The Expression of Matrix Metalloproteinases-2 and -9 and their Tissue Inhibitor 2 in Pancreatic Ductal and Ampullary Carcinoma and their Relation to Angiogenesis and Clinicopathological Parameters

GEORGE GIANNOPOULOS[1], KITTY PAVLAKIS[2], AIKATERINI PARASI[4], NIKOLAOS KAVATZAS[2], DINA TINIAKOS[3], ANTIGONI KARAKOSTA[4], NIKOLAOS TZANAKIS[1] and GEORGE PEROS[1]

[1]4th Surgical Department, Attikon Hospital, University of Athens, Athens;
[2]Department of Pathology and [3]Laboratory of Histology and Embryology, School of Medicine, University of Athens, Athens;
[4]Department of Pathology, St. Pantelehemon General Hospital, Piraeus, Greece

Abstract. *Aim: To investigate the expression of metalloproteinase (MMP) -2, MMP-9 and tissue inhibitor of MMP (TIMP) -2 in pancreatic ductal and ampullary carcinoma and to test the findings for correlation with angiogenesis and several clinicopathological parameters. Patients and Methods: Paraffin sections from 32 pancreatic ductal adenocarcinomas and 17 ampullary carcinomas were assessed for the expression of MMP-2, MMP-9 and TIMP-2 by immunohistochemistry. Stromal and epithelial staining was evaluated separately. Moreover, sections stained immunohistochemically with anti-CD34 antibody were evaluated by image analysis for the quantification of microvessel density (MVD). Results: In pancreatic ductal adenocarcinoma, lower levels of glandular TIMP-2 were found in poorly differentiated tumors, while high glandular TIMP-2 expression was significantly associated with better survival. The age of the patients and the degree of differentiation of the tumor were identified as independent prognostic parameters. No relation was found between the expression of MMPs, TIMP or angiogenesis and the parameters under consideration. In ampullary adenocarcinoma, strong expression of glandular MMP-2 was associated with higher MVD values. Moreover, lymph vessel invasion was associated with higher stromal TIMP-2. Conclusion: In pancreatic ductal adenocarcinoma, TIMP-2 may have a more crucial role in prognosis than MMP-2, MMP-9 or angiogenesis. In ampullary adenocarcinoma, MMP-2 expression correlated with MVD, supporting its postulated role in angiogenesis.*

Correspondence to: George Giannopoulos, Fourth Surgical Department, Attikon Hospital, University of Athens, Athens, Greece, Apostoli 2 st, 185 37 Piraeus, Greece. Tel/Fax: +30 210 4511451, e-mail: geogianno@med.uoa.gr

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It has long been postulated that matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play a crucial role not only in normal tissue remodeling, as in inflammatory procedures, fertilization and organogenesis, but also in cancer invasion and metastasis (1, 2). The ability of malignant cells to migrate has been attributed to an imbalance between the activity of MMPs and TIMPs (3, 4). MMPs, especially gelatinases (MMP-2 and MMP-9), are thought to be essential for degrading type IV collagen, which is the major component of basement membranes (5-7). Moreover, the degradation of the extracellular matrix is one of the key steps in the process of neovascularization. It has long been established that solid tumors must induce angiogenesis in order to grow and metastasize. Therefore, the correlation between the expression of MMPs, their tissue inhibitors and the degree of tumor angiogenesis might be of great importance in predicting the biological behavior of several types of cancer.

In pancreatic duct adenocarcinoma (PDAC), the influence of MMPs activity on invasiveness and prognosis has been demonstrated by several authors (4, 6, 8-10) with conflicting results. Only two studies have evaluated both MMPs and angiogenesis (5, 11), while there is only one study assessing the relation of ampulla Vater carcinoma (AVC) to the expression of MMPs and TIMPs (12).

PDAC and AVC are two distinct entities but still have several common characteristics. Similarities in their molecular fingerprint have been described (13, 14). Moreover, PDAC originating from the head of the pancreas shares the same lymphatic drainage with AVC and is surgically managed in the same way. Yet, the prognosis of AVC is much better than that of PDAC.

Taking into consideration the above data, we sought to investigate the expression of MMP-2, MMP-9 and their tissue inhibitor TIMP-2 in pancreatic and ampullary cancer and to test the findings for their correlation with the degree of angiogenesis, other clinicopathological parameters and survival.

Patients and Methods

Patients. The study comprised 51 patients with pancreatic and ampullary adenocarcinoma who had undergone resection at the 4th Department of Surgery, University of Athens, Athens, Greece, between December 1993 and September 2003. There were 32 men and 19 women. Two patients were excluded from the analysis (1 *in situ* ampullary carcinoma and 1 pancreatic carcinoma stage IV). The remaining patients were subdivided into two groups: the ampullary group comprising 17 patients and the pancreatic cancer group comprising 32 patients. The median follow-up time for all patients was 80.3 months. Out of the 31 patients with pancreatic cancer, 24 underwent a classic Whipple resection, 3 a pylorus-preserving pancreatico-duodenectomy (PPPD) and 4 a distal pancreatectomy (tumor in the tail of the pancreas). All patients with ampullary adenocarcinoma undertook a Whipple resection. None of them received preoperative chemotherapy and/or radiotherapy. Informed consent was obtained for all patients and the study was approved by the local Ethics Committee.

The staging of the tumors was performed according to the standards of the American Joint Committee on Cancer (15).

Immunohistochemistry. Immunohistochemical staining was performed on sections of formalin-fixed and paraffin-embedded specimens. Tissue sections of 4 μm thick were deparaffinized with xylene and rehydrated in ethanol. Antigen retrieval was performed at 120˚C for 30 min in 0.01 M sodium citrate buffer (pH 6.0) for anti-MMP-9, anti-MMP-2, anti-TIMP-2 (DAKO, Carpinteria, CA, USA) and anti-CD34 antibodies (Novocastra Ltd, UK). Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide for 30 min. Sections were incubated with anti-MMP-9 (diluted1/150), anti-MMP-2 (diluted 1/100), anti-TIMP-2 (diluted 1/100) and anti CD34 (diluted 1/50) antibodies at 4˚C overnight. The sections were washed and treated with peroxidase using the labeled polymer method with DAKO En Vision + Peroxidase, Mouse kit (DAKO) for 30 min. The peroxidase reaction was visualized with the liquid DAB substrate kit (Zymed Laboratories, San Francisco, CA, USA) and the sections were counterstained with hematoxylin. Immunohistochemical specificity was established using a negative control, in which the primary antibody was omitted. Sections from breast carcinoma were used as positive controls.

Scoring system. The sections were examined using light microscopy by two independent observers (K.P. and A.P.) who were unaware of the experimental data. Interobserver variation was resolved by simultaneous re-evaluation. Staining was evaluated over the entire tumor section, separately in the neoplastic epithelium and the stroma.

Immunoreactivity for MMP-2, MMP-9 and TIMP-2 was evaluated with reference to both the staining intensity and the positively stained area. Immunostaining intensity was scored as weak (1+), moderate (2+) and strong (3+). The positively stained area was graded as focal (10% or less), regional (11%-50%) or diffuse (more than 50%). Staining patterns of moderate and diffuse, intense and regional, or intense and diffuse were considered positive (Figure 1, 2).

Image analysis method. Images were obtained using a Zeiss Axiolab microscope (Carl Zeiss Jena GmbH, Jena, Germany) with a mechanical stage, fitted with a Sony-iris CCD videocamera (Sony Corporation, Tokyo, Japan). The video camera was connected to a Pentium II personal computer loaded with Image Scan Software (Jandel Scientific, Erkrath, Germany). Slides were examined carefully at a low power magnification (×40) to identify the areas with the highest density of capillaries and small vessels. In each case, the 3 - 4 most vascularized areas (hot spots) were selected and a ×200 field, representing an area of 0.128 mm^2 each, was stored as a JPEG file [(1550×1070 pixels, 16.7 million colors (24-bit)]. Single endothelial cells or clusters of endothelial cells positive for CD34 were considered as individual vessels. In each vessel, the outline was identified and traced. The presence of blood cells or fibrin without any detectable endothelial cells was not sufficient to define a microvessel. Areas with a dense leukocytic or hemorrhagic infiltration were excluded. Vessels with muscular wall were not counted; however, there was no restriction regarding the size of the countable vessels, so as not to underestimate longitudinal sections or bifurcations of microvessels (Figures 3 and 4).

Statistical analysis. Association between categorical variables was assessed using the Fisher exact test, while continuous variables were compared using the Mann-Whitney test. Survival curves were estimated by the Kaplan-Meier method and differences were assessed by the log-rank test. Univariate Cox regression analysis was performed separately for patients with pancreatic carcinoma and ampullary carcinoma. Variables included in this analysis were tumor size (T3 or T4 *versus* T1 or T2), lymph node metastasis (yes *versus* no), degree of differentiation (3 *versus* 2 or 1), lymphatic invasion (yes *versus* no), blood vessel invasion (yes *versus* no), MVD, stromal MMP2, glandular MMP2, stromal MMP9, glandular MMP9, stromal TIMP2 and glandular TIMP2. For the group of patients with pancreatic carcinoma, multivariate Cox regression was performed. The subclass of significant variables was identified using the backwards selection method. For the group of patients with ampullary carcinoma, multivariate analysis was not performed because of the small number of cases. Four postoperative deaths were not considered as events.

All statistical tests were performed at α=0.05 significance level. The analysis was performed using SPSS 10.0.1 (SPSS, Chicago, IL, USA) for Windows.

Results

In a total of 49 cases, 32 were pancreatic ductal carcinomas (mean age 62.6 years, range 9.6 years) and 17 ampullary carcinomas (mean age 60.3 years, range 12.5 years). The pancreatic cancer group had a significantly higher pT (p=0.003) according to the TNM classification system and was of higher stage (p<0.001) compared to the ampullary cancer group. Patients with ampullary cancer had positive (N1) regional lymph nodes at a higher rate than patients with pancreatic cancer, yet with marginal significance (p=0.065). No significant difference was noted between the two groups

Table I. *Clinical and morphological characteristics of ampullary and pancreatic carcinomas.*

	Ampullary* N=17		Pancreatic N=32		*p*
Gender					0.219
Male	13	(76.5%)	18	(56.3%)	
Female	4	(23.5%)	14	(43.8%)	
Age (years)					
Mean (SD)	60.3	(12.5)	62.6	(9.6)	0.708
Pathologic size					**0.003**
pT1 or pT2	10	(58.8%)	5	(15.6%)	
pT3 or pT4	7	(41.2%)	27	(84.4%)	
Regional lymph node metastasis					0.065
Yes	8	(47.1%)	8	(25%)	
No	9	(52.9%)	24	(75%)	
Lymphatic vessels invasion					0.217
Yes	4	(23.5%)	3	(9.4%)	
No	13	(76.7%)	29	(90.6%)	
Blood vessel invasion					0.999
Yes	4	(23.5%)	8	(25%)	
No	13	(76.7%)	24	(75%)	
Grade					0.371
1	2	(11.8%)	4	(12.5%)	
2	10	(58.8%)	12	(37.5%)	
3	5	(29.4%)	16	(50%)	
Stage					**<0.001**
I	8	(47.1%)	2	(6.3%)	
II	4	(23.5%)	30	(93.8%)	
III	5	(29.4%)			
Micro vessel density					
Mean (SD)	50.7	(24.9)	50.8	(21.7)	0.891
Stromal staining					
MMP-2					
1+ or 2+	13	(86.7)	28	(87.5)	0.999
3+	2	(13.3)	4	(12.5)	
MMP-9					0.533
1+ or 2+	7	(46.7)	19	(59.4)	
3+	8	(53.3)	13	(40.6)	
TIMP-2					0.999
Negative	13	(86.7)	27	(84.4)	
Positive	2	(13.3)	5	(15.6)	
Epithelial staining					
MMP-2					**0.026**
≤10%	11	(73.3)	11	(34.4)	
>10%	4	(26.7)	21	(65.6)	
MMP-9					0.055
≤10%	9	(60)	9	(28.1)	
>10%	6	(40)	23	(71.9)	
TIMP-2					0.461
≤10%	13	(86.7)	23	(71.9)	
>10%	2	(13.3)	9	(28.1)	

*In two cases, immunohistochemical data were not available and therefore they are not included in this table.

regarding the MVD values or the existence of lymphatic or blood vessel invasion.

Glandular MMP-2 and MMP-9 levels in pancreatic tumours were significantly higher than those in ampullary tumours (p=0.026 and p=0.055 respectively) (Table I).

Table II. *Association of glandular TIMP-2 expression with clinical parameters in pancreatic carcinomas.*

	TIMP2 glandular		*P*
	≤10% n (%)	>10% n (%)	
Size			0.999
pT_1 or pT_2	4 (17.4)	1 (11.1)	
pT_3 or pT_4	19 (82.6)	8 (88.9)	
Regional lymph node metastasis			0.999
No	6 (26.1)	2 (22.2)	
Yes	17 (73.9)	7 (77.8)	
Blood vessel invasion			0.999
No	17 (73.9)	7 (77.8)	
Yes	6 (26.1)	2 (22.2)	
Lymphatic vessel invasion			0.999
No	21 (91.3)	8 (88.9)	
Yes	2 (8.7)	1 (11.1)	
Grade			**0.015**
1 or 2	8 (34.8)	8 (88.9)	
3	15 (65.2)	1 (11.1)	

Table III. *Association of stromal TIMP-2 expression with clinical parameters in ampullary carcinomas.*

	TIMP-2 Stromal		*P*
	0+ n (%)	1+ n (%)	
Size			0.999
pT_1 or pT_2	7 (53.8)	1 (50)	
pT_3 or pT_4	6 (46.2)	1 (50)	
Regional lymph node metastasis			0.999
No	7 (53.8)	1 (50)	
Yes	6 (46.2)	1 (50)	
Grade			0.999
1 or 2	9 (69.2)	2 (100)	
3	4 (30.8)	0 (0)	
Lymphatic vessel invasion			**0.057**
No	11 (84.6)	0 (0)	
Yes	2 (15.4)	2 (100)	
Venous invasion			0.476
No	10 (76.9)	1 (50)	
Yes	3 (23.1)	1 (50)	

In the pancreatic cancer group, poorly differentiated carcinomas had significantly lower expression of glandular TIMP-2 compared to well and moderately differentiated carcinomas (p=0.015) (Table II). No association was found between MVD and MMPs or TIMP-2 in pancreatic adenocarcinoma.

In ampullary carcinomas, MVD values were significantly higher among patients who expressed glandular MMP-2 [median MVD 77 (68-85) *versus* 40 (12-98), p=0.018)].

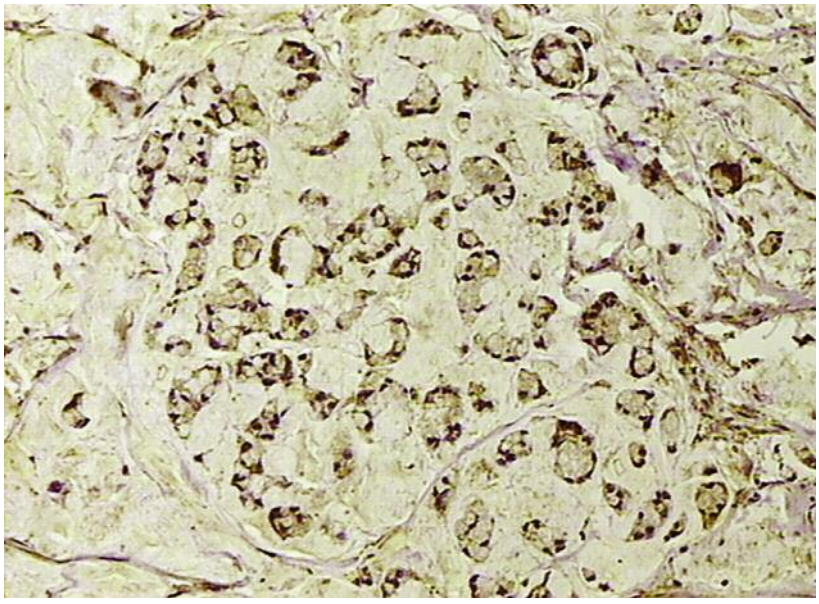

Figure 1. *Strong positive cytoplasmic MMP-2 expression in a mucinous pancreatic ductal adenocarcinoma (×40).*

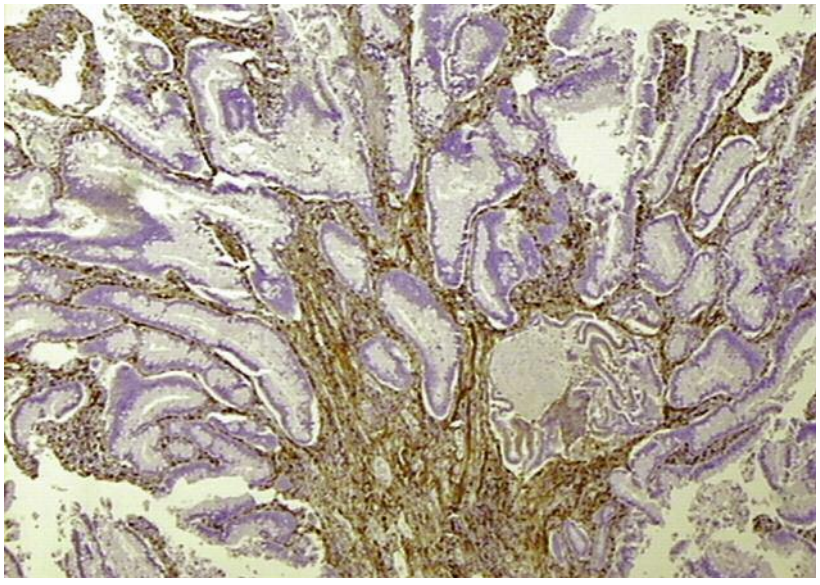

Figure 2. *Positive stromal MMP-9 expression in an ampulla Vater carcinoma. Absence of epithelial immunostaining (×200).*

Moreover, patients with lymph vessel invasion had a higher expression of stromal TIMP-2, although of marginal significance (p=0.057) (Table III).

Survival analysis. The median survival of the pancreatic group was 17 months (range 0.1-83) and the median survival of the ampullary group was 83 months (range 0.1-97) (p<0.001). Univariate Cox analysis for the PDAC group showed that glandular TIMP-2 was significantly associated with survival (p=0.019). In order to determine whether the clinicopathological characteristics of the tumours or the expression of MMP-2, MMP-9, TIMP-2 and MVD could be considered as possible independent prognostic factors for pancreatic cancer, multivariate Cox regression analysis was performed (Table IV). In the present study, only age and tumour differentiation were identified as independent prognostic values (p=0.033 and p=0.002, respectively).

The multivariate Cox regression analysis was not performed for the ampullary cancer group because of the small number of cases.

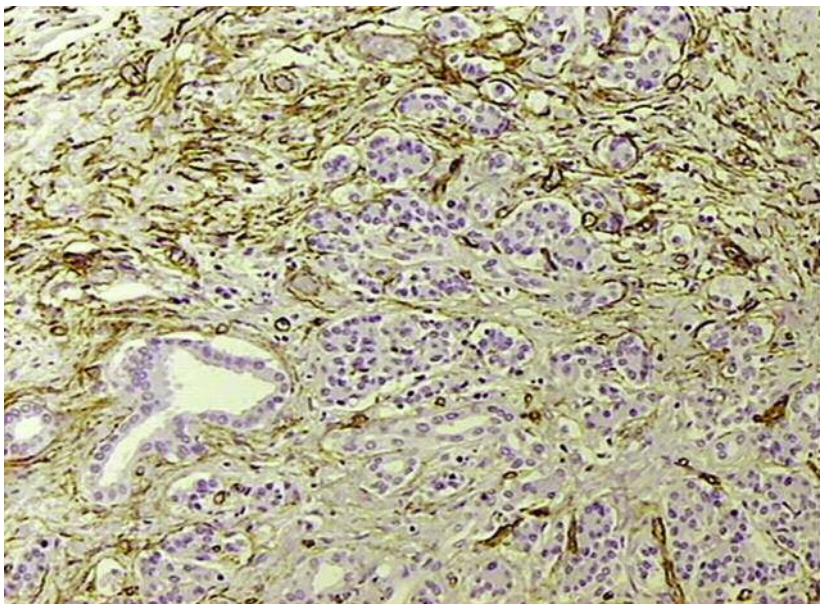

Figure 3. *High microvessel density in pancreatic ductal adenocarcinoma. Immunohistochemical staining with anti-CD34 antibody (×100).*

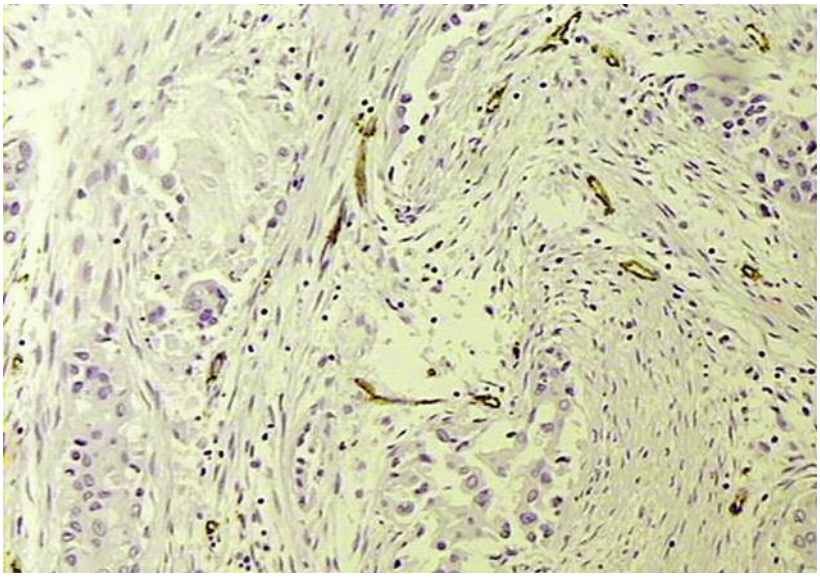

Figure 4. Low microvessel density in ampullary carcinoma. Immunohistochemical staining with anti-CD34 antibody (×100).

Discussion

The role of MMPs, especially MMP-2 and MMP-9, in the multistep process of metastasis has long been investigated and they have been implicated in the prognosis of various types of malignancies (16-19). Moreover, the balance between the function of MMPs and TIMPs is known to be crucial in both tissue remodeling and tissue invasion (1).

In pancreatic cancer, many studies have tried to determine the impact of MMPs on the metastatic potential of the tumors or on patients' survival, with conflicting results (4, 6, 8-10). In the present study, both MMP-2 and MMP-9 were found in the great percentage of tumors, mostly in the neoplastic epithelium but also in stromal cells, yet this immunohistochemical expression was not statistically correlated to tumor stage or survival. Nevertheless, these results should be considered with caution, since immunohistochemistry cannot discriminate between the latent and the activated form of gelatinases (4).

Another factor that has also been extensively investigated is the inhibition of MMPs action through TIMPs. Several early

Table IV. Cox analysis for survival in pancreatic carcinomas.

	Univariate			Multivariate		
	HR	95% C.I.	*P*	HR	95% C.I.	*P*
Age	0.96	0.90-1.01	**0.112**	0.93	0.87-0.99	**0.033**
Size	1.46	0.49-4.33	0.497			
Regional lymph node metastasis	1.27	0.51-3.17	0.613			
Grade	4.48	1.67-12	**0.003**	4.69	1.74-12.63	**0.002**
Vascular invasion	1.77	0.74-4.28	0.202			
MVD	1.01	0.99-1.03	0.350			
MMP-2						
stroma	1.48	0.50-4.37	0.475			
epithelia	1.01	0.46-2.21	0.985			
MMP-9						
stroma	0.55	0.25-1.20	0.132			
epithelia	1.12	0.50-2.54	0.782			
TIMP-2						
stroma	0.65	0.19-2.21	0.486			
epithelia	0.27	0.09-0.81	**0.019**			

HR: Hazard ratio; C.I.: confidence interval; MVD: microvessel density.

experimental studies *in vitro* and *in vivo*, confirmed the hypothesis that TIMP overexpression can reduce tumor aggressiveness, not only by inhibiting MMPs but also by reducing angiogenesis (20, 21). However, these data have not been corroborated by the results of clinical studies. Bloomston *et al*. (22) using cell lines showed that TIMP-1 downregulation greatly reduced the invasive potential of pancreatic cancer. Clinical studies on the expression of TIMPs in breast, gastric, cervical and colorectal cancer have shown conflicting results. Some authors advocate an anti-metastatic activity, others believe that high TIMP levels enhance tumor aggressiveness, while in the work of some, no correlation was found (23-25). In pancreatic cancer, the majority of studies revealed no significant correlation between the expression of any TIMP and tumor stage or survival (25-27). Yet, a lower expression of TIMP-1 in poorly differentiated tumors has been demonstrated (6). Likewise, in our study, epithelial TIMP-2 levels were significantly lower in poorly differentiated carcinomas. Furthermore, it was shown that high epithelial TIMP-2 levels were significantly associated with better survival. This finding is in agreement with the concept of an antitumor effect of TIMPs supported by early studies (1). As opposed to studies on breast carcinomas (28), stromal TIMP-2 was not found to correlate with tumour aggressiveness. The impact of TIMP-2 on prognosis and survival appears to be complex and unpredictable. Possibly, as in our study, high epithelial TIMP-2 levels and well-differentiated tumors can be considered as factors indicating a less aggressive behavior.

The existing literature on the relation of MMPs and TIMPs with ampullary cancer is very limited. In a single study, Bramhall *et al*. showed that high levels of MMP-2 were found in poorly differentiated tumors, while low levels of TIMP-1 were associated with N1 status (12). In our study, significantly lower immunohistochemical expression of epithelial MMP2 and MMP9 was found in ampullary carcinomas as compared to pancreatic duct carcinomas, yet in neither condition was the expression statistically related to the clinicopathological parameters under investigation. Only stromal TIMP-2 expression was associated (p=0.057) with lymph vessel invasion, indicating a possible role in the metastatic process.

In the present study, the expression of MMP-2 and MMP-9 was also investigated in relation to the degree of angiogenesis for both groups of tumors. In pancreatic duct adenocarcinoma, no relation was found between the expression of MMPs and angiogenesis. These tumors are considered hypovascular. Eventually, the hypovascular nature and the strong desmoplastic reaction of pancreatic cancer (6, 29) constitute a rather difficult environment for tumor angiogenesis and consequently create higher needs for MMPs. In the ampullary carcinoma group, high levels of epithelial MMP-2 were significantly correlated to high MVD. Although only MMP-9 has been shown to trigger the angiogenic switch during carcinogenesis (11), there are several reports supporting the belief that activated MMP-2 can further activate, among others, vascular endothelial growth factor and induce the formation of new blood vessels thus contributing to angiogenesis and the progression of carcinomas (30) .

Pancreatic cancer remains a highly incurable disease because of late diagnosis and tumour aggressiveness. Despite intensive research, the key step in invasion and metastasis remains unknown. MMPs, TIMPs and angiogenesis appear to be just pathways in the highway of malignancy. The results of the present study indicate that in pancreatic ductal adenocarcinoma, glandular TIMP-2 may have an important role in prognosis. In the ampullary cancer group the significant correlation of MMP-2 expression and MVD further supports its postulated role in angiogenesis. For the latter group of tumors, combined therapy with MMP inhibitors and anti - angiogenic factors may prove effective.

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