Glutathione and Glutathione S-Transferases as Early Markers for Ovarian Carcinomas: Case Series

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Aim. To determine the activity of glutathione (GSH) and concentrations of glutathione S-transferases (GST), urokinase type plasminogen activator (uPA), and plasminogen activator inhibitor type 1 (PAI-1), and to evaluate their diagnostic and prognostic value and possible correlation with clinical and histopathological prognostic factors for ovarian carcinomas.

Methods. The concentrations of GSH, uPA, PAI-1, and activity of GST were analyzed in 35 tissue samples taken from 10 normal ovaries, 10 benign, 10 primary malignant, and 5 metastatic ovarian tumors. The GSH level and GST activity were determined by spectrophotometric methods, and uPA and PAI-1 concentrations by ELISA commercial kits.

Results. GSH concentrations were significantly higher in primary malignant (126.3±12.8 nmol/mg protein) and metastatic (160.5±24.3 nmol/mg protein) ovarian tumor specimens than in normal ovarian tissue (48.9±8.1 nmol/mg protein, p<0.003 for both carcinoma groups) or benign ovarian tumor samples (35.2±5.0 nmol/mg protein, p=0.001). The GST activity was significantly higher in primary malignant (245.8±22.7 nmol/min/mg protein) and metastatic (303.7±48.8 nmol/min/mg protein) ovarian tumor tissues than in benign tumor specimens (105.9±16.2 nmol/min/mg protein, p=0.004 for both carcinoma groups) or normal ovarian tissue samples (133.2±32.0 nmol/min/mg protein, p=0.044 for both carcinoma groups). There were no statistical differences in uPA and PAI-1 concentrations between normal, benign, and malignant tumor samples. Concentrations of GSH, uPA and PAI-1, and activity of GST were independent from histopathological and clinical prognostic factors.

Conclusion. Increased GSH concentration and GST activity found in primary malignant and metastatic ovarian tumor samples were independent of histopathological and clinical prognostic factors, suggesting that they could be early markers for ovarian carcinomas.

Key words: carcinoma; glutathione; glutathione transferase; ovarian neoplasms; plasminogen activator inhibitor 1; urinary plasminogen activator

Ovarian cancer remains the leading cause of death among gynecological malignancies. Since no symptoms or signs are manifested at the early stages of the disease, it is no surprise that the vast majority of women are diagnosed after the cancer has spread to the peritoneum. The screening for early detection of the disease is not yet available, and the overall 5-year survival is low despite the advances in the conservative methods of treatment (invasive and noninvasive) (1-3). Although chemotherapy can improve long-term survival of patients with ovarian cancer, the major factor limiting the effectiveness of such treatment is the development of drug resistance (1,3-5). A better understanding of the molecular routes in normal ovarian tissues and in ovarian cancer would likely provide new approaches in diagnosis and therapy.

Intrinsic and acquired drug resistance is a complex phenomenon involving different molecular mechanisms (6,7), in which glutathione (GSH) may play an important role. GSH is the most abundant cellular thiol, essential for cellular homeostasis (8-10). It serves diverse physiological functions, such as detoxification of xenobiotics and protection of cells from oxidative stress. It also acts as a storage and transport form of cysteine and affects cellular thiol redox status. GSH may determine the response of tumor cells to various chemotherapy agents. Increased level of GSH may reduce cell-sensitivity to alkylating agents, platinum compounds, and anthracyclines in several ways (8,10-14), e.g., by catalyzed or non-catalyzed nucleophilic reaction of GSH with cytostatic or its metabolites in cytoplasm, or by detoxification of organic per-
oxides (8-10). GSH can combine with anticancer drugs to form less toxic and more water-soluble GSH conjugates. These conjugates can be exported from the cells by glutathione conjugate export pump or by multidrug resistance associated protein (15). In the nucleus, GSH may support the repair of the damaged DNA by maintaining functional repair enzymes or deoxyribonucleotide triphosphate pool size (16). Finally, GSH may act as a negative regulator of apoptosis (10,17).

Glutathione S-transferases (GST) are multifunctional enzymes that catalyze the conjugation of the glutathione with a variety of electrophilic compounds (including anticancer drugs), resulting in their inactivation and elimination from the body (18-21). The elevation of GST activity and changes in the expression of GST enzymes are frequently found in drug-resistant tumor cells (18-21).

Extracellular matrix degradation by tumor-derived proteases is an essential process for tumor cell invasion (22,23). Plasminogen activation cascade is one of the major proteolytic systems involved in this process.

Urokinase-type plasminogen activator (uPA) is a highly specific serine protease with an essential role in tumor invasion and metastasis (23-25). It converts plasminogen to plasmin, a trypsin-like protease, which degrades most components of the extracellular matrix directly or through the activation of some procollagenases. Inactive precursor of uPA is secreted as a soluble protein that binds to a high-affinity cell surface receptor. The zymogen form is converted by limited proteolysis to active uPA. Surface receptors focus uPA activity close to the plasma membrane, where invasive cells can use it. Since plasminogen also has a receptor on the cell surface, plasmin is generated at the cell-matrix interface, allowing localized pericellular proteolysis. In a variety of tumors, high levels of uPA are associated with poor prognosis, suggesting that uPA could be used as a prognostic factor (23,26).

Plasminogen activation is regulated by plasminogen activator inhibitors type-1 (PAI-1) and type-2 (PAI-2) (23,25,27,28). They are the members of the serine proteinase inhibitor (SERPIN) superfamily – specific, fast-acting inactivators, present in the most body fluids, tissues, and cell lines. Plasminogen activator inhibitors covalently bind to the active site of the receptor-bound and secreted plasminogen activator, neutralizing their activity. If exogenously added, PAI-1 (29) inhibits matrix protein degradation, invasion, and metastasis. Paradoxically, many clinical studies demonstrated that high rather than low PAI-1 levels predict poor prognosis for the survival of patients suffering from various cancers (25,27,30).

The aim of our study was to determine the activity of glutathione and concentrations of glutathione S-transferases, urokinase-type plasminogen activator, and plasminogen activator inhibitor type-1, and to investigate whether they may have diagnostic and prognostic values for ovarian cancers. Their levels were examined in normal ovarian tissue, benign, and malignant ovarian tumor specimens.

### Material and Methods

#### Tissue Samples

We analyzed 35 samples taken from 10 normal ovaries, 10 benign ovarian tumors, 10 primary ovarian carcinoma, and 5 metastatic ovarian carcinoma, obtained during surgical procedures at the Department of Obstetrics and Gynecology, Zagreb University Hospital Center, Zagreb, Croatia. Immediately after surgery, the samples were frozen in liquid nitrogen until biochemical analysis. Each patient received detailed information about the study and signed informed consent to participate. The Research Ethics Committee of the Zagreb University School of Medicine approved the protocol.

#### Tumor Samples

Ovarian tumor samples were classified by their histopathological characteristics (Table 1). Primary ovarian carcinomas were classified separately according to the International Federation of Gynecology and Obstetrics staging for gynecologic tumors (Table 2).

#### Control Samples

Normal ovarian tissue specimens were obtained during abdominal hysterectomy and oophorectomy due to uterine leiomyoma (n=4), and during vaginal hysterectomy and oophorectomy due to stress urinary incontinence (n=6).

### Table 1. Histopathological characteristics of ovarian tumor specimens, classified according to International Federation of Gynecology and Obstetrics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Primary malignant ovarian tumor (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor grade</td>
<td>GI or well differentiated (4)</td>
</tr>
<tr>
<td></td>
<td>G II or moderately differentiated (3)</td>
</tr>
<tr>
<td></td>
<td>G III or poorly differentiated (3)</td>
</tr>
<tr>
<td>Hystological type</td>
<td>mucinous (2)</td>
</tr>
<tr>
<td></td>
<td>serous (5)</td>
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<tr>
<td></td>
<td>endometrioid (1)</td>
</tr>
<tr>
<td></td>
<td>mixed (1)</td>
</tr>
<tr>
<td>Patient age</td>
<td>&lt; 50 (4)</td>
</tr>
<tr>
<td></td>
<td>&gt; 50 (6)</td>
</tr>
<tr>
<td>Parity</td>
<td>O (3)</td>
</tr>
<tr>
<td></td>
<td>≥ 1 (7)</td>
</tr>
<tr>
<td>GOG (Gynecology Oncology Group)</td>
<td>low risk – stage 1A and B, grade 1 or 2 (3)</td>
</tr>
<tr>
<td></td>
<td>high risk – stage IC or II, grade 3 (7)</td>
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</tbody>
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Tissue Processing

For biochemical analysis, the samples were prepared as described in detail elsewhere (31,32). Briefly, each specimen was minced, homogenized in a lysis buffer, and centrifuged for 45 min at 15,000 G. Supernatant (tumor tissue cytosol) was used for biochemical studies.

Glutathione Determination

The total intracellular glutathione level was measured by modified Tietze’s method (33). The samples were cut into small pieces, covered with buffer (50 mmol TRIS, 250 mmol saccharose, 13.8 mmol KCl, pH 7.6) (Kemika, Zagreb, Croatia) and homogenized on ice (Ika Kunker, Labor Technich FRG, Staufen, Germany, three strokes for 5 s). The suspension was centrifuged at 4°C for 45 min at 15,000 G. The total GSH content in the supernatant was determined by enzymatic recycling assay. The absorbance of 2-nitro-5-thiobenzoic acid (Boehringer Mannheim GmbH, Ingelheim am Rhein, Germany) at 412 nm was monitored spectrophotometrically. Values were normalized according to the total protein assessed by Bradford’s method (34). For each sample, the levels of GSH were determined in triplicate.

Glutathione S-Transferrases Determination

Intracellular glutathione S-transferases activity was determined as described by Habig and Jacoby (35). GST activity was measured in the supernatant prepared for GSH determination, with 1-chloro-2,4-dinitrobenzene (Boehringer Mannheim GmbH) used as the electrophilic substrate. GST activity was expressed in nanomoles of conjugated formed per min per mg protein. For each sample, GST activity was determined in triplicate.

uPA and PAI-1 Determination

uPA and PAI-1 were determined by an ELISA assay, according to the protocol given by the producer of IMUBIND®uPA and PAI-1 (American Diagnostic Inc., Greenwich, CT, USA). The uPA kit detects precursor of uPA, high molecular weight uPA, receptor-bound uPA, as well as uPA complexes with PAI-1 and PAI-2. By PAI-1 kit, latent and active forms of PAI-1 and PAI-1 complexes were detected. Values were normalized according to the total protein assessed by Bradford’s method, and expressed as ng/mg protein. For each sample, the levels of uPA and PAI-1 were determined in duplicate or triplicate.

Statistics

Statistically significant differences among the groups were tested by two-way ANOVA with Tukey HSD test, with correction for unequal group size for post hoc comparison. Their relationship to histopathological and clinical factors was calculated by Spearman’s rank correlation test. Data are presented as mean±SE. Differences were considered significant at p<0.05.

Results

GSH levels were significantly higher in primary malignant ovarian tumor tissues (126.3±12.8 nmol/mg protein) than in normal ovarian tissue specimens (48.9±8.1 nmol/mg protein, p=0.003) and benign tumor samples (35.2±5.0 nmol/mg protein, p=0.001). In metastatic ovarian tumor specimens, the concentration of GSH was significantly increased (160.5±24.3 nmol/mg protein) compared with corresponding normal ovarian tissue samples (p=0.001) and benign ovarian tumor specimens (p=0.001). However, there was no statistically significant difference in GSH levels between primary and metastatic ovarian tumor specimens (p=0.393), or between normal ovarian tissues and benign tumor specimens (p=0.500) (Fig. 1).

GST activity was significantly higher in primary malignant (245.8±22.7 nmol/min/mg protein) than in benign tumor specimens (105.9±16.2 nmol/min/mg protein, p=0.004) and normal ovarian tissue (133.2±32.0 nmol/min/mg protein, p=0.044).

The activity of GST was also significantly higher in metastatic ovarian tumor samples (303.7±48.8 nmol/min/mg protein) than in benign tumor specimens (105.9±16.2 nmol/min/mg protein, p=0.001) and normal ovarian tissue (133.2±32.0 nmol/min/mg protein, p=0.006). Similarly to GSH levels in examined groups of samples, there was no statistically significant difference in GST activities between primary malignant and metastatic ovarian tissue samples (p=0.668) or between normal and benign ovarian tissues (p=0.634) (Fig. 2).

Although the mean concentration of uPA was higher in primary malignant ovarian tissue specimens (2.2±1.0) than in metastatic ovarian tumor specimens (0.5±0.1), benign ovarian tumor specimens (0.4±0.1) and normal ovarian tissue samples (0.5±0.1), the difference was not statistically significant (Fig. 3).

Also, no statistically significant difference appeared found between the groups of samples in the mean concentration of PAI-1, although it was higher in primary malignant (7.6±1.8 ng/mg protein) and metastatic (5.0±1.0 ng/mg protein) ovarian tumor specimens than in benign ovarian tumor specimens (4.0±1.6 ng/mg protein) and normal ovarian tissues (3.6±1.0 ng/mg protein). There was no significant difference in the levels of PAI-1 between primary malignant and metastatic ovarian tumor samples (p=0.668; malignant vs metastatic p=0.668; malignant vs benign p=0.004; malignant vs normal p=0.444; metastatic vs benign p=0.001; and metastatic vs normal p=0.006, benign vs normal p=0.634.)
The results of two-way ANOVA analysis of variance: malignant vs metastatic $p=0.501$; malignant vs benign $p=0.364$; malignant vs normal $p=0.524$; metastatic vs benign $p=0.934$; metastatic vs normal $p=0.966$; benign vs normal $p=0.945$.

The results of two-way ANOVA analysis of variance: malignant vs metastatic $p=0.784$; malignant vs benign $p=0.589$; malignant vs normal $p=0.627$; metastatic vs benign $p=0.937$; metastatic vs normal $p=0.581$; and benign vs normal $p=0.822$.

The levels of GSH, uPA, and PAI–1, as well as GST activity, were independent of histopathological and clinical characteristics of tumor tissues.

The only significant correlation found in primary malignant and metastatic ovarian tumor specimens was between GSH concentrations and GST activity ($r=0.461$, $p=0.047$) and between uPA and PAI–1 concentrations ($r=0.385$, $p<0.001$).

**Discussion**

Despite some improvements in the survival of patients with ovarian cancer due to new chemotherapeutic regimens, intrinsic and/or acquired drug resistance still remains the major obstacle in improving the overall survival rate in those patients (1-5). Both glutathione and glutathione S-transferases are frequently found in drug resistant phenotypes and associated with poor disease outcome in patients with different malignant tumors (14–16, 18). The aim of this study was to determine whether the differences in the concentrations of glutathione, urokinase-type plasminogen activator, plasminogen activator inhibitor type-1, and glutathione S-transferases activities in ovarian tumor and normal tissues samples had relevant potential diagnostic and prognostic values for ovarian carcinoma and whether such differences were in correlation with histopathological and clinical factors.

We found significantly higher concentration of glutathione in primary and metastatic malignant carcinomas than in normal ovarian tissues or benign tumor samples. This is in agreement with the results of Ghahia et al (36), who found the highest GSH levels in ovarian tumors as compared with breast, prostatic, and liver neoplasms. However, in our previous research, we had not observed such differences in malignant tumors of the uterine corpus (37). GSH levels in tumor tissues were independent of histopathological or clinical parameters. Contrary to our results, Tanner et al (38) found that GSH concentrations significantly correlated with tumor stage as classified by the International Federation of Gynecology and Obstetrics, with higher levels found in stages III and IV. Maybe the explanation for the discrepant findings of these two studies might lie in a larger number of tumor samples in Tanner’s study.

GST activities were significantly elevated in primary and metastatic malignant ovarian tumor samples, compared to the corresponding normal ovarian tissues and benign ovarian tumor specimens. Matsumoto et al (39) obtained similar results. In our previous study, we also detected an increase in GST activity in malignant tumors of the uterine corpus (37). Higher GST activities were found in other cancers, including cancer of colon, rectum, stomach, lung, and breast, but not of kidney and liver (40–42). Similarly to our results, Wrigley et al (43) did not find any correlation in GST level with the clinical and pathological parameters tested.

Both total glutathione level and glutathione S-transferases activity are determinants of a tumor’s ability to detoxify chemotherapeutic agents. In vitro studies have demonstrated that increased levels of glutathione may be associated with the resistance to platinum compounds, alkylating agents, and anthracyclines (6, 10-14, 18, 20). It has been suggested that GSH may reduce the cytotoxicity of these drugs by forming inactive conjugates mediated by GST, by exporting the GSH-anticancer drug conjugates out of cells, by enhancing the repair of DNA damage, or by inhibiting apoptosis (8, 10, 15-17, 18-21).

Published reports on the levels of uPA and PAI–1 in ovarian cancers are conflicting. Some groups have found that the increase in levels of both uPA and PAI–1 was significantly associated with the malignant progression of ovarian tissues: their levels were the lowest in normal tissue and then increased from benign tumors, to primary and metastatic cancers (44, 45). Other researchers (46) did not detect any rise in uPA and PAI–1 content in malignant ovarian tissues, which is in agreement with our data. They also did not find uPA or PAI–1 significantly associated with the age of the patients, stage, or tumor grade classified according to International Federation of Gynecology and Obstetrics.
system can be useful target for more rational and clinically effective modulation chemotherapy of patients with ovarian carcinoma remains to be determined in a larger clinical study.

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References


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