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Ovarian carcinoma cells in serous effusions show altered *MMP-2* and *TIMP-2* mRNA levels

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Abstract

The expression of matrix metalloproteinases (MMP) and their inhibitor TIMP-2 in serous effusions from patients with ovarian carcinoma and its association with clinico-pathological parameters were analysed. The findings in carcinoma cells in effusions were compared with corresponding primary and metastatic lesions. Sixty-six effusions and 96 tissue sections were stained for MMP-1, MMP-2 and MMP-9 applying immunohistochemistry (IHC) and analysed for *MMP-2*, *MMP-9* and *TIMP-2* expression using mRNA *in situ* hybridisation (ISH). *MMP-2* and *MMP-9* mRNA levels in 30 effusions were subsequently analysed using reverse transcription- polymerase chain reaction (RT-PCR). MMP and TIMP expression was detected in both carcinoma and mesothelial cells in effusions. The levels were consistently higher in malignant cells, significantly so for MMP-1 ($P=0.016$) and MMP-2 ($P=0.036$) proteins, as well as for *TIMP-2* mRNA ($P=0.008$). In tissue sections, MMP-1, MMP-2 and MMP-9 protein expression was mostly localised to tumour cells, while *MMP-2*, *MMP-9* and *TIMP-2* mRNA were predominantly detected in stromal cells. Adenocarcinoma cells in effusions showed a significant upregulation of MMP-2 expression compared with primary tumours, with a concomitant downregulation of TIMP-2. RT-PCR demonstrated the presence of *MMP-2* and *MMP-9* in 28/30 and 0/30 specimens, respectively. MMP and TIMP are thus mainly synthesised by cancer cells in effusions, while stromal cells have a similar role in solid tumours. MMP-1 and MMP-2 production predominates over that of MMP-9 in effusions. Increased MMP-2 and reduced TIMP-2 levels are seen in ovarian carcinoma cells in effusions, possibly marking the acquisition of a metastatic phenotype.

Keywords: Immunohistochemistry; Matrix metalloproteinases; mRNA *in situ* hybridisation; Ovarian carcinoma; Serous effusions; Reverse transcription-polymerase chain reaction; Tissue inhibitors of metalloproteinases

1. Introduction

Ovarian carcinoma is the leading cause of death from gynaecological cancers in western countries [1], with approximately 450 new cases annually diagnosed in Norway [2]. Owing to their insidious development, two-thirds of these tumours are diagnosed at stage III or IV [3]. Abdominal discomfort is experienced by two-thirds of the patients, caused by the accumulation of ascitic fluid which often contains malignant cells [4]. The presence of ovarian carcinoma cells in peritoneal effusions is attributed to direct shedding from the ovarian tumour surface and their metastatic potential has not yet been fully defined. However, the presence of tumour-positive ascites has been shown to correlate with poor outcome [5].

The dissemination of malignant neoplasms is a multi-step process, involving degradation of the sub-epithelial and sub-endothelial basement membranes and modification of the extra-cellular matrix (ECM) [6]. Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent enzymes, are central mediators of these processes owing to their ability to degrade basement

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membrane and ECM components [7]. MMP-2 (Gelatinase A, 72kD type IV collagenase) and MMP-9 (Gelatinase B, 92kD type IV collagenase) are able to degrade collagen type IV, a component of all basement membranes, thereby facilitating stromal and vascular invasion by tumour cells [7]. The enzymatic activity of MMPs is controlled by tissue inhibitors of metalloproteinases (TIMPs), a family presently comprised of four proteins (TIMP1-4) with a molecular size of 21–28 kD with a different distribution in normal tissues [8]. Cell surface-mediated activation of MMP-2 and MMP-9 has been shown to involve the formation of a complex with TIMP and membrane-type MMPs (MT-MMP) [8].

The expression of MMPs and TIMPs in ovarian carcinoma cells in effusions has been the subject of several studies. The expression of MMP-2, MMP-9, MT1-MMP and TIMP-2 in ovarian and primary peritoneal carcinoma (PPC) cells using short-term cultures from peritoneal effusions and solid tumours has been previously studied [9–12]. MMP-2 was reported to be the predominant Gelatinase in ovarian carcinoma cells, and an increase in TIMP-2 and MT1-MMP levels was seen concomitantly to the activation of MMP-2 [10]. MMP-2 and MMP-9 were both detected in tumour cells in an additional study in which cells in short-term cultures showed gradual decrease in MMP-9, but not MMP-2 levels [11]. In two additional studies, enhanced expression of MMP-9 was detected in ovarian carcinoma cells cultured in a medium containing human peritoneal tissue, a phenomenon mediated by the presence of fibronectin [13,14]. However, no large-scale studies compared the expression of MMP and TIMP in ovarian carcinoma cells in peritoneal and pleural effusions with that in primary ovarian tumours and metastatic lesions, and the prognostic significance related to the expression of these molecules in effusions is unknown and has the relative contribution of mesothelial cells to the elaboration of these molecules been previously analysed.

The aim of this study was to analyse the cellular source and expression of MMP-1, MMP-2, MMP-9 and TIMP-2 in malignant effusions from patients diagnosed with ovarian and primary peritoneal carcinomas. In addition, to analyse the role of these proteins in tumour spread in ovarian carcinoma, through a comparative analysis of their presence in pleural and peritoneal effusions, primary tumours and metastatic lesions, and the prognostic role of MMP and TIMP expression in effusions.

2. Patients and methods

2.1. Effusion specimens

Sixty-six fresh non-fixed peritoneal and pleural effusions were submitted to the Division of Cytology, Department of Pathology, The Norwegian Radium Hospital, during the period of January 1998–March 1999. Fifty-eight malignant effusion specimens were obtained preoperatively, intra-operatively, or at disease recurrence, from 50 patients diagnosed with ovarian carcinoma and 3 patients diagnosed with primary peritoneal carcinoma (PPC). These consisted of 44 peritoneal and 14 pleural effusions. Eight reactive effusions, obtained from patients with various malignancies or a clinical suspicion of malignancy, were additionally studied.

Effusion specimens, as well as relevant clinical data, were obtained from the Department of Gynecologic Oncology, The Norwegian Radium Hospital. The distribution of the studied malignant effusions according to histological type and site is detailed in Table 1. All specimens were received as fresh non-fixed effusions, volume range 20–2000 ml. Cell block and frozen cell preparations were prepared as previously described [15]. Morphological and immunohistochemical evaluation of all specimens were performed as previously detailed [15] according to established criteria [16].

2.2. Tumour specimens

Ninety-six surgical specimens, consisting of primary tumours ($n=37$) and metastatic lesions ($n=59$) of the above patients were additionally studied. Formalin-fixed paraffin-embedded tissue blocks were obtained either from archival material at the Department of Pathology, Norwegian Radium Hospital or from other hospitals in Norway. All tissue specimens underwent microscopic confirmation of diagnosis, tumour type and histological grade following established criteria [17]. The distribution of biopsies according to site and histological type is shown in Table 2.

Table 1
The distribution of malignant serous effusions according to histological type and location

Histological type	Peritoneal effusion	Pleural effusion	Total
Serous	32	11	43
Mucinous	0	1	1
Clear cell	1	0	1
Combined	2	1	3
Undifferentiated	1	0	1
Non-epithelial[a]	2	0	2
Borderline[b]	2	1	3
Primary peritoneal carcinoma	4	0	4
Total	44	14	58

[a] One malignant endodermal sinus tumour and one malignant granulosa cell tumour.

[b] Unequivocally malignant cells of serous type were detected in the pleural effusion specimen, inconsistent with the primary tumour diagnosis of borderline mucinous carcinoma.

Table 2
Tissue distribution of the studied primary tumours and metastatic lesions according to histological type

Histological type	Ovary	Omentum	Peritoneum	Intestine	Lymph node	Other	Total
Serous	32	21	7	8	4	6	78
Mucinous	1	1	0	0	0	0	2
Combined	1	0	1	1	0	1	4
Undifferentiated	1	0	0	1	0	0	2
Non-epithelial	1	0	0	0	0	0	1
Borderline	1	0	0	0	0	0	1
Primary peritoneal carcinoma	2	3	1	1	1	0	8
Total	39	25	9	11	5	7	96

2.3. Immunohistochemical analysis

Immunohistochemistry (IHC) was performed on all specimens (total = 162) as previously described [18], using monoclonal antibodies against MMP-1 (clone 41-IE5), MMP-2 (clone 42-5D11) and MMP-9 (clone 56-2A4) (Calbiochem, La Jolla, CA, USA). Staining was evaluated as negative, weak or intense. The presence and intensity of immunoreactivity in effusion specimens were scored in both tumour and mesothelial cells. Similarly, staining was scored in tumour cells and stromal fibroblasts in tissue specimens. In cases of heterogenous staining in any of the above-mentioned cell classes, the more intense areas determined the score, provided that at least 5% of tumour cells displayed that level of staining.

2.4. Oligonucleotide probes

Specific antisense oligonucleotide DNA probes for mRNA transcripts of *MMP-2*, *MMP-9* and *TIMP-2* were obtained from Research Genetics (Huntsville, AL, USA). Probe sequences (5′-3′) were as follows:

MMP-2: TGGGCTACGGCGCGGCGGCGTGGC
MMP-9: CCGGTCCACCTCGCTGGCGCTCCGGU
TIMP-2: CCAGGAAGGGATGTCAGAGC

A poly d(T)20 oligonucleotide (Research Genetics) was used to verify the integrity and lack of degradation of mRNA in each sample. DNA probes for *MMP-2*, *MMP-9* and *TIMP-2* were hyperbiotinylated. The stock dilution was diluted with probe diluent (Research Genetics) immediately before use. Working dilutions of 1:200 for *MMP-2* and *TIMP-2* and 1:100 for MMP-9 were used. Specific sense oligonucleotides were used for the evaluation of non-specific activity for each probe.

2.5. *Colorimetric non-radioactive* in situ *mRNA hybridisation (ISH)*

Tissue sections (4 μ-thick) of formalin-fixed, paraffin-embedded specimens were mounted on ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Sectioning was performed in RNAase-free water. Slides were dewaxed and rehydrated using xylenes (2×10 min) and iso-propyl alcohol (5 min), and then hybridised. ISH was carried out by using the microprobe manual staining system (Fisher Scientific) [19,20]. Hybridisation of the probes was carried out as previously described in Ref. [21]. A positive enzymatic reaction in this assay stained red. Known positive controls were used in each hybridisation reaction. These consisted of 2 cases for which positive hybridisation was reproducible in pilot studies. Controls for endogenous alkaline phosphatase for all probes included treatment of the sample in the absence of the probe and use of chromogen alone. Hybridisation results for all probes were interpreted as either absent, weak or intense. Strong red staining was interpreted as intense.

2.6. Scoring of IHC and ISH results

Slides in pilot studies were evaluated by two experienced observers. In these sessions, consensus regarding the scoring procedure was achieved. Thereafter slides were evaluated by one observer blinded to all clinico-pathological data, including biopsy site, disease stage and outcome. Equivocal cases were discussed in a panel of three observers, in a manner similar to the morphological evaluation of cytology cases.

2.7. Reverse-transcription polymerase chain reaction (RT-PCR)

Thirty effusion specimens (27 malignant, 3 reactive) were analysed for the presence of *MMP-2* and *MMP-9*. All malignant specimens contained a large population of carcinoma cells, being more than 80% of the total cell population in 16/27 samples. The remaining 11 effusions contained a population of less than 80% cancer cells, with a significant population of both mesothelial and inflammatory cells. PCR analysis was performed on reversed transcribed mRNA using the following primers:

MMP-2:	sense:	5′ AACACAGCCTTCT CCTCCTG
	antisense:	5′ CACCTACACCAA GAACTTCC
MMP-9:	sense:	5′ AAACTGGATGAC GATGTCTGCGTCCCG
	antisense:	5′ ACCTGTTCCGCTAT GGTTACACCCGCGTA
Glyceralde-3-phosphate dehydrogenase (*GAPDH*) (control):	sense:	5′TTCACACCCATCA CAAACAT
	antisense:	5′ GCCATCAACGAC CCCTTCAT

Products were separated on 1% agarose gels. HT-1080 fibrosarcoma cell line was used as control in all reactions. Band intensity was evaluated using the *MMP*/*GAPDH* ratio.

2.8. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) PC package (version 9.0, SPSS, Chicago, IL, USA, 1999). Probability of <0.05 was considered statistically significant. Comparative analyses of malignant cytological specimens, primary tumours and metastatic lesions were executed using Wilcoxon Signed Ranks Test. In cases for which more than one metastatic lesion was available, the lesion showing the most intense staining was included in the statistical evaluation. Evaluation of the association between tumour grade, International Federation of Gynecology and Obstetrics (FIGO) stage, residual tumour mass, chemotherapy status and effusion site (pleural versus peritoneal) and IHC results in effusions, was executed using the Chi-square test. Comparative analyses of IHC and ISH results in malignant epithelial versus reactive mesothelial cells in effusions, as well as in tumour versus stromal cells in tissue specimens, were executed using Wilcoxon Signed Ranks Test. The association between IHC and ISH results and biopsy site (ovary, omentum, peritoneum, intestine or other) for solid specimens was studied using the two-sided Chi-square test. Univariate survival analyses for effusion specimens were executed using the Kaplan–Meier method and log-rank test.

3. Results

3.1. Clinicopathological data

Four of 58 malignant specimens originated from grade I, 27 from grade II, and 27 from grade III ovarian carcinomas. 34 patients were diagnosed at FIGO stage III and 24 at stage IV. Residual tumour mass ranged from 1 to 5 cm in diameter. Thirty-nine specimens were obtained after chemotherapy had been instituted, while the remaining 19 were obtained prior to chemotherapeutic treatment.

3.1.1. Serous effusions

All 58 malignant effusion specimens contained distinctive populations of carcinoma cells (Fig. 1a). Significant populations of reactive mesothelial cells were found in 34/58 of the latter, as well as in all eight reactive effusions (total = 42).

3.1.1.1. IHC. MMP-1, MMP-2, and MMP-9 were detected in both carcinoma cells and reactive mesothelial cells, but their levels were consistently higher in the carcinoma cells (Table 3) (Fig. 1b). These differences reached statistical significance for MMP-1 ($P=0.016$) and MMP-2 ($P=0.036$) protein expression, failing to do so for MMP-9 ($P=0.063$). The pattern of protein expression was cytoplasmic and/or membranous in the tumour cells, being exclusively cytoplasmic in the mesothelial cells.

3.1.1.2. ISH. A positive signal was detected in all specimens using the poly d(T) probe (data not shown). *MMP-2*, *MMP-9* and *TIMP-2* mRNA was found in both tumour and mesothelial cells. However, as with IHC, mRNA levels were higher in the tumour cells (50% versus 29% for total positive cases), reaching significance for *TIMP-2* ($P=0.008$) (Table 3) (Fig. 1c). mRNA expression was cytoplasmic and/or nuclear in both cell populations.

3.1.1.3. RT-PCR. MMP-2 mRNA was detected in 28/30 (93%) specimens (25/27 malignant specimens and 3/3 reactive specimens). *MMP-2*/*GAPDH* ratio ranged between 0.01 and 0.75) (Fig. 2). *MMP-2* mRNA was detected in tumour cells in 18 of the 25 (72%) positive malignant samples using ISH, while 17/25 (68%) specimens showed immunoreactivity for MMP-2 protein. None of the 30 specimens analysed was positive for *MMP-9* using RT-PCR, although low mRNA expression was detected in 17/30 specimens using ISH and 5/30 specimens using IHC.

Positive controls for IHC, ISH and PCR included in our detection systems were consistently positive. ISH sections hybridised with sense probes were negative.

3.1.2. Tumours

IHC and ISH results for tumour specimens (37 primary carcinomas and 59 metastatic lesions) are shown in Table 4 and Fig. 3a–e. mRNA integrity was demonstrated in all samples using the poly d(T) probe (data not shown). MMP and TIMP were localised to both tumour and stromal cells. However, marked differences in expression were observed in the two cell classes.

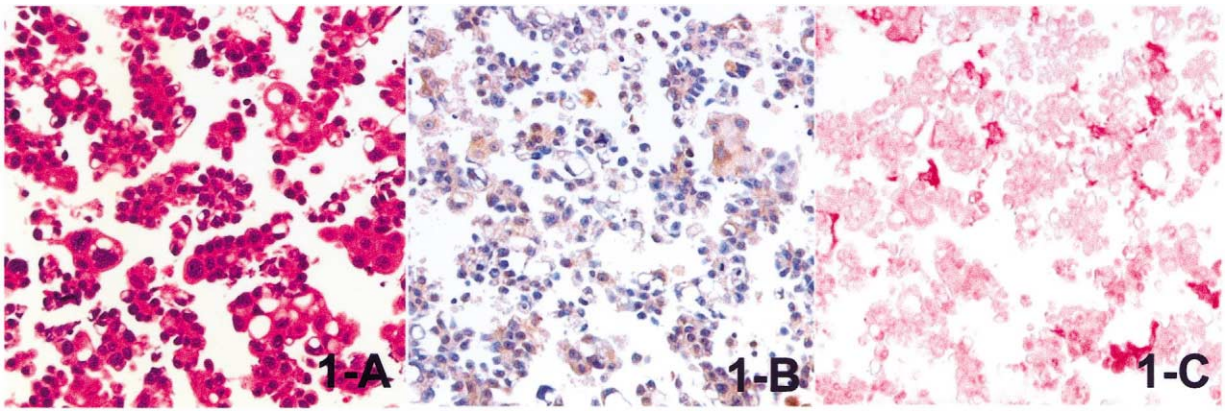

Fig. 1. Haematoxylin eosin (H&E)-stained section of a peritoneal effusion, consisting of ovarian carcinoma cells exclusively (a). (b) Shows immunoreactivity for MMP-2 in carcinoma cells. Intense mRNA expression of MMP-2 is present in the same specimen (c).

Table 3
IHC and ISH results in ovarian carcinoma and reactive mesothelial cells in effusion specimens

Marker	Level	Staining intensity		
		Absent (%)	Weak (%)	Intense (%)
Ovarian carcinoma cells ($n=58$ cases)				
MMP-1	Protein	22	16	62
MMP-2	Protein	34	21	45
MMP-9	Protein	79	16	5
MMP-2	mRNA	50	40	10
MMP-9	mRNA	52	40	8
TIMP-2	mRNA	50	34	16
Reactive mesothelial cells ($n=42$ cases)				
MMP-1	Protein	36	21	43
MMP-2	Protein	52	29	19
MMP-9	Protein	83	10	7
MMP-2	mRNA	65	33	2
MMP-9	mRNA	57	38	5
TIMP-2	mRNA	71	17	12

IHC, immunohistochemistry; ISH, *in situ* hybridisation; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases.

Stromal cells showed consistently higher levels of all markers on the mRNA level ($P=0.001$ for *MMP-2* and *MMP-9*, $P<0.001$ for *TIMP-2*). Conversely, higher levels of all three enzymes were detected in tumour cells using IHC ($P=0.001$ for MMP-1, $P=0.003$ for MMP-2, $P<0.001$ for MMP-9). As in the effusion specimens, the pattern of protein expression was cytoplasmic and/or membranous in the tumour cells and exclusively cytoplasmic in the stromal cells, while mRNA expression was cytoplasmic and/or nuclear in both cell populations. Endothelial cells showed intense immunoreactivity and/or mRNA signals in occasional cases, most often for *TIMP-2* mRNA.

3.1.3. Comparison of cancer cells in effusion specimens and tissue sections

IHC results for the total study cohort showed an upregulation in MMP-2 protein levels in carcinoma cells in effusions compared with solid lesions (66% versus 43% for total positive cases, 45% versus 30% for cases

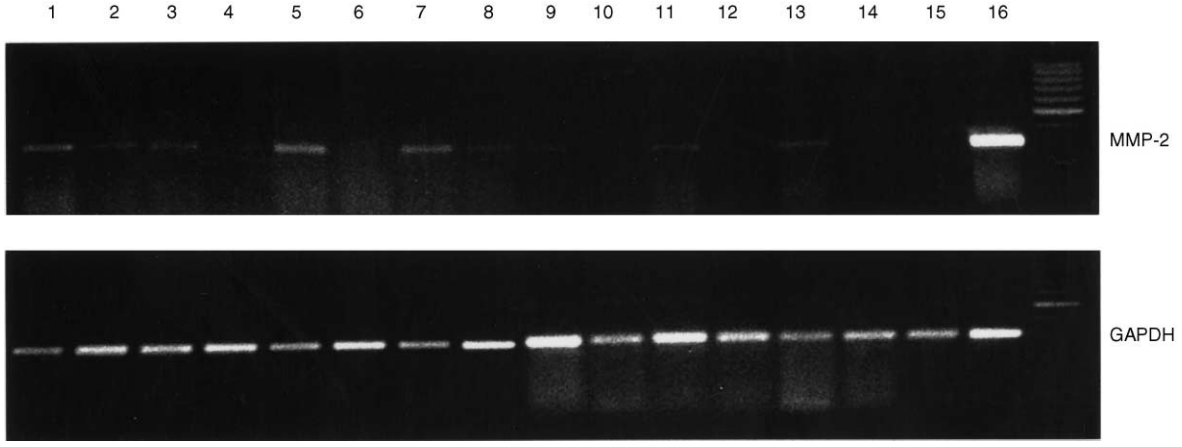

Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) results for *MMP-2* (product size = 327 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (product size = 314 bp) in effusion specimens. Lanes 1–15 represent 15 malignant effusions. Lane 16 is of a positive control. Weak signals were defined as MMP/GAPDH ratio $\leqslant 0.01$. Strong signals were defined as $MMP/GAPDH > 0.01$.

Table 4
IHC and ISH results in ovarian carcinoma and stromal cells in biopsies from primary and metastatic lesions (total = 96)

Marker	Level	Staining intensity		
		Absent	Weak	Intense
Ovarian carcinoma cells				
MMP-1	Protein	15	8	77
MMP-2	Protein	57	13	30
MMP-9	Protein	62	16	22
MMP-2	mRNA	72	22	6
MMP-9	mRNA	59	33	8
TIMP-2	mRNA	37	30	33
Stromal cells				
MMP-1	Protein	26	2	72
MMP-2	Protein	78	4	18
MMP-9	Protein	81	7	12
MMP-2	mRNA	52	32	16
MMP-9	mRNA	45	27	28
TIMP-2	mRNA	20	12	68

IHC, immunohistochemistry; ISH, *in situ* hybridisation; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases.

showing intense immunoreactivity, respectively). A similar trend was observed for *MMP-2* mRNA using ISH (50% versus 28% for total positive cases, 10% versus 6% for cases showing an intense signal). MMP-9 protein levels showed an opposite trend, manifested as a decrease in protein content in malignant cells in effusions (21% versus 38% for total positive cases, 5% versus 22% for cases showing intense immunoreactivity). Unlike protein levels, *MMP-9* mRNA levels were comparable in the two groups (49% versus 41% for total positive cases, 9% versus 8% for cases showing an intense signal). *TIMP-2* mRNA was detected less often in carcinoma cells in effusions compared with the corresponding cell population in solid lesions (50% versus 63% for total expression).

In the statistical evaluation, when only cases with corresponding effusion and solid lesions were evaluated, the following results were observed: Using IHC, an increase in MMP-2 protein expression was detected in tumour cells in effusion specimens compared with their respective primary tumours ($P = 0.01$). No significant association was observed for MMP-9 ($P = 0.07$) (Table 5). Using ISH, a similar finding was seen for *MMP-2* mRNA levels ($P = 0.036$). In addition, a

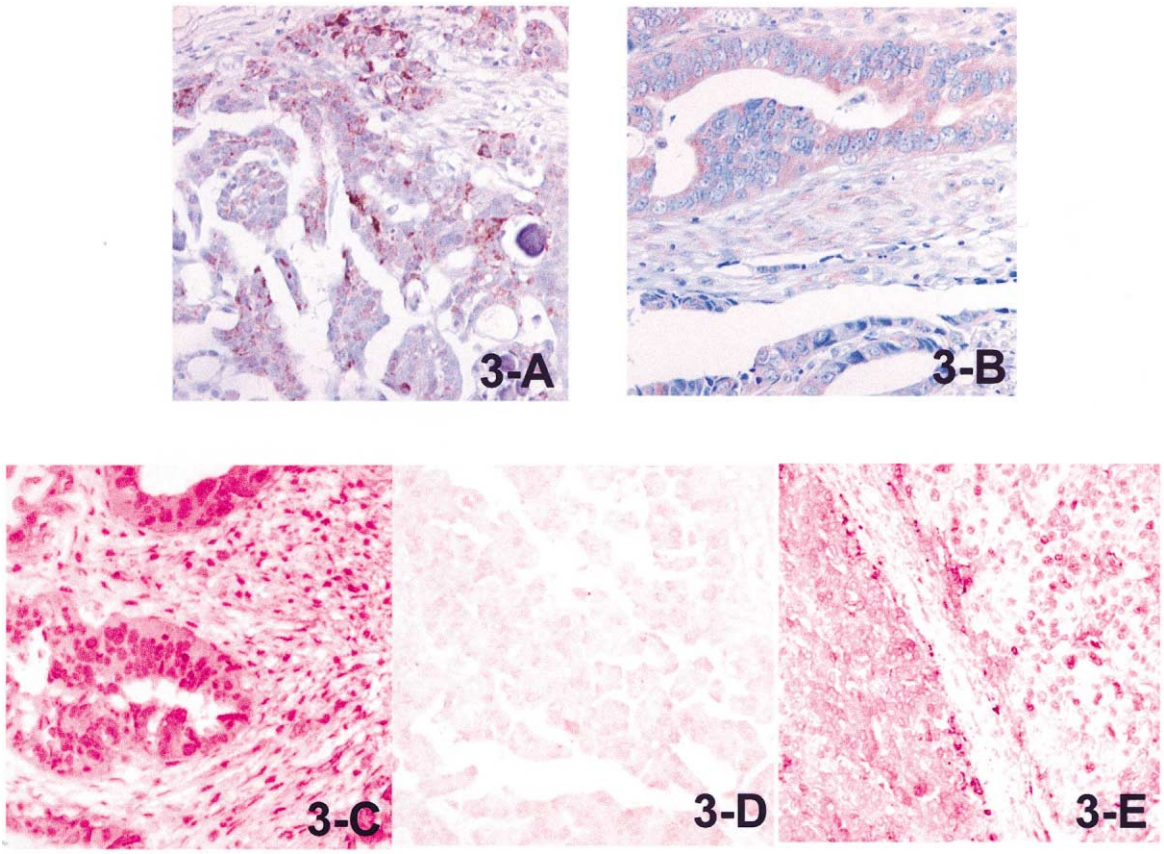

Fig. 3. MMP and TIMP expression in solid lesions. (a) Shows immunoreactivity for MMP-1 in tumour cells in an omental metastasis. In (b), immunoreactivity for MMP-9 in tumour and stromal cells in a peritoneal metastasis is shown. (c) Shows intense signals for *MMP-9* mRNA in tumour and stromal cells in a primary ovarian carcinoma. In (d), a weak signal for *MMP-9* mRNA is present in tumour cells in a primary ovarian carcinoma. (e) Shows an intense signal for *TIMP-2* mRNA in tumour cells in a primary ovarian carcinoma.

decrease in *TIMP-2* mRNA levels was detected in cancer cells in effusions ($P = 0.015$) (Table 5). Similar trends were observed when carcinoma cells in effusions were compared with those of metastatic lesions. However, the differences between these groups did not reach statistical significance on either the protein or mRNA level.

3.1.4. Comparison of cancer cells in peritoneal and pleural effusions

Cancer cells in peritoneal and pleural effusions showed comparable expression patterns for all molecules studied ($P > 0.05$). Analysis of serous carcinomas exclusively led to similar results in all comparative studies. Similarly, RT-PCR results in 20 peritoneal effusions were comparable to those in 10 pleural effusion specimens.

3.1.5. Comparison between cancer and stromal cells at different solid primary and metastatic sites

Carcinoma cells at all metastatic sites showed reduced expression of *MMP-9* and *TIMP-2* mRNA compared with primary tumours ($P = 0.025$ for both) (Table 6). Intense expression of MMP-9 protein in the tumour cells was twice as frequent in primary tumours, intestinal metastases and metastasis to various sites as in omental and peritoneal lesions ($P > 0.05$) (Table 6). Conversely, intense MMP-2 protein expression in the tumour cells showed a nearly 2-fold increase in omental and more than 3-fold increase in intestinal lesions, compared with the primary tumours ($P > 0.05$) (Table 6). In agreement with this finding, *MMP-2* mRNA expression (weak or intense) in the carcinoma cells showed a more than 2-fold increase in intestinal metastases and a larger than 50% increase in omental and peritoneal metastases compared with the primary tumours ($P > 0.05$) (Table 6). MMP and TIMP expression in the stromal cells was comparable at all sites (data not shown).

Table 5
IHC and ISH results in cancer cells in corresponding serous effusions, primary tumours and metastatic lesions

Marker	Level	Staining intensity		
		Absent (%)	Weak (%)	Intense (%)
Effusion specimens ($n = 36$ cases)				
MMP-1	Protein	22	11	67
MMP-2	Protein	28	28	44
MMP-9	Protein	72	20	8
MMP-2	mRNA	47	47	6
MMP-9	mRNA	53	39	8
TIMP-2	mRNA	44	39	17
Primary tumours ($n = 36$ cases)				
MMP-1	Protein	20	8	72
MMP-2	Protein	61	17	22
MMP-9	Protein	64	8	28
MMP-2	mRNA	77	17	6
MMP-9	mRNA	44	36	20
TIMP-2	mRNA	25	25	50
Metastatic lesions ($n = 34$ cases)				
MMP-1	Protein	15	3	82
MMP-2	Protein	47	12	41
MMP-9	Protein	59	15	26
MMP-2	mRNA	59	32	9
MMP-9	mRNA	62	35	3
TIMP-2	mRNA	26	35	39

IHC, immunohistochemistry; ISH, *in situ* hybridisation; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases.

3.1.6. Survival analysis

The follow-up period ranged from 1 to 91 months (mean = 23 months). Twenty-five effusion specimens were obtained from patients that died of disease, 23 from patients alive with disease, and 10 from patients with no evidence of disease at the time of control. In survival analysis, protein and mRNA expression of the molecules studied in the effusions showed no correlation with disease outcome ($P > 0.05$)(data not shown).

4. Discussion

The presence of malignant cells in the abdominal and chest cavities triggers the reactive accumulation of peritoneal and pleural effusions. Three cell populations—malignant cells, reactive mesothelial cells (mesoderm-derived resident cells covering the serosal surfaces) and inflammatory cells—are typically found in neoplastic serous effusions. However, the relative contribution of each of these cellular components to the effusion cell population may vary considerably in different specimens. Two studies of pleural effusions have previously demonstrated the presence of MMPs and TIMPs in both malignant and non-malignant exudates, as well as in transudates [22,23]. However, the methods employed (enzyme linked immunosorbent assay (ELISA), zymography and immunoblotting) did not render possible the detection of the cellular source of these proteins. Furthermore, the neoplastic exudate group in both studies included specimens with no evident tumour cells, even though they were obtained from patients with a documented malignancy. In our study, strict diagnostic criteria were applied, supported by immunohistochemical characterisation of all samples using epithelial and mesothelial markers.

In agreement with the ability of reactive mesothelial cells to participate in matrix turnover in a variety of conditions [16], we detected all three MMPs in both malignant epithelial and reactive mesothelial cells. However, cancer cells were the predominating cellular source on both the mRNA and protein level. Rather than the elaboration of large amounts of MMPs, the role of mesothelial cells may well be the induction of

Table 6
IHC and ISH results in cancer cells in primary and various metastatic lesions

Site		Level									Total no.
		MMP-1 (%)			MMP-2 (%)			MMP-9 (%)			
	Protein:	0[a]	1	2	0	1	2	0	1	2	
Ovary		18	10	72	64	15	21	64	8	28	39
Omentum		16	4	80	56	8	36	72	16	12	25
Peritoneum		0	11	89	67	11	22	67	22	11	9
Intestine		9	9	82	36	0	64	46	27	27	11
Other		17	8	75	50	25	25	50	25	25	12
		MMP-2 (%)			*MMP-9*[b] (%)			*TIMP-2*[b] (%)			Total no.
	mRNA:	0	1	2	0	1	2	0	1	2	
Ovary		80	15	5	44	38	18	27	27	46	39
Omentum		68	28	4	60	40	0	36	40	24	25
Peritoneum		67	22	11	78	11	11	44	12	44	9
Intestine		55	36	9	55	45	0	27	46	27	11
Other		75	17	8	92	8	0	75	25	0	12

[a] 0 = absent, 1 = weak, 2 = intense.
[b] $P = 0.025$. For all other comparative analyses: $P > 0.05$.

MMP production in ovarian carcinoma cells through the secretion of matrix components or cytokines, as was recently shown in the NOM1 ovarian carcinoma cell line through the action of fibronectin [13,14].

Production of both MMP-2 and MMP-9 by ovarian carcinoma cells was shown in a series of studies by Stack and co-workers using short-term cultures of peritoneal effusion specimens [9–11], as well as cultures from primary and metastatic lesions [9,10]. Cultures obtained from two patients suffering from primary peritoneal carcinoma (PPC) displayed the same enzymatic profile [12]. MMP-2 synthesis predominated in one of the above-mentioned reports [10] and a decline in MMP-9, but not MMP-2 levels was seen in repeated cultures [11]. Our findings support the postulated central role of MMP-2 in tumour invasion of ovarian carcinoma and PPC cells as an infrequent, weak and mostly focal expression of MMP-9 was seen on both protein and mRNA level. However, diffuse intense immunoreactivity for MMP-1, previously unreported, was additionally detected. Whereas the substrate profile of MMP-2 and MMP-9 overlap considerably, the additional expression of MMP-1 (interstitial collagenase) may facilitate the degradation of other collagens (types II, III and VII), thereby enhancing the invasive potential of malignant cells.

An overall agreement was observed between IHC, ISH and RT-PCR results for *MMP-2*. In contrast, MMP-9 mRNA was not detected using RT-PCR in a number of ISH- or IHC-positive specimens. All the latter discrepant cases had evidence of focal, mostly weak expression of MMP-9. Thus, small changes in cellular composition, a phenomenon often seen in comparative morphological analyses of cytological smears and cell blocks, could lead to different results. The combined use of ISH and PCR can thus be of a complementary nature.

As in effusion specimens, MMP-1 was the predominant enzyme detected in tissue sections, in both tumour and stromal cells, underscoring again its possible significance in ovarian cancer. When present, MMP-2 and MMP-9 protein expression was prominent in the tumour cells, while that of the mRNA was significantly higher in peritumoural stromal cells. Our mRNA ISH results are in agreement with previous reports. Autio-Harmainen and co-workers localised *MMP-2* mRNA predominantly to stromal and endothelial cells, whereas protein expression was detected in both stromal and tumour cells [24]. Localisation of *MMP-2* mRNA to peritumoral stromal cells was subsequently confirmed by two additional studies [25,26]. In the latter study [26], a similar distribution was observed for *TIMP-2* mRNA, although MMP-9 was present in both stromal and epithelial cells. Studying another cohort of advanced-stage ovarian carcinoma patients, we have recently reported expression of *MMP-2*, *MMP-9* and *TIMP-2* mRNA in both tumour and stromal cells in patients with poor survival [21]. Stromal production of MMPs and TIMPs thus appears to play a central role in the pathogenesis of ovarian carcinoma.

Comparative analysis of MMP and TIMP expression in solid specimens revealed significantly reduced expression of *MMP-9* and *TIMP-2* mRNA expression. In addition, reduced MMP-9 protein expression was seen in omental and peritoneal metastases. Put together with our observations in effusion specimens, these findings could support a lesser role for MMP-9 in both

effusion and solid metastatic lesions. The concomitant increase in MMP-2 protein and mRNA expression in solid metastases provides additional support to this hypothesis and provides a direct link between MMP expression profile in effusions and solid lesions. Interestingly, the increase in MMP-2 protein and mRNA expression was most pronounced in intestinal lesions, in which tumour cells show extensive, often through-and-through invasion of the wall, a process depending on heightened proteolytic activity. A more moderate, but clearly evident increase in MMP-2 expression was seen in deeply invasive omental (protein and mRNA) and peritoneal (protein) lesions. These findings suggest a central role of MMP-2 in metastatic ovarian cancer, whereas the role of MMP-9 may be mainly limited to local invasion in primary tumours.

Positive cytology in pleural effusion specimens, even in the absence of solid metastases, represents a stage IV disease and is associated with a poor prognosis [27], reflecting the presence of distant tumour spread. In contrast, the dissemination of ovarian carcinoma cells within the peritoneal cavity is postulated to be the result of direct shedding from the surface of the ovary, and may be associated with a localised disease, i.e. FIGO stage Ic. These facts are hard to reconcile with the reports associating the presence of large-volume ascites [5,28] and positive peritoneal cytology [5] with poor outcome in ovarian carcinoma, unless one assumes that cells with markedly different metastatic properties are found in peritoneal effusions. To date, no large-scale comparative studies of MMP and TIMP expression in effusion specimens and corresponding primary and metastatic tumours are available. Our findings point to a transient change in the metastatic phenotype of ovarian carcinoma cells in effusions, as an increase in MMP-2 and a decrease in TIMP-2 expression were observed compared with both primary and metastatic lesions, significantly so when compared with primary tumours. A 2-fold increase in protein content was concomitantly found in mesothelial cells in effusions compared with stromal cells in solid lesions, although mRNA levels did not differ considerably. We hypothesise that high levels of MMP-2 are necessary for the establishment of metastatic implants in the peritoneal cavity. As *TIMP-2* mRNA synthesis takes place predominantly in stromal cells in solid lesions, tumour cells in effusions may further reduce TIMP-2 production and rely on a stromal contribution for metastatic spread. Although a relatively small number of pleural effusions was analysed in this study, the similarities in cancer cell protease expression profile in the peritoneal and pleural cavity provide the first evidence suggesting that ovarian carcinoma cells at these sites do not differ from each other in this component of the metastatic phenotype. Added to our recent observations of similar carbohydrate antigen [29], CD44 [30] and E-cadherin complex expression [31] in this cohort, it is evident that further studies are necessary in order to determine the changes, if any, acquired in the transition from stage III to stage IV disease in ovarian carcinomas.

Protein and mRNA expression of MMP and TIMP failed to show correlation with clinico-pathological parameters and disease outcome in the present study. These results differ from those reported by us in a recent study of solid tumours from ovarian carcinoma patients, in which expression of *MMP-2*, *MMP-9* and *TIMP-2* mRNA was significantly higher in aggressive tumours, and was a marker of poor survival [21]. This difference may be attributed to the shorter follow-up period in the present study. Alternatively, it may reflect the frequency of the above-discussed changes in MMP and TIMP expression in effusions. Thus, a generalised and rather homogenous increase in MMP-2 and decrease in TIMP-2 levels, compared with primary tumours, will not allow for segregation according to disease outcome. Other markers may be more effective for this purpose, as evidenced by our recent study of cell cycle proteins, in which cyclin A levels were found to predict disease outcome (B. Davidson, the Norwegian Radium Hospital).

In conclusion, MMP-1, MMP-2, MMP-9 and TIMP-2 expression was studied in serous effusions from patients diagnosed with ovarian carcinoma, as well as in corresponding primary and metastatic lesions. The data presented suggest the acquisition of an altered protease phenotype for malignant cells in peritoneal and pleural effusions. Our studies thus far evaluated the expression of MMP and TIMP, CD44, carbohydrate antigens, E-cadherin complex proteins, angiogenic genes, multidrug resistance (*MDR-1* gene and protein), cell cycle proteins, integrins, and the transcription factor Ets-1 in effusions. Our results at present document extensive changes in tumour cells in effusions. These changes are likely to be, at least partially, induced by differences in the micro-environment and interactions with native cells of body cavities, i.e. mesothelial cells. However, one is more likely to believe that they are part of a major phenotypic change in ovarian carcinoma cells in effusions, which is directly linked to tumour progression of this cancer. We hope to be able to answer this question with more certainty in the near future.

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