Immunohistochemical Staining for Glutathione S-Transferase Predicts Response to Platinum-based Chemotherapy in Head and Neck Cancer


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ABSTRACT

The glutathione S-transferases (GSTs) play an important role in the cell's defense against toxic substances. The GSTs are a family of enzymes produced by several genes that interact with distinct but overlapping substrates and that may play a role in resistance of tumor cells to several chemotherapeutic agents.

We examined the correlation between expression of GSTs determined by immunohistochemistry and clinical response to platinum-based chemotherapy in 51 patients with head and neck cancer, who received a total of 56 courses of chemotherapy.

The overall response rate for the 56 chemotherapy treatment courses was 48%. The overall response rate (complete response + partial response) for patients with low GST scores was 88% (21 of 24), whereas among the patients with high GST scores, the overall response rate was 19% (6 of 32; P = 0.001). Patients with a low GST score were 4.7 times more likely to respond to chemotherapy than patients with high GST scores. GST scores corresponded to response in 84% of cases. Among 23 patients treated with neoadjuvant chemotherapy, the overall response rate for patients with low GST scores was 100% (14 of 14), whereas among the patients with high GST scores, the overall response rate was 44% (4 of 9; P = 0.002). Among 33 patients treated with chemotherapy for relapsed disease, the overall response rate for patients with low GST scores was 70% (7 of 10), whereas among the patients with high GST scores, the overall response rate was 8.6% (2 of 23; P < 0.001). We conclude that GST expression correlates well with response to platinum-based chemotherapy in head and neck cancer.

INTRODUCTION

In 1995, there were approximately 50,000 new cases of squamous cell cancer of the head and neck cancer diagnosed in the United States, accounting for an estimated 13,000 deaths (1).

Until recently, chemotherapy has been used almost exclusively for palliation in relapsed head and neck cancer and has had relatively little role in the curative therapy of these tumors. However, because approximately 20,000 patients per year will present with recurrent or metastatic disease, which is generally treatable only with chemotherapy, this is a large and important subset of patients (2). Partial response rates for methotrexate and most other single agents approximate 30% (3). In the 1970s, cisplatin was introduced into the chemotherapeutic armamentarium. Although the efficacy of cisplatin as a single agent in head and neck cancer was initially found to be only modestly better than other single chemotherapy drugs, it was soon appreciated that chemotherapy combinations based on cisplatin were highly active in head and neck cancers. Overall responses of 70–90% with complete responses of 20–30% have been reported (in untreated patients) when cisplatin is combined with 5FU (4) or other agents such as bleomycin (5–9).

These numbers shift dramatically downward in patients who present for chemotherapy in the setting of relapse. Jacobs et al. (2), in a well-documented study of more than 200 patients treated for relapse at the primary site or the local lymph node field following surgery and radiation, showed only a 32% response rate to the same cisplatin-based chemotherapy. The conclusion to be drawn from these studies is that squamous cell carcinoma of the head and neck in previously untreated patients is extremely chemotherapy sensitive, whereas the same cancer in patients who have relapsed after surgery and radiation is extremely chemotherapy resistant.

The majority of patients with head and neck cancer who receive chemotherapy are treated for recurrent or metastatic disease, and because only about one-third of them will respond to the treatment, it would be tremendously useful to be able to predict which patients are likely to benefit from chemotherapy and which are not. Given the cost (1) and morbidity of cisplatin-based chemotherapy in this disease, such information would, at a minimum, spare a portion of patients significant treatment-
associated discomfort and reduce considerable expenditure for unsuccessful therapy. Just as important, it would identify patients who should be considered for experimental therapies, including strategies that are designed to circumvent chemotherapy resistance.

A number of mechanisms have been studied regarding the ability of tumor cells to resist the cytotoxic effects of cisplatin (10). These include inactivation of cisplatin by naturally occurring thiol compounds such as metallothioneins and glutathione. Glutathione is a tripeptide thiol found ubiquitously throughout nature and is the most abundant nonprotein thiol in mammalian cells (11). Functionally, glutathione and enzymes important in the synthesis and consumption of glutathione play many roles, particularly in the protection of cells from oxidative insults and in the detoxification of noxious agents (12, 13).

Although glutathione physiology is complex and incompletely understood, a somewhat simplistic view of the relevant pathways can be divided into three principal components. First, there are several synthetic enzymes critical to the de novo and salvage synthesis of glutathione (γ-glutamylcysteine synthetase, γ-glutamyl transpeptidase, and glutathione synthetase). Second is glutathione itself. Third are the pathways involved in consumption of glutathione, most important of which are the conjugation reactions catalyzed by the GSTs.

The GSTs play an important role in the cell’s defense against numerous toxic substances. The GSTs are a family of isozymes produced by several genes, which react with distinct but overlapping substrates (14, 15). They are expressed at high levels in liver and placenta, among other tissues, and exist in several isoforms: α (basic), μ (neutral), and π (acidic; Ref. 16). More recently, a fourth class (θ) of GSTs have been described (17).

GSTs have been implicated in chemotherapy resistance for some time. GSTs have been shown to catalyze conjugation of glutathione to a number of chemotherapy agents, including alkylators such as cyclophosphamide (18) and thiotepa (19). Many tumors have altered expression of these enzymes when compared with their normal counterparts (16, 20). The GSTs are overexpressed in a number of tumor cell lines and react with a variety of agents (14). GST-π mRNA expression is increased in a variety of tumors (kidney, colorectal, head and neck, ovarian carcinomas, soft tissue sarcomas, and non-Hodgkin lymphomas) compared with available normal counterparts (21). Studies in ovarian cancer patients show that exposure to chemotherapy agents is followed by significant increases in GST levels (22). In head and neck cancer, elevated GST levels were associated with poorly differentiated tumors and were significantly higher than in adjoining normal tissues (21).

On the basis of these data, we hypothesized that increased expression of GST may be associated with a diminished clinical response rate to cisplatin-based chemotherapy. In the present study, we examine the correlation between expression of GSTs as determined by immunohistochemistry and response to platinum-based chemotherapy in a series of patients with head and neck cancer.

MATERIALS AND METHODS

Patient Selection. Fifty-one patients with head and neck tumors who had previously been treated with platinum-based chemotherapy at Georgetown University Hospital were selected for this retrospective analysis. Formalin-fixed, paraffin-embedded biopsy tissue was available for a total of 56 courses of therapy in these patients. (Five patients had neoadjuvant chemotherapy after initial biopsy and subsequently received salvage chemotherapy after biopsy-proven relapse.) Twenty-three patients received neoadjuvant chemotherapy after initial biopsy either for organ preservation or for unresectable disease. Thirty-three patients received chemotherapy for relapsed disease. In all cases, prechemotherapy biopsy tissue had been obtained as part of routine medical management either as the initial diagnostic biopsy specimen (for all patients treated with neoadjuvant chemotherapy) or as the biopsy specimen taken at the time of relapse. For all relapsed patients, the biopsy taken at the time of relapse was analyzed, instead of the initial diagnostic biopsy. Patient characteristics are described in Tables 1 and 2.

GST Immunohistochemistry. Paraffin blocks were obtained from the Department of Pathology, Georgetown University, or from the referring institution. Paraffin sections (5 μm) were stained with a commercially available rabbit polyclonal antibody (Ref. 23; Novocastra, Newcastle upon Tyne, UK) raised against all three principal isoforms of GST (α, μ, and π).

After analyzing the complete data set with the pan-GST antibody described above, 20 cases with an evenly distributed range of GST expression were reexamined with an isoform-specific antibody raised against human GST-π (Oncor, Gaithersburg, MD).

Immunohistochemistry was performed using a standard biotin-avidin complex method. In brief, the slides were depar-
affinized, washed in PBS, and blocked with normal goat serum for 20 min at room temperature. The primary GST antibody was diluted 1:3000 in PBS containing 1% BSA and 1% sodium azide and incubated on the tissue sections at 4°C for 6 h. The slides were then rinsed twice in PBS for 3 min each wash. The reaction was visualized with BioGenex multilink system (BioGenex, San Ramon, CA). The slides were incubated with biotinylated goat anti-rabbit immunoglobulin (37°C, 5 min) followed by horse-radish peroxidase-conjugated streptavidin (37°C, 5 min) according to the manufacturer’s protocol. The final immune complex was detected with diaminobenzidine solution (22°C, 10 min). The slides were counterstained with hematoxylin, then mounted.

Positive control for antibody staining consisted of normal kidney, which shows intense specific staining of proximal ductal epithelium. Glomerular and medullary tissue did not stain. Control staining of head and neck tumor tissues without primary antibody did not produce any visible staining, indicating that there was no significant endogenous peroxidase activity in the tumor tissue to generate a false positive result.

GST immunoreactivity within tumor cells in each section was scored as follows: grade 0, no immunoreactivity; grade 1, weak immunoreactivity slightly stronger than background staining; grade 2, clear immunoreactivity in more than half of the cancer cells; grade 3, strong immunoreactivity as dark as nuclear counter stain in the majority of cancer cells.

For the purposes of subsequent statistical analysis, grades 2 and 3 were classified as high GST immunoreactivity, and grades 0 and 1 were classified as low GST immunoreactivity. The investigator scoring the cases (T.N.) had no knowledge of clinical information or treatment response. To confirm the reproducibility of this scoring system, a board-certified pathologist (E.M.) analyzed all the cases independently, again without knowledge of patient identity or clinical response.

Clinical responses to chemotherapy were determined by retrospective chart review. Complete response was determined as complete disappearance of tumor by physical examination or radiographic exam. Partial response was 50% or greater reduction in tumor mass measured in two dimensions either by physical examination or radiographic exam, of more than 1 month duration. Stable disease represented no significant change in tumor dimension, whereas progressive disease represented a 25% or greater enlargement in tumor dimension while on therapy. Forty-eight patients received a minimum of two cycles of cisplatin/5FU (cisplatin 100 mg/m²/day 1, 5FU 1000 mg/m²/day for 4 or 5 days), except for the patient with the esophageal carcinoma, who received etoposide instead of 5FU.

Two patients received single-agent carboplatin.

**Statistical Analysis.** GST scores were compared with chemotherapy response data by $\chi^2$ analysis using Lab Stats Software (J. Codde, Floreat Park, Australia)

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**RESULTS**

Among 56 biopsy specimens examined, 24 had low GST scores (0 or 1), and 32 had high scores (2 or 3). Representative photomicrographs are shown in Fig. 1.

Variable cytoplasmic staining was seen in tumor epithelium, but uniformly intense staining was seen in areas of normal mucosa, possibly indicating a role for this group of enzymes in maintaining normal host defenses against ingested toxins at the mucosal level in the aerodigestive tract.

The overall response rate for the 56 corresponding chemotherapy treatment courses was 48%. The overall response rate for patients with low GST scores was 88% (21 of 24), whereas among the patients with high GST scores, the overall response rate was 19% (6 of 32; $P = 0.001$). The relative risk for the two groups is 4.7; i.e., patients with a low GST score were 4.7 times more likely to respond to chemotherapy than patients with high GST scores. GST scores accurately predicted response to chemotherapy in 84% of cases.

Among 23 patients treated with neoadjuvant chemotherapy for organ preservation or unresectable disease, the overall response rate was 78%. The overall response rate for patients with low GST scores was 100% (14 of 14), whereas among the patients with high GST scores, the overall response rate was 44% (4 of 9; $P = 0.002$). The relative risk for the two groups is 2.3; i.e., patients with a low GST score were a little more than twice as likely to respond to chemotherapy than patients with high GST scores. GST scores in this group accurately predicted response to chemotherapy in 82% of cases.

Among 33 patients treated with chemotherapy for relapsed disease, the overall response rate was 27%. The overall response rate for patients with low GST scores was 70% (7 of 10), whereas among the patients with high GST scores, the overall response rate was 8.6% (2 of 23; $P < 0.001$). The relative risk for the two groups is 8.1; i.e., patients with a low GST score were more than eight times as likely to respond to chemotherapy than patients with high GST scores. GST scores in this group accurately predicted response to chemotherapy in 85% of cases.

GST scores were less accurate predictors of survival than of chemotherapy response in this group of patients. The relapse-free and overall survival rates were 27 and 33%, respectively. Eleven of 22 patients (50%) with low GST scores are alive, whereas only 6 of 29 patients (20%) with high GST scores are alive ($P = 0.029$). The relative risk for these groups is 2.4. The correlation with relapse-free survival was similar, with low GST patients 2.4 times more likely to be relapse free. However, the difference was not statistically significant ($P = 0.11$).

The results for all these groups are summarized in Table 3. The GST scores obtained by a second observer produced similar results. The second observer was blinded to all patient data and to the first observer’s scores. The two observers produced the same GST scores (high versus low) in 88% of cases. Similar statistical relations were also seen between the second observer’s scores and chemotherapy response. Using the second observer’s ratings for the overall patient population, low GST scores were associated with a 2.9-fold higher response rate than high GST scores ($P = 0.001$). In the neoadjuvant patients, the response rate for low GST scores was 1.6-fold higher than for high GST scores ($P = 0.038$). For relapsed patients, the low
Fig. 1 GST immunohistochemistry in representative head and neck tumors. A, locally relapsed laryngeal cancer postlaryngectomy radiation therapy. GST score, 0. This patient had a complete response to CDDP/5FU. B, another locally relapsed laryngeal cancer postlaryngectomy radiation therapy. GST score, 3. This patient had progressive disease despite CDDP/5FU. C, primary laryngeal cancer. GST score, 1. This patient had a complete response to induction chemotherapy with CDDP/5FU. D, another primary laryngeal cancer. GST score, 2. This patient had a minor response to induction chemotherapy and wound up having a salvage laryngectomy after two cycles of CDDP/5FU. E, hyperplastic mucosa adjacent to the tumor shown in C. This photograph demonstrates the strong GST staining present in nonmalignant mucosa. F, primary T2N2M0 laryngeal cancer, 1992. GST score, 1. The patient had induction chemotherapy with CDDP/5FU, achieving a complete response, followed by radiation therapy. G, the same patient shown in F at the time of local relapse in 1994. He underwent salvage laryngectomy at that time. Note focally increased GST staining of tumor cells. H, the same patient shown in F and G at the time of a second local relapse in 1995. GST score, 3. The patient was unresponsive to further CDDP/5FU at this time.
GST scores were associated with a 5.7-fold higher response rate than patients with high GST scores ($P < 0.01$).

GST score was weakly associated with tumor differentiation. Well-differentiated tumors generally had low GST scores, whereas poorly differentiated tumors had higher GST scores ($P = 0.045$).

Finally, after the initial analysis of the 56 cases was completed with the pan-GST isofrom antibody, 20 cases with an evenly distributed range of GST scores (0, 1, 2, or 3) were reanalyzed with an isozyme-specific antibody raised against GSTP. The blinded scores obtained with the GSTP antibody were identical to the pan-specific antibody in all but one case ($P = 0.002$).

**DISCUSSION**

In the present study, we demonstrate a strong inverse correlation between expression of GST and response to platinum-based chemotherapy in head and neck cancer.

The family of GSTs represents a series of distinct gene products. The genes encoding GST α, π, ω, and θ are located on 6p11 (24), 11q13 (25), 1p13 (26), and 22 (27), respectively. The predominant GST isofrom in tumor tissues (including head and neck cancers) is GSTP. In a recent analysis of paired normal/tumor specimens derived from laryngeal and oropharyngeal squamous cell cancers, GSTP was present in much higher concentration than GSTα or GSTω. GSTP levels were elevated compared with normal tissues in 11 of 14 oral tumors. In contrast, GSTα and GSTω levels were diminished in tumor tissues compared with normals (17). In the same study, mean glutathione levels were nearly 2-fold elevated compared with adjacent normal tissues. Although this study showed some expression of GSTα and GSTω in these lesions, which can be detected by the antibody used in this study, we presume that the signal detected in our tumor samples largely represents GSTP. Analysis of a subset of our cases with a GSTP-specific antibody showed essentially identical staining to that produced by the pan-isoform antibody, further supporting the notion that GSTP is the important isofrom in these samples.

Similar results have recently been reported in gastric cancer, where GSTα and GSTω were lower in tumor than in normal mucosa, and GSTP was elevated in a significant proportion of tumors. High tumor levels of GSTP were associated with poor prognosis (28).

GSTP maps to chromosome 11q13, an area of frequent amplification in head and neck cancers (29, 30). Recently, amplification of this locus was associated with significantly diminished survival in head and neck cancer patients (31). Subsequently, the same group reported that there was no clear association between amplification of 11q13 and overexpression of GSTP measured by immunohistochemistry (32). Only 2 of 24 tumors analyzed showed amplification of the GSTP locus, whereas 24 of 64 cases showed amplification at some other 11q13 locus. However, 55 of 64 specimens analyzed were positive for GSTP by immunohistochemistry using an isozyme-specific antibody different from the one selected for the present study. In that study, no significant correlation was found between GSTP expression and survival, which is in agreement with our data. However, the authors did not examine treatment response in that series of cases. The percentage of positive cases and distribution of scoring intensity for GSTP immunohistochemistry were similar to the results seen in our series.

Our data support the hypothesis that constituents of the glutathione pathway may play a significant role in determining resistance to platinum-based chemotherapy in this family of tumors. It is not possible from these data to determine whether GST has a direct role in inhibiting the action of cisplatin in these tumors or whether it is a marker of some other cellular event that is more immediately responsible for cisplatin resistance.

The assay system used in this study is rapid, inexpensive, and uses commercially available reagents on routinely fixed and processed tumor tissue. The scoring system is simple and appears to have reasonable interobserver reproducibility. For all these reasons, it merits further study as a possible tool to help guide selection of chemotherapy.

The results appear to have the greatest potential utility for the group of relapsed patients. The response rate for patients in this subset with low GST scores was 70%, whereas the response rate for patients with high scores was only 8.3%. These sort of predictive data may ultimately be very helpful for treating physicians to advise patients more accurately before undertaking the morbidity and expense of this chemotherapy. Given the very low response rate in tumors with high GST levels, these patients may either elect not to receive chemotherapy or elect to be treated on a clinical trial.

In patients being treated with neoadjuvant chemotherapy, the response rate was 100% for patients with low GST scores. However, because a significant subset (4 of 9) of patients with high GST scores had partial or complete responses to therapy, larger sample numbers will be needed before we can determine what predictive value, if any, GST scoring may have in this group.

The data presented here also compare favorably with other
recent literature on prognostic indicators in head and neck cancer. The patient cohort from the Veterans Affairs Laryngeal Cancer Group Study was examined retrospectively for expression of p53 in an effort to determine whether expression of a mutated form of this tumor suppressor gene corresponded with treatment response or survival. Overexpression of p53 was seen in 61% of patients examined. Expression did not correlate with response to chemotherapy in the patients who were randomized to receive neoadjuvant treatment or in overall or disease-free survival in either the surgery or chemotherapy arms. However, elevated p53 expression was associated with an increased likelihood of successful larynx preservation in the chemotherapy arm (74% versus 52.5%, P = 0.03; Ref. 33). This finding is somewhat surprising, because increased expression of mutant p53 has been associated with advanced stage and poorer prognosis in head and neck tumors (34), as well as other malignancies. It may indicate a difference in the ability of tumor cells to resist and/or repair radiation damage.

The same group reported that response to induction chemotherapy in patients with advanced larynx cancer was higher in patients with elevated cellular DNA content measured by a microscopic image analysis technique. Patients with low tumor cell DNA content (diploid tumors) had poor response to chemotherapy, but no significant difference in survival or larynx preservation compared with patients with high tumor cell DNA content (aneuploid tumors; Ref. 35). Direct measurements of tumor cell kinetics may also permit prediction of response to radiation therapy. Corvo et al. (36) reported analysis of tumor cell potential doubling time, which was calculated by analysis of S-phase fraction and duration of S phase in biopsies obtained after the patient received bromodeoxyuridine to label DNA. Patients with a prolonged tumor cell doubling time showed enhanced local control and survival compared with patients with a short tumor potential doubling time.

GST expression in these tumors in part may reflect an adaptive cellular response to stress, as has been described for the family of heat shock proteins. Elevated heat shock protein levels have been associated both with resistance to chemotherapy and diminished survival (37, 38). Elevated GST expression may reflect the intact ability of the tumor cell to respond to cytotoxic injury from agents such as cisplatin.

Other markers of response to cellular injury may also predict response to therapy. Head and neck tumor patients who responded to chemotherapy treatment showed a significant induction of mRNA expression for both c-jun and gadd153, whereas patients who did not respond to treatment showed no change in steady-state mRNA levels (39). These two markers may indicate the presence of an unrecoverable injury following exposure to cytotoxic stress.

At the present time, treatment and prognosis in head and neck cancer are based almost entirely on clinical staging. As the biochemical and molecular mechanisms that determine response to therapy and survival are better defined, patients will receive treatments that more accurately reflect the nature of their tumors. Specific biochemical manipulations may enhance treatment efficacy, and better selection of treatment options will spare patients some of the considerable morbidity and cost of ineffective therapy.

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