Glutathione S-transferases P1-1 and A1-1 in ovarian cyst fluids

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Summary

Purpose: The purpose of the present study was to determine the gluthathione S-transferases (GST) P1-1 and A1-1 levels in cyst fluid from malignant, borderline, and benign ovarian tumors. The clinical relevance of these enzymes in cyst fluid was investigated, including the possible relation with resistance to chemotherapy.

Methods: A total of 90 ovarian cysts were punctured for cyst fluid collection. GSTP1-1 and GSTA1-1 concentrations were determined by ELISA in cyst fluid from 23 malignant, 9 borderline, and 51 benign primary ovarian tumors, and levels were correlated with histopathological data.

Results: Significantly higher GSTP1-1 concentrations were found in cyst fluid from malignant (median: 477 ng/ml), compared with benign (median: 52 ng/ml) ovarian cysts ($p < 0.0001$), as well as in fluid from borderline (median: 366 ng/ml) compared with benign cysts ($p < 0.0001$). No significant differences were found in cyst fluid GSTA1-1 concentrations between the histologic subgroups. In cyst fluid from malignant tumors higher GSTP1-1 and lower GSTA1-1 concentrations were found in patients with worse prognostic factors: FIGO II-III-IV, grade 2-3, residual tumor > 2 cm, presence of ascites, patients with recurrent disease, and survival, but differences were not significant. In the subgroup of patients that received cisplatin-based chemotherapy ($n = 14$) significantly higher GSTP1-1 ($p = 0.01$) concentrations were found in patients with recurrence compared with patients without recurrence. Considering only FIGO stage I patients, a differentiation could be made between patients with or without recurrence based on cyst fluid GSTP1-1 concentrations.

Conclusions: Determination of glutathione S-transferases P 1-1 in cyst fluid samples from ovarian tumors can be of additional value in the differentiation between histologic subgroups. In case of possible low malignant potential cysts where sampling of the most representative tissue can be an issue, determination of GSTP-1 concentrations in cyst fluid may optimise histopathologic classification. Cyst fluid GSTP 1-1 seems to be a good marker for aggressiveness of the ovarian tumor, and it may predict response to chemotherapy.

Key words: Ovarian tumor; Cyst fluid; Glutathione S-transferase; Chemotherapy; Drug resistance.

Introduction

Neoplastic ovarian pathology, benign as well as malignant transformation, consists of many types and subtypes. The most common tumors are of epithelial origin. Malignant epithelial ovarian tumors are the fourth most common malignancy in women, and have the highest incidence in gynecologic malignancy [1]. Therapy of first choice for malignant ovarian tumors consists of cytoreductive surgery followed by systemic chemotherapy in case of advanced disease. Despite this regimen the percentage of survivors > 5 years is low (15-20%) [2].

Glutathione (GSH) and GSH-related enzymes are involved in the metabolism and detoxification of cytotoxic and carcinogenic compounds [3, 4]. Glutathione S-transferases (GSTs) are cytosolic enzymes that play an essential role in the elimination of potentially toxic compounds by catalyzing the addition of GSH. In this way carcinogens, but also drugs such as anticancer drugs, can be eliminated. The group of GSTs consists of four main isoenzyme classes: Alpha, Mu, Pi, and Theta. These four classes are each divided into one or more isoforms [4]. The role of GSTs in relation to reproduction [3], hypertensive disorders in pregnancy [5, 6] or oncological disease [4, 7, 8] has been reviewed previously.

The expression of GST in ovarian carcinoma tissue and serum samples has been the subject of several studies, however has not as yet been fully established and results are conflicting [9-14]. Most studies agree on higher GST Pi (GSTP1-1) levels in malignant ovarian tissue as compared to benign. Some authors suggest prognostic significance of GSTP1-1 tissue levels [9, 11, 12], whereas others indicate no relation between tissue levels of GSTs and clinicopathologic or prognostic parameters [13, 14]. Furthermore, GSTs are thought to play a role in the multifactorial process of resistance of tumor cells to chemotherapy [9, 12, 15-18].

The majority of ovarian tumors, benign as well as malignant, are of epithelial origin and consist of solid and cystic components [19]. These cysts can be single or multiple and are filled with fluid. The metabolic composition of cyst fluid may provide a better representation of overall tumor biology than random tissue samples since it is not always clear where to take the most representative samples due to tumor heterogeneity. To our knowledge, determination of glutathione S-transferase levels in cyst fluid from ovarian tumors has never been performed.

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The aim of the present study was to determine the concentrations of glutathione S-transferases P1-1 and A1-1 in cyst fluids from malignant, borderline, and benign ovarian tumors, and to investigate the relation with clinicopathologic characteristics and resistance to chemotherapy.

Materials and Methods

Patients and cyst fluid collection

Ninety patients with an ovarian cyst planned for surgical removal were recruited for this study and cyst fluid was collected in the period January 1998 until December 1999. Immediately after surgical removal, the ovarian tumor was transported to the Pathology Department for cyst fluid collection by aseptic fine needle aspiration. After cooled transport to the laboratory the cyst fluid samples were centrifuged at 3,000 x g for ten minutes and the supernatant was stored at –35°C in small portions until use. Determination of the levels of glutathione S-transferases was performed without prior knowledge of the histological or clinical outcome. Histopathology was performed by an experienced gynecologic pathologist. Clinicopathologic characteristics were retrieved from patient medical records. Two cyst fluid samples were obtained after primary chemotherapy (one malignant, one borderline) and are presented separately. Two fluid samples were obtained from cysts where the primary location was elsewhere and not ovarian, and three samples could not be measured due to high viscosity of the fluid (one borderline malignancy, one mucinous cystadenoma, one dermoid). These five samples were excluded from further analysis.

ELISA procedures

ELISAs for glutathione S-transferase P 1-1 and A 1-1 were performed as described previously [20, 21]. Polystyrene microtiter plates were used. All incubations were performed at room temperature in a 100 µL/well, unless stated otherwise. In-between incubations, plates were washed five times with > 200 µL/well phosphate-buffered saline (140 mmol/L NaCL, 6.4 mmol/L Na_2HPO_4, and 1.3 mmol/L $Na_2H_2PO_4$, pH ~7.4; PBS) supplemented with 0.5 mL/L Tween 20 (PBS-T). Plates were coated overnight at 4°C with 10 mg/L purified anti-GSTP1-1 or GSTA1-1 monoclonal antibody in PBS and blocked with 200 µg/L well PBS-T supplemented with 1% (*w/v*) bovine serum albumin (PBS-T-BSA) for one hour. Standards (0.4-100 µg/L GSTP1-1; 0.04-20 µg/L GSTA1-1) diluted in PBS-T supplemented with 10 mmol/L EDTA and 10% (*v/v*) heat-treated normal human plasma (PBS-T-EDTA-NHP) and cyst fluid samples diluted with an equal volume of PBS-T-EDTA-NHP were then added to the wells. Plates were incubated overnight, washed, incubated with rabbit anti-GSTP1-1 and GSTA1-1 antiserum diluted (1/1000 and 1/4000, respectively) in PBS-T supplemented with 10% (*v/v*) heated normal human plasma for three hours, washed and subsequently incubated for two hours with peroxidase-labeled swine anti-rabbit (Dakopatts, Glostrup, Denmark) diluted 1/2000 in PBS-T-BSA. After a final wash, plates were incubated with 3.75 µmol/L o-phenylenediamine, 1 mmol H_2O_2 in 24 mmol/L sodium citrate, 51 mmol/L Na_2HPO_4, pH 5.0 for 15 minutes. The reaction was stopped by adding 100 µL 2mol/L H_2SO_4 and absorbance was read at 492 nm with a background subtraction at 620 nm. All standards and samples were measured in duplicate. A four-parameter weighted logistic regression model was used to calculate standard curves and unknowns.

The detection limit of the assays for GSTP1-1 and GSTA1-1, corresponding to 3 standard deviations above the mean signal of 5 zero standards was 0.4 and 0.04 ng/ml, respectively.

Clinicopathologic characteristics

From the medical records of 23 patients with malignant histopathologic diagnoses, the following clinicopathologic characteristics were retrieved: stage according to the International Federation of Gynecology and Obstetrics (FIGO), histopathologic grade, residual tumor after surgery, presence of ascites, tumor recurrence, disease-free survival (DFS), and overall survival (OS). Residual tumor was divided into two groups; ≤ 2 cm or > 2 cm. Histopathologic grade was defined as grade 1 (well differentiated), grade 2 (moderately differentiated), or grade 3 (poorly differentiated). Tumor recurrence was defined as evidence of disease within the follow-up interval between date of surgery and closure of the follow-up period (May 2000). Serum CA-125 concentrations (U/ml) after three courses of chemotherapy or, if not determined at that time, after six courses were recorded.

Statistics

Significance of the differences in concentrations were tested by using the Mann-Whitney U-test for two groups of independent samples (level of significance $p < 0.05$). Differences between more than two groups were tested by Kruskal Wallis analysis.

GSTP1-1 and GSTA1-1 concentrations are given as median, 25th and 75th percentiles, unless stated otherwise.

Results

The histopathologic diagnosis of ovarian tumors whose corresponding cyst fluids were used for assay of GSTP1-1 and GSTA1-1 concentrations, are listed in Table 1. The median GSTP1-1 and GSTA1-1 concentrations as found in the histologic subgroups are presented in Figures 1 and 2, respectively. The data comprised 23 malignant, 9 borderline, and 51 benign cysts.

Table 1. — *Histopathological diagnosis of ovarian tumors.*

	n
Malignant	
Serous cystadenocarcinoma	9
Mucinous cystadenocarcinoma	8
Endometrioid carcinoma	4
Undifferentiated cystadenocarcinoma	2
Total	23
Borderline	
Serous cystadenoma	1
Mucinous cystadenoma	8
Total	9
Benign	
Serous cystadenoma	13
Mucinous cystadenoma	18
Dermoid cyst	4
Functional cyst	16
Total	51
Total	83

n: number of tumors in each group.

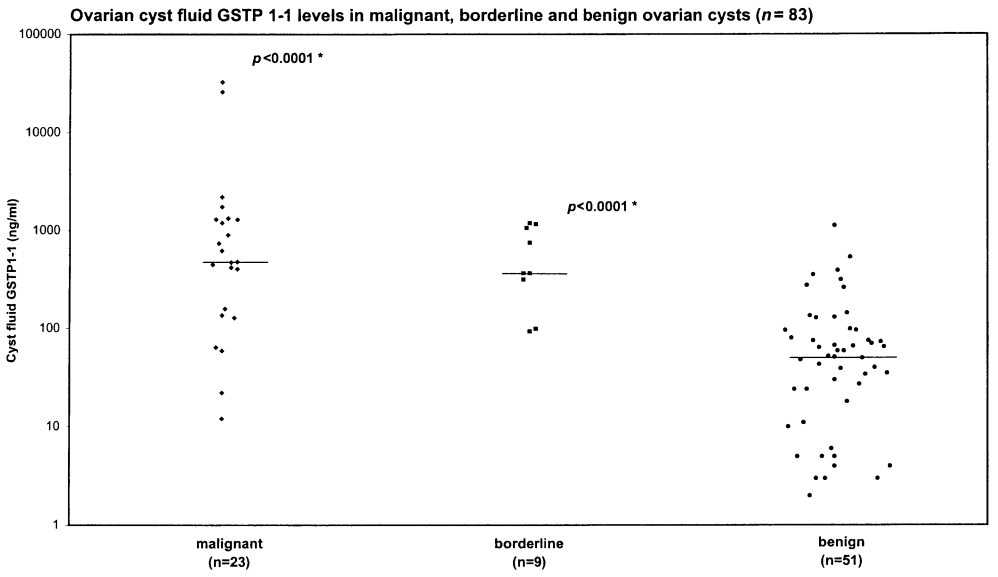

Figure 1. — Median values are illustrated as horizontal bars. * statistical significance compared to benign subgroup.

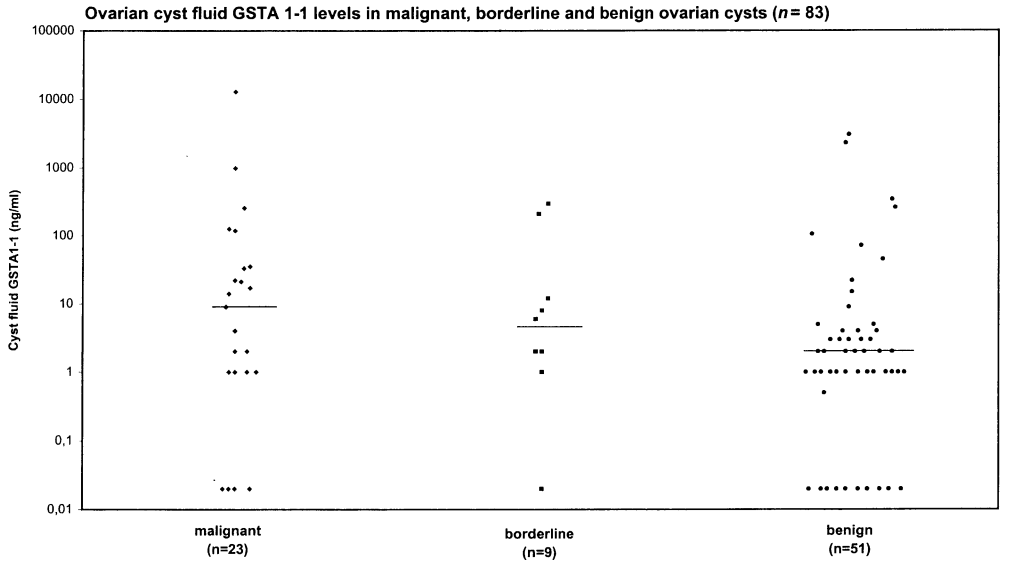

Figure 2. — Median values are illustrated as horizontal bars. No significant differences were found between histologic subgroups.

Median GSTP1-1 concentrations in cyst fluid from malignant, borderline, and benign ovarian tumors were 477 ng/ml, 366 ng/ml, and 52 ng/ml, respectively. Significantly higher median GSTP1-1 concentrations were found in cyst fluids from malignant compared with benign, and in cyst fluid from borderline compared with benign cysts (Figure 1).

Median GSTA1-1 concentrations in cyst fluid from malignant, borderline, and benign ovarian tumors were 9 ng/ml, 6 ng/ml, and 2 ng/ml, respectively. No significant differences in cyst fluid GSTA1-1 concentrations were found between the histologic subgroups (Figure 2).

Correlation with clinicopathologic characteristics

Clinicopathologic characteristics from 23 patients with a malignant ovarian cyst and their corresponding median, 25th-75th percentiles GSTP1-1 and GSTA1-1 concentrations are listed in Table 2. Higher median GSTP1-1 levels were found in FIGO stages II-III-IV, histologic grade 2-3, residual tumor > 2 cm, presence of ascites, patients with recurrent disease, DFS ≤ 14 months, and OS ≤ 24 months as compared with stage I, grade 1, residual tumor ≤ 2 cm, absence of ascites, patients without recurrence during follow-up, DFS > 14 months, and OS > 24 months, respectively. However, the differences did not reach significance. In con-

Table 2. — *GSTP1-1 and GSTA1-1 cyst fluid concentrations in clinicopathologic subgroups of patients with malignant ovarian tumors (n=23).*

		GSTP1-1		GSTA1-1	
	n	median (ng/ml)	(25th-75th percentile) (ng/ml)	median (ng/ml)	(25th-75th percentile) (ng/ml)
FIGO Stage					
I	13	419	(128-907)	17	(2-35)
II-IV	10	967	(448-1747)	1	(1-22)
Grading					
1	6	474	(64-1297)	34	(17-118)
2	5	1196	(419-1332)	1	(1-22)
3	12	535	(147-1553)	2	(0.5-15)
Residual tumor					
≤ 2 cm	18	474	(136-1297)	15	(2-35)
> 2 cm	5	738	(158-1332)	1	(0.02-1)
Ascites					
No	10	445	(136-1297)	27	(4-118)
Yes	13	622	(158-1332)	2	(1-17)
Recurrent disease					
No	10	304	(64-1297)	15	(2-35)
Yes	13	738	(419-1332)	2	(1-22)
Disease-free survival					
> 14 months	11	472	(64-1297)	21	(9-118)
≤ 14 months	12	680	(147-1539)	1	(1-13)
Overall survival					
> 24 months	11	477	(158-1297)	9	(1-21)
≤ 24 months	12	605	(132-1317)	18	(0.5-191)
All patients	23	477	(136-1302)	9	(1-35)

n: number of tumors in each group. - Note: clinicopathologic characteristics are defined in the materials and methods section.

Table 3. — *GSTP1-1 and GSTA1-1 cyst fluid concentrations and clinicopathologic characteristics in patients with malignant ovarian tumors, treated with cisplatin-based chemotherapy after primary surgery.*

Patient no.	Age	FIGO	Grade	Recurrence[c]	GSTP1-1 (ng/ml)	GSTA1-1 (ng/ml)	CA-125[a] (U/ml)
1	74	IA	3	yes	902	21	8.4
2	65	IA	3	no	136	4	nd
3	66	IC	3	no	12	0.02	nd
4	49	IC	1	no	64	14	11[b]
5	44	IC	3	no	128	2	2
6	57	IC	3	yes	404	1	3.3
7	52	IIIB	3	yes	622	2	19
8	31	IIIC	3	yes	32695	12900	285
9	51	IIIC	2	yes	1747	22	15
10	59	IIIC	2	yes	1196	1	17
11	52	IIIC	3	yes	738	0.02	22[b]
12	56	IIIC	3	yes	25963	987	7.6
13	63	IIIC	2	yes	59	1	nd
14	57	IV	3	yes	158	1	6.5

[nd]not determined; [a]after 3 courses of chemotherapy; [b]after 6 courses of chemotherapy; [c]GSTP1-1 was significantly higher (p = 0.01) in patients with than without recurrence.

trast, higher median GSTA1-1 levels were found in FIGO stage I, patients with residual tumor ≤ 2 cm, ascites without malignant cells, patients without recurrent disease, and DFS > 14 months as compared with stage II-III-IV, residual > 2 cm, presence of ascites, patients with recurrence, and DFS ≤ 14 months, respectively. However, differences did not reach significance. Significantly higher GSTA1-1 concentrations were found in histologic grade 1 compared with grade 2-3 ($p < 0.05$).

Response to chemotherapy

Cyst fluid GSTP1-1 and GSTA1-1 concentrations of the excluded two patients that received primary chemotherapy before surgery were 2,542 ng/ml and 25 ng/ml (malignant) and 2 ng/ml and 4 ng/ml (borderline), respectively. Adjuvant cisplatin/cyclophosphamide-based chemotherapy was given in 14 patients (6 stage I, 8 stage III-IV). Table 3 presents clinicopathological characteristics, GSTP1-1 and GSTA1-1 cyst fluid concentrations as well as the presence or absence of recurrent disease during follow-up. Median cyst fluid GSTP1-1 levels in ten patients with recurrence was 820 ng/ml and was significantly higher ($p = 0.01$) than the median level of 96 ng/ml which was measured in the four patients without recurrence (Table 3). No significant differences were found between these groups for GSTA1-1, although the median GSTA1-1 level was higher in patients without versus patients with recurrent disease (3 ng/ml versus 1 ng/ml, respectively).

Considering only stage I patients, two patients with recurrence were revealed with GSTP1-1 levels of 404 and 902 ng/ml, respectively, and four patients without recurrence with GSTP1-1 levels of 12, 64, 128, and 136 ng/ml, respectively (Table 3). GSTP1-1 and GSTA1-1 levels were compared with CA-125 concentrations after three courses of chemotherapy in 11 patients. Median CA-125 after three courses of chemotherapy in nine patients with recurrence was 15 U/ml. In the two patients without recurrence these concentrations were 2 and 11 U/ml, respectively. The highest CA-125 after three courses of chemotherapy was found in the one patient with the highest GSTP1-1 and GSTA1-1 concentrations of 32,695 ng/ml and 12,900 ng/ml, respectively. This patient developed recurrent disease within three months after surgery and died within four months.

Discussion

Studies in the past investigating the role of glutathione-S transferase isoenzymes in ovarian tumors were performed on tumor tissue samples, and immunohistochemistry results are qualitative and conflicting. Tumors are heterogeneous and most likely tumor biology is not optimally reflected by one tissue biopsy. Murphy *et al.* suggested homogenisation of several tumor specimens to overcome this problem [22]. Analysis of cyst fluid probably provides an even better overall representation of cellular processes of the tumor. Cyst fluid can be easily aspirated after surgery without the risk of spill, no tissue homogenization is required, and the problem of sampling in a heterogeneous tumor is avoided. Until now, no study on the levels of glutathione-S transferases in cyst fluid of ovarian tumors has been performed.

The aim of the present study was to perform a quantitative assessment of GSTP1-1 and GSTA1-1 in a large number of ovarian cyst fluid samples from malignant, borderline and benign tumors.

We found significant differences in GSTP1-1 cyst fluid concentrations between histopathologic subgroups. The highest levels were present in cyst fluid from malignant ovarian cysts. This was in line with several results from ovarian tissue studies [16, 18]. However, this seems to contradict the results of Djuric *et al.* who described lower GST activity in malignant compared with normal or benign ovarian tissues [10]. In the Djuric study however, overall GST enzyme activity was determined and no estimation of the main isoenzyme levels was made, whereas the "normal" ovarian tissues were taken from patients with cervical or endometrial cancer in which high GST expression was described [23, 24]. This may explain the high GST activity found by Djuric *et al.* in their group of normal ovarian tissue.

The significantly higher median GSTP1-1 concentrations in the present study as found in cyst fluid of tumors of low malignant potential (borderline malignancy) compared to benign cysts may reflect histopathological changes specific for this subgroup and emphasizes the relevance of including borderline malignancy as a separate entity. It would be worthwhile to analyse other prognostic factors that have been described in borderline malignancy, such as micro-invasive growth, in relation to the GSTP1-1 cyst fluid concentration in a larger series of patients with extensive follow-up.

Although differences were not significant, the higher median ovarian cyst fluid GSTP1-1 concentrations that we found in the malignant subgroups with worse prognostic factors, suggest that GSTP1-1 cyst fluid level is a possible measure of aggressiveness of the corresponding ovarian tumor. However, this difference in prognosis can also be the result of the already mentioned chemoresistance. A significant correlation between positive GSTP1-1 staining and shorter patient survival was also found by Green *et al.* [9], Hirazono *et al.* [12], and Hamada *et al.* [15].

In contrast to GSTP1-1, cyst fluid GSTA1-1 concentrations were higher in the subgroups with more favourable characteristics. GSTA1-1 seems to have the capability of suppressing tumor aggressiveness. In this study no correlation was found for disease-free survival or overall survival with either GSTA1-1 or GSTP1-1 cyst fluid concentrations.

Several authors reported a correlation between high tissue GSTP1-1 expression and resistance to chemotherapy [9, 15, 17]. However, it was suggested that the role of GSTP1-1 in the development of anticancer drug resistance is restricted to serous subtypes of cystadenocarcinoma of the ovary [16]. Other authors could not confirm the possible association between GST expression and response to chemotherapy [14, 25-27]. Conflicting data in the literature can be explained by differences in tissue sampling methods, the relatively small number of examined patients, and a large variation in clinicopathologic characteristics. Furthermore, resistance to chemotherapy is a multifactorial process with a number of potentially important determinants.

Our results support the prognostic value of GSTP1-1 cyst fluid concentrations in the prediction of response to chemotherapy. We found a relation between higher cyst fluid GSTP1-1 concentrations and tumor recurrence after chemotherapy. This indicates that higher GSTP1-1 cyst fluid levels reflect increased detoxification of anticancer drugs.

In summary, quantification of glutathione S-transferase P1-1 levels in cyst fluid samples from ovarian tumors can be of additional value in the differentiation between histologic subgroups. Ovarian cyst fluid GSTP1-1 levels seem to be a good marker for the aggressiveness of ovarian tumors, and may predict response to chemotherapy.

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