner, MD5
Wolfgang Doppler, MD, PhD5

1 Division of Maxillofacial Surgery, Biocenter, Innsbruck Medical University, Innsbruck, Austria.
2 Division of Hematology & Oncology, Biocenter, Innsbruck Medical University, Innsbruck, Austria.
3 Tyrolean Cancer Research Institute, Innsbruck, Austria.
4 Department of Pathology, Innsbruck Medical University, Innsbruck, Austria.
5 Division of Medical Biochemistry, Biocenter, Innsbruck Medical University, Innsbruck, Austria.

BACKGROUND. For patients with squamous cell carcinoma of the oral cavity, both locoregional and distant recurrences are common, and an appropriate adjuvant treatment modality has yet to be defined. Thus, there is an urgent need to identify novel molecular markers with potential prognostic and/or predictive value to improve treatment outcome in these patients. This retrospective study was designed to investigate the predictive and/or prognostic value of STAT1 activation in squamous cell carcinoma of the oral cavity.

METHODS. STAT1 expression and subcellular localization was examined immunohistochemically on a tissue microarray of paraffin-embedded tumor specimens from 89 patients who underwent surgical treatment in the period between 1980 and 1997. A nuclear staining score of greater than 35% was defined as high STAT1 activation.

RESULTS. According to study criteria, 18% of analyzed tumor samples exhibited high STAT1 activation. High STAT1 activation was associated with negative lymph node status. Moreover, in the subgroup of patients who received chemotherapy, high nuclear STAT1 staining in the tumor was associated with good prognosis.

CONCLUSIONS. This is the first report demonstrating the potential predictive value of STAT1 activation status in patients with squamous cell cancer of the oral cavity. If confirmed in large prospective trials, this molecular marker could help in guiding therapeutic decisions in these patients. Cancer 2007;110:326–33. © 2007 American Cancer Society.

KEYWORDS: STAT1, oral squamous cell carcinoma, chemotherapy, predictive marker, tissue microarray.

Squamous cell carcinoma of the oral cavity, as a subgroup of head and neck cancers, accounts worldwide for about 4% to 5% of all carcinomas in men and 2% in women, with geographically varying frequency.1,2 A high incidence of oral squamous cell carcinoma (OSCC) has been reported in India,3 where chronic consumption of tobacco, especially the chewing of tobacco leaves, in combination with alcohol abuse are essential etiological factors.4 Alcohol intake increases the permeability of the oral mucosa, which, in turn, enhances carcinogenic effects of nitrosamines and polycyclic hydrogen contained in tobacco. Insufficient oral hygiene, chronic pressure caused by dental prostheses, infection with papilloma virus, and chronic diseases (such as Plummer-Vinson-syndrome or Lichen planus) are considered further etiological factors.5–7 Clinical stage (according to American Joint Commission on Cancer TNM classification) and tumor grade5 are established prognostic indicators for OSCC. Cervical lymph node metastases are rather common,
whereas distant metastases, involving especially the lung, are seen in advanced stages of disease.9

Surgery is the primary therapy modality for both early stage and locally advanced head and neck cancer, followed by radiotherapy.10 In the case of resectable OSCC, the use of adjuvant chemotherapy is controversial: Some institutions propose the use of neoadjuvant chemoradiation, whereas others follow the surgery and/or radiotherapy alone concept.11,12 Established chemotherapy regimens contain a combination of 5-fluorouracil (5-FU) with platinum-containing drugs, such as cisplatin and carboplatin. During past years, various strategies combining chemotherapy with radiotherapy were tested for their capability to improve treatment outcome.13 In a study on high-risk head and neck cancer patients, chemotherapy in combination with radiation led to significant improvement in local cancer control and an increase in disease-free survival, but this combination failed to show an advantage in terms of overall survival.14,15 Improvements in local cancer control with concurrent postoperative chemotherapy and radiation therapy were described in some studies.16,17 However, a comparison of data that were obtained in a nonrandomized study with OSCC patients treated with surgery alone or adjuvant chemotherapy revealed no evidence for improved cancer control or disease-specific survival in patients who received adjuvant therapy.11,18 Similarly, a meta-analysis of adjuvant, neoadjuvant, and concomitant chemotherapy for OSCC and other forms of head and neck cancer revealed only a small survival benefit in favor of chemotherapy.19 However, the heterogeneity of these studies did not allow firm conclusions on this issue.

One hypothesis for the treatment of OSCC is that only a fraction of patients with chemosensitive tumors may benefit from chemotherapy. Responsiveness to chemotherapy may, in turn, depend on tumor phenotype. It would, therefore, be of interest to find molecular markers that define this subpopulation of chemosensitive OSCC to specifically select patients for adjuvant chemotherapy. The signal transducer and activator of transcription 1 (STAT1) has frequently been found to be constitutively activated in a great variety of tumors,20 including head and neck cancer,21,22 and has been implicated in triggering apoptosis and/or cell-cycle arrest.23 Effector molecules that stimulate apoptotic action of STAT1 comprise the death receptor Fas,24 the death receptor ligand TRAIL,25 2,5 oligoadenylate synthase, which activates the tumor suppressor gene Rnasel,26 and p21.22 Activation of STAT1 within the tumor can be triggered by cell endogenous signaling pathways or by autocrine or paracrine growth factors. Further-

| **TABLE 1** Clinical and Pathological Features of Patients With Either High or Low STAT1 Activation |
|----------------|----------------|----------------|
| **Characteristics** | **Low** | **High** | **P** |
| Total no. of patients | 73 | 16 | |
| Sex | | | NS |
| Women | 16 (80%) | 4 (20%) | NS |
| Men | 57 (82.6%) | 12 (17.4%) | |
| Recurrence | 23 (88.5%) | 3 (11.5%) | NS |
| Grading | | | |
| I | 12 (66.7%) | 6 (33.3%) | NS |
| II | 41 (87.2%) | 6 (12.8%) | |
| III | 20 (83.3%) | 4 (16.7%) | |
| Clinical stage | | | NS |
| I | 4 (80%) | 1 (20%) | |
| II | 4 (80%) | 1 (20%) | |
| III | 15 (68.2%) | 7 (31.8%) | |
| IV | 50 (87.7%) | 7 (12.3%) | |
| Lymph node involvement | 51 (92.7%) | 4 (7.3%) | .001 |
| Site | | | NS |
| Tongue | 7 (63.6%) | 4 (36.4%) | |
| Floor of mouth | 29 (87.9%) | 4 (12.1%) | |
| Gum/cheek | 22 (88%) | 3 (12%) | |
| Trigonum retromolare | 5 (83.3%) | 1 (16.7%) | |
| Total palate | 6 (75%) | 2 (25%) | |
| Other parts of mouth | 4 (66.7%) | 2 (33.3%) | |
| NS indicates not significant. Percentages are calculated for each subgroup. *P compares low versus high STAT1 activation. |

more, STAT1 can be activated by immune cells that secrete interferons, thus resulting in antitumor immunosurveillance action.27 In this study, we investigated whether the activation status of STAT1 in OSCC can serve as a predictive marker for response to adjuvant chemotherapy.

**MATERIAL AND METHODS**

**Patients**

The study was conducted according to regulations of the local ethics committee and Austrian Law. All patients who underwent surgery between 1980 and 1997 at the Innsbruck Medical University Department of Maxillofacial Surgery and who had available clinical follow-up data and tissue specimens from the local pathology repository were included. Seventy-nine (77.5%) were men, and twenty (22.5%) were women. The median age was 63.3 years (range, 25.6 years–85.2 years). Fifty-five (61.8%) patients presented with the primary diagnosis of lymph node-positive disease. Fifty-eight (64.0%) were classified stage IV, 22 (24.8%) as stage III, 5 (5.6%) as stage II, and 5 (5.6%) as stage I (Table 1). All patients underwent neck dissection and careful evaluation of
lymph-node status. The decision for adjuvant therapy was made according to individual considerations based on performance status, patient compliance, and clinicopathological features. Thirty-three (37.1%) patients received 5-FU in combination with cisplatin (25 patients), carboplatin (4 patients), and mitomycin (4 patients) in 3 to 5 treatment cycles (Table 2).

**Histopathology**

All tumor samples were formalin-fixed, embedded in paraffin, and stored at the local pathology repository. From each tumor sample, a hematoxylin and eosin-stained slide was prepared, by using routine methods, and then examined by light microscopy. Tumor grade was assessed by 2 pathologists in a blinded fashion by using standard pathology criteria. Low, moderate, and high differentiation grade was observed in 18 (20.2%), 47 (52.8%), and 24 (27%) of patients, respectively.

**Tissue Microarray (TMA)**

For TMA construction, the pathologist (P.O.) used hematoxylin and eosin-stained slides from each tumor block to select morphologically representative tumor area. Tissue cylinders with a diameter of 2 mm were punched from the marked tumor areas of each block (donor block) and brought into a recipient paraffin block (recipient block) by using a precision instrument (Manual Tissue Arrayer, MTA-1, Beecher Instruments, Sun Prairie, Wis). Three different TMAs were constructed, each containing about 30 punches of oral squamous cell carcinoma in a specific array pattern. Sections from these blocks were cut with a microtome and mounted on TMA-specific, adhesive-coated, glass slides, which were used for immunohistochemical analysis.

**Immunohistochemistry**

The expression of STAT1 was determined by immunohistochemistry by using a rabbit polyclonal antibody directed against Stat1α p91 at a dilution of 1:100 (C-24, Santa Cruz Biotechnology, Santa Cruz, Calif). Sections measuring 5 μm were cut from recipient paraffin blocks, mounted on micro tissue array-specific, adhesive-coated, glass slides (Instrumedics, St. Louis, Mo), deparaffinized, and rehydrated. Endogenous peroxidase was blocked with methanol containing 3% hydrogen peroxide for 20 minutes. Slides were then incubated with peroxidase-conjugated goat antimouse antibody (EnVision; Dako, Vienna, Austria) for 60 minutes and treated with chromogen diaminobenzidine in a solution containing 3% hydrogen peroxide. Finally, slides were counterstained with Mayer's Hemalum solution and rinsed with water. Representative micrographs of tumors with predominant nuclear or cytoplasmic staining of STAT1 are shown in Figure 1. As a positive control for cells that contained a high proportion of activated STAT1 in the nucleus, T47D mammary carcinoma cells were treated with 10 ng/mL human IFN alpha (Preprotech EC, London, UK) for 20 minutes in culture and compared with cells that remained untreated and that contained only a small percentage of nuclear STAT1. Cells were then washed with phosphate-buffered saline, scraped from the tissue plate, formalin-fixed, embedded in paraffin, and processed for immunohistochemistry in the same way as the micro tissue array.

**Evaluation of Slides**

The fraction of cells with specific staining of the nucleus was determined as a measure for STAT1 activation. The sections were observed under a light microscope individually by 2 pathologists who were not aware of the clinical characteristics of the patients. By using high magnification power (×400), the 4 fields of each punched representative tumor section were evaluated. In total, 50 tumor cells were counted in each field. The percentage of immunoreactive tumor cells with nuclear staining ranged from 0 to 70%. The mean was determined as an optimal cutoff value to separate high and low STAT1 activation categories, thus, tumors with STAT1 staining in greater than 35% of the nuclei were considered to exhibit high STAT1 activation, whereas those with 35% and less were scored as tumors with low STAT1 activation.

**Statistical Methods**

All calculations and the statistical analyses were performed by using the statistical software program SPSS for Windows (SPSS, Inc. Chicago, Ill). Differences between groups were examined for statistical significance by using the chi-square test. Survival curves were calculated according to the method of Kaplan and Meier. For this method, P values were
evaluated by the log-rank test for censored survival data. Follow-up time was censored if the patient was lost during follow-up. The significance of various parameters for survival was analysed by the Cox proportional hazards model. The final model adjusted for tumor grade, clinical stage, lymph-node metastasis (positive vs negative), and STAT1 activation status. Correlations between parameters were assessed according to the Spearman nonparametric test. For all analyses a $P$ value of $<.05$ was defined as statistically significant.

RESULTS
The median overall survival in this study cohort was 20.8 months (range, 1 month to 245 months). Forty-seven (52.8%) patients received adjuvant therapy. Tumor grade ($P < .008$) and clinical stage ($P = .037$), but not STAT1 activation status ($P = .108$), were predictors of survival in the entire patient cohort. There was a tendency toward longer survival for lymph node-negative patients. However, in contrast to other studies in head and neck cancer patients, this did not reach statistical significance, which may be because of the relatively small number of patients in the study. In a multivariate Cox regression model, only tumor grade was statistically significant (hazard ratio [HR], 1.65; 95% confidence interval [CI], 1.13–2.39; $P = .009$). STAT1-activation was assessed by immunohistochemistry as described in the Methods section through evaluation of the percentage of cells with nuclear STAT1. Sixteen (18%) of the analyzed tu-

FIGURE 1. Examples for OSCC tumors with low or high STAT1 activation as determined by immunohistochemistry. In Panels A and B, tumors classified to have low STAT1 activation are shown, whereas in Panels C and D, tumors with high STAT1 activation are depicted. (A) Moderately differentiated squamous cell carcinoma with strong cytoplasmic STAT1 staining. (B) Squamous cell carcinoma with an inflammatory infiltrate. STAT1 staining is mostly restricted to the cytoplasm in both tumor and infiltrate. (C) Moderately differentiated tumor with strong STAT1 staining both in the cytoplasm as well as in the nucleus of the cancer cells. (D) Tumor with strong nuclear STAT1 staining in the cancer cells. In stromal tissue, nuclear staining is absent.
mor samples were classified as tumors with high STAT1 activation. Representative examples for the immunohistochemical staining of tumors with either low or high STAT1 activation are shown in Figure 1.

Characteristics of the study population segregated by STAT1 activation are shown in Table 1. Low-grade tumors tended to have a higher rate of STAT1 activation and were associated with good prognosis. High STAT1 activation was associated with negative lymph node status (Table 1; \( P < 0.001 \)). There was neither a positive nor a negative correlation between epidermal growth factor receptor (EGFR) expression levels, as determined in a previous study,\(^{28}\) and nuclear STAT1 levels (data not shown). This is in contrast to the findings with STAT3, where a positive correlation between STAT3 activation and EGFR expression has been described.\(^{29}\)

We further evaluated the correlation of STAT1 activation with overall survival in different treatment groups of patients who were receiving adjuvant chemotherapy, radiotherapy, both treatment modalities, or no adjuvant therapy. The numbers of patients in each of these subgroups are shown in Table 2. Strikingly, STAT1 activation was a significant predictor for better survival in patients who received adjuvant chemotherapy (HR, 0.3; 95% CI, 0.1–0.92; \( P = .03 \)). This suggests that the determination of STAT1-activation status in the tumor enables identification of patients who have an increased likelihood of responding to chemotherapy. The correlation of STAT1 activation and good prognosis was confined to patients who were receiving adjuvant chemotherapy and was not observed for the group of patients who were not receiving chemotherapy (Fig. 2). This explains the lack of statistically significant correlations between STAT1 and survival in the entire patient cohort (Table 1).

When stratification for STAT1-activation status was not applied, chemotherapy did not prove to be of benefit to patient survival. This is in accord with previous studies that have demonstrated no favorable effect of neoadjuvant chemotherapy on patient survival.\(^ {14}\) It should be noted that in the subgroup of patients with low STAT1 activation, administration of chemotherapy even slightly decreased overall survival. Although this effect did not reach statistical significance, the results suggest that treatment of patients with low STAT1 activation by chemotherapy may be inefficient.

**DISCUSSION**

So far, decisions on therapeutic modalities for patients with SCC of the oral cavity are mainly influenced by node status, histological features, local extension, and patient performance status. However, these clinicopathological parameters do not sufficiently predict response to chemotherapy. This has promoted extensive investigation into a great variety of tissue biomarkers for their suitability as molecular

---

**FIGURE 2.** STAT1 as predictor for response to chemotherapy. Kaplan-Meier survival curves are compared for patients with either high or low STAT1 expression. In Panel A, all patients were treated with chemotherapy after tumor surgery. Because patients with clinical stage I and II usually do not receive chemotherapy, only patients with clinical stage III and IV were included. In Panel B, patients did not receive chemotherapy.
In a high percentage of SCC of the head and neck region (SCCHN), the EGFR is overexpressed in an early stage of carcinogenesis.21 Stimulation of EGFR by an autocrine TGF-β signal results in activation of STAT3.29 Sustained activation of the EGFR pathway together with inactivation of the endogenous STAT3 inhibitor SOCS-3 by methylation31 appear to be important for the constitutive STAT3 activation found in the majority of SCCHN cancers.28 A preceding study at our institution revealed that EGFR is also frequently overexpressed in the same patient material described in our present study and that high expression represents a prognostic marker that predicts low overall survival.28 STAT3 activation has been shown to be crucial for tumor growth in animal models of SCCHN, and inhibition of STAT3 activity is an interesting therapeutic option.32–34 However, there are no data that suggest usefulness of STAT3 as a marker to predict response to conventional chemotherapy in SCCHN patients.

In contrast to STAT3, STAT1 expression and activation are usually associated with growth inhibition and apoptosis in tumors. This notion appears to also be relevant for head and neck cancer: Higher STAT1 expression levels were found in well differentiated compared with poorly differentiated tumors.35 Furthermore, as shown in our study, STAT1 activation also was predominantly observed in tumors with a high differentiation grade. Moreover, an important role for STAT1 as a tumor suppressor in SCCHN carcinogenesis has been suggested by a recent study that demonstrated decreased STAT1 expression as a result of promoter methylation and a growth inhibitory effect of STAT1 overexpression in cell lines derived from SCCHN.22 Finally, we show here that patients with squamous cell cancer of the oral cavity, who have retained STAT1 expression and exhibit STAT1 activation in their tumor, respond better to adjuvant 5-FU/platinum-based chemotherapy. By contrast, without adjuvant chemotherapy, patients with high STAT1 activation did not have significantly better outcomes in terms of relapse of disease or survival. This suggests that high STAT1 activation can sensitize the tumor to 5-FU/platinum-based chemotherapy.

Which molecular mechanisms are likely to be responsible for enhanced chemosensitivity of STAT1 positive cancer cells? One line of evidence indicates synergism between proapoptotic signaling pathways triggered by chemotherapeutic drugs and STAT1. In tumor cell lines, type I interferons activate STAT1 and synergistically induce apoptosis with chemotherapeutic drugs such as 5-FU and doxorubicin.36,37 Most interestingly, apoptosis induction in SCCHN-derived tumor cells lines by cisplatin was enhanced after treatment of cells with the demethylating drug azacytidine, which increased STAT1 and p21 expression.32 Furthermore, the actions of the topoisomerase inhibitor CPT-11 (irinotecan) and the antifolate raltrexed were shown to be dependent on STAT1 expression.38 Cell-cycle checkpoint pathways are another site of interaction between STAT1 and chemotherapeutic agents: Many chemotherapeutic drugs, including 5-FU and platinum-containing compounds, act by inducing DNA damage and genotoxic stress,39,40 and they activate Ataxia telangiectasia mutated (ATM)-triggered and Ataxia telangiectasia, Rad3-related (ATR)-triggered cell-cycle checkpoint pathways as part of their antitumor action.41 Activation of these cell-cycle checkpoints promotes DNA repair of the damaged cells. In case DNA repair is not possible, these checkpoints initiate apoptosis to remove damaged cells.39 Another report demonstrated that STAT1 is required for the transcriptional up-regulation of 2 important mediators of ATM checkpoint activation, 53BP1 and MDC1,42 suggesting the hypothesis that STAT1 can enhance the action of DNA-damaging drugs by its influence on the ATM checkpoint pathway.

A third intriguing explanation of why patients with activated STAT1 in the tumor may benefit from chemotherapy is its role as an immunomodulator: STAT1 has been found to be an indicator and possible mediator of response to immunotherapy.27 In accord with this notion, loss of STAT1 activation in tumors has been demonstrated to be part of an immune escape mechanism of aggressive tumor variants.43 Because some chemotherapeutic drugs can augment antitumor immune responses,44 it is tempting to speculate that OSCCs with activated STAT1 respond better to chemotherapy, because STAT1 signaling promotes immunosurveillance mechanisms. Patients with low STAT1 expression of the tumor did obviously not benefit from adjuvant chemotherapy with 5-FU and platinum drugs (Fig. 2). This could be the result of a defective ATM checkpoint pathway, requiring STAT1 signaling,42 leading to an increased accumulation of drug-induced mutations. It remains to be seen whether these patients experience a tumor. Indeed, STAT1 knock-out mice are more likely to form tumors in response to chemotherapeutic drugs.45

In summary, this is the first report of an investigation of the role of STAT1 activation for response to 5-FU/platinum adjuvant chemotherapy in patients.
with OSCC. In our retrospective study, STAT1 activation was a potent predictor for response to chemotherapy. However, it has to be noted that treatment protocols of included patients were not completely uniform in terms of the use of platinum compounds, and the number of patients who received adjuvant chemotherapy was limited. We, therefore, conclude that determination of STAT1 activation could be potentially useful as a predictive clinical marker. The hypothesis generated in the current study has to be further validated by prospective, randomized, clinical trials.

REFERENCES


