Role of Matrix Metalloproteinases 2 and 9 in Determination of Invasive Potential of Pancreatic Tumors

L. E. Gurevich

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Our findings indicate that expression of matrix metalloproteinase-2 is an important prognostic marker of invasive potential of pancreatic tumors. Expression of this marker in neuroendocrine tumors (particularly in gastrinomas) can be used as a differential diagnostic criterion characterizing more malignant phenotype of tumors with the presence of ductal component. On the contrary, weak expression matrix metalloproteinase-9 is characteristic of less invasive phenotype of tumor cells (neuroendocrine and solid pseudopapillary tumors, mucinous cystadenomas). Co-expression of matrix metalloproteinases 2 and 9 is an unfavorable prognostic sign.

Key Words: *metalloproteinases; pancreatic tumors; invasive properties; cell-to-cell interactions; extracellular matrix destruction*

Matrix metalloproteinases (MMP) belong to the family of extracellular zinc endoproteases capable of destroying extracellular matrix glycoproteins (collagens, proteoglycanes, elastin, laminin, fibronectin, *etc.*) [3,13].

All MMP can be divided into specific collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), and membrane metalloproteinases (MMP-14, MMP-15, MMP-16, and MMP-17). MMP-7 and MMP-12 are characterized by special properties. All MMP are regulated by specific tissue metalloproteinase inhibitors. MMP expression is regulated at three levels: modulation of gene expression, activation of latent zymogens, and inhibition by tissue metalloproteinase inhibitors [4]. Activation of metalloproteinases is modulated by growth factors and cytokines (fibroblast-derived insulin-like growth factor, epidermal growth factor, integrins).

Cell migration through extracellular matrix at the interface of different types of tissues is the most important biological mechanism participating in regeneration, angiogenesis, and some other physiological processes. MMP released by migrating cells increase permeability of the extracellular matrix [1]. The release of MMP-1, MMP-2, MMP-9, MT1-MMP by endothelial cells promotes their migration through basal membranes, formation of tubular structures, and survival in the new environment [13]. Active MMP are stored in 300-600-nm cytoplasmic vesicles located near the endotheliocyte plasma membrane. Normally, the vesicles migrate from endotheliocyte surface only in response to specific angiogenic stimuli and only at a certain site of the cell membrane, while in tumor cells this migration is incessant and is observed all over the cell surface. Angiogenic serum factors, such as endothelial growth factor and fibroblast growth factor-2, promote rapid (within 4 h) and intensive release of MMP-containing vesicles. The rate of this release markedly increases in proliferating cells, while in tumor cells, particularly in the invasion zone, these vesicles are released continuously [7]. It is known that close contact of tumor cells with endotheliocytes of blood or lymph vessels is an essential condition for tumor invasion and formation of metastases at the interface between the tumor and adjacent or target

M. F. Vladimirskii Moscow Regional Research and Clinical Institute, Moscow; Institute of Human Morphology, Russian Academy of Medical Sciences, Moscow

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organ tissue [1]. Cells of different malignant tumors produce MMP-2 and MMP-9 participating in degradation of the main component of basal membranes (collagen IV), though the mechanisms of activation of these proteinases are different. MMP-2 promotes cleavage of extracellular matrix proteins and is intensively expressed by both neoplastic and stromal components of many tumor types, while MMP-9 modulates permeability of the vascular endothelium [2].

The correlation between superexpression of MMP-2 and MMP-9 and malignant potential of the tumor was demonstrated for many types of human tumors. Superexpression of MMP-1 by cells in colorectal cancer is associated with worse prognosis and Dukes' stage [12], while superexpression of MMP-9 is associated with the presence of synchronous distant metastases [5]. Superexpression of MMP-1 is an independent prognostic marker of esophageal cancer, that of MMP-2 for stomach cancer (rarely MMP-1 and MMP-9) [1]. Activation of MMP-2 correlates with 3-6-fold higher risk of lethal outcome in breast cancer [12] and with higher malignancy of the tumor in lung cancer of all histological types [5]. Increase in the metastatic potential and the degree of invasiveness of ovarian cancer is associated with expression of MMP-2 and MMP-9 by tumor cells [8].

The growth of many pancreatic adenocarcinomas is associated with pronounced desmoplastic stromal reaction, which is characterized by decreased content of collagen IV in the extracellular matrix, the content of laminin is inversely proportional to the degree of differentiation of tumor cells [11,14,15]. More aggressive phenotype of pancreatic tumors is associated with superexpression of MMP-2, while the presence of metastases in lymph nodes is associated with inhibited expression of tissue inhibitor of metalloproteinase-1 (TIMP-1) [1,6]. Almost nothing is known about MMP-2 and MMP-9 expression in pancreatic neuroendocrine tumors.

We studied the relationship between MMP-2 and MMP-9 expression and pancreatic tumor type and malignancy by the immunohistochemical (IHC) method.

MATERIALS AND METHODS

Biopsy material for IHC studies was fixed in 10% formalin buffered by Lilly's method (pH 7.2), treated in alcohols and xylols, and embedded in paraffin (melting point 54°C). Serial paraffin sections (3-5 μ) were prepared on a microtome and transferred onto polylysin-coated slides. The sections were deparaffinized, washed in deionized distilled water and phosphate buffer (pH 7.4) for 5 min. The study was carried out using L. A. Sternberger's method [10]. Antibodies against MMP-2 (clone A-Grl VC2, working solution 1:10; BD PharMingen Technical) and MMP-9 (clone 2C3, working dilution 1:50; Novocastra) served as the first antibodies. Before incubation with first antibodies, the sections in citrate buffer (pH 6.0) were processed twice in a microwave oven (5+5 min) at 750 W (MW processing) as described elsewhere [9]. The specific reaction was intensified by prolonged incubation with first antibodies (30 min at 20°C, then 14-18 h at 4°C). At the next stage the EnVision system (anti-mouse; DAKO) was used. Diaminobenzidine (DAB; DAKO) solution served as the visualization agent. All reagents were dissolved in deionized water. The reaction was evaluated visually under a microscope. After attaining the required intensity of staining, the sections were washed in distilled water, the nuclei were post-stained with Mayer hematoxylin (2-5 min), washed in distilled water, plunged in flowing water, to which several drops of ammonium hydroxide were added for creating alkaline medium. After the sections turned bluish, the slides were removed, routinely dehydrated in alcohols and xylenes, and embedded in balm. The intensity of MMP-2 expression in tumor cells was heterogeneous at different sites of the tumor and was scored as follows: 0) no reaction; 1) few marker-positive cells in the zone of growth and invasion; 2) positive cells in the growth zone and few positive cells in more central areas of the tumor; and 3) the majority or all cells are positive to MMP-2. The reactivity to MMP-9 in the tumors was more uniform and was located in the cytoplasm; it differed only by staining intensity, which was also scored (0: no reaction; 1: weak expression; 2: moderate reaction; and 3: intensive reaction).

RESULTS

Histological study and IHC verification of 77 pancreatic tumors of different genesis were carried out: 31 adenocarcinomas, 5 mucinous cystoadenoma (MCA), 5 solid pseudopapillary (SPPT), and 36 neuroendocrine tumors (NET), 15 of these were insulinomas, 3 glucagonomas, 8 gastrinomas, 7 nonfunctioning tumors, and 3 endocrine carcinomas of high malignancy without hyperfunction syndrome. The expression of MMP-2 and MMP-9 was studied in pancreatic tissue at the interface with the tumor, in tumor cells in zones of tumor growth and/or invasion and in the tumor center, and in the stromal extracellular component of the tumor.

In the majority (93.5%) of pancreatic adenocarcinomas MMP-2 was intensively expressed (2-3 points). In highly differentiated adenocarcinomas formed by large duct-like structures surrounded by solid fibrous stroma the expression of MMP-2 was seen in small foci of cells in the zone of tumor growth and invasion.

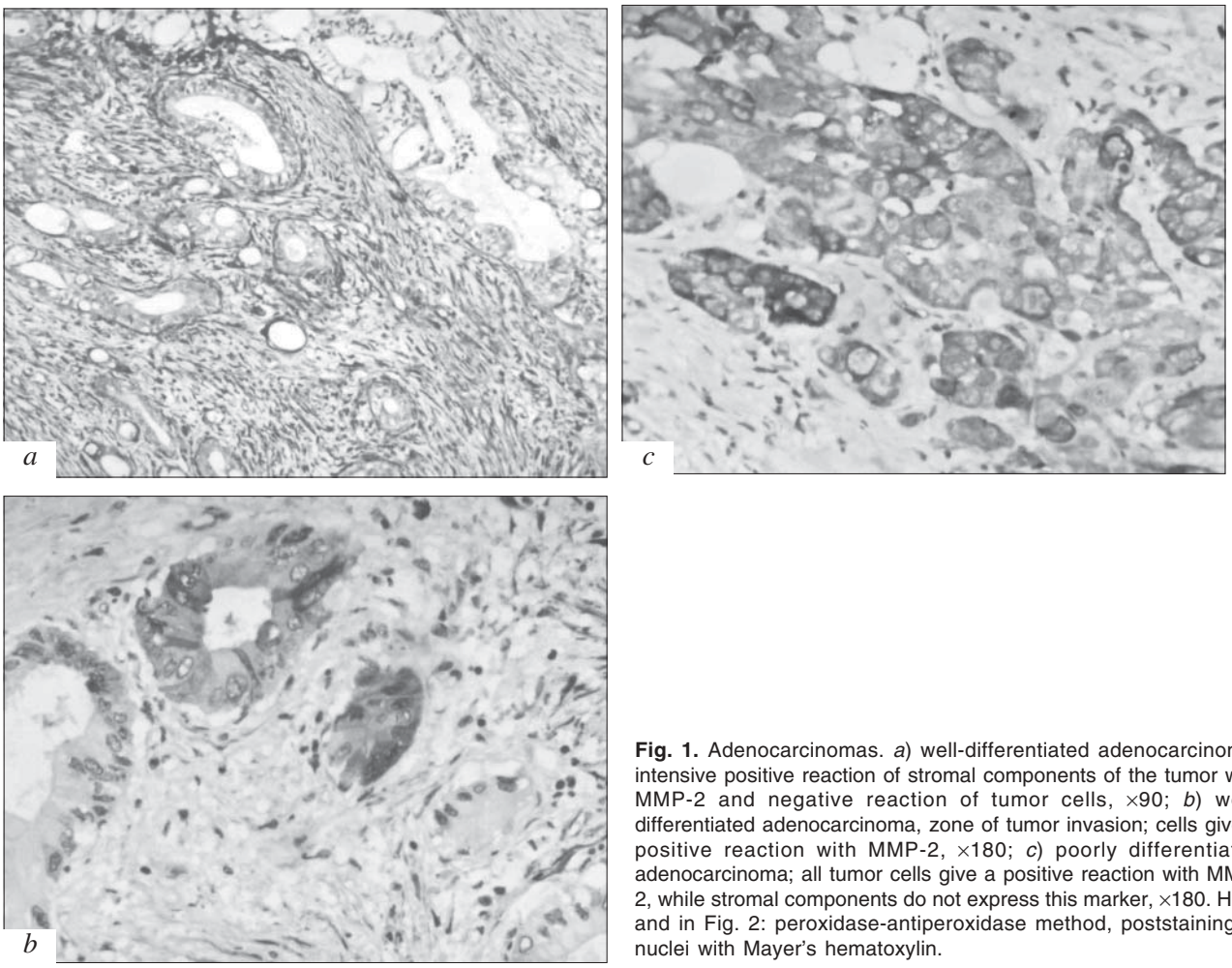

Fig. 1. Adenocarcinomas. *a*) well-differentiated adenocarcinoma; intensive positive reaction of stromal components of the tumor with MMP-2 and negative reaction of tumor cells, ×90; *b*) well-differentiated adenocarcinoma, zone of tumor invasion; cells give a positive reaction with MMP-2, ×180; *c*) poorly differentiated adenocarcinoma; all tumor cells give a positive reaction with MMP-2, while stromal components do not express this marker, ×180. Here and in Fig. 2: peroxidase-antiperoxidase method, poststaining of nuclei with Mayer's hematoxylin.

By contrast, the extracellular matrix (stroma) of these tumors was characterized by intensive expression of MMP-2 (Fig. 1, *a*). Moderately differentiated adenocarcinomas were characterized by medium and small duct-like and tubular structures. The expression of MMP-2 in these tumors was more intensive and was

TABLE 1. Expression of MMP-2 and MMP-9 in Pancreatic Tumors

Tumor type	MMP-2 expression						MMP-9 expression				
	0	1	2	3	%	pos/ total	0	1-2	3	%	pos/ total
Adenocarcinomas (*n*=13)	2	8	6	13	93.5	29/31	13	16	2	58.1	18/31
MCA (*n*=5)	5	0	0	0	0	0/5	2	2	1	60	3/5
SPPT (*n*=5)	5	0	0	0	0	0/5	0	5	0	100	5/5
NET (*n*=36)					16.7	6/36				58.3	21/36
of these:											
insulinomas (*n*=15)	14	1	0	0	6.7	1/15	6	9	0	60	9/15
glucagonomas (*n*=13)	3	0	0	0	0	0/3	2	1	0	33.3	1/3
gastrinomas (*n*=8)	5	3	0	0	37.5	3/8	1	7	0	87.5	7/8
nonfunctioning tumors (*n*=7)	1	1	0	0	14.3	1/7	4	3	0	42.9	3/7
endocrine carcinomas (*n*=3)	2	1	0	0	33.3	1/3	2	1	0	33.3	1/3

Note. pos/total: ratio of marker-positive tumors to total number of observations.

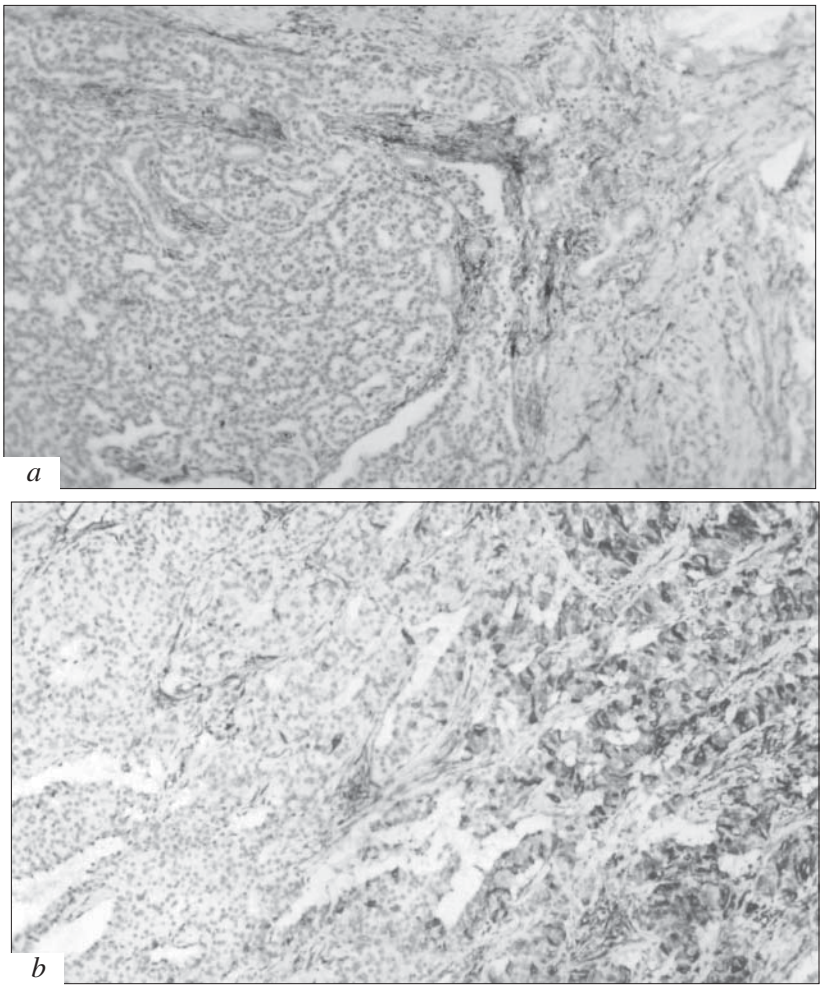

Fig. 2. Neuroendocrine tumors. *a*) nonfunctioning tumor; cells do not express MMP-2 and stromal elements give a positive reaction with this marker, ×90; *b*) gastrinoma: focal positive reaction of its cells with MMP-2, ×90.

detected in different sites of the tumor; expression of the marker in the extracellular matrix considerably decreased (Fig. 1, *b*). Intensive immunoreactivity of tumor cells to MMP-2 and the absence of this reaction in the extracellular matrix were characteristic of virtually all differentiated adenocarcinomas with diffuse, solid, microtubular, and alveolar growth types (Fig. 1, *c*). MMP-2 expression was not characteristic of pancreatic SPP tumors, was rarely detected in MCA and only in less differentiated sites. MMP-2 was expressed in few fibrillar structures and vascular walls of these tumors. Focal expression of MMP-2 was detected in only 5 of 36 NET (3 gastrinomas, insulinoma of low malignancy, and carcinoma of high malignancy, all tumors with metastases into lymph nodes or liver) (Fig. 2, *b*). Other NET (the greater part of insulinomas, glucagonomas, nonfunctioning tumors, and some gastrinomas) were MMP-2-negative. The expression of MMP-2 in NET stroma was observed only in vascular walls and fibrous pseudocapsule of the greater part of tumors (Fig. 2, *a*). By contrast, intensive expression of MMP-2 was observed in the stromal component of the majority of gastrinomas and some NET of other functional activity, which was paralleled by predominance of type IV collagen in their stroma.

Although expression of MMP-9 was detected in 58.1% adenocarcinomas, only weak focal diffuse cytoplasmic staining (1, rarely 2 points) was observed (Table 1). In poorly differentiated adenocarcinomas (staining intensity 2-3 points) almost all tumor cells were immunoreactive to MMP-9. In the tumor stroma this marker was expressed by cells of lymphoid infiltration, nerve elements, and some fibrillar structures of the extracellular matrix. The cytoplasm of all (100%) relatively benign SPP tumors was MMP-9-positive: 2 points in 4 of 5 tumors and 1 point in 1 tumor; 3 of 5 MCA were MMP-9-positive. In general, most NET showed weak (1-2 points) cytoplasmic expression of MMP-9, which was primarily characteristic of gastrinomas (87.5%) and insulinomas (60%) and to a lesser extent of nonfunctioning tumors (42.9%).

Our results indicate that the expression of MMP-2 is an important prognostic marker of invasive potential of pancreatic tumors. The expression of this prognostic marker in NET can be used as a criterion characterizing more malignant phenotype of tumor cell. By

contrast, weak expression of MMP-9 alone is characteristic of more benign phenotype of tumor cells (NET, SPPT, MCA), while co-expression of MMP-2 and MMP-9 is most likely an unfavorable prognostic sign.

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