Glutathione S-Transferase π Amplification is Associated with Cisplatin Resistance in Head and Neck Squamous Cell Carcinoma Cell Lines and Primary Tumors

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Abstract

Purpose: The purpose is to evaluate the association of glutathione S-transferase π (GST-π) amplification and cisplatin resistance in head and neck cancer.

Experimental Design: An analysis of chromosomal abnormalities in 10 head and neck cancer cell lines by comparative genomic hybridization was performed. GST-π amplification and expression were evaluated in head and neck cell lines and paraffin-embedded tissue by fluorescence in situ hybridization (FISH) and immunohistochemistry.

Results: Changes in the DNA copy number were seen in all 10 cell lines by comparative genomic hybridization. The most frequent chromosomal alterations were: gain at 3q; loss at 3p; gain at 8q; loss of 18q; gain at 20q; and gain of 11q11-13. Using FISH, 9 of 10 cell lines showed increased GST-π copy number. GST-π amplification was detected in 7 of 10 cell lines. Five were relatively cisplatin resistant, and 2 were relatively cisplatin sensitive (median IC50, 11.2 and 2.75 μg/mL). Two relatively cisplatin-sensitive cell lines showed GST-π gain and another relatively cisplatin-sensitive cell line had predominantly two copies of the gene. In 10 tumor specimens, 4 had two copies of GST-π. All 4 had a complete response to neoadjuvant chemotherapy, 3 of whom are alive >50 months from treatment compared with 2 patients showing GST-π amplification. Neither responded to chemotherapy, and both died of disease <9 months from diagnosis.

Conclusions: Using FISH, GST-π amplification is a common event in head and neck squamous cell carcinoma and may be associated with cisplatin resistance and poor clinical outcomes in head and neck cancer patients treated with cisplatin-based therapy.

Introduction

CDDP is the most important chemotherapeutic agent involved in the treatment of HNCAs, and despite excellent responses in previously untreated patients (1), patients treated for relapsed or recurrent disease usually have only a 20–30% response rate (2). CDDP resistance in HNCAs may be mediated by a number of different mechanisms, including drug detoxification, up-regulation of DNA repair enzymes, and overexpression of antiapoptotic proteins such as bcl-2 and detoxifying enzymes such as GST-π (3). GST-π, located on chromosome 11q13, is a member of a family of isozymes (α, μ, π, and θ) that plays an important role in the detoxification of many xenobiotic substances through conjugation to glutathione [GSH]; Refs. 4 and 5]. Chromosome 11q13 amplification has been reported in a variety of malignancies including lung, esophagus and head and neck (6–8). Amplification of 11q13 has been detected using a number of techniques, including CGH and FISH. Increased copy numbers of 11q13 have been seen in as much as 50% of HNCAs by CGH (9).

In addition to the GST-π gene, several other genes are located on 11q13, including the cyclin D1 gene (CCND1), the gene containing the BCL-1 translocation breakpoint found in many B-cell lymphomas, the FGF-3 and FGF-4 genes, and the EMS-I gene (10–13). GST-π overexpression has been associated with increased resistance to various chemotherapeutic agents (5). We have previously shown that in patients with advanced HNCA, elevated expression of GST-π measured by immunohistochemistry has been associated with a poor response to CDDP-based chemotherapy and that GST-π overexpression is relatively common, especially in relapsed tumors (14, 15). In contrast, GST-π amplification has rarely been reported in HNCA (8, 10–13). Gaffey et al. (10) examined GST-π amplification by Southern blot analysis and found amplification in only 3% (2 of 64) of patients, whereas GST-π expression was seen in 86% (55 of 64) of patients by immunohistochemistry. Using Southern blot analysis, Yellin et al. (11) did not see any amplification of GST-π and found no correlation between expression and activity of GST-π and CDDP sensitivity in 14 head and neck cell lines. Wang et al. (13) also used Southern blot analysis to examine expression and amplification of GST-π in primary squamous cell carcinomas of the head and neck. Only 3 of 36 tumors (8%) showed GST-π gene amplification.

The present study was undertaken to characterize chromosomal abnormalities in head and neck cell lines of known CDDP responsiveness using CGH. In addition, FISH analysis and immunohistochemistry were used to examine gene amplification and protein expression of GST-π both in the cell lines, as well as in a group of pretreatment biopsy specimens from HNCA patients treated with CDDP and 5FU for advanced disease. The goal of the study was to correlate these findings with response to CDDP therapy to identify any genetic similarities and differences between cell lines exhibiting varying degrees of sensitivity to CDDP.

Materials and Methods

Patient Selection. Ten patients previously treated at Georgetown University Hospital with neoadjuvant CDDP/5FU for advanced (stages II, III, or IV) HNCA were selected for analysis. All patients received neoadjuvant chemotherapy for unresectable disease or organ preservation therapy after initial biopsy. Six of the patients were responsive to CDDP-based therapy and 4 were not. Patient characteristics are described in Table 2.

Cell Line Selection and Growth Assays. Ten HNCA cell lines were studied. The JSQ-3 HNCA cell line was provided by Dr. Esther Chang (Lombardi Cancer Center, Washington, DC); SCC4, SCC9, and SCC25–2, isolated by Dr. James G. Rheinwald, and FaDu, isolated by Dr. S. R. Ragan, were obtained from the Lombardi Cancer Center Tissue Culture Shared Resources Facility (Washington, DC) and maintained in 1:1 DMEM:Ham’s F-12, supplemented with 10% fetal bovine serum (FBS) and 400 ng/mL hydrocortisone. Five cell lines [HN 17B (SCC17B), HN 22A (SCC22A), PCI 13, SCC25–1, and SCC25CP] have been described previously (16). CDDP cytotoxicity assays were carried out as reported previously (16). All assays were performed in triplicate.
GSH Assay. Total intracellular GSH was determined using a modification of the enzymatic method of Tietze (17). A standard curve was run using purified GSH (Sigma, St. Louis, MO). Total intracellular GSH was normalized to 25 μg of protein. All assays were carried out in triplicate.

Western Blot Assay. Cell lines to be analyzed were grown to 80% confluence. Total protein was extracted using a cell lysis buffer and quantitated using a modification of the Bradford method, with BSA as a standard. Thirty μg of protein were applied per lane to a NuPAGE 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). The blot was probed for the GST-π protein using a commercially available mouse monoclonal antiserum (Novocastra, Peterborough, United Kingdom) at a 1: 500 dilution, followed by a goat antirabbit antibody conjugated to horseradish peroxidase (1:20,000; Amersham, Buckinghamshire, United Kingdom).

CGH. CGH was performed as described previously (18). In brief, normal control DNA was prepared from peripheral blood lymphocytes of a normal donor, and test DNA was extracted from the cultured cell lines using standard protocols. Nick translation was performed to label the test DNA with biotin-16-dUTP (Roche, Indianapolis, IN) and control DNA with digoxigenin-11-dUTP (Roche). Five hundred ng of each of the labeled genomes were hybridized in the presence of excess Cot-1 DNA (50 μg; Invitrogen) to metaphase chromosomes prepared from a normal donor. The biotin-labeled tumor genome was visualized with avidin conjugated to FITC (Vector Laboratories, Inc., Burlingame, CA), and the digoxigenin-labeled control DNA was detected with a mouse antidigoxigenin antibody (Sigma-Aldrich, St. Louis MO) followed by detection with a goat antiantibody conjugated to tetramethylrhodamine isothiocyanate (Sigma-Aldrich). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and embedded in antifading agent to reduce photobleaching. Gray scale images of the FITC-labeled tumor DNA, the tetramethylrhodamine isothiocyanate-labeled control DNA, and the DAPI counterstain were obtained at least eight metaphase spreads from each hybridization were acquired with a cooled charge-coupled device camera (CH250: Photometrics, Tucson, AZ) connected to a Leica DMRBE microscope equipped with fluorescence-specific optical filters TR1, TR2, TR3 (Chroma Technology, Brattleboro, VT). Quantitative evaluation of the hybridization was done using commercially available software (Applied Imaging, Pittsburgh, PA). Average ratio profiles were computed as the mean value of at least eight ratio images to identify chromosomal copy number changes in all cases.

FISH. A GST-π-specific BAC clone was obtained from BACPAC Resources (Oakland, CA) for use as a FISH probe. BAC clone DNA was prepared and labeled with biotin-11-dUTP (Roche) using nick translation as described previously (19). Biotin-labeled DNA was detected with fluorescein-Avidin (Vector Laboratories, Burlingame, CA), and the digoxigenin-labeled control DNA was detected with a polyclonal antibody (Novocastra) has been described previously (14, 15). A positive control tissue was included in each set of sections stained: normal kidney known to be positive for GST-π was used. In negative controls, PBS with 1% BSA and 1% sodium azide replaced the primary antibody solution. Immunohistochemical staining was assessed by at least two independent observers, each of whom was blinded to patient identity and outcome. Criteria for positive staining for GST-π expression have been described previously (14, 15).

Statistical Analysis. Total cellular GSH, GST-π protein expression, and GST-π gene amplification were compared with the IC_{50} for the 10 head and neck cell lines. Statistical significance was calculated using GraphPad Prism Statistical Software (version 3.02, San Diego, CA).

Table 1: Cisplatin response along with GST-π FISH analysis, CGH at 11q13, GST-π protein levels and total intracellular glutathione levels in cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin mean IC_{50} (μM)</th>
<th>GSH mean level (nmol/mg protein)</th>
<th>11q13 gain by CGH</th>
<th>Relative GST-π gene expression (densitometry)</th>
<th>GST-π FISH</th>
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<tbody>
<tr>
<td>PCI 13</td>
<td>0.25</td>
<td>12.3</td>
<td>No</td>
<td>1</td>
<td>2 copies</td>
</tr>
<tr>
<td>HN 22A</td>
<td>2</td>
<td>25.8</td>
<td>No</td>
<td>1.7</td>
<td>Gain</td>
</tr>
<tr>
<td>HN 17B</td>
<td>2.25</td>
<td>34.5</td>
<td>No</td>
<td>3.6</td>
<td>Gain*</td>
</tr>
<tr>
<td>SCC 25-2</td>
<td>2.5</td>
<td>17.2</td>
<td>Yes</td>
<td>2.2</td>
<td>Amplified**</td>
</tr>
<tr>
<td>SCC 4</td>
<td>3</td>
<td>8.4</td>
<td>Yes</td>
<td>2.2</td>
<td>Amplified**</td>
</tr>
<tr>
<td>SCC 25-1</td>
<td>6</td>
<td>32.6</td>
<td>Yes</td>
<td>2.1</td>
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</tr>
<tr>
<td>FaDu</td>
<td>6</td>
<td>181.7</td>
<td>Yes</td>
<td>4.2</td>
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</tr>
<tr>
<td>JSQ-3</td>
<td>13</td>
<td>144.5</td>
<td>Yes</td>
<td>3.8</td>
<td>Amplified**</td>
</tr>
<tr>
<td>SCC 9</td>
<td>14</td>
<td>17.8</td>
<td>No</td>
<td>1.8</td>
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<tr>
<td>SCC25/CP</td>
<td>17</td>
<td>53.2</td>
<td>Yes</td>
<td>2.1</td>
<td>Amplified**</td>
</tr>
</tbody>
</table>

Results

Cytotoxicity Assays and GSH Levels in Head and Neck Cell Lines. Total GSH expression and CDDP sensitivity data are shown in Table 1. There was variable CDDP sensitivity among the 10 cell lines, with a ~70% fold difference between the most sensitive (PCI 13) and the most resistant (SCC 25CP) cell lines. In addition, cell lines showed relatively more resistance to CDDP (such as SCC 25CP and JSQ-3) were associated with higher GSH levels than more sensitive cell lines (such as PCI-13 and HN22A). The average GSH level of the more sensitive cell lines was 19.64 nmol/mg protein, compared with an average of 89.42 nmol/mg protein for the relatively more resistant cell lines.

CGH, GST-π FISH Analysis, and GST-π Western Blot for Head and Neck Cell Lines. CGH analysis revealed changes in the DNA copy number in all 10 cell lines. Overall, chromosomal gains were twice as frequent as losses. The most frequent chromosomal alterations observed were: gain at 8q (minimal overlapping region 8q23-qter; 8 cell lines); gain at 20q (minimal overlapping region 20q13; 8 cell lines); gain at the 3q region (minimal overlapping region 3q26-qter; 7 cell lines); loss at 3p (minimal overlapping region 3p13; 7 cell lines); gain of 11q1-q13 (6 cell lines); loss of 18q (6 cell lines); and loss at 8p (minimal overlapping region 8p22-pter; 5 cell lines). Changes in the cell lines were relatively similar regardless of CDDP sensitivity, except for the gain at 9q, which occurred in 4 of the 5 more sensitive lines versus 1 of the 5 relatively resistant lines, and the loss of 3p, which occurred in all of the 5 more sensitive cell lines and only in 2 of 5 relatively resistant ones. The karyogram of chromosome gains and losses observed in all of the cell lines analyzed is summarized Fig. 1.

FISH analysis using a GST-π-specific probe showed increased copy number in 9 of the 10 cell lines (Table 1). Clear amplification of the GST-π gene was detected in 7 of the 10 cell lines, where the majority of cells had four or more copies of the gene. Of these 7 cell lines with GST-π amplification, 5 had a CDDP IC_{50} ≥ 6 μM (SCC9, HN 22A, SCC 4, SCC 25-2, and PCI 13 (IC_{50} ≤ 6). Relative GST-π expression is determined based on assigning the lowest GST-π expressing cell line (PCI 13) a value of 1 relative to expression of the control protein β-actin.
All 4 patients with two copies of the GST-π gene had a complete response to neoadjuvant chemotherapy. Three of those 4 patients are alive >50 months from treatment. One patient relapsed and died >2 years from his initial therapy. Of note, all 4 patients in the nonresponder group showed gain or amplification of GST-π by FISH, whereas only 2 of 6 patients in the complete responder group showed gain and none showed amplification (Table 2, \( \chi^2, P < 0.05 \)).

Four of 4 patients in the nonresponder group with gain or amplification showed increased GST-π expression, with an intensity score of 2 compared with 1 of 2 patients exhibiting gain in the complete responder group. Examples of GST-π FISH and immunohistochemistry in complete responders and nonresponders are shown in Fig. 3.

Discussion

The reasons patients fail chemotherapy are unclear. Given the cost and morbidity associated with all forms of therapy for HNCA, the ability to properly triage patients to the most appropriate treatment protocol would be invaluable. Because CDDP is the most commonly used agent in treatment of HNCA, understanding the molecular mechanisms that lead to resistance may provide potential targets for intervention.

In the present study, we investigated the relationship of chromosomal abnormalities, GST-π amplification, and GST-π expression to CDDP sensitivity. We used CGH to detect chromosomal aberrations in 10 head and neck cell lines of known CDDP response. We found that 6 of 10 HNCA cell lines (60%) showed increased copy number at 11q13, the locus where GST-π maps. Increased copy numbers of 11q13 occur frequently in HNCAs and may be associated with a more aggressive phenotype (9, 21). Similar to other studies, several cell lines also showed gains on 3q, 5p, 8q, and 20q and losses on 3p, 8p, 9p, and 18q (22). We observed a similar pattern of chromosomal changes in all of the cell lines regardless of CDDP sensitivity, except for the gain at 9q, which occurred in 4 of the 5 more sensitive cell lines versus 1 of the 5 more resistant cell lines, and the loss of 3p, which occurred in all 5 of the more sensitive cell lines and in only 2 of 5 more resistant ones.

One of the mechanisms by which chemotherapy resistance may occur is by gene amplification or the overexpression of gene products (such as GST-π) that provide a tumor cell with survival advantage relative to normal cells. In the past, other studies of 11q13 amplification in HNCAs have focused on the amplification of the cyclin D1 gene (CCND1) and other genes more centromeric than GST-π. CCND1 amplification and cyclin D1 overexpression are commonly accepted to be associated with tumor progression, higher tumor grade, increased rates of recurrence, and decreased survival in HNCA studies (23, 24). The role of GST-π gene amplification is less clear. We have previously reported that expression of GST-π correlates with prognosis and response to therapy in HNCA patients (14, 15). In an analysis of >50 patients with advanced HNCA treated with CDDP-based neoadjuvant therapy for unresectable disease or organ preservation therapy (14, 15), high GST-π expression was associated with poor overall survival. GST-π expression was also associated with a lack of response to therapy, with high GST-π-expressing tumors having a response rate of 19% compared with 87% for low GST-π-expressing tumors (relative risk = 4.7, \( P = 0.001 \)). Although convincing evidence for chromosomal gain at 11q13 has been demonstrated by several groups using different approaches, the few available studies looking specifically at GST-π amplification (none of which used FISH) have concluded that this is a rare event and failed to show any important clinical correlation between amplification of the gene and/or protein expression (8, 10–13).

In the present study, we found that 9 of 10 cell lines showed either
**GST-**π gain (2 cell lines) or frank amplification (7 cell lines) by FISH. This correlated well with the patient data presented here, with 60% of the specimens examined showing either gain or amplification using the same GST-**π**-specific BAC clone. Given the frequency with which 11q13 amplification has been seen in HNCA, it is unclear why previous studies have failed to show a higher incidence of GST-**π** amplification. One reason for the discrepancy between these results and those of previous studies may involve the different techniques used to detect amplification. Previous studies assessing 11q13 amplification and GST-**π** amplification in HNCA have used Southern blot analysis, which may be inadequate in its ability to detect subtle differences in gene amplification secondary to stromal contamination.

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**Table 2** **GST-**π** evaluation by FISH, GST-**π** expression, and clinical response to CDDP-based chemotherapy in primary tumors**

Immunohistochemical analysis was performed using a polyclonal antibody to GST-**π** and FISH analysis using a GST-**π** BAC clone. NED, no evidence of disease; DOD, dead of disease; CR, complete response; NR, no response.

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Primary site</th>
<th>Stage</th>
<th>Therapy</th>
<th>Response</th>
<th>Status</th>
<th>Overall survival (mo.)</th>
<th>Relapse-free survival (mo.)</th>
<th>GST-<strong>π</strong> IHC scoring</th>
<th>GST-<strong>π</strong> FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hypopharynx</td>
<td>4</td>
<td>CDDP/5FU</td>
<td>CR</td>
<td>NED</td>
<td>20</td>
<td>20</td>
<td>2</td>
<td>2 copies</td>
</tr>
<tr>
<td>2</td>
<td>Supraglottic</td>
<td>3</td>
<td>CDDP/5FU</td>
<td>CR</td>
<td>NED</td>
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<td>33</td>
<td>0</td>
<td>Gain</td>
</tr>
<tr>
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<td>Tonsil</td>
<td>2</td>
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<td>CR</td>
<td>NED</td>
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<td>31</td>
<td>2</td>
<td>Gain</td>
</tr>
<tr>
<td>4</td>
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<td>CR</td>
<td>DOD</td>
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<td>Amplification</td>
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</tr>
<tr>
<td>10</td>
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<td>CDDP/5FU</td>
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<td>DOD</td>
<td>11</td>
<td>11</td>
<td>2</td>
<td>Amplification</td>
</tr>
</tbody>
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Fig. 2. FISH analysis using a GST-**π** probe of cell lines PCI 13 (Fig. 2A) and SCC25/CP (Fig. 2B). Both figures show metaphase (left) and interphase (right) results. FISH analysis of the PCI 13 line (relatively cisplatin sensitive) shows two copies of the gene. SCC25/CP is a squamous cell carcinoma cell line that has been developed to be relatively resistant to cisplatin. FISH analysis shows amplification of the gene in this line.
in tissue samples. In contrast, FISH is a very sensitive and rapid method for detecting genetic abnormalities associated with malignancy and has been shown to detect gene amplification even when Southern blotting has not (25). Using FISH, we have been able to effectively detect frequent variances in the GST-π gene copy number at a cell by cell level.

Although squamous cell carcinoma of the head and neck is histologically the most common type of cancer of the upper aerodigestive tract, it is very clear clinically that squamous cell carcinomas behave differently depending on the primary site. It is reasonable to assume that some of these differences may be mediated by specific genetic alterations and may be detectable by CGH. Site-specific genetic abnormalities have been identified for the oral cavity, pharynx, and larynx and are associated with higher tumor grade and nodal spread (22). We did not see any specific subsite associated with GST-π amplification or increased expression in our pilot study group, although this may be a result of sample size. Additional investigation is warranted.

Among the head and neck cell lines, there was not a consistent correlation between GST-π amplification and expression as detected by Western blot analysis (Table 1). This finding is similar to what has been reported by other groups for HNCA (11, 14). Reasons for this are unclear. Enzyme activity may be important in mediating resistance in pathologic samples (23). Recent data have shown that the activity may play a role in mediating CDDP sensitivity (26). We saw a better correlation in clinical tumors between GST-π expression and GST-π activity.

In conclusion, GST-π amplification is a frequent event in HNCA cell lines and tumors when studied using FISH and is associated with increased expression in tumors poorly responsive to CDDP-based chemotherapy. Total cellular GSH expression and total cellular GST-π expression are not predictive of CDDP sensitivity in head and neck cell lines. However, given the frequency of GST-π amplification detected by FISH, additional investigation of potential mechanisms is warranted.

References


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