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Molecular Prognostic Markers in Recurrent and in Non-recurrent Epithelial Ovarian Cancer

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Abstract. *Background: The outcome and prognosis of ovarian cancer is highly variable. The objective of this study was to compare survival and clinicopathological prognostic factors with the expression levels of two matrix metalloproteinases (MMP) and fibronectin as tumor invasion and metastasis markers in ovarian cancer patients. Materials and Methods: Histologically-verified epithelial ovarian tumours from 27 patients were studied. The latent and the activated forms of MMP-2 and MMP-9 were measured as gelatinase activity from tumour extracts and from serum and ascites samples by a zymographic technique. The fibronectin content was quantified by immunoblotting and densitometric analysis. Molecular marker levels were correlated to clinicopathological parameters such as survival and disease recurrence during the median postoperative follow-up period of 30 months. Results: The levels of MMP-9 and fibronectin, but not those of MMP-2, were significantly higher in tumour tissues and in the ascites fluid of the recurrent patient group and the patient group who did not survive, as compared to the non-reccurent cases. Conclusion: Our data support that high expression of MMP-9 and fibronectin indicate poor prognosis for ovarian cancer patients who have similar clinicopathological prognostic factors.*

Among gynecological malignancies, ovarian cancer is the leading cause of mortality in the industrialised countries (1). The majority of ovarian cancers are epithelial and show an advanced stage at the time of diagnosis (1). Previously, we have shown that thymidine kinase activity of ovarian carcinomas is at least 12-fold higher than that of normal ovarian tissue, demonstrating the high proliferation activity of this type of tumour (2). The progression of epithelial ovarian cancer (EOC) is distinct from that of most epithelial tumours (*e.g.* breast, colon, lung), because its dissemination occurs mainly by intraperitoneal spreading of the cancer cells, resulting in multiple metastatic nodules on both the visceral and parietal peritoneum (1, 3). Peritoneal tumour implantation may lead to increased surface area and increased plasma transudation, producing ascites – a common manifestation in advanced ovarian cancer (1, 3). The outcome and prognosis of apparently similar cases of advanced ovarian cancer with the same clinicopathological prognostic factors are highly variable (1, 3). The objective of this study was to compare survival and clinicopathological prognostic factors with molecular markers of invasion and metastasis in patients with ovarian cancer during a 30-month follow-up period.

The development and spread of malignant tumours is thought to be a multistep process, which depends on the activity of many mediators (4). A prerequisite of invasion and metastasis of EOC cells is the expression of specific proteolytic enzymes that degrade components of the basement membrane and the extracellular matrix (ECM) and participate in the process of tumour angiogenesis (5-7). One of the most important groups of ECM proteases are matrix metalloproteinases (MMPs), which also play indispensable roles in wound healing, angiogenesis and embryogenesis (5-7). A common feature of MMPs is the presence of a zinc ion in the active centre, but their domain structure varies (5-7). Some MMPs possess gelatine-binding domains (therefore known as gelatinases), such as MMP-2 and MMP-9 (5-7). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are also secreted as latent enzymes with a M_r of 72,000 and 92,000 kDa,

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respectively, and are activated by plasminogen activator-like processes to degrade type IV collagen, a major component of the basement membrane (5-7).

The fact that ECM degradation represents an essential step in tumour invasion is also supported by studies revealing high MMP-2 and MMP-9 activities in EOC cells (8-14). Further data on the relevance of MMP-9 and MMP-2 levels in the metastatic spread of ovarian tumour are required to decide whether the activity of these proteases, either in the serum (ascites) or in the tumour, could better indicate tumour progression.

Fibronectins (FN) are a family of glycoproteins that mediate cell-cell and cell-matrix interactions and are important in physiological and pathological processes, including cell-matrix interactions in metastasis (15). It has been reported, by Shibata *et al.,* that fibronectin secretion from peritoneal tissue induces MMP-9 expression in ovarian cancer cell lines in tissue culture (16). An adverse association between fibronectin expression and prognosis in ovarian carcinoma has also been shown by Franke *et al.* (17).

In the present work, the latent and activated MMP activities and fibronectin levels were compared in normal and cancerous ovarian tissues as well as in body fluids (serum, ascites) of patients. We found significant elevations of both MMP-9 and fibronectin levels in recurrent tumours, implying their use as valuable prognostic factors in epithelial ovarian cancer.

Materials and Methods

Patients and tumour sampling. The study presents data from patients who had been surgically treated and followed-up at the First Department of Obstetrics and Gynecology of Semmelweis University, Budapest, Hungary. None of the patients had been subjected to neoadjuvant chemo- and/or radiotherapy, and all of them had undergone debulking surgery followed by platinum- and taxane-based chemotherapy. Ovarian tumour samples, serum and ascites fluid, if available, were obtained during surgery. Representative areas of each tumour were sampled by a gynaecological pathologist, both for histological assessment according to the WHO classification and for biochemical studies. Written informed consent was obtained from the patients, and tissue procurement was approved by the Ethics Committee of Semmelweis University. The median follow-up time for patients was 30 months.

Histology. Tissue sections from formalin-fixed, paraffin-embedded tumour samples were stained with H&E. According to histological examinations, the samples were classified as follows: i) normal ovarian tissues from (n=5) patients who had undergone hysterectomy for non-malignant disease; ii) low malignant (n=5) potential ovarian tumours (3 serous and 2 mucinous); iii) sex-cord stromal ovarian tumours (n=5); iv) primary EOCs (n=17).

Tissue extraction and gelatin zymography. Tissue specimens were snap-frozen in liquid nitrogen and pulverized in a mortar, then resuspended in a hyperosmotic lysis buffer containing 50 mM Tris-HCl pH 7.6, 500 mM NaCl, 5 mM $CaCl_2$ and rotated gently at 4˚C for 10 min. Lysates from the tumour samples were centrifuged at 11,000 x g (30 min, 4˚C) and the supernatants were applied for gelatin zymography. Serum and ascites fluids were diluted 40 times, and the protein concentration was adjusted to 5 mg/ml (BioRad Hercules, CA, USA: DC reagent).

Gelatin zymography is a suitable technique for simultaneously distinguishing between the pro- and active forms of MMPs, according to their M_r as follows: MMP-2 (72 kDa/62 kDa) and MMP-9 (92 kDa/82 kDa), respectively. The assays were performed as described previously (18). Briefly, tumour extracts, ascites and serum samples (50 μg total protein) were resolved by SDS-PAGE containing 0.3 mg/ml gelatin. The gel was then incubated with 2.5% Triton X-100 to remove SDS, and was subsequently incubated overnight at 37˚C in a buffer consisting of 5 mM $CaCl_2$, 500 mM NaCl and 50 mM Tris-HCl pH 7.6. Proteins were stained with Coomassie Brilliant Blue R-250. Zones of gelatinase activity appeared as white bands against a blue background. The intensity of MMP-9/-2 gelatinolytic zones was quantified using the Eagle Eye II (Stratagene, La Jolla, CA, USA) densitometric scanner with a Scananalytics software van-dscan. Gelatinase activities were expressed as integrated arbitrary units (IU) per 10 μg of total protein (18).

Table I. *Clinicopathological prognostic factors of patients with recurrent or non-recurrent epithelial ovarian cancer.*

Clinicopathological factors	Recurrent group (n=8)	Non-recurrent group (n=9)
Age		
Younger than 60 years	3	4
Older than 60 years	5	5
Stage		
I	-	2
II	-	-
III	7	7
IV	1	-
Histological subtype		
Serous	6	9
Mucinous	1	-
Endometrioid	1	-
Grading		
G1	-	2
G2	1	-
G3	7	7
Ascites		
Not present	-	2
Present	8	7
Surgery		
Optimally resected	4	5
Suboptimally resected	4	4
Deaths due to EOC	4	-

Table II. *Activities of the latent and active forms of MMP-2 and MMP-9 in serum, ascites and tumour samples from different groups of EOC patients.*

	Non-recurrent patients (n=9)			
	MMP-2		MMP-9	
	72 kDa	62 kDa	92 kDa	82 kDa
Serum	1.06±0.87	0.00	2.78±2.21	0.00
Ascites	2.10±1.60	0.00	2.28±1.53	0.14±0.08
Tumour	1.41±1.09	0.15±0.05	1.52±1.13	0.00
	Recurrent patients (n=8)			
	MMP-2		MMP-9	
	72 kDa	62 kDa	92 kDa	82 kDa
Serum	0.16±0.06	0.00	5.10±3.73	0.00
Ascites	0.87±0.33	0.04±0.02	3.19±2.64	0.75±0.55*
Tumour	0.89±0.69	0.51±0.22	5.35±3.66*	1.11±0.87*
	Patients who died of EOC (n=4)			
	MMP-2		MMP-9	
	72 kDa	62 kDa	92 kDa	82 kDa
Serum	0.08±0.03	0.00	2.02±1.55	0.00
Ascites	0.94±0.54	1.18±1.05	3.44±2.32	0.97±0.45*
Tumour	0.98±0.55	0.85±0.55*	5.85±3.67*	3.37±2.25*

Collagenase activities of the samples were tested by gelatin zymography as decribed in Methods. The different molecular weight forms (latent: 72 or 92 kDa; and active: 62 or 82 kDa, for MMP-2 and -9, respectively) were separated by gel electrophoresis as described. Enzyme activities are expressed as arbitrary units (IU) per 10 μg of total protein; data represent means and standard deviations. Significance levels ($p<0.05$) relative to corresponding values of the non-recurrent group are indicated by asterisks.

Determination of fibronectin concentration. The Western blot technique was applied to measure fibronectin content, as reported previously (18). Ovarian tumour extracts, serum and ascites fluid samples were prepared as described above. Samples were separated by SDS-PAGE, transferred to nitrocellulose membranes that were blocked overnight, then washed and probed with an anti-fibronectin antibody (Sigma, St. Louis, Missouri, USA: F3648) (1:1000 dilution). As for the secondary antibody, biotinylated goat anti-rabbit immunoglobulin (Dako, Carpinteria, CA, USA: E0432) was applied, and the blots were probed with streptavidin-horseradish peroxidase conjugate (1:200 dilution). Immunocomplexes were detected with the DAB reagent. The fibronectin concentration was quantitated by densitometric scanning, as described above.

Statistical analysis. Variances between groups were analysed with ANOVA. Differences were evaluated using the Student's *t*-test. $p<0.05$ was considered to be significant and is indicated as*.

Table III. *Expression of fibronectin in serum, ascites and tumour samples from different groups of EOC patients.*

	Tumour	Ascites	Serum
Non-recurrent (9)	1.87±2.21	4.92±3.53	4.34±2.35
Recurrent (8)	6.92±3.64*	5.99±4.61	4.55±2.45
Died of EOC (4)	6.67±3.55*	6.14±4.55	2.07±1.56

Fibronectin concentrations were determined by immunoblotting and densitometric evaluation as described in Methods. Data are expressed as arbitrary units (IU) per 10 μg of protein, representing means and standard deviations. Significance levels ($p<0.05$) relative to corresponding values of the non-recurrent group are indicated by asterisks.

Results

Clinicopathological parameters along with MMP-2, MMP-9 and fibronectin levels were tested in 32 ovarian samples histologically classified into four groups, as described in the Materials and Methods section. There were no significant differences either between clinicopathological characteristics or between invasion markers with respect to the i) normal (n=5), ii) low malignant (n=5) and iii) sex-cord tumour (n=5) groups (data not shown).

Considering the patients with primary EOC (n=17), recurrent disease was observed in eight individuals and four of them died during the 30-month follow-up period. Importantly, no significant differences were found between the clinicopathological parameters of recurrent and non-recurrent ovarian cancer cases (Table I). Most carcinomas were serous, anaplastic and diagnosed in stage III in both groups. Regarding the most important therapy-related prognostic factor, approximately half of the patients in both groups underwent optimal resection (Table I).

MMP-2 and MMP-9 activities in recurrent and non-recurrent EOCs. MMP-2 activities in serum, ascitic fluid and ovarian tissues were slightly higher in samples from ovarian cancer patients (n=17) as compared to the normal (n=5) group. Nevetheless, due to great variances among the individual data, the changes are not significant, even though the increase in MMP-2 activities in the cancerous ovarian tissues and in the ascitic fluid was considerable (data not presented).

Both the latent and active MMP-9 levels were significantly higher in EOC tumour samples from patients who had developed recurrent disease relative to those without recurrence (Table II). Moreover, elevated active MMP-9 levels were also found in the ascites of recurrent patients. Notably, active forms of MMPs were never detected in the serum (Table II).

Fibronectin concentrations. Although the fibronectin content was markedly elevated in EOC tissues, there was no statistically significant difference between the fibronectin concentrations in normal ovaries, in borderline tumours (groups ii and iii) and in ovarian carcinomas (data not shown). As observed for MMP-9 activity, the fibronectin concentrations in recurrent tumors (but not in the ascites or serum) were also significantly higher relative to tumours without early recurrence (Table III).

Patient survival. During the follow-up, four of the seventeen patients died due to EOCs. All of them had verified recurrent tumours, and each tumour was anaplastic and diagnosed in an advanced stage. Both fibronectin concentrations and MMP-9 activities were significantly elevated in the tumours of patients who died, compared to the non-recurrent group (Tables II and III), as observed in the recurrent group as well. MMP-9 enzyme activity was also significantly elevated in the ascites fluid in the same patients. Moreover, even MMP-2 levels were significantly increased in these tumours (Table II).

Discussion

It is widely accepted that the outcome of EOCs with similar clinocopathological parameters may be very variable, and that traditional prognostic factors often fail to predict disease outcome in a clinically reliable manner (Table I). Therefore, much is expected from such molecular prognostic markers that may help to identify EOCs with an aggressive phenotype (3). MMPs and fibronectin are among the candidate prognostic factors in ovarian cancers (5-17).

In the present study, the question was raised as to whether MMP activities differed in normal ovaries, in borderline (low malignant potential) tumours and in EOCs. Since MMPs play a critical role in the dissemination of tumours (5-9), their activities might be correlated with the prognosis of ovarian tumours.

We found no correlation between the histology of ovarian tumours and the levels of MMP activities, although the activated forms of both MMP-9 and MMP-2 were slightly higher in ovarian cancers than in normal ovaries, but the differences were not significant (data not presented). On the other hand, pro-MMP-9 levels were significantly elevated and the activated forms of both MMP-9 and MMP-2 were more frequent in EOC patients who developed recurrent disease or died, compared with the non-recurrent group (Table II). In addition, the MMP-9 levels of ascites fluid from patients with recurrence were also significantly higher than in non-recurrent ovarian tumours. On the contrary, pro-MMP-2 levels showed no increase in this group of patients; indeed, there was a tendency for lower activity (Table II).

Wu *et al.* (11) showed that activated MMP-2 might be a potential marker to predict EOC outcome. In contrast, our results suggest that, out of the two extracellular collagenases, MMP-9 seems to be a more reliable prognostic marker. Similar results were obtained by Lengyel *et al.* (13), who concluded that latent MMP-9 activity might be a good candidate to predict survival in advanced ovarian carcinoma. Herrera *et al.* (19) recommended using the sum of MMP-2 and MMP-9 expression as a prognostic factor in epithelial ovarian tumours.

Elevated levels of MMPs in serum have been reported in patients with other solid tumours (20, 21). Our data support the findings of Manenti *et al.* (22), who found slightly increased levels of pro-MMP-9 in the serum of patients with malignant ovarian disease; however, this difference was not significant. Therefore, serum MMP activities should not be considered as prognostic markers in ovarian malignancies.

Fibronectin expression has previously been found to be an important prognostic factor in ovarian cancer, and its central role in tumour progression was suggested (17). In agreement with this, we detected significantly elevated fibrinectin levels in recurrent ovarian tumours (including cases with fatal outcome) relative to the non-recurrent ones (Table III). It should be noted, however, that no increase of fibronectin concentration was found in body fluids (Table III). The parallel elevation of MMP-9 activities and fibronectin levels in recurrent ovarian cancers are in accord with previous observations that fibronectin secretion from human peritoneal tissues induces MMP-9 expression and enhances the invasiveness of ovarian cancer cells (16). Thant *et al.* revealed that fibronectin promotes MMP-9 expression through activation of the classic MEK1-MAPK cascade and the PI3K-Akt survival signalling pathway in ovarian tumours (23). It is tempting to speculate that the metastatic potential of aggressive ovarian tumours with high fibronectin and MMP-9 expression might be attenuated by targeted disruption of these intracellular signal transduction systems.

In conclusion, our data suggest that the determination of MMP-9 activities and fibronectin expression in surgically removed epithelial ovarian tumours might provide novel prognostic factors that help to predict tumour recurrence and disease-free survival.

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