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Original Paper

Significance of Cathepsin-D Expression in Uterine Tumours

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Cathepsin-D (Cath-D) expression was evaluated by an immunoradiometric assay in 67 primary endometrial carcinomas and 70 cervical cancers. In the endometrial tumours, an inverse correlation was observed between Cath-D levels and stage ($P = 0.027$) and myometrial invasion ($P = 0.046$). A significant correlation between Cath-D levels and hormone receptor status was demonstrated ($P < 0.05$). In cervical cancer, no differences in the distribution of Cath-D levels according to clinicopathological parameters and hormone receptors were observed. However, patients not responding to neoadjuvant chemotherapy had significantly lower Cath-D values than those showing complete or partial response ($P = 0.011$). As far as prognostic significance is concerned, it appears that Cath-D expression might have a different role in the two uterine neoplasias. While our preliminary data in endometrial cancer suggest that high Cath-D levels may be a favourable prognostic indicator, cervical cancer patients with Cath-D+ tumours had a shorter disease-free survival than those with Cath-D− tumours ($P = 0.017$).

Key words: cathepsin-D, prognostic factors, cervical cancer, endometrial cancer
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INTRODUCTION

THE DEVELOPMENT of human neoplasia is accompained by changes in the extracellular matrix due, at least in part, to the activity of proteolytic enzymes secreted by cancer cells. Proteases permit the invasion of blood and lymphatic vessels, contributing to the diffusion and metastasis of tumour cells [1].

Cathepsin-D (Cath-D) is an acidic lysosomal endopeptidase secreted by normal and neoplastic cells [2]. Enhanced production of Cath-D has been demonstrated in neoplastic cells [3] and in human tumours [4–6], suggesting a role of this protease in cancer onset and spread. This is further supported by the finding that Cath-D has shown a mitogenic action on human breast cancer cells [7]. The production of Cath-D is regulated by oestrogen in hormone-responsive breast and ovarian cancer cells [3, 8], although it is also constitutively produced in oestrogen-receptor (ER)-negative breast cancer cells [9]. In contrast, Cath-D release is under progesterone regulation in normal human endometrium [10] and in rat uterus [11], while oestrogen has no effect [10].

High Cath-D levels have been correlated with a poor prognosis in breast and ovarian cancer patients [12–14].

At present, only scanty data have been reported on the expression and significance of Cath-D in other tumour types. Variable Cath-D levels have been found in human endometrial and cervical cancer [10, 15, 16]. Maudelonde and associates [10] reported higher Cath-D levels in endometrial carcinomas than in normal endometrium suggesting that Cath-D may be a marker of transformation.

In this study, Cath-D levels were measured in a large series of cervical and endometrial tumours. Our results show that Cath-D expression is related to steroid hormone receptor status in endometrial cancer. Moreover, our findings suggest that Cath-D may represent a new prognostic factor in uterine tumours.

PATIENTS AND METHODS

The study was conducted on a group of 67 consecutive primary endometrial cancer patients and 70 consecutive patients with cervical cancer admitted to the Department of Gynaecology of the Catholic University of Rome, Italy.

All patients were staged according to the FIGO classification [17] and their tumours were graded as well (G1), moderately (G2) or poorly (G3) differentiated.

The characteristics of patients with endometrial cancer are presented in Table 1. The median age of these patients was 62 years (range 30–84). All tumours were histologically classified as endometrioid, except one case of adenosquamous carcinoma. In keeping with the FIGO classification [17], myometrial invasion was classified as M1 when tumour infiltration was <50% of myometrial thickness and M2 when it exceeded 50%.

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Table 1. Cathepsin-D levels according to clinicopathological parameters in primary endometrial cancer

	No. of patients	Median (pmol/mg protein)	Range	No. of cases >cut-off † (%)	*P* value*
Total	67	15.0	0.3–94.5	29 (43)	
Histology					
Endometrioid	66	15.0	0.3–94.5	28 (42)	
Adenosquamous	1	2.5	—	—	ns
Age					
<60 years	32	16.5	3.3–61.0	11 (34)	
≥60 years	35	14.4	0.3–94.5	14 (40)	ns
Menopausal Status					
No	12	15.4	5.9–60.9	2 (17)	
Yes	55	14.9	0.3–94.5	23 (42)	ns
Stage					
I	52	17.4	0.3–94.5	26 (50)	
II	4	13.6	7.8–26.8	1 (25)	
III	11	10.7	4.3–25.7	1 (9)	0.027
Grade					
G1	16	15.4	4.3–40.0	3 (19)	
G2	37	17.4	0.3–94.5	19 (51)	
G3	14	13.3	2.4–28.6	4 (29)	ns
Myometrial invasion					
<50%	39	16.9	3.3–64.0	19 (49)	
≥50%	28	13.3	0.3–94.5	10 (36)	0.046
Lymph node involvement					
No	18	15.1	5.0–94.5	7 (39)	
Yes	4	13.6	8.1–25.7	1 (25)	ns

*The *P* value was calculated by the Mann–Whitney rank-sum non-parametric test. †The cut-off value for Cathepsin-D was 17 pmol/mg protein. ns, non-significant.

39 patients were M1 and 28 patients were M2. Patients were treated by abdominal hysterectomy plus bilateral salpingo-oophorectomy. In 22 cases, systematic pelvic and para-aortic lymphadenectomy was performed.

The characteristics of the cervical cancer patients are listed in Table 2. 52 patients received cisplatin-based neoadjuvant chemotherapy [18]. Cervical tumour size was measured by gynaecological examination, colposcopy and pelvic ultrasonography. Stability, progression and regression of disease were defined according to World Health Organization criteria. The neoadjuvant chemotherapy and radical surgery protocols were approved by the Investigational Review Board and patients gave their informed consent. 18 patients received conventional exclusive radiotherapy.

Cath-D assay and oestrogen and progesterone receptor measurements

All tissue specimens were frozen on dry ice shortly after surgical removal and stored at −80°C until processed. A representative section of the specimens was retained for histopathological examination. Cytosol and membrane fractions of the tissue specimens were separated as previously described just prior to assay [19]. Cath-D concentration was assayed using a solid-phase two-site immunoradiometric assay (CIS Bioindustries, Giftsur Yvette, France) in which the first monoclonal antibody (MAb), D7E3, is coated on the ELSA solid phase and the second MAb, M1G8, radiolabelled with ^{125}I, is used as a tracer [20]. Cytosol protein concentration was measured by the Bradford method [21]. Results were expressed as pmoles/mg of protein (pmol/mg protein). Intra- and interassay variations were 6.4 and 8.5%, respectively. In a preliminary evaluation we analysed our prognostic data using different cut-off values (10, 15, 17, 20 and 25 pmoles/mg protein). The value of 17 pmol/mg protein was the best prognostic discriminator, therefore, all subsequent analyses were conducted using this cut-off value.

Oestrogen receptor (ER) and progesterone receptor values (PR) were measured by the dextran-coated charcoal (DCC) assay, according to the EORTC protocol [22]. Results were expressed as fmoles/mg protein. The cut-off values of 10 and 20 fmol/mg protein were chosen for ER and PR, respectively.

Statistical analysis

The Mann–Whitney rank-sum non-parametric test was used to analyse the relationship between Cath-D levels and clinicopathological characteristics.

Survival analysis was performed by the Kaplan and Meier method [23], and the curves were examined by means of the log-rank test [24]. Multivariate analysis was performed by Cox's proportional hazards model [25].

RESULTS

Cath-D expression according to clinicopathological parameters and steroid receptors

Figure 1 shows the distribution of Cath-D levels in our series of 67 endometrial carcinomas and 70 cervical tumours. The median Cath-D value was 15.0 pmol/mg protein for endometrial tumours (range 0.3–94.5) and 14.9 pmol/mg protein for cervical tumours (range 2.2–95.5). Using an arbitrary cut-off value of 17 pmol/mg protein, 29 (43%) and 31 (44%) cases were Cath-D+ in endometrial and cervical tumours, respectively.

Tables 1 and 2 show the distribution of Cath-D levels according to clinicopathological parameters. In endometrial cancer, patients with advanced stage disease tended to express lower Cath-D levels than those with early stage disease (median values were 17.4 pmol/mg protein for patients with stage I, 13.6 pmol/

Table 2. Cathepsin-D levels according to clinicopathological parameters in primary cervical cancer

	No. of patients	Median (pmol/mg protein)	Range	No. of cases >cut-off † (%)	*P* value*
Total	70	14.9	2.2–95.5	31 (44)	
Histology					
Squamous	67	15.6	2.2–95.5	30 (45)	
Adenosquamous	3	13.2	11.0–17.5	1 (33)	ns
Age					
<50 years	31	14.2	4.0–95.5	13 (42)	
≥50 years	39	15.7	2.2–95.0	18 (46)	ns
Stage					
I–II	36	16.3	4.0–95.5	17 (47)	
III–IV	34	14.9	2.2–64.2	14 (41)	ns
Grade					
G1–2	43	16.3	4.0–95.5	16 (37)	
G3	27	17.1	4.0–64.2	12 (44)	ns
Tumour size					
<5 cm	34	14.9	4.0–95.5	11 (32)	
≥5 cm	36	14.1	2.2–74.6	13 (36)	ns
Lymph node involvement					
No	27	14.9	4.0–95.5	11 (41)	
Yes	13	17.5	2.2–95.0	7 (54)	ns
Response to chemotherapy					
CR–PR	44	14.8	2.2–95.5	19 (43)	
NC–P	8	9.2	4.0–17.3	1 (13)	0.011

*The *P* value was calculated by the Mann–Whitney rank-sum non-parametric test. †The cut-off value for Cathepsin-D was 17 pmol/mg protein. CR, complete response; PR, partial response; NC, no change; P, progression; ns, non-significant.

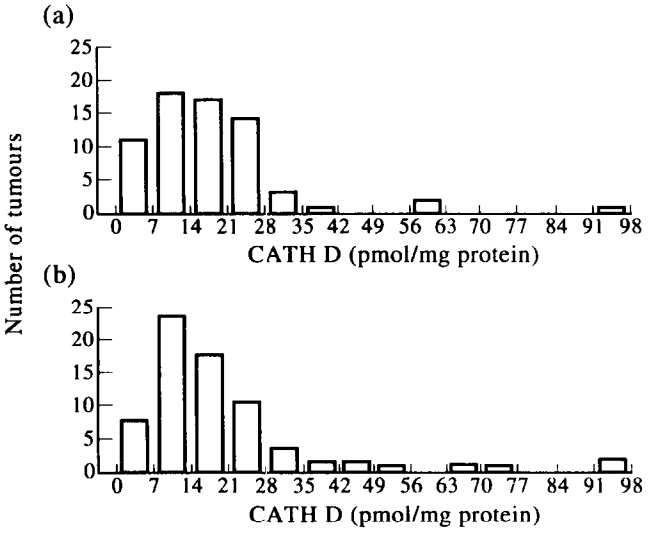

Figure 1. Distribution of Cathepsin-D levels in 67 primary human endometrial cancers (a) and in 70 primary human cervical tumours (b).

mg protein for stage II and 10.7 pmol/mg protein for stage III disease; $P = 0.027$). Moreover, patients with myometrial invasion <50% showed higher Cath-D levels than those with myometrial infiltration ≥50% (median 16.9 pmol/mg protein, range 3.3–64.0 versus median 13.3 pmol/mg protein, range 0.3–94.5; $P = 0.046$). There was no correlation between Cath-D levels and age, menopausal status, grade of differentiation and lymph node involvement.

In cervical cancer, no difference in the distribution of Cath-D levels according to histological classification, age, stage, grade and lymph node involvement was found. However, Cath-D levels were correlated with clinical response to chemotherapy since patients not responding to neoadjuvant chemotherapy had significantly lower Cath-D values than those showing complete or partial response (median 9.2 pmol/mg protein, range 4.0–17.3 versus median 14.9 pmol/mg protein, range 2.2–95.5; $P = 0.011$).

Table 3 shows a significant correlation between Cath-D levels and ER and PR status ($P = 0.05$) for endometrial cancer. In particular ER−/PR− endometrial tumours had lower Cath-D levels than cases with at least one receptor present ($P = 0.013$). No correlation between Cath-D and ER and PR expression was found in cervical tumours (data not shown).

Survival analysis

As far as the survival analysis was concerned, reliable data were obtained only in cervical cancer. In endometrial cancer, the small sample size and the small number of deaths and recurrences occurring in this population did not allow an adequate calculation of survival curves. However, it is interesting to note that only 2 of 7 endometrial cancer patients (29%) who recurred were Cath-D+ compared to 27 of 60 (45%) patients without recurrence. Moreover, all patients with Cath-D+ tumours are still living, while the 6 patients who died were Cath-D−.

The survival analysis of cervical cancer patients was performed on all patients, except 3 who were lost to follow-up. 23 patients died of disease during the follow-up period. Of the 52 patients with locally advanced disease who received neoadjuvant chemotherapy, 44 underwent complete or partial response and were, therefore, subjected to radical surgery. After surgery, 14 of these patients developed recurrent disease.

Figure 2 shows the survival curves of cervical cancer patients according to Cath-D status. Patients with high Cath-D values had a shorter survival than patients with low levels, although the

Table 3. Distribution of Cathepsin-D levels according to oestrogen receptor (ER) and progesterone receptor (PR) status for patients with endometrial cancer

	No. of patients	Median (pmol/mg protein)	Range	No. of cases >cut-off † (%)	*P* value*
ER−/PR−	18	8.4	0.3–40.0	5 (28)	<0.05
ER+/PR+	25	17.4	3.2–94.5	13 (52)	
ER−/PR+	15	19.3	12.0–64.0	8 (53)	
ER+/PR−	9	16.0	5.9–27.3	3 (33)	

*The *P* value was calculated by the Mann–Whitney rank-sum non-parametric test comparing all groups simultaneously. †The cut-off value for Cathepsin-D was 17 pmol/mg protein.

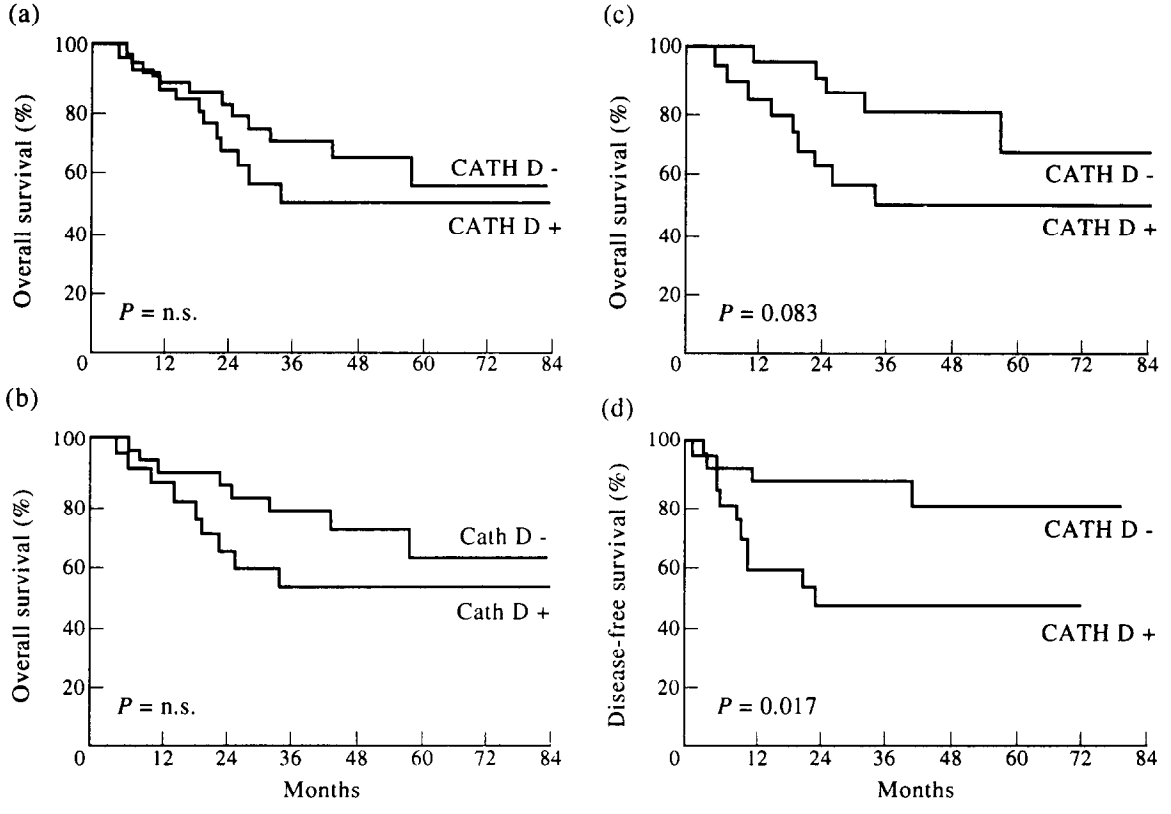

Figure 2. Survival of cervical cancer patients according to Cathepsin-D status defined by a cut-off value of 17.0 pmol/mg protein: (a) overall survival rate (*n* = 67); (b) overall survival rate of patients who received neoadjuvant chemotherapy (*n* = 52); (c) overall survival rate and (d) disease-free survival rate of patients who responded to neoadjuvant chemotherapy and therefore underwent radical surgery (*n* = 44).

difference was not statistically significant. The 3-year survival of Cath-D+ patients was 50% versus 73% for Cath-D− patients (Figure 2a). When only patients who received neoadjuvant chemotherapy were considered, the 3-year survival of patients with high Cath-D values was 52% as compared to 78% of patients with low Cath-D levels (Figure 2b). We also examined the disease-free and overall survival of patients undergoing surgery after complete or partial response to neoadjuvant chemotherapy. In these patients, Cath-D status seems to discriminate a group of subjects with poor prognosis ($P = 0.083$) (Figure 2c). In particular, patients with Cath-D+ tumours had a shorter disease-free survival than those with Cath-D− tumours ($P = 0.017$) (Figure 2d).

A multivariate analysis performed on disease-free survival data confirmed the negative prognostic role of high Cath-D levels (Table 4).

DISCUSSION

Previous studies have reported a wide range of Cath-D concentrations in endometrial [10, 15, 16], ovarian [5, 14] and breast [13, 20, 26–28] cancers. Our report demonstrates that Cath-D is widely expressed in a large series of endometrial and cervical tumours. Compared to the concentrations reported in breast cancer tissues, uterine tumours show lower Cath-D levels. In endometrial tumours, we report an inverse correlation between Cath-D levels, stage and myometrial invasion. This is in contrast with Nazeer and associates [16] who reported that high cytosolic Cath-D levels are correlated with deep myometrial

Table 4. Multivariate analysis of disease-free survival in 44 cervical cancer patients

	Univariate *P* value	Multivariate *P* value
Stage I–II vs Stage III–IV	0.007	0.25
Grade 1–2 vs Grade 3	ns	ns
Tumour size <5 cm vs Tumour size ≥5 cm	ns	ns
Cathepsin-D− vs Cathepsin-D+	0.017	0.043

ns, non-significant.

invasion. However, in this study very few cases were examined and the patient population was heterogeneous with regard to tumour histological classification.

Some evidence indicates that Cath-D is a hormone-regulated protein in various experimental models [8, 10, 11]. In endometrial tumours, we observed a positive association between Cath-D and steroid hormone receptor expression, which suggests a hormonal regulation of Cath-D levels in this tumour. This is consistent with the finding that Cath-D gene expression is increased by progestin in rat uterus [11] and in normal endometrial cells [10]. A similar correlation between Cath-D and steroid hormone receptors has been reported in breast cancer [27, 28], although data are conflicting [13, 26].

As expected, no association between Cath-D and steroid hormone receptors was observed in cervical cancer. Biological and clinical evidence suggests that steroid hormones only play a minor role, if any, in the development and spread of this disease [29]. Therefore, our data are consistent with the hypothesis that Cath-D regulation is tissue-specific [2]. It is conceivable that in hormone-dependent tissues Cath-D activity is modulated by steroid hormones, while in non-target organs other factors may be involved. In particular, the finding of high levels of epidermal growth factor receptor in cervical cancer [19] suggests that growth factors such as epidermal growth factor and/or transforming growth factor-α could be more important than hormones in the regulation of Cath-D synthesis [30].

Several clinical studies have demonstrated high Cath-D concentrations in primary breast cancer to be a significant independent predictor of poor prognosis [12, 13, 28]. Our previous studies reported higher levels of Cath-D in omental metastases than in the corresponding primary ovarian tumours [5], and a significant correlation between Cath-D levels and prognosis in ovarian cancer patients [14].

To our knowledge, this is the first study analysing the correlation between Cath-D levels and survival in patients with uterine tumours. Although our data need further confirmation in larger clinical trials, it can be suggested that Cath-D activity plays a different role in hormone-independent squamous tumours than in hormone-sensitive adenocarcinomas. In cervical tumours, high Cath-D levels seem to be correlated with poor prognosis. However, in analysing the survival data, it should be noted that in our series all patients had locally advanced, inoperable, cervical cancer, and that response to neoadjuvant chemotherapy is one of the major prognostic determinants in these patients [18]. The fact that a significant survival difference was only reached in patients responding to neoadjuvant chemotherapy can be explained by the finding that almost all patients unresponsive to chemotherapy had lower Cath-D values. Therefore, it is conceivable that a subset of patients with low Cath-D levels has a bad prognosis due to the presence of chemoresistance. This is in keeping with our previous report on ovarian cancer [14], in that low Cath-D levels were correlated with poor response to chemotherapy. It is also worth noting that in both cervical and ovarian cancer, chemotherapy consisted of cisplatin-containing regimens [14, 18].

Further studies using *in vitro* models are needed in order to clarify whether Cath-D is linked to mechanisms of chemoresistance and/or sensitivity of tumour cells.

In our series of cervical cancer patients, Cath-D levels were not correlated with clinicopathological parameters, in accordance with previous reports on ovarian [14] and breast [20, 26–28] cancer, thus suggesting that the presence of high Cath-D levels is an unfavourable parameter unrelated to other common prognostic features. This hypothesis is further confirmed by the results of the multivariate analysis showing that Cath-D is an independent prognostic factor.

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

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