Comparison of Circulating MMP-9, TIMP-1 and CA19-9 in the Detection of Pancreatic Cancer

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Abstract. *Background/Aim: The performance of the circulating tumor markers carbohydrate antigen 19-9 (CA19-9), matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinase 1 (TIMP-1) were evaluated separately and in combination for their potential value in detecting pancreatic ductal adenocarcinoma. Patients and Methods: The patients had symptoms of pancreatic cancer. The discriminative strength of MMP-9 and TIMP-1 were compared to that of CA19-9 using receiver operating characteristics curves, area under the curves (AUC), specificity and sensitivity. Results: The sensitivities of MMP-9, TIMP-1 and CA19-9 in detecting pancreatic ductal adenocarcinoma were 58.82%, 47.1% and 86%, respectively, with specificities of 34.6%, 69.2% and 73%. The AUCs of MMP-9, TIMP-1 and CA19-9 were 0.50, 0.64 and 0.84, respectively. Combining the three markers did not significantly improve detection of pancreatic ductal adenocarcinoma compared to CA19-9 used alone. Conclusion: Circulating MMP-9 and TIMP-1 were inferior to CA19-9 as markers for detecting pancreatic ductal adenocarcinoma and did not improve the diagnostic accuracy when combined with CA19-9.*

Pancreatic cancer is the fifth leading cause of cancerrelated death and has the lowest survival rate of any solid cancer (1). Despite increasing understanding of etiology and pathogenesis of pancreatic ductal adenocarcinoma (PDAC), the 5-year survival has only increased marginally above 3-4% over the last two decades (2). Patients with surgically resectable PDAC may achieve a 5-year survival of 15-40% (3). However, only 10-15% of the patients present with resectable PDAC at time of diagnosis (1). Diagnosis is often delayed because patients present with nonspecific symptoms. An accurate serological test might facilitate the early diagnosis of pancreatic cancer. Such a test might also help in the screening of high-risk populations such as patients with hereditary pancreatitis and patients predisposed for familial pancreatic cancer. Carbohydrate antigen (CA) 19-9 is the most commonly used tumor marker for the diagnosis, prognosis and monitoring of the clinical course of PDAC (4), although the definitive diagnosis of PDAC still has to be verified by pathology. Approximately 5-10% of the general population have the Lewis a-b- phenotype and do not synthesize the CA19-9 antigen, and will therefore not present with elevated CA19-9 levels in case of PDAC (5). Moreover, CA19-9 is frequently elevated in other abdominal malignancies and in benign pathological conditions such as pancreatitis and cholestasis (6). Determination of circulating CA19-9 is capable of detecting PDAC with a sensitivity of 58-87% at a specificity of 93% (7). Thus alternatives or adjuncts to CA19-9 are needed.

Since destruction of the basement membranes and extracellular matrix are essential for cancer invasion and metastases to occur, the involvement of matrix metalloproteinases (MMPs) in neoplasia has attracted a lot of attention. Gelatinase B (MMP-9) and gelatinase A (MMP-2) digest type IV collagen, the main component of basement membranes (8) and they thereby contribute to the invasion and metastasis of various human malignancies including PDAC (9, 10). This results in an intense desmoplastic stromal reaction by the tumor (11), with subsequent cancer cell invasion into the surrounding stroma (12). Experimental evidence supports the belief that the expression of MMP-2 and -9 is correlated with aggressiveness of pancreatic carcinoma cells *in vitro* and/or in mouse models (13, 14). MMPs are secreted as proenzymes and require activation by proteinases or organic mercurials (15). On activation, the predomain (10 kDa) is cleaved from the proteinase, freeing the zinc ion to participate in proteolytic cleavage. The final

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Table I. *Patient characteristics, frequency (%) and [95% CI].*

	Cases	Controls	*P*-value
Patient characteristics			
Number of patients (%)	51 (49.5)	52 (50.5)	
Gender (male(%): female(%))	28:23 (54.9:45.1)	27:25 (51.9:48.1)	0.76
Median age at inclusion	66.38 [60.62-69.31]	60.36 [53.20-63.08]	0.01
Median age at symptom debut	66.19 [60.55-68.80]	59.84 [53.14-62.58]	0.01
Died during follow-up			
Median age at death	49 (96.1)	11 (21.2)	0.0001
Median time period from	67.1 [61.38-70.06]	65.83 [56.67-74.25]	0.67
symptom debut to death (years)	0.96 [0.7-1.07]	1.68 [0.77-2.55]	0.02
Biochemistry			
Median CA19-9 (U/ml)	166.3 [84.4-216.9]	25.9 [17.7-30.4]	0.00001
Median MMP-9 (ng/ml)	529.2 [384.7-597.9]	523 [454.5-609.8]	0.99
Median TIMP-1 (ng/ml)	197.3 [175.9-241.8]	162.6 [130.6-196.8]	0.02
Median bilirubin (μmol/l)	22 [14.0-48.9]	9 [8.0-11.2]	0.0001
Histopathology and final diagnosis			
	PDAC verified	Chronic pancreatitis 21 (40.4)	
	by histology: 43 (84.3)	No pathology 10 (19.2)	
		Benign cysts 4 (7.7)	
		Gall stones 4 (7.7)	
		Adenocarcinoma metastasis from colon 2 (3.9)	
	TMN:	Cholangiocarcinoma 2 (3.9)	
	1: 0	Periampullary cancer 2 (3.9)	
	2: 6 (11.8)	Sequelae after acute pancreatitis 2 (3.9)	
	3: 22 (43.1)	Suspicion of mucinous cystic neoplasm 1 (1.9)	
	4: 23 (45.1)	Metastasis from lung cancer 1 (1.9)	
		PDAC not verified by histology: 8 (15.7)	
		Primary sclerosing cholangitis 1 (1.9)	
		Epitheloid sarcoma 1 (1.9)	
		Vascular malformation 1 (1.9)	

domain, the hemopexin/vitronectin-like domain, has a sequence similar to the heme-binding protein and is found in almost all MMPs except MMP-7, MMP-23 and MMP-26. The function of this domain is to bind tissue inhibitors of metalloproteinases (TIMPs) (16). TIMPs are capable of forming noncovalent bonds with the active forms of MMPs and the latent form of MMP-9. Once bound to MMPs, TIMPs inhibit their activity (17) and thereby impart an antitumor effect; but they are also involved in the activation of MMPs, thus potentially promoting tumor progression. The exact role of TIMPs in tumorgenesis is not completely understood (18). For example, TIMP-1 also has an antiapoptotic effect, and high levels of TIMP-1 may thus result in a poor response to chemotherapy (19).

As MMPs and their natural inhibitors may be implicated in the progression of pancreatic cancer and as CA19-9 is a suboptimal marker of PDAC, we found it of interest to study the concentration of these proteins in plasma of patients with and without PDAC. In the present study, we thus evaluated if determination of circulating MMP-9 and TIMP-1, alone or in combination with CA19-9, could improve the detection of PDAC.

Patients and Methods

The present study was a prospective case-control study. The study population comprised patients referred for endoscopic ultrasonography of the pancreas or endoscopic retrograde cholangio pancreaticography because of suspicion of pancreatic cancer (mass in the pancreas, pain and weight loss). Cases were patients diagnosed with PDAC after 12 months of follow-up (Figure 1).

The upper reference range for CA19-9 in healthy controls is 37 U/ml according to the manufacturer's specifications. It is often used as the cut-off level in tumor marker studies. No cut-off value for MMP-9 or TIMP-1 indicating the presence of PDAC exists. Testing 100 healthy donors revealed that the TIMP-1 ELISA method used in the present study (see below) identified a reference range for plasma TIMP-1 levels from 58.0 to 91.8 ng/ml with a mean of 73.5±14.2 (SD) ng/ml (20). There is no reference concentration for MMP-9 in serum. We used the generated ROC curves to estimate the associations between sensitivities and specificities and to determine cut-off values for MMP-9 and TIMP-1 where the discriminative strength of the marker was best.

Sample size calculations were performed according to Flahault *et al*. (21). The prevalence of PDAC in the test population was estimated to be 0.5. By testing 51 cases with PDAC and 52 controls without PDAC, the lower 95% confidence interval (CI) for an expected sensitivity of 0.85 would be above 0.65. With a sensitivity

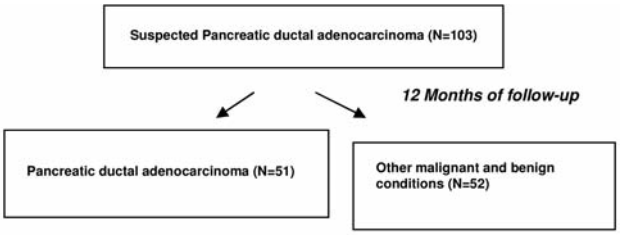

Figure 1. *Study design: 103 patients were included, 51 were found to have pancreatic ductal adenocarcinoma (PDAC) and 52 not.*

of CA19-9 at approximately 0.80, this sample size would allow us to demonstrate a difference in sensitivity of 0.20 with reliability (alpha) of 0.05 and 90% power.

Blood samples were collected as serum and EDTA plasma after an overnight fast and in the time period 8 a.m. until 1 p.m. The blood samples were centrifuged at 2608 × g, for 7 min and the supernatant was removed within 120 minutes. The samples were kept at –80˚C until analysis.

Total MMP-9 (pro- and active) concentration in serum was determined by ELISA (Quantikine kit, R&D Systems, Minneapolis, USA). Serum was diluted 100-fold. With this assay, the minimum detectable concentration of MMP-9 is 0.156 ng/ml (22). The test kit requires 100 µl diluted serum per assay. According to the manufacturer, the intra-assay precision was 1.9-2.9% and the inter-assay precision was 6.9-7.9%.

Total TIMP-1 levels (uncomplexed and complexed TIMP-1) were determined by ELISA (20). This assay detects TIMP-1 with high sensitivity and specificity. Inter- and intra-assay variations were below 10%.

CA19-9 was measured in sera by an immunofluorescent assay (Brahms, Henningsdorf, Germany) run on the Kryptor analyser using a time resolved amplified cryptate emission technology. This is a routine analysis carried out at Statens Serum Institute, Copenhagen, Denmark, and the normal upper level is 37 U/ml. The coefficient of variation (combined intra- and inter-assay variation) for the CA19-9 measurements was 6.7%.

All samples were assayed in duplicate, and they were analysed without knowledge of the final diagnosis of the patients.

The PDACs were graded according to the UICC classification. Stage I: tumor confined to the gland. Stage II: advanced local extension, no nodal involvement. Stage III: Regional lymph nodes involved and stage IV: distant metastasis (23).

All data analyses were performed using STATA 9.2 (Stata Corp, Tx USA). Since data were not normally distributed, non-parametric tests were used. Categorical data were compared using Fishers Exact test or Chi-squared test. Continuous data were compared using Mann-Whitney or Kruskal-Wallis test. Spearman rank-order correlation coefficient was calculated to assess association. Subsequently, logistic regression models were applied to adjust for confounders and for calculation of odds ratios. Receiver operating characteristic (ROC) curves were used to describe the performance of CA19-9, MMP-9 and TIMP-1, separately and in combination, in detecting PDAC and identifying the best cut-off value for each marker. *P*-values <0.05 were considered statistically significant.

The study was approved by the Scientific Ethics Committee (S-VF-20040031) and the Danish Data Protection Agency (2007-41-1479). All patients gave their informed consent to participate in the study.

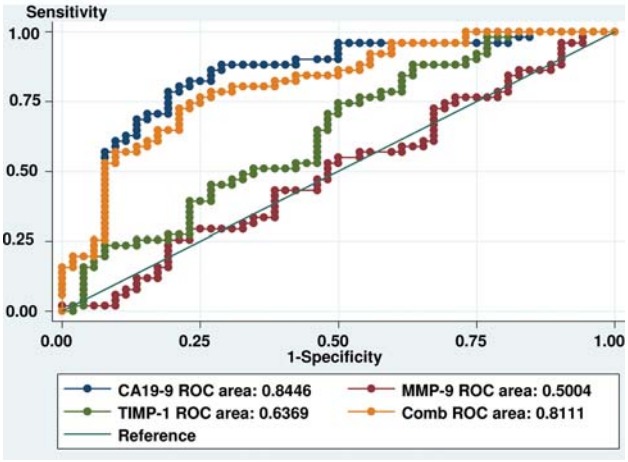

Figure 2. *Receiver operating characteristic curves of CA19-9, MMP-9 and TIMP-1 for the diagnosis of pancreatic ductal adenocarcinoma. Combining the 3 tumor markers did not improve the diagnostic capability compared to use of CA19-9 alone (p=0.18).*

Results

Patient demographics. A total of 103 patients, 51 cases and 52 controls, were included in the study (Table I). The female:male ratio was identical in cases and controls and there was no sex-dependent difference in age at onset of symptoms, neither among cases nor among controls. Cases were 6.35 years (median) older than controls (p=0.01). The age ranged from 43.5 to 81.8 years in cases, and from 36.4 to 83.8 years in controls. Age at death did not differ significantly between females and males, neither in cases nor in controls. The time span from symptom onset until death was 0.7 years shorter among cases than among controls (p=0.02). Loss in body weight was significantly higher in cases (10 kg, 95% CI: 7-10 kg) than in controls (5 kg, 95% CI: 4.4-7 kg; p=0.03). Six (11.8%) cases and 21 (40.4%) controls had chronic pancreatitis (p=0.001). Ten (17.5%) cases and 8 (15.4%) of the controls had diabetes mellitus (p=0.61). Similar levels of CA19-9 (p=0.48), MMP-9 (p=0.88) or TIMP-1 (p=0.29) were found in females and males.

Analysis of MMP-9, TIMP-1 and CA19-9. Due to the flat shape of the ROC curves for MMP-9 and TIMP-1 (Figure 2), it was difficult to decide an optimum cut-off level for the markers when analyzed for their value in the detection of PDAC. For MMP-9, sensitivity of 58.82% with a specificity of 34.62%, giving the best discriminative strength, gave a cut-off of 450 ng/ml (discriminating cases from controls). The ROC curve for TIMP-1 showed an optimum (best sensitivity and specificity) at a sensitivity of 47.1% with a specificity of 69.2%, which resulted in a cut-off level at 207.3 ng/ml. The optimal cut-off for CA-19-9 in this study

Table II. *Correlations between CA19-9, MMP-9, TIMP-1, p-bilirubin and tumor stage. r, Spearman rank-order correlation coefficient.*

	TIMP-1	MMP-9	CA19-9	Bilirubin
TNM	p=0.91	p=0.86	p=0.89	p=0.82
Bilirubin	r=0.6, p=0.00001	r=0.02, p=0.16	r=0.38, p=0.006	
CA19-9	r=0.37, p=0.007*	r=0.14, p=0.34		
MMP-9	r=0.08, p=0.58			

was 39.1 U/ml using the ROC curve, resulting in a sensitivity of 86.3% at a specificity of 73.1%. However, in order to compare our results with others, we decided to use the cut-off value of 37 U/ml commonly agreed on. If sensitivity, with the purpose of comparison, in this study was fixed at 90%, then cut-off levels and specificities for MMP-9, TIMP-1 and CA19-9 were 201.2 ng/ml and 9.6%, 116.2 ng/ml and 26.9%, and 27.8 U/ml and 57.7%, respectively. If specificity was set at 90%, the cut-off levels and sensitivities for MMP-9, TIMP-1, and CA19-9 were 1298 ng/ml and 5.9%, 392.8 ng/ml and 23.5%, and 102.1 U/ml and 60.8%, respectively. The median CA19-9 level was significantly higher among cases than among controls, approximately 140 U/ml (p=0.00001) (Table I). The median TIMP-1 level was also significantly (p=0.02) higher (approximately 35 ng/ml) in cases than in controls. In contrast, the median level of MMP-9 in cases was not statistically different from that found in controls (p=0.99). No correlation was found between CA19-9 and tumor stage in patients with PDAC (Table II), but positive correlations were identified between CA19-9 and bilirubin level, and CA19-9 and TIMP-1 level.

MMP-9 did not correlate with bilirubin, CA19-9 or TIMP-1. The level of TIMP-1 also did not correlate with tumor stage, but did with bilirubin level and CA19-9 as mentioned. Adjusting for bilirubin, the odds ratio for an elevated CA19-9 (>37 U/ml) in PDAC was 15 (95% CI: 5.2-43.3). Levels of CA19-9 were also elevated in patients with chronic pancreatitis (p=0.001), and adjusting for this in addition to the bilirubin level gave an odds ratio for an elevated CA19-9 among cases of 14 (95% CI: 4.7-41.1). The presence of chronic pancreatitis was not correlated with TIMP-1 (p=0.89). The odds ratio for having an elevated TIMP-1 (>207.3 ng/ml) in cases was not significantly different from 1.00 (OR=0.9, p=0.73, 95% CI: 0.3-2.2) after adjusting for levels of bilirubin and CA19-9. When establishing a sensitivity specificity relationship for CA19-9, MMP-9 and TIMP-1, we used ROC curves (Figure 2). CA19-9 (area under the curve (AUC) =0.84, 95% CI: 0.77-0.92) had a superior sensitivity in comparison to both MMP-9 (AUC=0.50, 95% CI: 0.39-0.61) and TIMP-1 (AUC=0.64, 95% CI: 0.53-0.74) (Figure 2).

The AUC for combined markers was 0.81, which is significantly higher than that for MMP-9 alone (p=0.0002) and TIMP-1 alone (p=0.0006) but less than that for CA19-9 alone (p=0.18). When TIMP-1 and CA19-9 were combined, AUC was still 0.81. If the markers are studied in detail, 2 (3.92%) patients with PDAC had an MMP-9 level above 450 ng/ml while the CA19-9 level was lower than 37 U/ml. If both MMP-9 and CA19-9 were used these 2 patients would undergo more thorough examinations in search for PDAC, while some 41 (39.81%) controls would undergo more examinations because of their elevated MMP-9 levels (false positive). PDAC would not have been detected in five (9.80%) patients with if the diagnosis of PDAC relied on elevations of CA19-9 and MMP-9 levels. One patient with PDAC (1.96%) had a TIMP-1 level above 207.3 ng/ml, while the CA19-9 level was lower than 37 U/ml. Twenty-four (23.30%) controls would undergo unnecessary examinations because of elevated TIMP-1. Relying on TIMP-1 and CA19-9, 6 (11.76%) patients with PDAC would not have been diagnosed.

Combining MMP-9, TIMP-1 and CA19-9, 4 (7.84%) patients with PDAC would not have been diagnosed and 46 controls (44.66%) would have undergone unnecessary examinations. The positive predictive values of CA19-9, MMP-9 and TIMP-1 in this clinical setting were 0.76, 0.48 and 0.59 respectively. The negative predictive values for CA19-9, MMP-9 and TIMP-1 were 0.84, 0.48 and 0.56 respectively.

Using the previously reported upper reference range of 91.8 ng/ml for TIMP-1 found in healthy donors (23), all patients with any malignant disease (n=60 or 58.25%) in our study had elevated TIMP-1 levels. However, only four (9.3%) patients with no malignant disease had normal TIMP-1 levels and 39 (90.7%) had elevated TIMP-1 levels falsely deemed; to be indicative of malignancy. The proportion of patients with elevated TIMP-1 was significantly higher in patients with malignant disease than in patients with non malignant disease (p=0.028). Because no patients with malignant disease had normal TIMP-1 levels it was not possible to calculate an odds ratio for having an elevated TIMP-1 level.

Discussion

In this prospective study including a selected cohort of patients with symptoms of PDAC, the use of measurements of MMP-9 and TIMP-1 as tumor markers in a diagnostic test for PDAC was evaluated and compared with measurements of the established tumor marker CA19-9 using ROC curves, AUC and sensitivity and specificity.

The choice of a cut-off is not solely a statistical decision but will rely on the clinical action following a positive test, especially considering whether the test is a screening or a diagnostic test. For screening tests, it is important to have a high sensitivity, as we are willing to have a moderate number of false positives but do not accept any false negatives. Those who turn out positive will be tested again with a diagnostic test with a higher specificity and positive predictive value. The sensitivity and specificity do not assess the accuracy of the test in a clinically useful way as the predictive value would have done – but this value depends strongly on the prevalence (24), which in our study is very high. This means that predictive values observed in a study like ours is not to be applied universally and if the predictive values should have any meaning they should be calculated evaluating the diagnostic test on patients with the same prevalence of disease as those for whom the test will be used in the future. Thus we only compared sensitivity and specificity measurements in our study in addition to the AUC of the ROC curves. The ROC curve does not take account of the prevalence of the disease being tested for. The AUC is a measurement given using ROC statistics; it is a combined measure of sensitivity and specificity, or, in other words, the overall performance of a diagnostic test.

Based on the AUC of the ROC curve, CA19-9 was a better marker diagnosing PDAC than were MMP-9 and TIMP-1, separately and combined. Comparing sensitivity when the specificity was fixed at 90%, approaching an optimal diagnostic test, CA19-9 also proved to be the best marker, followed by TIMP-1. Comparing specificities when sensitivity was fixed at 90%, approaching an optimal screening test, CA19-9 again was superior to the other two markers, again followed by TIMP-1. Because PDAC is a lethal disease, we are not willing to accept any false negative using the markers. Therefore, the specificity of the marker at 90% (or higher) sensitivity is the value we think evaluates the usefulness of the markers best.

No correlation between the levels of any tumor marker and the TNM stage was found. This is in contrast to several immunohistochemical studies, in which it was reported that increased staining of mRNA for MMP-2, MMP-9, TIMP-1 and TIMP-2 in carcinoma *in situ* correlated with the existence of regional lymph node metastasis, distant metastasis and post resection recurrence, but not with patient survival in pancreatic cancer (25). Some studies have also reported that MMP-9 expression in tumor correlates with a shorter survival time (26). On the other hand TIMP- 1 in plasma may not necessarily correlate with the content of TIMP-1 in tumor (27). Controlling for confounders such as CA19-9 and bilirubin by logistic regression did not improve the diagnostic characteristics of the TIMP-1 assay.

One of the few studies on serum MMP-9 and PDAC is by Tian *et al*. They recently reported significantly higher serum MMP-9 levels in patients with PDAC (median=255.14 ng/ml, interquartile range 125.4 ng/ml) when compared to patients with chronic pancreatitis (median=210.22 ng/ml, interquartile range 12.48 ng/ml) and healthy controls (median=203.77 ng/ml, interquartile range 17.04 ng/ml). Serum levels were analyzed with a commercially available ELISA kit (Biotrak). The sensitivity of this MMP-9 ELISA was 0.08 ng/ml (28). However, the study only included a small number of patients (8 PDAC patients, 9 patients with chronic pancreatitis and 8 healthy controls) and sensitivity and specificity of the test were not stated. We found an optimal cut-off value of 207.3 ng/ml for TIMP-1. Koopmann *et al*. have reported a cut-off value for TIMP-1 of 1564 ng/ml comparing patients with PDAC and those with chronic pancreatitis. They found that the sensitivity and specificity of TIMP-1 in discrimination was 50% and 72%, respectively, and that the AUC was 0.66 (29), which is similar to our results. The difference in cut-off values may be explained by the use of serum instead of plasma or another reference standard.

Our results do not indicate that MMP-9 and TIMP-1 are valuable circulating markers for PDAC; CA19-9 is better but also not good enough to be used solely in screening or diagnosing. The diagnosis still rely on pathology and if used, all three markers should only be used as a supplement to more specific and sensitive radiological investigations such as endoscopic ultrasound. However, an elevated TIMP-1 level was found in all patients with malignant disease and consequently measurement of circulating TIMP-1 may have a role as an indicator of patients who need further investigations quickly. In conclusion: This study does not support the measurement of MMP-9 and TIMP-1 in serum and plasma as useful markers for PDAC, neither for screening nor for diagnosis.

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