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ORIGINAL ARTICLE

**Myriam Polette · Béatrice Nawrocki · Christine Gilles
Hiroshi Sato · Motoharu Seiki · Jean-Marie Tournier
Philippe Birembaut**

MT-MMP expression and localisation in human lung and breast cancers

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Abstract Thirteen primary pulmonary squamous cell carcinomas, 4 specimens of normal lung from around tumours, 4 benign proliferations of the mammary gland and 16 breast carcinomas were analysed by in situ hybridisation, Northern blot and immunohistochemistry for the expression of a recently described metalloproteinase (MMP), the MT-MMP (membrane-type matrix metalloproteinase). This MT-MMP can activate gelatinase A, involved in the degradation of basement membranes. In situ hybridisation revealed MT-MMP transcripts distributed in both tumour and stromal cells in squamous cell lung cancers, whereas these mRNAs were principally detected in stromal cells in close contact to tumour clusters in breast carcinomas and in lung adenocarcinomas. Northern blot analysis showed a parallel expression of MT-MMP and gelatinase A transcripts in both lung and breast cancers. Immunohistochemistry displayed a more extensive distribution of MT-MMP in pulmonary and mammary carcinomas with numerous labelled preinvasive and infiltrating cancer cells and stromal cells near the tumour cells. The large degree of expression of MT-MMP in these cancers indicates a potential role of this enzyme in tumour progression. The finding of MT-MMP transcripts in stromal cells in the vicinity of lung and breast tumour cells emphasises the cooperation between these cells and cancer cells for the expression of MT-MMP and in tumour invasion in vivo.

M. Polette (✉) · B. Nawrocki · J.-M. Tournier · P. Birembaut
I.N.S.E.R.M. U.314, C.H.R. Maison Blanche,
45, Rue Cognacq-Jay, F-51100 Reims, France

C. Gilles
Department of Cell Biology,
Vincent T. Lombardi Cancer Research Center,
Georgetown University Medical Center,
3800 Reservoir Road N.W., Washington, DC 20007, USA

H. Sato · M. Seiki
Department of Molecular Virology and Oncology,
Cancer Research Institute, School of Medicine,
Kanazawa University, Takara-machi 13-1, Kanazawa,
Ishikawa, 920 Japan

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Introduction

Proteolytic extracellular matrix degradation is a major step in tumour invasion. Among the various proteinases involved in this process, matrix metalloproteinases (MMPs) represent a family of enzymes that are able to degrade all components of the extracellular matrix. The MMPs are divided into three major subclasses: interstitial collagenases, gelatinases (also called type IV collagenases) and stromelysins. Two types of gelatinases, A and B (MMP-2 and MMP-9, respectively) have been isolated and characterised.

Gelatinase A degrades basement membrane collagens, especially type IV collagen and denatured collagens [21]. Therefore, this MMP could participate in the various steps of tumour progression in cancers: basement membrane disruption, stromal invasion and metastasis. The role of gelatinase A in the progression of breast cancers is supported by various in vitro and in vivo studies. Indeed, cultured breast tumour cell lines have been shown to secrete gelatinase A. Increased gelatinase A expression is associated with increased metastatic potential in a variety of systems [10, 13]. In vivo, elevated gelatinase A levels have been detected in breast cancers, but not in the epithelial component of premalignant disease or normal breast [5, 6, 8, 11, 12, 21]. Davies et al. [7] using quantitative zymography, have shown increased ratios of activated to latent gelatinase A in breast carcinomas of increasing grade. However, in situ hybridisation studies performed on breast carcinomas have revealed stromal cell expression of gelatinase A mRNAs which argues in favour of cooperation between stromal cells and cancer cells which do not express these mRNAs [15–17, 19].

Gelatinase A, like all MMPs, is secreted as a proenzyme and requires activation into a mature form before it can degrade its substrates. Binding sites for gelatinase A

have been described in vitro MCF7 and MDA-MB-231 suggesting that tumour cells might bind the enzyme [9]. Furthermore, recent studies have reported a membrane associated activation pathway which is sensitive to MMP inhibitors and have suggested the existence of MMP-2 activator [2, 4, 23]. The mechanisms responsible of the activation of progelatinase A in vivo are still unresolved [1, 20]. Recently, Sato et al. [18] have discovered a new MMP, called MT-MMP (membrane-type MMP), which might be a candidate for the progelatinase A activator. Indeed, the expression of MT-MMP at the surface of in vitro transfected cells induces specific activation of progelatinase A and enhances the invasion of matrigel. Moreover, they demonstrated the expression of MT-MMP on the surface of invasive tumour cells of lung cancers. MT-MMP may thus play a crucial role in tumour invasion by activating progelatinase A on the tumour cell surface.

The aim of the present work is to study the expression and localisation of MT-MMP in human breast and lung carcinomas with various complementary morphological studies applied on the same tumours. Lung carcinomas are known to express MT-MMP [18] but at the present time MT-MMP transcripts have not been localised in these tumours. The data obtained with these various approaches indicate the large degree of expression of MT-MMP in both lung and breast cancers and its production principally by stromal cells.

Materials and methods

Tissue was obtained from 13 lungs resected for carcinomas (9 squamous cell carcinomas and 4 adenocarcinomas), from 4 normal lung around tumours, from 4 benign breast proliferations (2 fibrocystic diseases and 2 fibroadenomas) and 16 breast cancers (5 lobular and 11 ductal carcinomas). All lung tumours were of stage T2 and breast cancers were grade 1 (2 cases), grade 2 (8 cases) and grade 3 (1 case) ductal carcinomas according the Scarf and Bloom classification.

A portion of each of the samples was frozen in liquid nitrogen for Northern blot analysis while the remainder was fixed in formalin and embedded in paraffin for in situ hybridisation and immunohistochemical studies.

Immunohistochemistry

Tissue sections (5 μm) were deparaffinised, rehydrated and treated with 0.3% hydrogen peroxide for 5 min to quench endogenous peroxidase activity. Non-specific binding was blocked with serum (Dako, USA) for 20 min. Slides were incubated with monoclonal antibody against MT-MMP (2 μg/ml) for one night at 4° C. As previously described [18], the MT-MMP antibody prepared specifically recognises the extracellular domain of MT-MMP (clone 113-5B7, directed against an oligopeptide of residue 319–333 of MT-MMP). Negative controls consisted of slides incubated with non-immune mouse IgG. After 10 min washes in PBS, the following steps were performed with peroxidase LSAB kit (labelled streptavidin biotin method; Dako, USA). Peroxidase activity was revealed with hydrogen fluride and 3-amino-9-ethylcarbazole.

All slides were briefly counterstained with Mayer's haematoxylin, mounted and examined under a Zeiss Axiophot microscope.

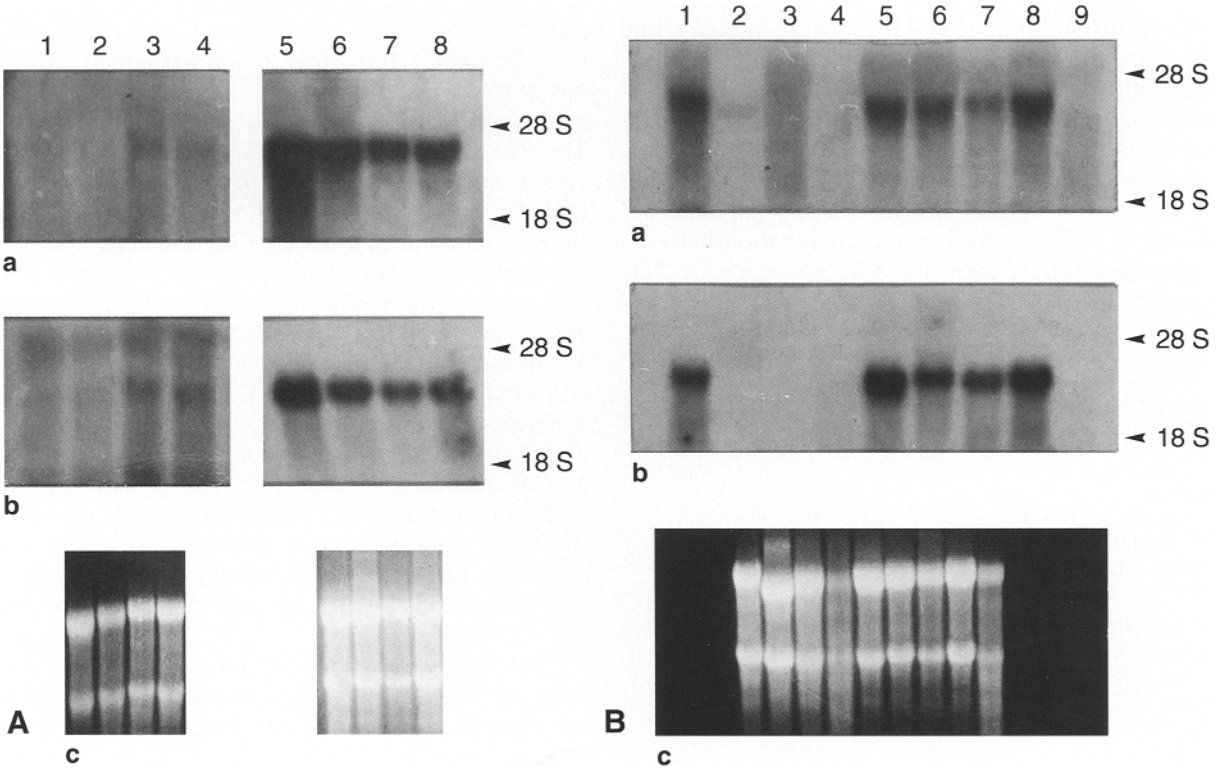


Fig. 1A, B Northern blot analysis of membrane-type metalloproteinase (MT-MMP) and gelatinase A mRNAs in lung cancers. **A** *Lanes 1–4* peritumoral normal lung, *lanes 5–8* adenocarcinomas. *a* MT-MMP, *b* gelatinase A. **B** *Lanes 1–9* squamous carcinomas. *a* MT-MMP, *b* gelatinase A, *c* Ribosomal RNA bands visualised under ultraviolet light after staining with ethidium bromide show amounts and quality of the RNAs. There is a coordinate expression of gelatinase A and MT-MMP mRNAs in these lesions

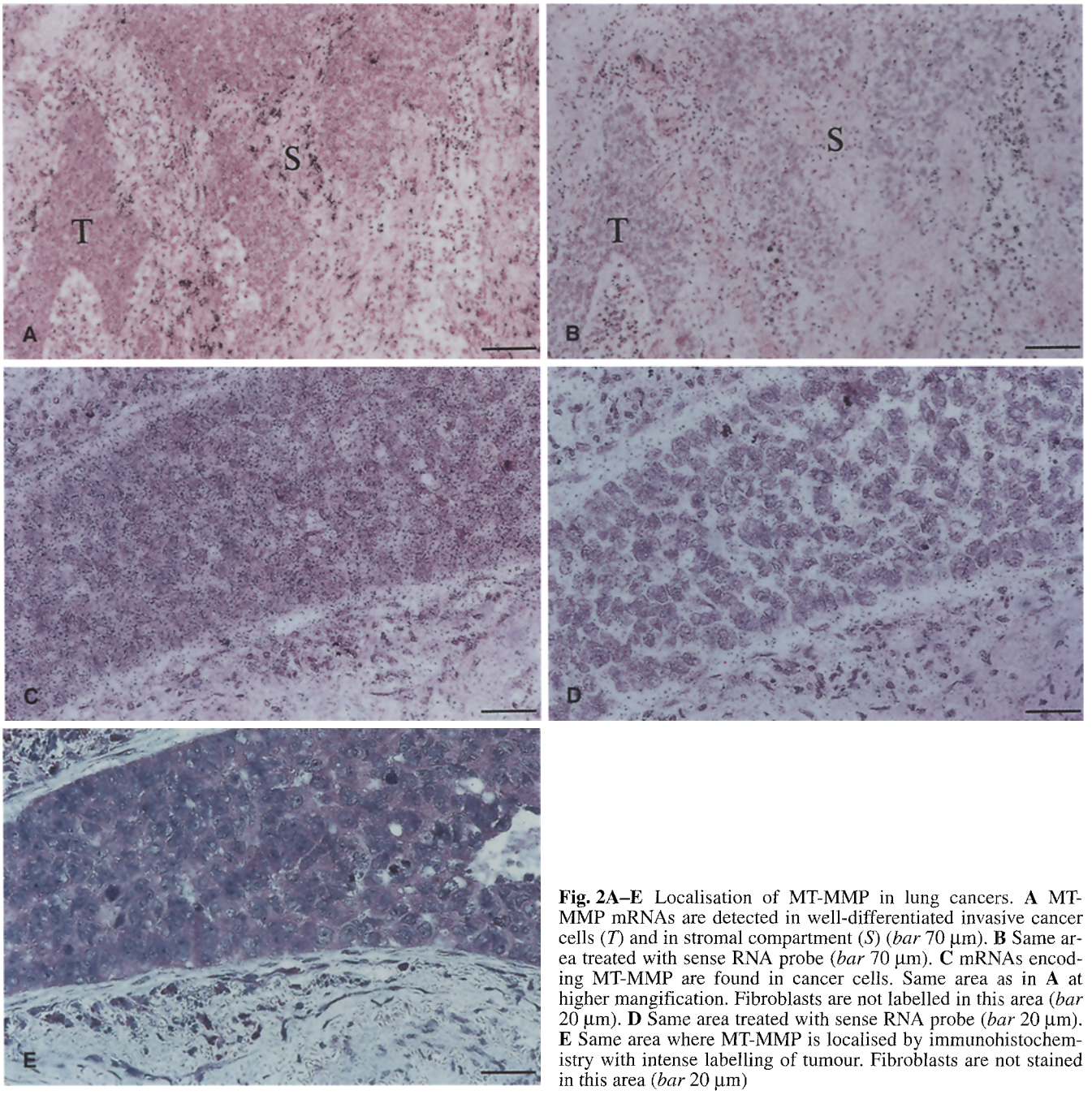


Fig. 2A–E Localisation of MT-MMP in lung cancers. **A** MT-MMP mRNAs are detected in well-differentiated invasive cancer cells (*T*) and in stromal compartment (*S*) (*bar* 70 µm). **B** Same area treated with sense RNA probe (*bar* 70 µm). **C** mRNAs encoding MT-MMP are found in cancer cells. Same area as in **A** at higher mangification. Fibroblasts are not labelled in this area (*bar* 20 µm). **D** Same area treated with sense RNA probe (*bar* 20 µm). **E** Same area where MT-MMP is localised by immunohistochemistry with intense labelling of tumour. Fibroblasts are not stained in this area (*bar* 20 µm)

In situ hybridisation

Tissue sections (5 µm) were deparaffinised, rehydrated and treated with 0.2 N hydrochloric acid for 20 min at room temperature, followed by 15 min in 1 µg/ml proteinase K (Sigma, St. Louis, Mo., USA) in TRIS-EDTA-NaCl, 37° C, to remove basic proteins. The sections were washed in 2×sodium saline citrate (SSC), acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and were hybridised overnight with ^{35}S-labelled (50° C) antisense RNA transcripts. A MT-MMP cDNA insert (1200 bp) was subcloned into pGEM4 plasmid and used to prepare ^{35}S-labelled RNA probes. Hybridisations were followed by RNase treatment (20 µg/ml, 1 h, 37° C) to remove unhybridised probe and two stringent washes (50% formamide/2×SSC, 2 h at 60° C) before autoradiography using D 19 emulsion (Kodak). Slides were exposed for 15 days prior to development. The controls were performed under the same conditions using ^{35}S-labelled sense RNA probes. All these controls gave negative results.

All slides were counterstained with HPS (haematoxylin-phloxin-safran), mounted and examined under a Zeiss Axiophot microscope.

Northern blot analysis

Extraction of total RNA from tissues was performed by RNAzol treatment (Biogenesis, Bournemouth, UK). Ten micrograms of each RNA was analysed by electrophoresis in 1% agarose gels containing 10% formaldehyde and transfered onto nylon membranes (Hybond-N, Amersham, UK). The membrane was hybridised with cDNA probes labelled with ^{32}P using random priming synthesis (5×10^8 cpm/µg) (Dupont de Nemours, Brussels, Bel-

gium). The filters were exposed 5 days for MT-MMP and 5 days for gelatinase A (1500 pb).

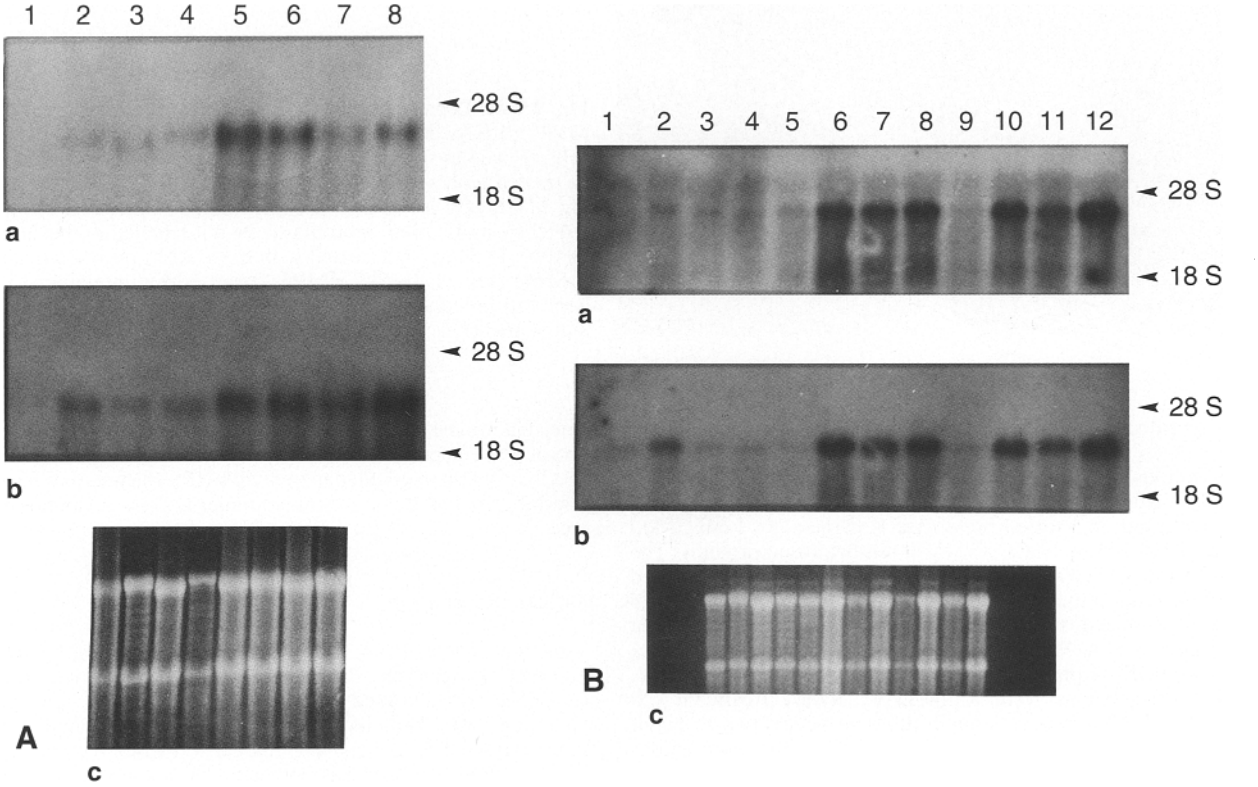
Results

Northern blot analysis showed that lung carcinomas (9 of 13) expressed high levels of MT-MMP mRNAs compared to peritumour lung tissue (Fig. 1A, B). There was a similar pattern of expression of gelatinase A and MT-MMP mRNAs in the tumours studied (Fig. 1A, B). In situ hybridisation revealed mRNAs encoding MT-MMP in most cancer cells (Fig. 2C) and in elongated stromal cells considered to be fibroblasts (Fig. 2A), with an intense labelling in close contact to tumour clusters. The samples treated with the sense RNA probe did not show specific hybridisation grains (Fig. 2B, D). Some of these stromal cells were in close contact to eroded bronchial cartilage and were particularly rich in hybridisation grains. By contrast, in adenocarcinomas, stromal cells near tumour clusters only expressed MT-MMP mRNAs. By immunohistochemistry, the cancer cells were uniformly stained (Fig. 2E), whereas some fibroblasts near the cancer nests were stained, with a distribution analogous to those expressing MT-MMP mRNAs in squamous cell carcinomas and adenocarcinomas.

In benign breast lesions, MT-MMP mRNAs were not detected by in situ hybridisation whereas Northern blot analysis showed a faint expression of MT-MMP mRNAs (Fig. 3A). Nevertheless, in some adjacent fibrocystic lesions near cancer areas, we found MT-MMP mRNAs in fibroblasts. Immunohistochemistry displayed some rare positivities in epithelial cells and some fibroblasts in benign lesions. The same pattern of distribution was observed in fibrocystic areas near carcinomas.

All carcinomas were positive for MT-MMP with the various methodologies used. MT-MMP mRNAs were detected by Northern blot analysis. There was a similar pattern of expression of gelatinase A and MT-MMP mRNAs in the different samples studies (Fig. 3A, B). Ductal carcinomas expressed more gelatinase A and MT-MMP mRNAs than lobular carcinomas. In situ hybridisation showed MT-MMP mRNAs principally in elongated stromal cells, with numberous grains in the fibroblasts and/or myofibroblasts in very close contact to tumour clusters, especially around preinvasive (intraductal) lesions or well-differentiated territories (Fig. 4A, B, F). The samples treated with the sense RNA probe did not show specific hybridisation grains (Fig. 4D). The labelling of tumour cells was faint and limited to some invasive cells and in most cases it may be confused with background. The most intense labelling was observed in ductal carcinomas as compared with lobular cancers. In contrast immunohistochemistry displayed an extensive staining in most non-invasive and infiltrating cancer cells (Fig. 4C). Stromal cells near these tumour cell nests and

Fig. 3A, B Northern blot analysis of MT-MMP and gelatinase A mRNAs in breast cancers. **A** *Lanes 1, 2* fibrocystic diseases; *lanes 3, 4* fibroadenomas; *lanes 5–8* ductal cancers. *a* MT-MMP, *b* gelatinase A. **B** *Lanes 1–5* lobular cancers, *lanes 6–12* ductal cancers. *a* MT-MMP, *b* gelatinase A. *c* Ribosomal RNA bands visualised under ultraviolet light after staining with ethidium bromide show amounts and quality of the RNAs. There is a coordinate expression of gelatinase A and MT-MMP mRNAs in these lesions



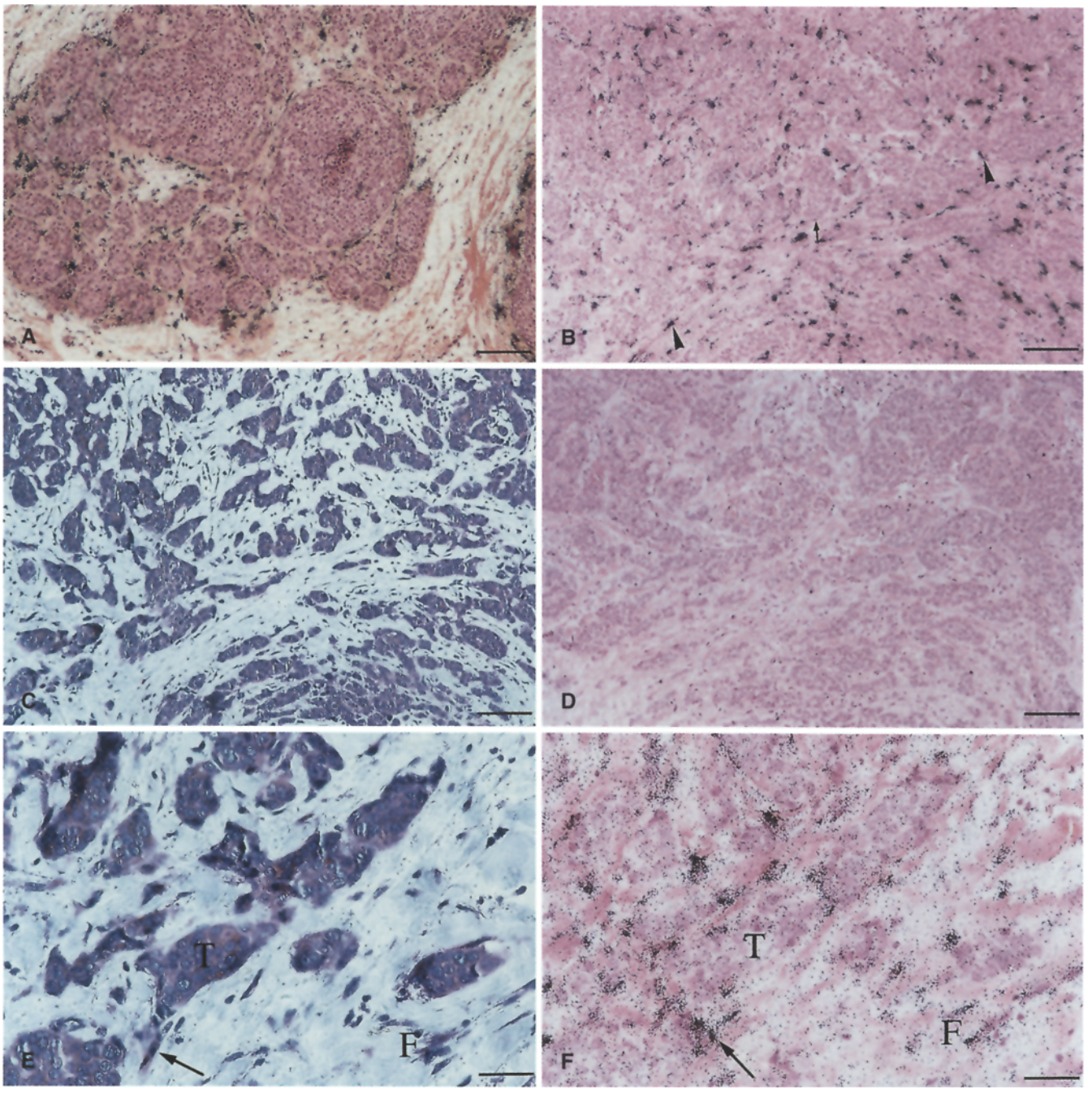


Fig. 4A–F Localisation of MT-MMP in breast cancers. **A** mRNAs encoding MT-MMP are expressed in stromal cells in close contact with the cancer cells in the intraductal area of ductal cancer (*bar* 70 μm). **B** Infiltrating ductal cancer area where MT-MMP mRNAs are detected in stromal compartment (*arrowheads*) near tumour clusters (*arrow*) (*bar* 70 μm). **C** Same area where MT-MMP is localised by immunohistochemistry in both tumour and stromal cells (*bar* 70 μm). **D** Same area treated with sense RNA probe (*bar* 70 μm). **E** MT-MMP is observed in all cancer (*T*) and fibroblastic (*F*) cells (*bar* 20 μm). **F** Same area where fibroblasts (*F*) (*arrow*) in close contact with tumour clusters (*T*) express MT-MMP mRNAs (*bar* 20 μm)

strands were also marked just as some endothelial cells (Fig. 4E).

Discussion

The present work clearly demonstrates the expression of MT-MMP in vivo, in both human breast and lung carcinomas. In breast cancers, MT-MMP mRNAs are only detected in stromal cells as previously shown by Okada et al. [14]. We have made the same observations in lung adenocarcinomas. Okada et al. [14] did not find any MT-MMP mRNAs in tumour cells in their squamous cell carcinomas of head and neck but in our squamous cell car-

cinomas of the lung we found different results. As expected, according to the data of Sato et al. [18], MT-MMP mRNAs and MT-MMP protein were detected in numerous tumour cells but stromal cells also expressed large amounts of MT-MMP mRNAs. Similarly, Urbanski et al. [22] detected gelatinase A transcripts in both tumour and stromal cells in such cancers. It is thus likely that, in lung carcinomas, both cancer and stromal cells produce gelatinase A and MT-MMP and would therefore cooperate in the degradation of the extracellular matrix components associated wilth tumour invasion.

In benign lesions of the mammary gland, Northern blot analysis has demonstrated the presence of MT-MMP transcripts. This MMP like gelatinase A may participate in the remodelling of the extracellular matrix. In breast cancer, the presence of MT-MMP could be related to malignancy since the level of expression of this matrix metalloproteinase is lower in adjacent normal tissues and benign proliferations than in both peinvasive and invasive areas. However, there were no correlations between the expression of MT-MMP and the size, grade of the tumours, the number of lymph node metastasis and the status of hormonal receptors (data not shown).

Immunohistochemistry displays a more extensive distribution of MT-MMP, when compared with the data observed with in situ hybridisation, particularly in breast cancers. Indeed, numerous stromal, endothelial and cancer cells as well as some normal ducts and lobules were stained. In contrast, in situ hybridisation principally shows hybridisation grains in stromal cells in close contact to tumour clusters, especially along preinvasive and well-differentiated areas, with a faint and limited labelling of cancer cells. This disparity in the distribution of this MMP and their mRNAs may be explained by differences in the regulation of transcription, half-life of mRNAs, rates of mRNA translation and capacity for intracellular storage of the protein. The threshold of detection in the breast cancer cell compartment for MT-MMP mRNAs in this system may not be reached so that only the more abundant mRNAs are demonstrable. However, our results obtained by in situ hybridisation and immunohistochemistry of human lung squamous cell cancers indicate that tumour cells produce MT-MMP protein. Therefore, considering these findings together with our observations by immunochemistry, we cannot exclude the possibility that breast cancer cells and lung adenocarcinoma tumour cells may also produce MT-MMP. The hypothesis that stromal cells may secrete MT-MMP protein and deliver it to tumour cells in breast cancers in questionable since MT-MMP is a transmembrane protein and has to be cleaved to be released. Our immunohistochemical observations using an antibody directed against the extracellular domain of this protein cannot exclude this hypothesis. However, Sato et al. [18] found that MT-MMP without a transmembrane domain could not activate progelatinase A.

The principal source of MT-MMP appears to be of stromal origin in both types of cancer strongly suggesting an active role of stromal cells in these pathologies. Moreover, stromal cells are known to be able to produce other MMPs like gelatinase A [21] and stromelysin 3 [3]. Indeed, fibroblasts are likely the principal source of gelatinase A in breast carcinomas [15–17, 19]. It is therefore relevent to conclude that in vivo, fibroblasts and/or myofibroblasts, surrounded by an abundant type I collagen matrix, in close contact to tumour clusters, produce MT-MMP which could then activate progelatinase A. Gelatinase A would then be involved in the invasion process by degrading the extracellular matrix components near cancer cells. In addition to the stromal localisation, Northern blot analysis reveals the same pattern of MT-MMP and gelatinase A mRNAs expression. This observation argues in favour of a coordinate transcriptional regulation of the genes encoding both MT-MMP and gelatinase A in breast and lung cancers, in vivo.

Taken together, our observations on lung and breast cancers, describing an association of MT-MMP with invasiveness, strongly support a role of this matrix metalloproteinase in tumour invasion. In both types of cancer, it is clear that cancer cells cooperate with stromal cells to produce MT-MMP which may therefore participate in the invasion process.

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