ONCOLOGY REPORTS 18: 425-431, 2007

SPANDIDOS PUBLICATIONS

Gelatinolytic activities (matrix metalloproteinase-2 and -9) and soluble extracellular domain of HER-2/neu in pleural effusions

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Received January 12, 2007; Accepted February 20, 2007

Abstract. Matrix metalloproteinases (MMPs) are proteolytic enzymes that are implicated in multiple stages of cancer progression including invasion and metastasis. MMPs exert these effects by cleaving a diverse group of substrates, which include not only structural components of the extracellular matrix, but also growth factor receptors. By gelatin zymography we verified MMP activity in the pleural effusions of patients with benign and malignant disease. Of these patients, 32 had malignant pleural effusion, consisting of 20 breast cancer, 6 non-small cell lung carcinoma, 4 ovarian carcinoma, and 2 colonic adenocarcinoma, and 10 had benign pleural effusion (5 pleurisy and 5 cirrhosis). Zymography showed the constant presence of a substantial amount of MMP-2 in all samples analyzed, whereas MMP-9 was present to lesser quantities. MMP-2 activity was enhanced in pleural effusions from patients with benign diseases compared with cancer patients. MMP-9 was present in 59% of cancer patients and the lytic activity was enhanced in pleurisy and absent in cirrhosis. Furthermore, we determined the pleural effusion levels of the soluble extracellular domain of HER-2/neu. The levels of HER-2/neu ECD were above the cut-off value in breast cancer patients. No correlation between gelatinolytic activities and high HER-2/neu ECD values was found.

Introduction

Pleural effusion represents a common and challenging diagnostic problem in the practice of pulmonary medicine with diverse and non-similar etiologies. One of the main causes of pleural effusions is malignancy. In fact, several types of carcinoma develop a pleural effusion in the course of the disease. Metastasis from breast or ovarian carcinoma are the most frequent etiology for the presence of malignant pleural effusions in female patients, whereas lung carcinoma and malignant mesothelioma account for a relatively large number of cases in both sexes (1). Involvement of the pleural cavity by carcinoma may occur at any point of the clinical course and may be the first symptom of metastatic disease (2). Usually diagnosis is carried out by cytological examination of pleural fluid. This is a simple and reliable method for the diagnosis of malignant effusions, but its sensitivity is only 40-60% (3), even lower in clinical practice. Cytological examinations are based on the cellular morphology, which may raise difficulties in distinguishing carcinoma cells from reactive mesothelial cells. Sometimes, carcinoma cells without typical morphological changes or enough number could not be diagnosed cytologically (4). Closed pleural biopsy confers a small additive diagnostic value. Thoracoscopy is the preferred diagnostic procedure in patients with cytology-negative pleural effusion who are suspected of having pleural malignancy (5,6). This method has a great sensitivity but, in addition to being expensive, it imposes physical and mental stress to the patient. The determination of tumor markers in pleural effusions has been proposed as an alternative, non-invasive way of establishing a diagnosis of pleural malignancy and a number of tumor markers has been intensively evaluated (7-11). However, their precise sensitivities in identifying malignant pleural effusion remains controversial.

It is generally believed that the oncogene family of the growth factor receptor (HER family) play an important role in the development of human cancer (12). An important member of this family, the human epidermal growth factor receptor-2 (HER-2), which is also referred to as HER-2/neu or c-erbB-2, is structurally and functionally related to the v-erbB retroviral oncogene and has intrinsic tyrosine kinase activities (13). Recent evidence, however, indicates that certain matrix metalloproteinases (MMPs) are implicated in multiple stages of cancer progression including invasion and metastasis (14-16). Indeed some of the c-oncogenes may contribute to tumorigenesis by regulating the expression of MMPs. For

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Key words: matrix metalloproteinase-2, matrix metalloproteinase-9, soluble extracellular domain of HER-2/neu, pleural effusions, cancer

example, transfection of MCF-10A breast cancer cells with either *c-erbB-2* or *c-ras* resulted in increased expression of MMP-2 (17), whereas transfection of MCF-7 cells with the *ets* gene led to increased production of MMP-9 (18). Furthermore, it is known that the heavily glycosylated extracellular domain (ECD, p105) of the HER-2 is shed in plasma and serum from healthy individuals and patients with cancer (19,20). The cleavage of the HER-2/neu ECD involves MMP activity and this process is inhibited by the MMP inhibitor TIMP-1 but not by TIMP-2 (21).

In the present preliminary study, we measured the MMP-2 and MMP-9 (also known as gelatinase A and gelatinase B) activity levels in the pleural effusions using gelatin zymography. These levels were compared with the pleural fluid content of soluble extracellular domain of HER-2/neu with the following objectives: i) to support the diagnostic and prognostic value of pleural fluid gelatinolytic activities; and ii) to determine the possible association between pleural effusion forms of MMP-2 and -9 with the levels of HER-2/neu.

Materials and methods

Pleural effusion samples. Pleural effusions were collected from patients admitted, between September 2004 and September 2005, for diagnosis, to the Surgery Department of the Faculty of Medicine of the University 'Federico II' of Naples. Patient evaluation included anamnesis, physical examination, chest-X-ray thoracentesis with biochemical cytologic and bacteriology study of pleural fluid. When the result of the cytologic examination was negative or in doubt patients underwent blind pleural biopsies and/or thoracoscopic guided biopsies. All pleural effusions had definite etiologies. Informed consent was obtained according to institutional guidelines. Patients' age ranged from 32 to 78 years at diagnosis. Fresh pleural fluid was obtained by thoracentesis, collected in plastic sterile tubes without coagulation accelerators to prevent the release of gelatinases during platelet activation and rapidly brought to our laboratory. Tubes were centrifuged at 1600 x g for 10 min. supernatants were aliquoted and stored at -80˚C until use. For each sample, determination of protein concentration was performed using the method of Bradford (22). Each aliquot was used only once in order to prevent enzyme activation due to freeze-thawing processes.

Materials. Gelatinase A (from human fibrosarcoma cells) and gelatinase B (isolated from human blood) were purchased from Hoffmann-La Roche, Ltd. (Basel, Switzerland). Triton X-100, calcium chloride ($CaCl_2$), glycerol, gelatin, ethylenediaminetetraacetic (EDTA), and phenylmethylsulphonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, USA). The protein assay reagent was from Bio-Rad Laboratories. All other reagents were available from commercial sources.

Gelatin zymography. MMP activity in each sample was determined through zymographic analysis under denaturating but non-reducing conditions as previously described (15). Briefly, 15 μg of the total protein of each sample were mixed with sample buffer and applied directly without prior heating or reduction to 7.5% (w/v) acrylamide gels containing 0.1% (w/v) of gelatin. After removal of SDS from the gel by incubation in 2.5% (v/v) Triton X-100 for 1 h, the gels were incubated at 37˚C for 18 h in 50 mM Tris-HCl pH 7.6 containing 0.2 M NaCl, 5 mM $CaCl_2$, and 0.02% (w/v) Brij 35. Gels were stained for 1 h in 30% methanol, 10% glacial acetic acid containing 0.5% (w/v) Coomassie Brilliant Blue G 250 and destained in the same solution without dye for several hours. The gelatinolytic activity of each collagenase was evident as a clear band against the blue background of stained gelatin. The molecular sizes of bands displaying enzymatic activity were identified by comparison with prestained standard protein, as well as with purified gelatinase A or gelatinase B. To normalize the possible difference between zymograms an internal effusion sample from a patient was incorporated in every gel.

Control gels for MMPs. Control gels contained either of the MMP selective inhibitors, 20 mM EDTA or 10 mM 1,10 phenanthroline, in the MMP incubation buffer to confirm that lysis bands were the results of MMPs. Furthermore, the character of proteolytic bands was analyzed by incubating the identical zymograms in 0.1 mg/ml of PMSF, a serine protease inhibitor; or 2 mM Pefabloc, an irreversible serine protease inhibitor.

Analysis of the gels. Following zymography, the degree of gelatin digestion was quantified as previously described (23). Briefly, we used image analysis software (ImageQuant TL; Amersham Bioscience, Chicago, IL, USA) according to the manufacturer's specifications. The image of the gel was inverted to reveal dark bands on a white background. The molecular weight, volume and background of each band were determined. The relative amounts of the different forms of the proteinases were expressed as the integrated density x 10^{-3} (volume) of all the pixels above the background of each band per microgram of protein and as percentage of the total gelatinolytic activity.

Measurement of HER-2/neu. The soluble extracellular domain of epidermal growth factor-2 (HER-2/neu) was detected with the automated ADVIA Centaur analyzer (Bayer Diagnostic SpA, Milan, Italy), according to the manufacturer's specifications. This immunoassay utilized two monoclonal antibodies (TA-1 and NB3) which are specific for two different epitopes of the extracellular domain of the HER-2/neu oncoprotein (glycoprotein p105 with a molecular weight of 97-115 kDa).

Results

During a 1-year period, a total of 42 patients with pleural effusion were evaluated. Standard criteria were used to establish the etiology and the nature (malignant or benign) of pleural effusions (24). Of these patients 32 (6 males and 26 females) had a malignant pleural effusion: 20 breast cancer, 6 non-small lung cancer, 4 ovary cancer, 2 colon adenocarcinoma. Ten patients had benign pleural effusion: 5 pleurisy and 5 cirrhosis.

To investigate gelatinolytic activity present in pleural effusions, substrate gel zymography was performed. This method allows the detection of the metalloproteinases that exhibit significant gelatinolytic activity. Representative

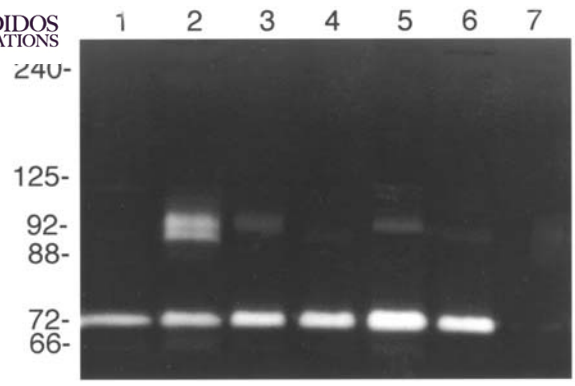

Figure 1. Gelatin zymography of pleural effusion specimens. Molecular weight standards are shown on the left. In all effusion samples, 15 μg of protein was loaded onto the gel. Lanes 1-6, lung cancer (patients AA, PG, AP, CG, DF, DS); lane 7, lung cancer in presence of 1.1 phenathroline 10 mM (patient PG).

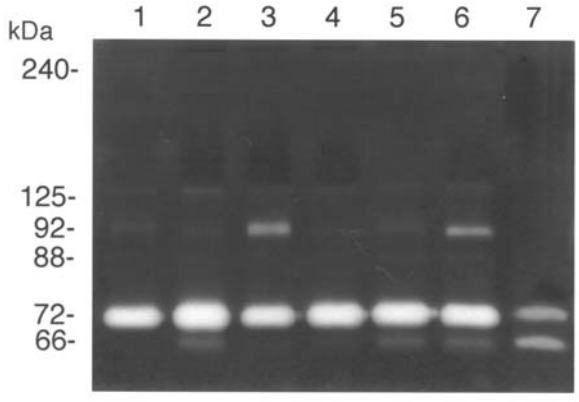

Figure 2. Gelatin zymography of purified gelatinase A and pleural effusion specimens. Molecular weight standards are shown on the left. In all effusion samples, 15 μg of protein was loaded onto the gel. Lanes 1-6, breast cancer (patients NC, NG, CC, DG, DA, PP); lane 7, gelatinase A (MMP-2) 120 mU.

zymography results are shown in the figures. Polyacrylamide gels were evaluated for the presence of a clear zone representing degradation of gelatin by proteolysis. The nature of the lytic bands was confirmed by inhibition assays with selective inhibitors of MMPs (Fig. 1, lane 7) and with selective inhibitor of serine protease (data not shown). As shown in the figures zymographic analysis revealed the existence of a strong sharp band of gelatinolytic activity migrating at ~72 kDa, a lytic band migrating at ~92 kDa and faint lytic bands with apparent molecular weight of 125, 88 and 66 kDa. Comparison of these gelatinolytic bands with prestained standard protein and purified gelatinase A (MMP-2) and gelatinase B (MMP-9) clearly identified the MMP-costituting bands as gelatinase A (proMMP-2, 72 kDa; and activated MMP-2, 66 kDa) (Fig. 2, lane 7) and gelatinase B (proMMP-9, 92 kDa; and activated MMP-9, 88 kDa) (Fig. 3, lane 1). The clear zones with a molecular weight higher than 92 kDa might represent complexes of MMPs that are not dissociated in zymography. In fact, MMP-9 can be associated with a 25-kDa protein with a band at 125 kDa (23,25,26) that can form dimer or multidimer with lytic bands at ~215 and 240 kDa (27). Following gelatin zymography, proteolytic bands were subjected to densitometric

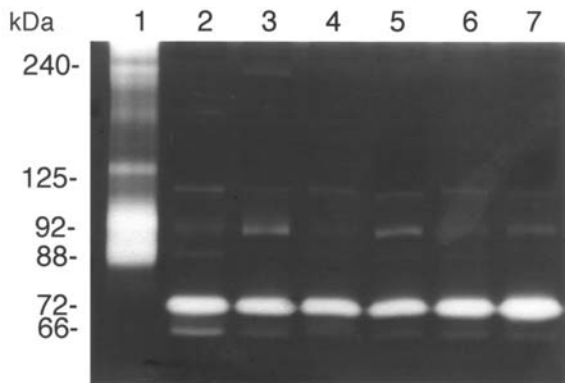

Figure 3. Gelatin zymography of gelatinase B and pleural effusion specimens. Molecular weight standards are shown on the left. In all effusion samples, 15 μg of protein was loaded onto the gel. Lane 1, gelatinase B (MMP-9) 20 μU; lanes 2-7, breast cancer (patients IR, CA, DL, OF, PI, RM).

analysis and the data expressed as the integrated density of all the pixels of each band (volume x $10^{-3}/\mu$g proteins), and as the percentage of the total gelatinolytic activity. A summary of expression patterns of proteinases in both benign and malignant pleural effusion specimens is shown in the tables. In the benign pleural effusions the total gelatinolytic activity ranged from 37 to 227 volume x $10^{-3}/\mu$g protein with a mean value ± SE of 119±19; whereas in malignant pleural effusions it ranged from 18.8-78.1 volume x $10^{-3}/\mu$g protein (mean, 43±1.8 volume x $10^{-3}/\mu$g protein). MMP-2 was detected in all specimens analyzed. In the benign pleural effusions the lytic activity (range, 37-227; mean, 96.8±19 volume x $10^{-3}/\mu$g protein) was 2.5-fold higher than that observed in malignant effusion specimens (range, 15.3-77; mean, 38.1±1.9 volume x $10^{-3}/\mu$g protein). MMP-9 was detected in pleurisy and in 19/32 (59%) of the malignant effusion specimens with a mean value ± SE of 4.8±0.8 volume x $10^{-3}/\mu$g protein (range, 1-17.5); however the lytic activity of gelatinase B was ~8-fold higher in pleurisy than in malignancy. Finally, in the patients with cirrhosis zymographic analysis revealed the existence of only the 72-kDa band. As it concerns pleurisy, 4 out of 5 samples (80%) showed lytic activity at 66 kDa (active proMMP-2), presumably due to an autoactivation during the renaturation period. All pleurisy specimens had MMP-9 ranging in density between 6.8 and 77.7 volume x $10^{-3}/\mu$g protein with a mean value of 37.7±14. Of these, 2 samples (40%) showed a faint lytic band with a molecular weight at ~88 kDa (Table I). Moreover, one pleurisy specimen (patient MV) showed a small lytic activity at 240 kDa (2.3 volume x $10^{-3}/\mu$g protein, 2.5%) (Fig. 4, lane 4). As shown in Table II, in patients with breast carcinomas the density of the 72-kDa lytic band ranged from 18.5 to 77 volume x $10^{-3}/\mu$g protein (mean, 41.3±2.9), and the faint lytic band with lower molecular weight (66 kDa) was detected in 15/20 (75%) of samples. The 92-kDa lytic band (pro MMP-9, gelatinase B) was detected in 14/20 (70%) samples with a value ranging from 1 to 13.3 volume x $10^{-3}/\mu$g protein (mean, 4±0.7 volume x $10^{-3}/\mu$g protein). Moreover, 4 out of 14 (28%) of these specimens showed a faint lytic band of 88 kDa (active MMP-9), and 8 (57%) showed a band at 125 kDa. As it concerns the non-small cell lung carcinomas, the density of the 72-kDa lytic band ranged from 15.3 to 69.5

Table I. Pleural effusion MMP content (volume x $10^{-3}/\mu$g protein and percentage) related to HER-2/neu in benign disease.

Patient	Sex	Age (years)	Disease	Total MMPs (vol x $10^{-3}/\mu$g pr)	MMP 92 kDa		MMP 88 kDa		MMP 72 kDa		MMP 66 kDa		HER-2/neu (ng/ml)
					A	B	A	B	A	B	A	B	
MM	F	67	Pleurisy	113.0	40.2	35.5	1.5	1.3	67.7	61.6	2.2	1.9	5.4
ML	M	75	Pleurisy	75.2	6.8	9.0	0	0	68.4	91.0	0	0	9.5
BN	M	72	Pleurisy	118.0	48.5	41.1	15.3	12.9	39.0	33.0	15.8	13.3	3.9
MV	M	43	Pleurisy	92.0	15.3	16.6	0	0	71.0	77.0	3.3	3.6	4.9
OA	M	42	Pleurisy	161.0	77.7	48.2	0	0	82.6	51.0	1.3	0.8	5.1
DV	F	67	Cirrhosis	85.5	0	0	0	0	85.5	100.0	0	0	4.5
IG	M	68	Cirrhosis	227.0	0	0	0	0	227.0	100.0	0	0	0.3
FD	M	63	Cirrhosis	37.0	0	0	0	0	37.0	100.0	0	0	4.3
FI	F	69	Cirrhosis	93.0	0	0	0	0	93.0	100.0	0	0	4.0
CA	F	63	Cirrhosis	195.0	0	0	0	0	195.0	100.0	0	0	0.5

A, volume x $10^{-3}/\mu$g protein; B, percentage. pr, protein.

Table II. Pleural effusion MMP content (volume x $10^{-3}/\mu$g protein and percentage) related to HER-2/neu in breast cancer.

Patient	Sex	Age (years)	Total MMPs (vol x $10^{-3}/\mu$g pr)	MMP 125 kDa		MMP 92 kDa		MMP 88 kDa		MMP 72 kDa		MMP 66 kDa		HER-2/neu (ng/ml)
				A	B	A	B	A	B	A	B	A	B	
NC	F	44	36.1	0	0	1.9	5.3	0	0	34.0	94.0	0	0	21
NG	F	71	44.6	0	0	1.0	2.2	0	0	42.1	94.4	1.5	3.4	10
CC	F	63	39.7	0	0	7.6	19.0	0	0	32.0	81.0	0	0	93
DG	F	72	48.6	0	0	0	0	0	0	48.6	100.0	0	0	46
DA	F	62	63.6	0	0	0	0	0	0	60.1	94.5	3.5	5.5	11
PP	F	39	50.4	0	0	7.0	13.8	0	0	40.8	81.0	2.6	5.1	73
DM	F	58	78.1	0	0	0	0	0	0	77.0	98.5	1.1	1.5	15
DR	F	59	67.4	3.5	5.2	2.8	4.1	1.4	2.1	56.3	83.5	3.4	5.0	45
ML	F	64	26.2	1.2	4.6	2.6	9.9	0.6	2.3	20.8	79.4	1.1	4.2	59
GA	F	62	31.4	0	0	0	0	0	0	31.4	100.0	0	0	29
RM	F	41	25.0	0	0	0	0	0	0	18.5	74.0	6.5	26.0	25
PR	F	58	29.0	0	0	1.1	3.8	0	0	27.2	94.0	1.0	3.4	41
EM	F	47	21.4	0	0	0	0	0	0	21.4	100.0	0	0	60
IR	F	34	45.3	1.3	2.8	1.6	3.5	0.5	1.1	40.2	88.7	1.5	3.3	42
CA	F	44	59.4	0	0	6.7	11.3	0	0	52.0	87.0	0.7	1.2	38
DL	F	53	53.5	1.4	2.6	1.7	3.2	0	0	49.5	92.5	2.3	4.3	20
OF	F	32	49.3	0.4	0.8	4.9	9.9	0	0	42.5	86.0	1.5	3.0	27
PI	F	60	77.1	0.75	1	2.2	2.8	0	0	73.0	94.0	1.0	1.3	12
RM	F	66	38.8	0.44	1.1	1.6	4.1	0	0	36.0	93.0	0.83	2.1	29
VM	F	54	51.7	4.5	8.7	13.3	25.7	2.9	5.6	22.8	44.0	8.2	15.8	17

A, volume x $10^{-3}/\mu$g protein; B, percentage. pr, protein.

volume x $10^{-3}/\mu$g protein and the 66-kDa lytic activity was observed only in one sample; whereas the 92-kDa lytic band was detected in 3 (50%) samples (Table III) (Fig. 1, lanes 1-6). Three out of four ovary carcinomas showed only a strong lytic activity at 72 kDa (Fig. 5, lanes 2-4), and one sample (patient GM) showed lytic bands at 240, 125 and 92 kDa as well as at 72 and 66 kDa (Fig. 5, lane 1). In colon cancer, one specimen showed only the 72-kDa lytic band (Fig. 5, lane 5) and the other sample had also a faint lytic band corresponding to MMP-9 (Table III).

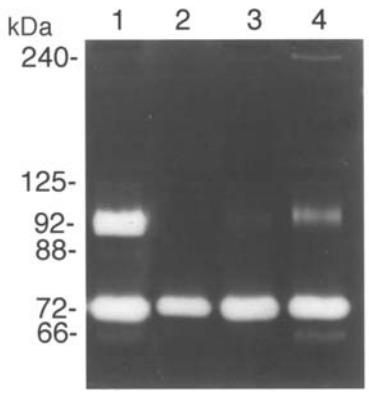

Figure 4. Gelatin zymography of pleural effusion specimens. Molecular weight standards are shown on the left. In all effusion samples, 15 μg of protein was loaded onto the gel. Lane 1, pleurisy (patient OA); lanes 2 and 3, cirrhosis (patients CA, IG); lane 4, pleurisy (patient MV).

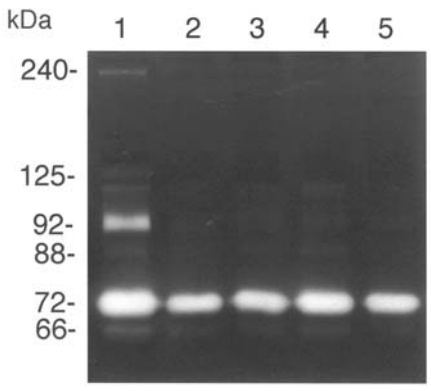

Figure 5. Gelatin zymography of pleural effusion specimens. Molecular weight standards are shown on the left. In all effusion samples, 15 μg of protein was loaded onto the gel. Lanes 1-4, ovary cancer (patients GM, TA, PF, CA); lane 5, colon cancer (patient TM).

Soluble extracellular domain of epidermal growth factor-2 (HER-2/neu) was measured on the pleural effusions of all subjects studied (Tables I-III). Since normal pleural fluids were not available for HER-2/neu we established the cut-off value by the mean ± 2SD of the value obtained in the sera of 103 healthy subjects of the same age and the cut-off value of 15 ng/ml was considered. The samples with HER-2/neu value higher than the cut-off were considered positive. In the benign effusion specimens, the mean value ± SE (4.2±0.9 ng/ml; range, 0.3-9.5) was lower than the cut-off value; whereas, in the specimens from cancer patients, the mean value (25±2.9 ng/ml; range, 0.8-93) was higher than the cut-off value. However, when we considered the different origin of neoplasias we observed that in the breast carcinomas, HER-2/neu ranged from 10 to 93 ng/ml with a mean value ± SE of 35.6±4.1 ng/ml (Table II); whereas, in the patients with lung, ovary or colon cancer the pleural fluid values of HER-2/neu (range, 0.8-14.2; mean, 7.5±1.1 ng/ml) were included in the normal range (Table III). Finally, we observed that HER-2/neu was positive in 80% (16/20) of breast cancer samples and negative in all the specimens from all the other types of tumors analyzed. We found no association between gelatinolytic activities and high HER-2/neu ECD values.

Discussion

Despite the sophisticated diagnostic methods, definitive diagnosis of pleural effusions may still be difficult. In addition, the nature of pleural effusions is of particular importance in cancer patients and needs to be clarified, as a co-existing malignant pleural effusion implies an advanced stage (6). Thus, there is a great interest in identifying 'biomarkers' that can be used as an altenative method to enhance the sensitivity of cytological examination in the diagnosis of pleural effusions and/or add more information on the undiagnosed pleural effusions in order to select patients for thoracentesis.

Table III. Pleural effusion MMP content (volume x $10^{-3}/\mu$g protein and percentage) related to HER-2/neu in cancer.

Patient	Sex	Age (years)	Site of tumor	Total MMPs (vol x $10^{-3}/\mu$g pr)	MMP 92 kDa		MMP 88 kDa		MMP 72 kDa		MMP 66 kDa		HER-2/neu (ng/ml)
					A	B	A	B	A	B	A	B	
AA	M	53	Lung	18.8	0	0	0	0	18.8	100.0	0	0	12.7
PG	M	70	Lung	32.8	17.5	53.0	0	0	15.3	46.6	0	0	3.2
AP	M	78	Lung	70.5	1.0	1.4	0	0	69.5	98.6	0	0	0.8
CG	F	52	Lung	29.7	0	0	0	0	29.7	100.0	0	0	6.2
DF	M	70	Lung	35.6	1.8	5.0	0	0	33.7	95.0	0	0	14.2
DS	M	63	Lung	44.8	0	0	0	0	39.5	88.0	5.2	12.0	3.1
GM	F	61	Ovary	61.0	15.0	24.5	0	0	43.0	70.5	1.5	2.4	4.0
TA	F	69	Ovary	23.5	0	0	0	0	23.5	100.0	0	0	9.8
PF	F	74	Ovary	28.5	0	0	0	0	28.5	100.0	0	0	8.0
CA	F	72	Ovary	39.5	0	0	0	0	39.5	100.0	0	0	8.7
TM	M	70	Colon	27.6	0	0	0	0	27.6	100.0	0	0	10.5
MA	F	50	Colon	26.0	1.2	4.6	0	0	24.8	95.4	0	0	9.0

A, volume x $10^{-3}/\mu$g protein; B, percentage. pr, protein.

Tumor progression is a multistep process. MMPs can promote cancer progression by increasing cancer-cell growth, migration, invasion metastasis and angiogenesis. MMPs exert these effects by cleaving a diverse group of substrates, which includes not only structural components of the extracellular matrix, but also growth-factor-binding proteins, growth-factor precursors, receptor tyrosine kinases, cell-adhesion molecules and other proteinases (14,16). A member of the epidermal-growth-factor receptor (EGF-R) family, HER-2/neu (also known as erb-B2), is substrate for MMPs. In this case, the extracellular domain of the receptor is released, and this might function as a decoy receptor for the ligand. Moreover, the expression of MMPs is regulated by a c-oncogene such as c-erb B2 (20,21,28,29).

In the present report, we measured in the pleural effusions, in which diagnosis has already been made, the gelatinolytic levels of MMP-2 and MMP-9 by zymography and related the data with the soluble extracellular domain of HER-2/neu. Zymography has some advantages over immunological assay, such as lower cost, a more rapid time of execution and the possibility of simultaneously detecting multiple forms of the same enzyme. Our results demonstrated that substantial amounts of MMP-2 and, to a lesser degree MMP-9 are present in pleural effusions. MMP-2 lytic activity was present in all specimens analyzed and the values were 2.5-fold higher in benign effusions than in malignant effusions. MMP-9 was detected in 59% of cancer patients and it was undetectable in cirrhosis specimens. Moreover, MMP-9 lytic activity was 8-fold higher in pleurisy than in malignancy.

The gene product of the HER-2/neu functions as a normal epithelial protein in cell growth and proliferation. HER-2/neu gene amplification and protein overexpression contribute to converting healthy cells to cancer cells; however, the exact timing of HER-2/neu involvement in neoplastic transformation has not been determined. Nowadays, the HER-2/neu oncoprotein is recognized as an important factor in cancer development and has become established as an important diagnostic tool in the investigation of patients with cancer. The soluble extracellular domain (ECD) of HER-2/neu, cleaved by a mechanism involving MMP activity, is shed in human fluids and serum and it is measurable by immunoassay. HER-2/neu ECD has been proposed as a tool for assessing prognosis and early dectection of cancer progression in sera of breast cancer patients (30) and in pleural effusions from patients with lung carcinoma (31). The data shown here indicate that HER-2/neu ECD is positive in 80% of breast cancer samples and negative in all the specimens from all the other types of tumor analyzed as well as in the benign pleural effusions. Although, we found no correlation between gelatinolytic activity and HER-2/neu ECD value in pleural effusions, our data suggest that the determination of these parameters might help to differentiate between malignant and benign effusions. To our best knowledge, others showed enhanced MMP-9 lytic activity in parainflammatory effusions and the MMP-9 value correlated with those of the C reactive protein, a systemic marker of inflammation (32). Furthermore, Davidson and colleagues reported increased levels of MMP-2 in cells by effusion from patients with ovarian and breast carcinoma and linked this factor to poor prognosis (33,34). In regard to HER-2/neu ECD, there are different and contrasting reports in the literature. Hung *et al* proposed HER-2/neu ECD as a potential tumor marker for a diagnosis of pleural effusions on patients with lung adenocarcinoma (31), whereas Porcel and colleagues reported that HER-2/neu ECD has poor diagnostic performance in patients with malignant effusions (35). We found that HER-2/neu ECD in pleural effusion might help to identify breast cancer and that the determination of MMPs might be useful to identify inflammatory diseases. Nevertheless, there are some limitations of our study: i) due to the small number of patients included in this observational study, the conclusion of our study may not be transferable to the general population and therefore need further evaluation; ii) we performed our experiments on pleural fluid supernatants and we were not able to establish the cellular source of the proteins analyzed. In fact, it is well known that in malignant effusions three cell populations (malignant cells, reactive mesothelial cells, inflammatory cells) are typically found. Therefore the relative contribution of each of these cellular components to the effusion cell population may vary considerably in different specimens. In our future research, the next step will be the analysis of the cellular content of MMP-2 and -9 in the cells of pleural effusions after the identification of each cell type.

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