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Cathepsin D Immunoreactivity in Ovarian Cancer: Correlation with Prognostic Factors

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In view of the somewhat inconclusive nature of reports of the role of Cathepsin D (Cath D) in ovarian carcinomas and its relationship with various other parameters of malignancy, the present study was performed to aid in the further clarification of this role. One hundred freshly resected primary ovarian carcinomas of various histological types were studied for ER, PR and Cath D status and the results examined with respect to menopausal status, histology, size and lymph node invasion. In our series Cath D positivity was more frequent in serous than in other types of ovarian cancer, but this positivity was not related to the frequency of lymph node invasion regardless of the size of the tumor. Furthermore, no association was observed between Cath D positivity and ER or PR status of the tumors or the menopausal state of the patients. The reported prognostic value of Cath D, ER and PR is discussed as well as the distinction between tumor invasion by lymphatic channels and direct interstitial infiltration. It was concluded that Cath D may not play role in the former mode but, as might be expected from its proteolytic properties, in the local spread by means of tissue destruction. (Pathology Oncology Research Vol 4, No 2, 103–107, 1998)

Key words: ovarian carcinoma, cathepsin D, immunocytochemistry, prognostic factors

Introduction

The growth of a primary tumor and the spread of tumor to distant sites depend on the ability to disguise its differences from natural host immunological defenses and also to overcome normal mechanical tissue barriers.[7,9] Some tumors are dependant for their growth on certain circulating hormones which are not essential for the homeostasis of normal tissues. Suppression of these hormones thus provides a non destructive method of tumor growth inhibition.[22,23] Apart from immunological disguise another means of facilitating tumor spread reported is the production by tumors of certain proteolytic enzymes among which Cathepsin D (Cath D) has been most studied.[11,21]

Cath D is a lysosomal aspartyl proteinase which could play an active role in the malignant progression of some human cancers.[3] Its presence may promote tumor cell proliferation by acting as an autocrine mitogen and may facilitate metastasis because it degrades extracellular matrix and frees growth factors from the matrix.[18] Several factors, including intracellular pH, hormones, growth factors and endogenous inhibitors seems to regulate the mechanisms of proteolytic activity of Cath D.[4,15,25,27]

One of the biological activities of this protease is the mitogenic on estrogen depleted cells suggesting that it is an estrogen regulated autocrine mitogen.[17] Cath D is reported to be associated with estrogen receptor (ER) positivity but while in many tumors this latter is associated with improved prognosis[6,20] tumors with Cath D overexpression have been reported to have shorter disease free and overall survival times.[12,16]

We have attempted to add to the existing information in literature by studying ER, PR and Cath D in 100 primary ovarian cancers. ER and PR were determined in tissue homogenates, Cath D on imprint smears from fresh tumor material using the Avidin-Biotin immunoperoxidase technique.

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Materials and Methods

Imprint smears were obtained by touching the cut surface of 100 fresh surgically resected ovarian cancers. The mean age of the women was 51.56±10.2 years. From them 39 were premenopausal (mean age 49.92±4.94 years), 61 were postmenopausal (mean age 57.08±8.77 years). The largest diameter of the tumors were noted and the tumors were then divided. ER and PR concentrations were measured by the dextran coated charcol technique in tumor tissue homogenates according to EORTC protocol.[2] Specimens were considered ER or PR positive if they contained at least 57 fmol of specific binding sites of protein. One part was submitted for histological diagnosis, while another portion was used for the immunocytochemical demonstration of Cath D overexpression. All smears were air dried for 15 min, fixed in cold acetone (–10°C) for 10 min and stored at –70°C until used. Immunocytochemical staining was performed by the Avidin-Biotin complex (ABC) immunoperoxidase method.[10] Smears were incubated for 45 min with normal rabbit serum (Dakopatts, Denmark) diluted 1:50 in PBS. The smears were then rinsed in three changes of PBS for 5 min each and then incubated overnight in Cath D antibody (1:40) commercially available murine monoclonal antibody (Cis Bio Int, France). After washing in PBS the smears were incubated with rabbit anti-mouse biotinylated immunoglobulins (Dakopatts, Denmark) diluted 1:200, followed by the ABC complex/HDR (Dakopatts, Denmark). Visualization was achieved by a final incubation in diaminobenzidine (Sigma, UK). The resulting preparations were counterstained with Mayer's haematoxylin, dried and mounted. Non immune mouse serum was used for negative controls in place of the primary antibodies. The immunocytochemical staining was considered positive when staining reaction (brown granules) was observed in the cytoplasm as well as on the cell membrane *(Figure 1)*. For the assessment of staining, random fields were sampled; 500 tumor cells were counted in at least five high power representative fields and the number of cells with positive staining was divided by the total number of cells counted. In cases where staining was heterogeneous on the slide, examined fields included those with the highest and those with the lowest percentage of stained cells. The intensity of staining was scored on a four-point scale: 0 no staining, 1 weak but unequivocal staining, 2 definite staining of moderate intensity, 3 strong staining. Only tumor cell scoring 2 or more were considered Cath D positive, regardless of the number of cells stained.

The results of all the above were recorded, and statistical analysis using the X^2 test with Yates correction was performed to examine the possibility of relationships between tumor size, histology, menopausal status, lymph node invasion, ER, PR and Cath D status.

Figure 1. *Clusters of serous adenocarcinoma cells of ovarian carcinoma showing positive reaction for Cath D (X500).*

Table 1. **Relationship between Cathepsin D and menopausal status, tumor size, lymph node invasion, ER and PR positivity of 100 patients' ovarian cancers**

	No	*Cath D⁺*	*Cath D⁻*	X^2	*P*
Postmenopausal	61	42	19	1.63	NS
Premenopausal	39	22	17		
>2 cm	56	39	17	1.9	NS
<2cm	44	25	19		
LN^+	62	40	22	0.02	NS
LN^-	38	24	14		
PR^+	17	13	4	1.37	NS
PR^-	83	51	32		
ER^+	20	15	5	1.29	NS
ER^-	80	49	31		

NS – non significant

Results

Fifty six of the tumors had diameter larger than 2 cm (>2cm). Histologically 53 of the tumors were serous, 12 endometrioid, 12 mixed, 13 clear cell and 10 mucinous carcinomas. Sixty two of the patients had evidence of lymph node invasion (LN+ve). Twenty of the tumors were positive for estrogen receptors (ER+ve), 17 for progesterone receptors (PR+ve) and 64 showed positive staining for Cathepsin D (Cath D+ve).

All 17 of the PR+ve tumors were also ER+ve. Thirteen patients were positive for ER, PR and Cath D and 50 patients were positive for Cath D but negative for ER and PR. *Table 1* demonstrates the relationships between Cath D positivity and the other parameters together with the results of the X^2 analysis.

Table 2 shows the relationships between tumor size, lymph node invasion and Cath D status. Lymph node inva-

Table 2. **Relationship between tumor size, lymph node invasion and Cathepsin D status (X^2 and Yates correction)**

	No	*Cath D⁺*	*Cath D⁻*	X^2	*P*
LN^+>2cm	47	8	7	0.58	NS
LN^->2cm	9	7	2		
LN^+<2cm	15	8	7	0.00	NS
LN^-<2cm	29	16	13		
		>2 cm	<2 cm		
LN^-	62	47	15	27.6	<0.001
LN^-	38	9	29		

NS – non significant

sion was a significantly more frequent observance in cases in which the tumor was greater than 2 cm in diameter. No other significant associations between the parameters were demonstrated.

For the purpose of further analysis the different histologies were divided into two groups, the 53 serous carcinomas forming one group, the 47 other cancers forming the second group. The results of the X^2 analysis of this grouping are shown in *Table 3*. It can be seen that Cath D positivity and lymph node invasion are significantly more frequent in this histological type of ovarian cancer than in the others. No such relationship was observed, however, for tumor size or menopausal status, ER or PR positivity.

At the time of writing 50 patients had died from their disease. The mean survival of these patients was 28.5 months while the mean survival, of those still alive stands now at 49.6 month. Comparing Cath D status, between those alive and died a significant difference is observed (X^2 = 5.2, $p<0.05$), i.e. Cath D positivity was more frequently associated with poor prognosis. There is however no such association concerning other parameters including the histology.

Within the group of patients who died the mean survival was compared with the histology, Cath D status and lymph node metastases at surgery and the results are presented in *Figure 2*. This diagram shows that the best possible survival was achieved in patients with Cath D negative, non serous tumors who had no lymph node invasion at surgery while the worst possible outcome was in patients whose tumors were Cath D positive with serous histology and lymph node metastases at surgery. Three of the patients who died had no lymph nodes at diagnosis and had small, Cath D negative non serous tumors.

There were 1 clear cell and 2 mixed type tumors and 7 others who died had no lymph nodes at diagnosis and had small Cath D positive tumors. These all were serous carcinomas. There were two patients with serous tumors who were negative for all parameters studied and are living at 5 years. One of the patients with endometrioid metastases Cath D, ER and PR positive in a premenopausal patient the other 11 are still alive.

In 26 of 30 patients with non-serous tumors who are still alive the ER and/or PR (ER/PR) showed negativity while in the group of died patients 7 out of 10 had ER/PR positivity (X^2 = 3.2). This figure is almost statistically significant. In the group of serous tumors no such relationship was found because the 9 ER/PR positive tumors were evenly divided between those who alive and those who had died.

Table 3. **Relationship between histological type of ovarian carcinoma and Cathepsin D status, tumor size, lymph node invasion, menopausal and ER and PR status**

	No	*Serous*	*Other*	X^2	*P*
Cath D^+	64	43	21	12.8	<0.05
Cath D^-	36	10	26		
>2cm	56	28	28	0.00	NS
<2cm	44	24	20		
LN^+	62	28	34	4.0	<0.05
LN^-	38	25	13		
Postmenopausal	61	30	31	0.89	NS
Premenopausal	39	23	16		
ER^+	20	9	11	0.57	NS
ER^-	80	44	36		
PR^+	17	8	9	0.27	NS
PR^-	83	45	38		

NS – non significant

Discussion

The obvious manipulation of the biochemical hormonal and physical environment by malignant tissues has been one of the most exciting and rewarding fields of study over the last 10 years. The plethora of genetic tricks called into play by these iniquitous cells constantly reminds us that „there are more things, in heaven and on earth than are dreamed of in our philosophy" and yet sets us scientists on new tracks every day to elucidate the intricacies in the hope of more effective treatments and/or life saving techniques.

In spite of some previous reports in which Cath D positivity has been associated with ER positivity (mainly in breast tumors)[6,8,13,14,20] the results of our study show no significant correlation between Cath D positivity and ER positivity. The same applies to PR positive tumors[4,14,20,21] which in our series were almost invariably also ER positive.

In view of the suggested role of Cath D as a substance facilitating early spread of primary disease it might have been expected to find some relationship between lymph node invasion and Cath D status but in our series there was

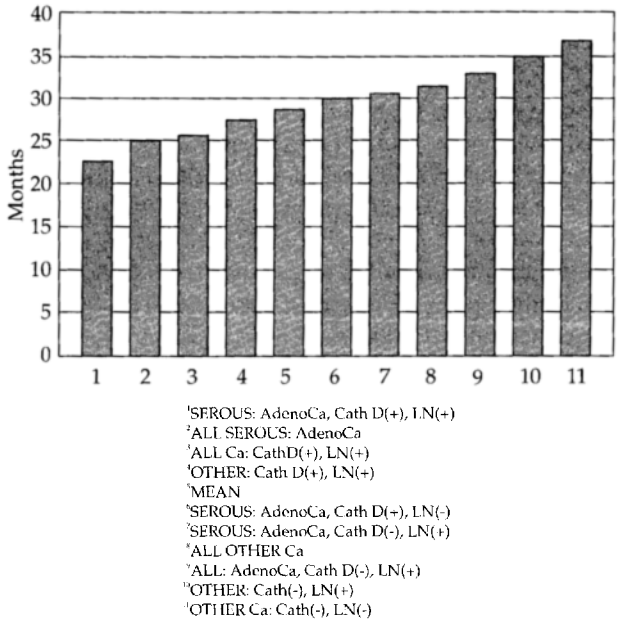

Figure 2. *Survival in months of the 50 patients who died in relation to their histology, Cath D and lymph node status.*

no indication of such a relationship. Also even when the size of the primary tumor at surgery was taken into account *(Table 2)* there was no observable relationship between Cath D status and lymph node invasion. Here though, it might be of value to separate the idea of early invasion of primary disease into two distinct entities. One via lymphatic or venous channels which might occur whether or not the primary tumor exhibited Cath D over-expression – as these roads are in any case open – and the other of direct invasion through adjacent connective tissue and peritoneum, which of course would probably not be discernable from a simple positive or negative lymph node survey. Thus, while the fact remains that Cath D in our series was not associated with lymph node infiltration, it would seem that this does not retract from the idea of Cath D encouraging local tumor invasion by its proteolytic properties and still remaining a poor prognostic indicator.[1,11,26] The only positive correlation which was observed in this field – and which of course was to be expected – was a very strong correlation between primary tumor size and frequency of lymph node metastases at the time of surgery ($X^2 = 30.17$, $p<0.05$).

There was also no relationship between tumor size and Cath D positivity, which was expectable considering that Cath D positivity is a characteristic of the malignant cell rather than an acquired feature dependant on the age of tumor.

A further indication of the independence of Cath D from estrogen and progesterone is provided by the lack of association between pre- or postmenopausal patients and the incidence of Cath D positivity in the ovarian carcinomas studied. Looking at the new data, however, it is interesting to note that 46 of 53 (86.7%) serous carcinomas were Cath D positive while the percentages in the other groups were 58% for mixed and between 25% and 40% in the other types of carcinomas. This led us to examine these data more closely, with the result that significant associations were found for serous carcinomas with Cath D positivity ($X^2 = 4.0$, $p<0.05$) and also with lymph node invasion, regardless of the size of the primary tumor ($X^2 = 4.0$, $p<0.05$). That Cath D positivity was not primarily associated with lymph node positivity in this situation can only be inferred from the results in the other types of carcinomas and the group as a whole. This would tend to support the hypothesis that serous carcinomas demonstrate both early lymph node invasion and Cath D positivity, another possible agent favouring local invasion thus in part accounting for at least the relatively poor prognosis of this histological type of ovarian carcinoma.

If we review the data related to the patients who died in comparison with those still surviving the only signifi-

Table 4. **Comparison of frequencies of the parameters studied between the surviving patients (living) and those who died**

	No	*Living (m)*	*Dead (m)*	X^2	*p*
Cath D^+	64	24	40	5.2	<0.05
Cath D^-	36	26	10		
>2 cm	56	26	30	0.36	NS
<2 cm	44	24	20		
LN^+	62	28	34	1.06	NS
LN^-	38	22	16		
Postmenopausal	61	28	33	0.67	NS
Premenopausal	39	22	17		
ER^+	20	9	11	0.0006	NS
ER^-	80	41	39		
PR^+	17	7	10	0.002	NS
PR^-	83	43	40		
Serous AdenoCa	53	25	28	0.001	NS
Other	47	25	22		
Cath D/Serous AdenoCa	43	17	26	0.02	NS
Cath D/Other	21	7	14		
Serous/ Cath D^+/LN^+	23	8	15	0.36	NS
Serous/ Cath D^-/LN^+	2	2	0		
Serous/ER/PR^+	9	4	5	0.00	NS
Serous/ER/PR^-	44	21	23		
Other/ER/PR^+	11	4	7	3.2	NS
Other/ER/PR^-	36	26	10		

m – months, NS – non significant

cant association is found for Cath D positivity. This would indicate that Cath D is not dependent on lymph node invasion for its lethal action and also that Cath D positivity is an equally bad prognostic indicator in all histological tumor types *(Table 4)* and supports the previously mentioned hypothesis of Cath D favouring direct invasion.[11] Tumor cells might more easily escape directly through the outer edge of the primary tumor and travel to distant sites by this means even without a large primary. Therefore, the fact that there was no relationship between Cath D positivity and primary tumor size is not against this hypothesis.

In view of the fact that 3 of the patients with non-serous Cath D negative tumors and were lymph node negative at surgery died indicates that other parameters should work apart from Cath D status and lymph node invasion. In this respect it is important that while in the group of patients with serous tumors ER/PR positivity was equally divided between the survivors and the non survivors, in the patients with non serous tumors ER/PR negativity was more frequently observed in the survivors. Though this was not significant statistically the difference in the X^2 values *(Table 4)* is considerable and requires further study.

In conclusion it may be stated that the results of the present study indicate that Cath D positivity of ovarian carcinomas is not associated with ER or PR positivity and is unrelated to lymph node invasion. At the same time Cath D positivity is observed more frequently in serous carcinomas than in other histological types and that in these serous carcinomas early lymph node involvement is more common but unrelated to either Cath D positivity or the ER or PR status. In non serous tumors however where Cath D is not so frequently found ER/PR positivity in the group studied was more frequently observed in the patients who died than in the group surviving. The significance of this finding awaits confirmation and investigation.

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