Immunohistochemical Determination of Glutathione S-transferases in Gastric Carcinomas and in Adjacent Normal Gastric Epithelium

D. LUCETTE SCHIPPER1,2, MURIEL J.M. WAGENMANS1,2, URBAN VAN HAELEST3, WILBERT H.M. PETERS2, THEO WOBSES4, ALBERT A.J. VERHOFSTAD3, WIL P.H. LANGE3, D.J. THEO WAGENER1

1Departments of Medical Oncology, 2Gastroenterology, Pathology and 4Surgery. University Hospital Nijmegen, The Netherlands

Abstract. Glutathione S-transferases (GSTs) are a family of isoenzymes that play an important role in protecting cells against cytotoxic and carcinogenic agents.

The distribution and levels of GST Alpha and Pi in normal and malignant gastric tissue of 34 patients with gastric cancer were examined immunohistochemically. Expression of GST Alpha and Pi was observed in 47 and 100 percent of the tumors, respectively. In normal mucosa both enzyme classes were present in 100 percent of the specimens. Mucous cells showed staining for GST Alpha and Pi in 88 and 97 percent, parietal cells in 93 and 67 percent, and chief cells in 82 and 30 percent, respectively. No correlation was observed between the amount or pattern of GST Alpha or Pi in carcinomas and the clinical and pathological characteristics of the patients. So it can be concluded that both GST Alpha and Pi cannot be considered as prognostic factors for gastric cancer.

The incidence and mortality associated with gastric adenocarcinoma has decreased in many countries during the past five decades (1). However, despite new diagnostic and therapeutic techniques, the 5-year survival rate of patients with advanced stage of gastric adenocarcinoma continues to be poor (2).

Two main histological types of gastric carcinoma were characterized by Lauren (3), the intestinal type, resembling small bowel mucosa; and the diffuse type, infiltrating the stomach wall. The diffuse cancer has a poorer prognosis and is more common in women and younger patients (4). Biotransformation enzymes, and in particular glutathione S-transferases (GSTs) are present in most epithelial tissues of the human gastrointestinal tract (5-8). Their presumptive function is to protect tissues against toxic or carcinogenic compounds, entering the body as food components, food additives or drugs (5,9). Four classes of cytosolic GST isoenzymes exist in man: Alpha, Mu, Pi and Theta (10,11). Expression of class Alpha and Pi enzymes is different in the various tissues. For instance, class Alpha enzymes are present in high levels in the liver (12,13), stomach (14) and small intestine (15), while class Pi enzymes are expressed in many organs, other than the adult liver (16,17). Increased expression of class Pi enzymes has been reported in a wide variety of human tumors, compared to the normal surrounding tissue (17-21). Interestingly, GST Pi expression in patients with node negative breast cancer (22) has been reported as a prognostic factor recently. To better understand the role of GSTs in gastric cancer and to study whether they have a predictive value, immunohistochemical expression of GST class Alpha and Pi was studied in gastric carcinomata and adjacent normal mucosa, in relation to patient and tumor characteristics, such as tumor type, stage, and the length of survival.

Patients and Methods

Patients and tumor samples. Tumor specimens and normal gastric tissue from 34 patients, who underwent primary surgery for gastric cancer between 1985 and 1989, were included in this study. Patient data are summarized in Table I. Tumor stage was classified according to the criteria of the American Joint Committee on Cancer (23). Selection for this study was based on the following criteria: formalin-fixed, paraffin-embedded specimens with both assessable tumor and normal mucosa must be available, patients must have undergone primary
Table 1. Patient characteristics (n = 34).

<table>
<thead>
<tr>
<th>Category</th>
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</tr>
<tr>
<td>Female</td>
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<tr>
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<td>Survival (months)</td>
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<td>Median</td>
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<tr>
<td>Range</td>
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surgery, and clinical information of status at presentation and follow-up must be available. The study was approved by the local ethical committee on human experimentation.

Immunohistochemical staining. From each specimen three 4 μm thick slices were used: one for standard haematoxalin eosin staining and two for immunohistochemical investigation of GST class Alpha and Pi. For immunohistochemical assays, sections of formalin-fixed paraffin-embedded tissue were dewaxed in xylol, rehydrated in ethanol and immersed in methanol with 2% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Subsequently the sections were preincubated with phosphate-buffered-saline (PBS) containing 4% bovine serum albumin (BSA; Boehringer, Mannheim, Germany) and 0.1% Triton X-100 (BDH Chemicals Ltd., Poole, England) to block nonspecific binding. The slides were incubated overnight at 4°C with primary antibodies against GST class Alpha (monoclonal antibody), as developed by us recently (Peters et al., 1992), diluted 1:5000 in PBS containing 4% BSA and 0.1% Triton X-100 (buffer A) and GST class Pi (polyclonal antibody, Biotrin International, Dublin, Ireland) diluted 1:2400 in buffer A. Subsequently a 45 minute incubation period at room temperature with peroxidase conjugated rabbit-anti-mouse immunoglobulin (Dakopatts) diluted 1:100 in buffer A or peroxidase conjugated swine-anti-rabbit immunoglobulin (Dakopatts) diluted 1:400 in buffer A was performed for GST Alpha or Pi immunodetection, respectively. In order to enhance the intensity of the final staining a third incubation step was used: peroxidase conjugated swine-anti-rabbit immunoglobulin (Dakopatts) diluted 1:40 in buffer A for GST class Alpha and peroxidase conjugated rabbit-anti-mouse immunoglobulin (Dakopatts) diluted 1:100 in buffer A for GST class Pi. Staining was performed using 0.1% 3,3'-diaminobenzidine (Sigma Chemical Company, St. Louis, MO, USA) in PBS containing 0.01% hydrogen peroxide as peroxidase substrate. The slides were counterstained with haematein. Between each step the sections were washed three times each for 5 minutes in PBS.

Human liver tissue and human colon tissue were used as a positive control for GST Alpha and Pi, respectively. Omission of primary antibodies served as negative controls.

Scoring. Both the intensity of staining and the proportion of stained cells were scored by three independent individuals. The intensity of staining was graded as follows: (-) negative, (+) weakly positive, (++) moderately positive, (+++) strongly positive. The proportion of cells showing staining was scored as follows: (0) <1 percent stained cells, (1) 1-5 percent stained cells, (2) 6-25 percent stained cells, (3) 26-50 percent stained cells, (4) 51-75 percent stained cells, (5) >75 percent stained cells. The distribution of staining was assessed by scoring tumor cells, mucous cells, parietal cells and chief cells, separately.

Statistics. Correlation between parameters was evaluated using the Spearman rank correlation test. In order to evaluate the differences in expression of the various cell types Friedman two-way Anova was used. In case of significant results (p < 0.05) it was followed by the sign test. Kaplan Meier survival functions were constructed, and the relation between survival and expression of GST Alpha and Pi was analyzed using the generalized Wilcoxon test. Localization of the tumor, age, tumor differentiation grade and Lauren classification were included as covariates in the analysis.

Results

GST Alpha. The results of scoring the proportion of cells staining for GST Alpha are summarized in Table II while intensity of staining is reported in Table III.
GST Alpha was present in 16 carcinomas (47 percent). Staining was generally focal and cytoplasmatic with staining of more than half the tumor cells in only 8 cases (Figure 1a).

The adjacent normal mucosa showed positive immunoreactivity in all cases (Figure 1b). Mucous cells were present in 33 specimens. Positive immunostaining of mucous cells was seen in 29 specimens (88 percent). In 20 sections (61 percent) more than half of the mucous cells gave positive staining. Staining was predominantly cytoplasmatic and localized both in surface epithelium and in cells localized deeper within the crypts. In areas with intestinal metaplasia all affected cells showed very strong immunoreactivity (Figure 1c). Parietal cells were seen in the normal mucosa of 28 cases. In 26 cases (93 percent) they showed diffuse cytoplasmatic and nuclear positivity for GST Alpha. Strongly positive immunoreactivity with dark staining of more than half the parietal cells was present in 21 cases (75 percent).

Chief cells were present in 28 sections and positive staining was observed in 23 sections (82 percent), with a generally weak to moderate strong cytoplasmatic staining pattern. Staining of more than 50 percent of the chief cells could be observed in only 7 cases (25 percent).

Intensity of staining for GST Alpha and proportion of cells stained was significantly higher in parietal cells and in mucous cells than in chief cells and tumor cells (p < 0.05).

Connective tissue components (i.e. collagen, muscle etc.) were consistently negative.

GST Pi. Results of scoring the proportion of cells, staining for GST Pi are summarized in Table II, while intensity of staining is given in Table III.

In all cases carcinoma showed positivity for GST Pi. Staining was predominantly cytoplasmatic, with additional nuclear staining in some cells (Figure 1d). Staining intensity for GST Pi showed heterogeneity, showing tumor cells either negative, or positive with moderate to high intensity within the same tumor. In 28 cases (82 percent) more than half of tumor cells were positive.

Normal mucosa showed positive immunoreactivity in all cases. Mucous cells were positive in all sections in which they were present. In 32 cases (97 percent) more than half of the mucous cells were positive. The surface epithelium was strongly positive, while mucous cells located deeper within the crypts showed less intense staining (Figure 1e). In sections with intestinal metaplasia all affected cells showed immunoreactivity (Figure 1f).

Parietal cells stained positive in 18 of the 27 sections in which they were present (67 percent). Staining was generally weak to moderate, with more than 50 percent positive in only 2 cases. Chief cells gave a weak immunoreactivity in only 8 of the 27 sections (30 percent) in which they were present.

Intensity of staining for GST Pi and the proportion of cells stained was significantly higher in mucous cells and in tumor cells than in chief cells and parietal cells (p < 0.05). Connective tissue components (i.e. collagen, muscle etc.) were consistently weakly positive. Plasma cells and lymphocytes showed strong nuclear and cytoplasmatic positivity.

### Correlation between GST Alpha or Pi expression with clinical and pathological findings

In Table IV and V the clinical and pathological characteristics of the tumors are compared with the proportion of tumor cells with detectable staining for GST Alpha and Pi, respectively. There was no significant correlation between expression of GST Alpha or Pi and clinical stage, tumor differentiation and Lauren classification of the tumor (all p < 0.2). In addition, expression of GST Alpha or Pi in the tumor was not related with length of survival.

### Discussion

Gastric tumors are known to contain GST activity and previous studies have indicated that most of them express GST Pi and to a lesser extent GST Alpha (14,17). However these studies were done on tissue homogenates which inevitably contain non-neoplastic elements such as stroma and which may even contain normal mucosa. Immunohistochemical analysis of GST Pi in gastric carcinoma was performed by Tsutsumi et al (24). They described high levels of GST Pi in all except for signet ring cell carcinomas. In our study GST Pi was detected in 100 percent of gastric tumors, whereas GST Alpha was found in only 47 percent. In many tumors there was a high expression of GST Pi in the accompanying stromal cells and inflammatory infiltrate. Such observations emphasize the usefulness of immunohistochemical techniques in demonstrating the distribution of GST activity.
Figure 1. Immunostaining of GST Alpha and Pi in gastric adenocarcinoma and gastric mucosa. Space bar=1081 μm. A. Tumor cells (T), heterogeneously positive for GST Alpha in gastric carcinoma. B. Normal gastric tissue showing strong positivity for GST Alpha in mucous cells (M) and parietal cells (P), while chief cells (C) were moderately positive. C. Intestinal metaplasia showing strong immunoreactivity for GST Alpha. D. Diffuse staining for GST Pi in tumor cells (T). E. GST Pi expression in normal gastric tissue, showing intense positivity in mucous cells (M). F. GST Pi expression in intestinal metaplasia.
enzymes and other proteins in tissues and tumors, composed of a variety of different cell types.

Knowledge of the distribution of GST tissue may improve our understanding of their function. For example, what is the reason for the high expression of GST Alpha found especially in parietal cells, and of GST Pi in mucous cells of the surface epithelium? Furthermore, the biological significance of the heterogeneity in distribution of GST isoenzymes in gastric cancer is unclear. Intra-tumor variation of GSTs was also described in immunohistochemical studies on human carcinomas of the cervix (25), esophagus (8), breast (22) and kidney (26). This variation causes a complicating feature in the interpretation of GST expression. Until now there was no evidence concerning the heterogeneity of GST in gastric tumors. Since GST activity may be relevant for detoxification of antineoplastic drugs (18) the variability of GST expression suggests that different parts of the same tumors may have varying response to chemotherapy. In addition glutathione, the cofactor for GSTs (9), is known to have a 2-3 fold variation in concentration within a single tumor (27).

Immunohistochemical techniques also provide information on intracellular localization. Antibodies against GST Alpha and Pi gave both cytoplasmic and nuclear staining, which has also been described in immunohistochemical studies of GSTs in the esophagus (8), cervix (28) and breast (29). The significance and function of the nuclear localization is unclear and remains to be clarified.

In contrast to the results of Gilbert et al (22) for breast cancer patients, in patients with gastric carcinoma no apparent correlation of GST Alpha or Pi expression with clinicopathological features or survival could be detected. The high levels of GST Pi in all normal mucosa specimens investigated suggests that GST Pi expression is not directly related to malignancy in gastric cancer. However it should be noted that the normal gastric mucosa used in this study was obtained from patients with gastric carcinoma. Although these tissues were microscopically normal, it is possible that (part of) the tissue already has undergone changes in GST Pi expression. Therefore the examination of normal gastric tissue from patients without gastric diseases would help to
resolve this question. Further investigations are now been carried out to clarify the significance and function of the heterogeneous GST expression in normal gastric tissue and in gastric carcinoma, especially in relation to anti-cancer drug resistance.

Acknowledgements

This study was supported by grant NUKC 92-64 of the Dutch Cancer Society.

References


Received October 23, 1995
Accepted December 5, 1995

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