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Clinical Significance of Cathepsin D in Primary Ovarian Cancer

G. Scambia, P.B. Panici, G. Ferrandina, G. Salerno, G. D'Agostino, M. Distefano, R. De Vincenzo, A. Ercoli and S. Mancuso

Cathepsin D (Cath D) levels were assayed in a prospective series of 72 patients with primary ovarian carcinoma, by using an immunoradiometric assay. Cath D levels ranged from 2.00 to 45.60 pmol/mg protein with a median value of 15.80 pmol/mg protein. Cath D levels were higher in metastatic deposits than in primary tumors (median 24.12, range 9.33–98.33 pmol/mg protein versus median 12.76, range 2.00–45.20 pmol/mg protein; $P = 0.04$). The cut-off levels of the lower, median and upper quartiles of the distribution of Cath D were identified to distinguish patients with low, intermediate, and high Cath D content. Cases with low Cath D content showed a lower percentage of complete response to chemotherapy than cases with intermediate and high Cath D content (22% versus 65% and 47%, respectively) ($P = 0.003$). Moreover cases with high Cath D content showed a worse prognosis with respect to patients with intermediate Cath D levels ($P = 0.09$). Interestingly, cases with low Cath D content had a shorter progression-free survival with respect to cases with intermediate Cath D content ($P = 0.04$). Cath D status retained an independent prognostic value when assessed in the multivariate analysis.

Key words: cathepsin D, ovarian cancer, prognosis
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INTRODUCTION

CURRENT RESEARCH has been focused on the contribution of biological factors in affecting the prognosis of cancer patients. In particular, secreted proteases are thought to have important functions in carcinogenesis, facilitating tumour growth and invasion [1]. Among these, special interest has been pointed out for Cathepsin D (Cath D). It was originally identified as a Mr 52 000 secreted protein induced by oestrogens in MCF-7 [2–4], but it may also be constitutively secreted by oestrogen receptor negative breast cancer cells [5, 6]. Cath D is active against the extracellular matrix components, and is able to display mitogenic activity *in vitro* on oestrogen-depleted MCF-7 cells, although there are not unequivocal findings [6, 7].

The clinical role of Cath D in predicting clinical outcome has been extensively investigated in breast cancer. Most of the studies have reported that high cytosolic Cath D content is

associated with an unfavorable outcome [8–10], although there are conflicting data [11, 12].

There is evidence supporting the hypothesis that cathepsins may play a role in the onset and spread of ovarian tumors. It has been reported that ovarian cancer cells under oestrogen stimulus are able to secrete Cath D in the culture medium [13]. Cathepsin B has been found to be overproduced by ovarian tumours, and secreted in ascites where it can be activated by Cath D [14]. Moreover, our previous report demonstrated the presence of scattered levels of Cath D in cytosols from ovarian tumours, and higher levels of Cath D in omental metastases than in corresponding primary tumour [15].

At present, there are no data on the possible prognostic role of Cath D in ovarian cancer.

This study aims to assess the clinical significance of Cath D content in a series of 72 primary ovarian cancer patients. Moreover, the correlation between Cath D and steroid hormone receptors was also investigated.

MATERIALS AND METHODS

Between 1987 and 1993, 75 previously untreated patients with histologically-confirmed ovarian carcinoma were admitted to the study. 3 patients were lost to follow-up. Clinicopathological parameters are listed in Table 1. Chemotherapy was started 2–3 weeks after surgery. All patients received cisplatin-containing regimens. 36 patients received high-dose cisplatin (200 mg/m^2) for three courses [16]. Gynaecological examination, abdominopelvic ultrasonography, CA 125 assay and radiological investigations, if necessary, were performed monthly for the clinical assessment of response, which was recorded according to WHO criteria [17]. Approximately 28 days after the last course, clinical complete responders underwent second-look laparoscopy. In laparoscopy-negative cases, second-look laparotomy was performed for the assessment of pathological response.

Patients who initially had only an explorative laparotomy underwent a second laparotomy after chemotherapy, and a second cytoreduction was attempted. Pathological complete responders received no further therapy, and all other patients were treated according to ongoing phase II studies.

Table 1. Patients' characteristics

	Number of patients (%)
Entered	75
Evaluable	72
Age (years)	
<60	43 (60)
≥60	29 (40)
Menopause	
No	24 (33)
Yes	48 (67)
FIGO stage	
I	10 (14)
II	4 (6)
III	50 (69)
IV	8 (11)
Grade of differentiation	
1	11 (15)
2	3 (4)
3	58 (81)
Histology	
Serious	53 (74)
Mucinous	3 (4)
Endometrioid	8 (11)
Undifferentiated	4 (6)
Other	4 (6)
Ascites	
No	24 (33)
Yes	48 (67)
Residual tumour after surgery	
<2 cm	55 (76)
≥2 cm	17 (24)
Response to chemotherapy	
CR	40 (56)
PR	17 (24)
NC–P	15 (21)

CR, complete response; PR, partial response; NC–P, no change–progression.

Preparation of cytosol and membrane fractions

Tissue specimens, frozen on liquid nitrogen shortly after surgical removal, were stored at −80°C until assay. Briefly, tumour specimens were finely minced and homogenised in 5 volumes of ice-cold buffer [25 mmol Tris, 1.5 mmol EDTA, 5 mmol NaN_3, 0.1% monothioglycerol and 20% glycerol (TENMG), pH = 7.4] by applying several intermittent bursts of an Ultra-Turrax homogeniser. The crude homogenate was centrifuged at 7000 *g* for 20 min at 0°C. The supernatants were then centrifuged at 105 000 *g* for 75 min at 0°C, obtaining the cytosolic fraction and the membrane pellet.

Cath D measurement

Cath D concentration was assayed in the cytosolic fraction using a solid phase two-site immunoradiometric assay (CIS bioindustries, Gift-sur-Yvette, France), in which the first monoclonal antibody (D7E3) is coated on the ELISA solid phase and the second one, M1G8, radiolabelled with ^{125}I, is used as a tracer [18, 19]. Cytosol protein concentration was measured by the Bradford method [20], using bovine serum albumin as standard and was reset to approximately 1 mg/ml before Cath D assay. Cytosols were then diluted 1/40 and 1/80 with the dilutent contained in the kit. Radioactivity was measured in a gamma-counter for 1 min. Intra- and interassay variations were 6.4% and 8.5%, respectively.

Oestrogen (ER) and progesterone (PR) receptor measurement

ER and PR were assayed with a single-point saturation assay, using the dextran-coated charcoal (DCC) method according to EORTC protocol [21–23]. Results were expressed as fmoles per mg of membrane protein (fmol/mg protein).

Statistical analysis

Signed Wilcoxon rank-sum test was used to compare Cath D levels in primary ovarian cancer and metastases. The Wilcoxon rank sum test and χ^2 test were used to analyse the relationship between Cath D status and tumour characteristics or response to chemotherapy.

All medians and life tables were computed using the product-limit estimate by Kaplan and Meier [24], and the curves were examined by means of the log-rank test [25]. The risk of progression was estimated by Cox's proportional hazards model

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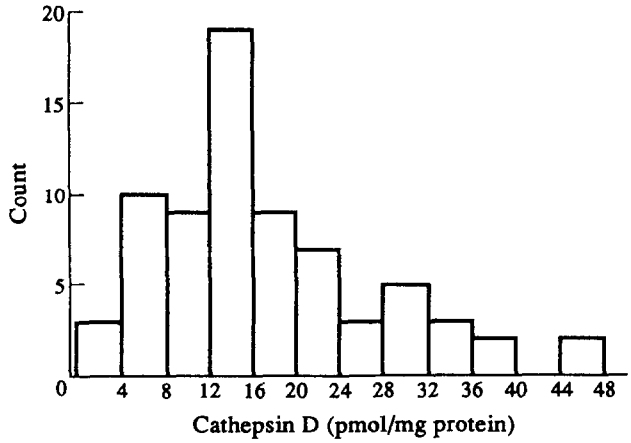

Figure 1. Distribution of Cath D levels in 72 primary ovarian tumours. The class interval is 4 pmol/mg protein.

[26]. Multivariate analysis was performed with BMDP statistical software. A backward stepwise procedure was used to identify the major prognostic factors. Progression-free survival (PFS) was calculated from the date of the first surgery to the date of clinical/pathological progression. The median follow-up was 19 months (range 2–104).

RESULTS

The distribution of Cath D levels in 72 primary ovarian tumours is shown in Figure 1. Cath D levels distributed in an asymmetrical way, and were skewed toward the lower values. Cath D values range from 2.00 to 45.60 pmol/mg protein, with a median value of 15.80 pmol/mg protein.

In 12 patients, Cath D was measured in the primary tumour and in simultaneous omental metastasis. In all cases except two, higher Cath D concentrations were found in metastatic deposits than in primary tumours (median 24.12, range 9.33–98.33 pmol/mg protein versus median 12.76, range 2.00–45.20 pmol/mg protein; $P = 0.04$) (Table 2).

Based on the results of the survival analysis (see below), the cut-off levels of the lower, median and upper quartiles of the distribution of Cath D were identified as the best discriminating values. Therefore, these values were chosen to distinguish patients with low (<10 pmol/mg protein), intermediate (10–21.5 pmol/mg protein), and high (>21.5 pmol/mg protein) Cath D content.

Table 2. Cathepsin D content in primary epithelial ovarian carcinoma and in specimens from metastases obtained concomitantly

Patient number	Cathepsin D content (pmol/mg protein) Primary ovarian cancer	Omental metastasis
1	3.83	12.00
2	2.00	98.33
3	14.20	19.45
4	18.00	39.68
5	45.20	28.80
6	6.57	31.40
7	8.07	34.21
8	28.40	13.82
9	7.09	9.33
10	11.33	14.88
11	19.87	54.00
12	14.83	17.92

Signed Wilcoxon rank sum test, $P = 0.04$.

The association between Cath D content and recognised prognostic factors and steroid hormone receptor status is shown in Table 3. No correlation was found between Cath D levels and stage, grading, histology, presence of ascites and residual tumour after surgery.

There is no apparent association between Cath D content and ER and PR positivity. The linear plot, comparing Cath D with the absolute levels of ER and PR, also did not show any association (data not known).

The correlation between Cath D status (low, intermediate, high) and response to chemotherapy is reported in Table 4. For this analysis, only patients with stages II–III–IV of disease were included. Interestingly, cases with low Cath D content showed a statistically significant lower percentage of complete response with respect to cases with intermediate or high Cath D content ($P = 0.051$).

Survival analysis

During follow-up, progression of disease was observed in 38 patients. Figure 2 shows the PFS curves in relation to Cath D status defined using the cut-off values which have been demonstrated to be the best discriminant in survival analysis.

Cases with high Cath D content showed the worst prognoses with respect to patients with intermediate Cath D levels. The 36-month PFS was 30% for patients with high Cath D content compared with 58% for those with intermediate Cath D content ($P = 0.09$). Median PFS was 13 months for cases with high Cath D content compared with 42 months for cases with intermediate Cath D levels. Interestingly, cases with low Cath D content showed a PFS curve rather similar to cases with high Cath D levels (median 16 months versus median 13 months; P = n.s.). There was a statistically significant difference between cases with low and cases with intermediate Cath D content. In fact, patients whose tumours contained low Cath D levels had a shorter PFS (36 months 12%) compared with intermediate Cath D content ($P = 0.04$).

Due to the very similar behaviour of PFS curves of patients with high and low Cath D content, the subsequent analyses were carried out comparing these patients as a distinct group with respect to those with intermediate Cath D levels. In the univariate analysis (Table 5), FIGO stage, ascites, post-operative residual tumour and Cath D status were found to have a role in predicting ovarian cancer patient prognosis. In the multivariate analysis (Table 5), FIGO stage and ascites remained significantly associated with progression, with Cath D status being the next most significant variable.

DISCUSSION

To our knowledge, this is the first report which has analysed the clinical significance of Cath D in ovarian tumours. In this series, both high and low Cath D content were associated with a worse prognosis than those cases with intermediate Cath D content.

The association between high Cath D levels and poor prognosis has been extensively investigated in breast cancer, for which there is general agreement that high Cath D identifies more aggressive breast tumours [8–10]. The reasons for this aggressiveness may be found in the mitogenic activity of Cath D demonstrated *in vitro* [6], and its involvement as an extracellular protease in tumour invasiveness [1, 6]. It is worth noting that,

Table 3. Cathepsin D levels according to clinico-pathological parameters and ER and PR status in primary ovarian cancer

	Number	Median	Range	Cathepsin D content number Low	Intermediate	High
Age (years)						
<60	43	15.55	3.90–45.20	11	25	7
≥60	29	17.08	2.00–45.60	7	10	12
Menopause						
No	24	15.37	3.90–45.20	5	15	4
Yes	48	16.00	2.00–45.60	13	20	15
FIGO stage						
I	10	16.17	10.54–28.80	—	6	4
II	4	13.27	6.54–13.77	1	3	—
III	50	16.00	2.00–45.60	15	24	11
IV	8	21.77	6.08–36.87	2	2	4
Grade of differentiation						
1	11	15.40	5.65–27.80	1	8	2
2	3	19.84	9.66–28.18	1	1	1
3	58	16.00	2.00–45.60	16	26	16
Histology						
Serous	53	15.68	2.00–45.60	13	26	14
Mucinous	3	16.00	13.35–23.33	—	2	1
Endometrioid	8	10.77	3.90–31.36	4	2	2
Undifferentiated	4	18.57	14.54–30.31	—	3	1
Other	4	15.41	8.00–21.50	1	2	1
Ascites						
No	24	16.20	2.00–28.80	4	14	6
Yes	48	15.19	3.83–45.60	14	21	13
Residual tumour after surgery						
<2 cm	55	16.00	2.00–45.60	12	28	15
≥2 cm	17	12.00	3.83–36.87	6	7	4
ER status*						
Negative	48	15.44	2.00–45.60	14	23	11
Positive	24	16.00	6.57–38.28	4	12	8
PR status						
Negative	53	16.00	2.00–45.60	13	23	17
Positive	19	14.55	3.90–27.50	5	12	2

*ER and PR status was defined using the value of 10 fmol/mg protein.

in our series, omental metastases generally showed higher Cath D levels than the corresponding primary tumour. Moreover, Cath D concentrations were found to be higher in the invaded lymph nodes than in non-invaded lymph nodes in breast cancer patients [18]. Experimental evidence demonstrated that tumour cells which overexpress Cath D, following transfection of its cDNA, show an increased metastatic ability [27]. Therefore, it is conceivable that Cath D may represent a biochemical characteristic of biological aggressiveness of the tumour. We reported that cases with low Cath D content have a bad prognosis

Table 4. Cathepsin D status according to response to chemotherapy

	Cathepsin D content Low	Intermediate	High
CR	4	19	7
PR	8	6	3
NC–P	6	4	5

CR, complete response; PR, partial response; NC-P, no change—progression. $P = 0.051$.

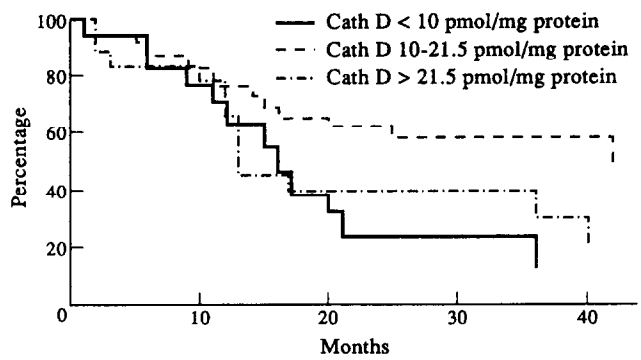

Figure 2. Progression-free survival by Cath D status. Cath D<10 pmol/mg protein: 18 patients entered, 12 progressed with a median of 16 months; Cath D 10–21.5 pmol/mg protein: 35 patients entered, 14 progressed with a median of 42 months; Cath D>21.5 pmol/mg protein: 19 patients entered, 12 progressed with a median of 13 months.

Table 5. Univariate and multivariate analysis of prognostic factors for progression-free survival in 72 ovarian cancer patients

	Univariate (*P* value)	Multivariate (*P* value)
FIGO stage		
I–II		
III–IV	0.0017	0.025
Grade of differentiation		
1		
2		
3	0.15	—
Ascites		
No		
Yes	0.0009	0.020
Residual tumour		
<2 cm		
≥2 cm	0.0071	0.64
Cath D status		
Low–high		
Intermediate	0.023	0.06

with respect to cases of intermediate levels. A possible explanation of this paradoxical result may be the fact that cases with low Cath D content are less likely to respond to chemotherapy. Alternatively, this finding is very intriguing since it supports the potential usefulness of Cath D assessment in predicting responses to treatment.

As previously demonstrated for breast cancer [9, 18, 28], Cath D is not related to any clinico-pathological prognostic characteristics of ovarian tumours. This finding suggests that Cath D assessment may provide additional prognostic information.

It is well known that Cath D undergoes a very complex regulation, which involves steroid hormones and peptides, and is subject to tissue specificity. Oestradiol is able to induce Cath D mRNA in human breast cancer cells [29], while progesterone but not oestradiol induces the accumulation of Cath D mRNA in normal rat uterine cells [30]. Galtier-Dereure and colleagues [15] demonstrated that oestradiol stimulates secretion of procathepsin D in human ovarian cancer cells BG-1. In this report, we confirmed our previous observations regarding the absence of a correlation between Cath D levels and ER or PR expression. These results are in agreement with findings reported in human breast cancer [9, 10, 28], and suggest that, in ovarian tumours, Cath D may escape from steroid hormone control thus being constitutively produced.

In conclusion, our data indicate that Cath D content may represent a possible prognostic factor, eventually linked to tumour chemoresistance in ovarian cancer patients. This should be investigated in larger clinical trials with a longer follow-up period and including other biological parameters in the multivariate analysis.

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Low-dose γ-Interferon Therapy is Ineffective in Renal Cell Carcinoma Patients with Large Tumour Burden

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The efficacy and immunomodulatory effects of low-dose γ-interferon (γIFN) were investigated in an unselected population of patients with metastasising renal cell carcinoma. 36 patients suffering from metastasising renal cell carcinoma with a performance status exceeding Karnofsky index of 50 were entered into the open phase I/II trial. The majority of the patients recruited displayed a large tumour burden, and 28 patients (78%) had metastases involving two to six organ sites. Treatment was started with a 2-week cycle of either daily or weekly subcutaneous administration of either 100, 200 or 400 μg γIFN. After a therapy-free interval of 2 weeks treatment was switched to the alternate mode of administration. Subsequently, treatment was continued with the same dose applied once a week for a minimum of 3 months. Serum levels of neopterin and β-2-microglobulin, as well as flow cytometric analyses of peripheral blood mononuclear cells, were used for the assessment of biological response. Minimal antitumour activity was observed in this high-risk patient group and only 1 patient experienced a partial response (PR) lasting 36+ months. Comparison of the patients' characteristics to those of other low-dose γIFN trials revealed a highly significant difference in the tumour burden and clinical response. We conclude that patient selection is a decisive parameter for the outcome of treatment with low-dose γIFN, and that patients with poor prognostic features and a large tumour burden are not likely to respond to this almost atoxic treatment.
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INTRODUCTION

RENAL CELL carcinoma (RCC) is generally considered to be a promising target for treatment with biological response modifiers [1]. This assumption is based on two observations: spontaneous regressions are observed in a minority of patients and a variety of biological response modifying agents are effective in a minority of patients [2]. Comparable response rates with overlapping 95% confidence intervals have been reported for treatment with interleukin 2, α-interferon (αIFN), γIFN, adoptive immunotherapies with lymphokine activated killer cells, and various combinations of these substances [3–9]. However, the majority of the tumour responses are not durable. In addition, most of these therapies have applied doses close to the maximum tolerated dose. Therefore, for the entire group of patients treated, the clinical value of such therapies is limited by their significant toxicity, which severely compromises the quality of life in the majority of patients [10–12].

Recently, we and others have explored the antineoplastic activity and toxicity of biologically defined low doses of γIFN in patients suffering from metastasising RCC [13, 14]. In both clinical trials published, the patients were treated with 100 μg γIFN once a week. This dose was chosen as a 'biologically active dose', because a single injection of 100 μg γIFN caused nearly maximum biological response, defined by induction of β-2-microglobulin (β_2m), and was associated with minimal toxicity [15].

It was the objective of the present study to answer two questions: (i) to further define the optimal biological regimen