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Blood expression of matrix metalloproteinases 8 and 9 and of their inducers S100A8 and S100A9 supports diagnosis and prognosis of PDAC-associated diabetes mellitus

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ABSTRACT

Background: Based on the knowledge that matrix metalloproteinases (MMPs) and S100A8/A9 synergistically work in causing PDAC-associated type 2 diabetes mellitus (T2DM), we verified whether tissue and blood MMP8, MMP9, S100A8 and S100A9 expression might help in distinguishing PDAC among diabetics.
Methods: Relative quantification of MMP8, MMP9, S100A8 and S100A9 mRNA was performed in tissues obtained from 8 PDAC, 4 chronic pancreatitis (ChrPa), 4 non-PDAC tumors and in PBMCs obtained from 30 controls, 43 T2DM, 41 ChrPa, 91 PDAC and 33 pancreatic–biliary tract tumors.
Results: T2DM was observed in PDAC (66%), in pancreatic–biliary tract tumors (64%) and in ChrPa (70%). In diabetics, with or without PDAC, MMP9 tissue expression was increased ($p < 0.05$). Both MMPs increased in PDAC and MMP9 increased also in pancreatic–biliary tract tumors PBMCs. In diabetics, MMP9 was independently associated with PDAC ($p = 0.025$), but failed to enhance CA 19-9 discriminant efficacy. A highly reduced S100A9 expression, found in 7 PDAC, was significantly correlated with a reduced overall survival ($p = 0.015$).
Conclusions: An increased expression of tissue and blood MMP9 reflects the presence of PDAC-associated diabetes mellitus. This finding fits with the hypothesized role of MMPs as part of the complex network linking cancer to diabetes.

1. Introduction

The death rate from pancreatic ductal adenocarcinoma (PDAC), unlike that from all cancer types combined, has been increasing since the early 2000s among whites [1,2]. This is mainly due to the failure in achieving PDAC specific primary prevention and to the spread of some PDAC-related risk factors, such as obesity, chronic pancreatitis and type 2 diabetes mellitus (T2DM) [3–10]. Although a comorbidity of obesity, T2DM independently increases the risk of PDAC [9], probably for the pro-proliferative and pro-oncogenic effects of hyperinsulinemia [11–14]. The duration of T2DM has differential effects on the risk of PDAC: while a long-standing history (>10 years) has a negligible effect [8,15], the odds ratio of a >2 to 8 year history is close to 1.8, and is greater than 2 when T2DM is of recent onset (<3 years from PDAC diagnosis) [15–18]. The strong association between PDAC and recent onset T2DM probably reflects the fact that diabetes is a manifestation of PDAC [18,19]. Accordingly, although T2DM reportedly enhances the risk of lung, breast, colon–rectum and prostate cancer, its prevalence in PDAC, which ranges from 50 to 80%, is much higher than that found in other cancer types [20]. Based on the premise that recent onset T2DM might be an early symptom of PDAC, it has been suggested that it may be of clinical value as a first filter criterion in identifying asymptomatic PDAC, the diagnosis being confirmed by means of sensitive and specific blood biomarkers [18]. However, markers' availability is still limited and their identification remains a relevant clinical challenge. For example, transcriptomic and/or proteomic expression profiling have recently allowed the identification of a number of potentially useful biomarkers, including S100A9 [21], FABP-1 [22], adrenomedullin [23], apolipoprotein A1 [24], Vanin-1 and matrix metalloproteinase 9 (MMP9) [25], glucagon/insulin ratio or CA19-9 with a 500 U/mL cut-off [26,27]. However, both sensitivity (0.36 to 0.96) and specificity (0.69 to 0.77) of the above-suggested markers appear rather unsatisfactory.

An understanding of PDAC-associated diabetes pathophysiology might lead to the identification of new potential biomarkers able to identify PDAC in patients with T2DM. We demonstrated that tissue

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specimens from patients with PDAC-associated diabetes mellitus are enriched of the N-terminal fragment of the calcium binding protein S100A8 [28], and this peptide was shown to alter beta cell function and reduce insulin release [29]. More recently, we found that S100A8 N-terminal fragments are released from the entire S100A8 molecule through the action of PDAC-derived proteases, including matrix metalloproteinases [30]. Once released, the S100A8 N-terminal fragments incur in a fast degradation kinetic in the PDAC milieu; this probably does not interfere with their action on the adjacent endocrine tissue, but might limit the bulk of their transfer into the bloodstream, where their concentration remains below the detection limit also of sensitive analytical methods, such as mass spectrometry [24]. The master molecules involved in this complex biological scenario, i.e. S100A8 and matrix metalloproteinases, might alternatively be taken into consideration as possible surrogate markers of PDAC-associated diabetes mellitus.

The S100A8 and its binding partner S100A9, are expressed by PDAC and stromal cells and their mRNA levels are reportedly higher in PDAC tissues of patients with associated diabetes mellitus [21]. Matrix metalloproteinases, a family of zinc-dependent endopeptidases consisting of at least 28 known members, are synthesized by both tumor and peritumoral stromal cells and their primary function is to degrade ECM proteins [31]. It was believed that MMPs played an exclusively facilitator role in tumor cell invasion and metastasis, but recent data suggest that some of them, such as MMP8 and MMP9, may have both pro- and anti-tumor effects [32,33]. In the PDAC setting, MMP9 is overexpressed and its presence correlated with an increased tumor invasiveness [34–36]. Moreover the expression of MMP9 by pancreatic tumor cells appears to be an early phenomenon, since it occurs not only in advanced, but also in borderline tumors and non-invasive IPMN [37]. MMP8 appears involved in the course and progression of colorectal cancer by influencing the anti-tumor immune response [38]. Moreover, in breast cancer cells MMP8 induces the expression of the tumor promoting cytokines IL8 and IL6 [39], the former found enhanced in the portal sera of PDAC patients [40]. Currently, no data is available on the role of MMP8 in PDAC.

The aim of the present study was to ascertain whether the mRNA tissue and blood expression levels of S100A8, S100A9, MMP8 and MMP9 and the serum levels of apolipoprotein A1, alone or in combination, could be candidate markers of PDAC-associated diabetes mellitus.

2. Materials and methods

2.1. Patients

Sixteen pancreatic tissue samples were obtained from a series of patients with localized PDAC, chronic pancreatitis and other tumors involving the pancreas (See details in Supplemental Table 1). Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until they were homogenized (Ultra-Turrax X T25, IKA®-Werke GmbH & Co. KG, Germany) for 5 min in a guanidine-HCl 4.5 M solution. Buffy coats from 8 healthy blood donors were used for "in vitro" experiments. Two hundred thirty eight consecutive patients were enrolled. The study consisted of a series of patients with PDAC, chronic pancreatitis (ChrPa), pancreatic–biliary tract tumors (other tumors), type II diabetes mellitus (T2DM) and healthy controls (see details in Table 1). The patients gave their fully informed written consent to the study, which was approved by the Local Institutional Ethic Committee ("Comitato Etico per la Sperimentazione, Azienda Ospedaliera di Padova", ce.sperimentazione@sanita.padova.it).

From all patients a fasting blood sample was collected to obtain sera (1200 ×*g* for 10 min) and peripheral blood mononuclear cells (PBMCs) (gradient centrifugation, Histopaque 1077, Sigma-Aldrich, Milano, Italy). In sera CA 19-9 (ADVIA Centaur CP Immunoassay System; Siemens, Deerfield, Ill) and Apo-A1 (Dimension Vista; Siemens) were assayed. Biochemical data were collected from clinical records.

2.2. mRNA expression analyses

Total RNA from PBMCs and tissue samples was isolated (High Pure RNA Isolation Kit, Roche, Germany) according to the manufacturer's instructions. Three micrograms of total RNA were reverse transcribed into cDNA (Random primers and Superscript TM II RNasiH-Reverse Trascriptase, Invitrogen, Italy). Relative quantification of S100A8, S100A9, MMP8 and MMP9 mRNA was undertaken by RT-PCR with ABI Prism 7900 HT (Applied Biosystems, CA, USA). Primers and fluorogenic probes for S100A8, S100A9, MMP8 and MMP9 and PCR details are reported in Supplementary Table 2.

2.3. "In vitro" experiments with PBMCs

After being isolated from the buffy coat of 8 healthy donors (gradient centrifugation), PBMCs were seeded (3×10^6 cells per well) in six well culture plates (RPMI 1640, 0.1% Gentamycin, 10% FCS, 1% L-Glutamine) and kept in continuous culture at 37 °C in a humidified atmosphere (5% CO_2) for 24 h. Media were then replaced with fresh cell culture media in the absence (control) or in the presence of 50 mU of insulin (Insuman Rapid, Sanofi-Aventis, Germany), 10 nM S100A8, 10 nM S100A9 and 10 nM S100A8/S100A9 complex (DBA Italia srl, Italy) for 1, 3, 6 or 24 h. MMP8 and MMP9 expression levels were analyzed in the same conditions described above.

2.4. Statistical analysis

The association of categorical variables was assessed by means of χ^2 test. Distributions of continuous variable were initially evaluated by

Table 1
Clinical characteristics of patients and control subjects enrolled for the analyses of S100A8, S100A9, MMP8 and MMP9 mRNA expression in PBMCs.

	HC	T2DM	ChrPa	PDAC	Other tumors#	Statistical analysis
Cases (Nr)	30	43	41	91	33	
Gender (F/M)	22/8	35/8	12/29	37/54	18/15	Chi-square = 33.22, $p < 0.001$
Age (years) Mean ± SD	67 ± 12	67 ± 8	57 ± 12*	68 ± 9^	62 ± 13	$F = 8.95$, $p < 0.005$
T2DM/RGT	0/0	43/0	20/9	35/25	10/11	Chi-square = 121.39, $p < 0.001$
Stage				IA = 3, IB = 1, IIA = 10, IIB = 21, III = 28, IV = 29		
Follow-up (months)				N = 89		
Median (IQR range)				10.47 (5.03–17.4)		
PDAC Surgery				PD = 32; DP = 11; PR = 30; no surgery = 18		

HC = healthy controls; T2DM = type 2 diabetes mellitus; ChrPa = chronic pancreatitis; PDAC = pancreatic ductal adenocarcinoma; #pancreatic–biliary tract tumors [serous cyst adenocarcinoma = 5; pancreatic neuroendocrine tumors (NETs) = 1; bile duct tumors = 6; duodenal tumors = 4; papillary tumors = 13; intraductal papillary mucinous neoplasm = 1; mucinous cystic neoplasm = 1; mesenteric hematoma = 1; mesenchymal tumors = 1]; RGT = reduced glucose tolerance; PD = Pancreatoduodenectomy; DP = Distal pancreatectomy; PR = palliative resection.
Bonferroni's test for pairwise comparisons: ^$p < 0.05$ with respect to other tumors; *$p < 0.05$ with respect to PDAC, ChrPa, T2DM and HC.