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Research Article

Co-Variation of Serum Osteoprotegerin and Pigment-Epithelial Derived Factor as Biomarker of Pancreatic Cancer

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Abstract

Pancreatic cancer is one the most lethal cancers. Currently, there are reliable predictive markers to assess cancer development. Widely used CA19-9 molecular marker has been less effective in the diagnosis of early stages of cancer.

Objective: To study if the soluble Osteoprotegerin (OPG) and pigment-epithelial derived factor (PEDF) levels in serum will be an indicator of cancer progression.

Methods: Soluble OPG and PEDF were measured from human pancreatic cancer patients by ELISA.

Results: We show that while OPG has been less predictive features, PEDF is more sensitive than CA19-9 in cancer detection. More importantly, PEDF and CA19-9 as combined markers showed higher sensitivity in stratifying early stages of pancreatic cancer.

Conclusion: Results from the pilot studies suggest that PEDF is useful biomarker for pancreatic cancer.

Keywords: Pancreatic cancer; Pigment-epithelial derived factor; Osteoprotegerin

Introduction

Pancreatic cancer has extremely poor prognosis and is the fourth leading cause of cancer-related death in the US [1]. Pancreatic ductal adenocarcinoma (PDAC) comprises more than 85% of all pancreatic cancer and the five-year survival rate for pancreatic cancer patients is estimated at a mere 6-7.7%, and complete cures are rarely if ever achieved [2].

A major challenge in the clinics is the lack of effective methods for early detection of the disease. Currently, CA19-9 (carbohydrate antigen 19-9, also called cancer antigen 19-9 or Sialyl-LewisA antigen) is the recommended marker for pancreatic cancer diagnosis in symptomatic patients and for monitoring therapy. However, it has been shown to be less reliable in predicting the progression of pancreatic cancer. Other modalities are imaging with computed tomography, magnetic resonance imaging, endoscopic ultrasound and positron emission tomography. However, these imaging tools are expensive and not suitable for early diagnosis in a wider population. There is an urgent need to identify biomarkers that can augment detection of pancreatic cancer and guide therapeutic options.

Malignant pancreatic cancer progression depends on tumor cells' ability to disseminate *via* blood and lymph vessels and the perineural space; this process is facilitated via disrupted structural elements of basement membranes and extracellular matrix (ECM) by heparinase, enzymes produced by the cancer cells [3]. The main components of basement membranes and ECM are glycoproteins decorated with glycosaminoglycan (GAG) that are largely consist of the heparan sulfate (HS) type (HSPG) [4]. HSPGs containing HS are negative-charged and bind to several factors that carry positively-charged proteins, termed as "Heparin binding proteins (HSBD)" [5,6]. These proteins binding to HSPG can regulate many diverse functions from blood coagulations, infection, immunity and cancer [7] Among HSBD, of particular interest related to pancreatic cancer are the secreted proteins that contains heparin binding motif, XBBBXXBX and XBBXBX, where B is Arg/Lys are Osteoprotegerin (OPG) [8,9] and pigment epithelial-derived factor (PEDF) [10,11]. These blood proteins can competitively bind to extracellular matrix collagen and HSPG through heparin-binding motifs and regulate fibrosis, inflammation, and metastasis. For example, IL-6 which plays a critical role in PDAC progression and metastasis has been shown to bind GAG through haparin-binding-domain [12]. These observations suggest that dysregulated soluble Heparin- Binding Domain containing Proteins (sHBDP) will influence the development of pancreatic cancer.

Osteoprotegerin (OPG) is a pleotropic factor that regulates dendritic cell maturation, osteoblast and vascular functions. It is a major decoy receptor to RANKL and TRAIL, a proapoptotic cytokines through N-terminal domain. The c-terminal domain contains putative GAG binding signature. The precise role of the C-terminus of sOPG is not known. In pancreatic cancer, OPG is overexpressed in pancreatic cancer patients' serum [8,13]. Since OPG is also secreted by immune cells such as dendric cells, we wanted

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to know whether the source of secreted/soluble OPG (sOPG) is pancreatic specific. For this purpose, we investigated the expression of OPG in panel of pancreatic cancer cells. We have shown that K-ras activation is positively correlated with the secretion of sOPG. By cancer cells, and not by normal cells. More importantly, it was shown that overexpression of sOPG promote pancreatic cancer growth by blocking TRAIL-induced apoptosis [9]. These observations suggested that one of the major sources for the observed excess secretion of OPG is by pancreatic cancer cells [8].

Pigment-epithelial derived factor (PEDF) is a pleotropic heparin-binding soluble protein, and it has been implicated in development of cancer by acting as linker in ECM; PEDF could link collagens with GAGs by simultaneously binding to both collagens and GAGs and could be important mediator of angiogenesis [14]. The expression of PEDF in prostate and lung cancers show that lower expression in cancer cells compared to normal cells indicated poor prognosis. In pancreatic cancer, as in other cancers, PEDF plays an important role in cancer development; unlike sOPG which is overexpressed, the loss of PEDF is correlated with invasive pancreatic cancer presumably modifying pancreatic cancer stroma to become highly vascularized (i.e. fibrotic and angiogenic). Due its role in cancer development, we investigated soluble factor sOPG and sPEDF in serum of pancreatic cancer patients. Here, we show that monitoring the expression of PEDF in solid tumor can be a useful biomarker. Based on these observations we hypothesized that the co-variation of heparin-binding proteins, in particular, the sOPG and the sPEDF expression level in serum will be a novel biomarker to stratify PDAC progression.

Materials and Methods

Quantitative real-time PCR (RT-PCR)

Total RNA was extracted using Trizol (ThermoFisher, Canoga Park, CA, USA), and reverse transcription reaction was carried out using High-Capacity Reverse Transcription Kit (Thermo Fisher, Canoga Park, CA, USA). Real-time quantitative PCR (RT-qPCR) was used for quantifying mRNA levels using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and BioRad cfx96 platform according to the manufacturer's protocol. Gene expression levels were normalized to that of GAPDH. Primers were purchased from Integrated DNA technologies (IDT), Coralville, IA, USA. The sequences of primers used for RT-PCR were as follow: h-PEDF-F; T A T G A C C T G T A C C G G G T G C G A T, h-PEDF-R; C C A C A C T G A G A G G A G A C A G G A G C [6], h-OPG-F; A A G A C C G T G T G C G C C C C T T G, h-OPG-R; A C G C G G T T G T G G G T G C G A T T [15], h-GAPDH-F; C C A G G T G G T C T C C T C T G A C T T C A A C A, h-GAPDH-R; A G G G T C T C T C T C T T C C T C T T G T G C T C.

Collection of human blood samples

Blood samples were collected from patients during their routine blood collections. The normal control group had no history of acute or chronic pancreatitis, diabetes, pancreatic surgery, and no family history of cancer. Patients with pancreatic cystic lesions, benign tumors, or marked pancreatic atrophy or fat degeneration on MRI images were excluded. This prospective study was approved by the local institutional review board. Written informed consents were obtained from all participants. Blood samples were provided by the Pancreatic Biomarker Bank, or "Panc-Bank," at the Cedars-Sinai Medical Center (CSMC) with IRB protocol number: 41571.

PEDF and OPG ELISA measurement

PEDF and OPG levels were measured in the patient's serum using the PEDF ELISA kit (Cat #: MBS760087, MyBiosource, San Diego, CA) and the OPG ELISA kit (Cat #: MBS2508007, MyBiosource, San Diego, CA), respectively following the protocol instructions.

Results

PEDF is increased in PDAC tissues compared to normal pancreatic tissues

First, we measured the mRNA level pf PEDF and OPG in mouse and human pancreatic normal and cancer tissues. We found a significant increase in PEDF levels in PDAC samples compared to normal pancreatic tissues in mice. The increase in PEDF mRNA levels was 10-fold in mice (Figure 1A) and 2-fold in human tissues (Figure 1C). Differently from PEDF, OPG levels showed a different ratio in mice and human tissues. In mice we found a significant increase in OPG in PDAC tissues compared to normal tissues (Figure 1B); while in humans, OPG was lower in PDAC tissues compared to normal tissues (Figure 1D).

PEDF is significantly increased in blood samples from PDAC patients compared to normal patients.

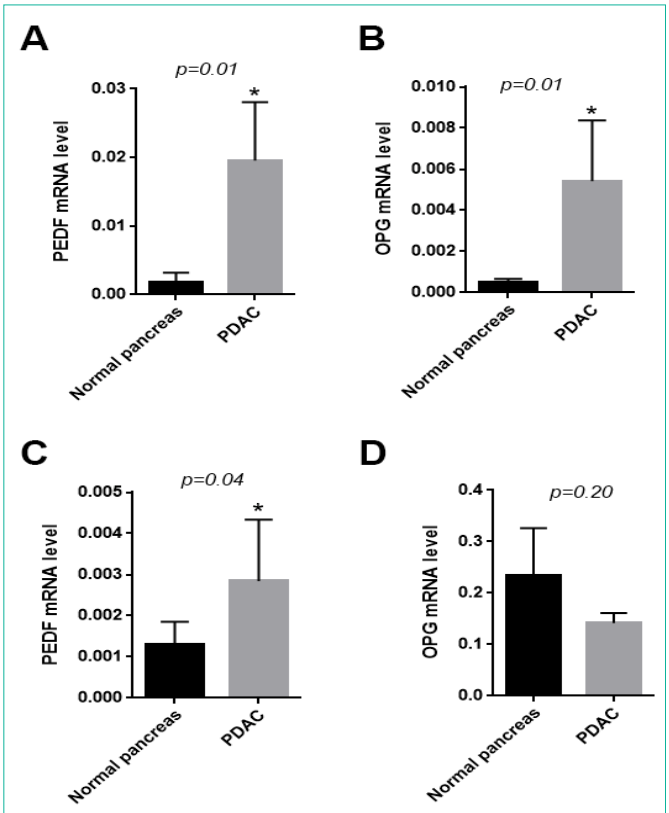

Figure 1: Expression levels of messenger RNA (mRNA) of PEDF and OPG in tissues. (A) Expression level from mouse tissue. Both PEDF and OPG are increased in pancreatic cancer tissues compared to normal pancreatic tissues. (B) Expression levels of PEDF and OPG in human tissue. Unlike in mouse tissue, only mRNA levels of PEDF, but not OPG, is increased in pancreatic cancer tissues compared to normal tissues. mRNA level was measured by RT-PCR. The significance of the difference is indicated by p-values.

Table 1: Human subjects used in the study.

Subject ID	New Subject ID	Diagnosis	PDAC stages (e.g: I,II,III, etc)	Tumor size	Age	Average (Age)	Gender	Ethnicity
PDACCS00#067	1	Healthy			57	61	Male	White; Non-Hispanic
PDACCS00#096	2	Healthy			47		Female	White; Non-Hispanic
PDACCS00#097	3	Healthy			54		Male	White; Non-Hispanic
PDACCS00#100	4	Healthy			57		Female	White; Non-Hispanic
PDACCS00#101	5	Healthy			57		Male	White; Non-Hispanic
PDACCS00#116	6	Healthy			73		Female	
PDACCS00#135	7	Healthy			43		Male	
PDACCS00#136	8	Healthy			77		Female	
PDACCS00#144	9	Healthy			69		Female	
PDACCS00#188	10	Healthy			76		Female	White; Non-Hispanic
PDACCS00#007	11	Pancreatitis			25	56.5	Male	White; Non-Hispanic
PDACCS00#013	12	Pancreatitis			50		Male	White; Hispanic
PDACCS00#026	13	Pancreatitis			42		Female	White; Non-Hispanic
PDACCS00#028	14	Pancreatitis			81		Male	White; Non-Hispanic
PDACCS00#031	15	Pancreatitis			59		Male	White; Non-Hispanic
PDACCS00#039	16	Pancreatitis			67		Male	White; Non-Hispanic
PDACCS00#053	17	Pancreatitis			54		Female	White; Non-Hispanic
PDACCS00#064	18	Pancreatitis			79		Female	White; Hispanic
PDACCS00#094	19	Pancreatitis			41		Male	White; Hispanic
PDACCS00#140	20	Pancreatitis			67		Female	
PDACCS00#030	21	PDAC			79	70.9	Male	White; Non-Hispanic
PDACCS00#057	22	PDAC	Stage IV	3.9x4.5 cm	70		Male	White; Non-Hispanic
PDACCS00#068	23	PDAC	T3N1M0=Stage IIB	8.4x5.8 cm heterogeneous mass	65		Female	White; Non-Hispanic
PDACCS00#081	24	PDAC	Metastatic=Stage IV	Not Available	46		Male	White; Hispanic
PDACCS00#091	25	PDAC	IA		78		Female	White; Non-Hispanic
PDACCS00#099	26	PDAC	Metastatic=Stage IV	3x5 cm in diameter with indistinct margins	70		Female	White; Non-Hispanic
PDACCS00#115	27	PDAC	Locally advanced (Stage IB)	Large	60		Female	
PDACCS00#126	28	PDAC	IA		79		Male	
PDACCS00#147	29	PDAC	Metastatic to liver=Stage IV		79		Male	
PDACCS00#148	30	PDAC	Localized to head of the pancreas (Stage IB)	2.5x2.3 cm	64		Male	White; Non-Hispanic
PDACCS00#151	31	PDAC	Metastatic to liver=Stage IV	3.6x3.0 cm	69		Female	
PDACCS00#152	32	PDAC	Metastatic to lung=Stage IV	3.5x2.5 cm	65		Female	
PDACCS00#153	33	PDAC	Invasive pancreatic tumor=Stage III	1.8x1.2 cm	74		Male	
PDACCS00#156	34	PDAC	IV		74		Male	White; Non-Hispanic
PDACCS00#158	35	PDAC	IV	40mm was identified in the pancreatic body	80		Female	White; Non-Hispanic
PDACCS00#159	36	PDAC	IV	6.6x3.8 cm	86		Male	White; Non-Hispanic
PDACCS00#161	37	PDAC	IIB	17mm x 16mm	76		Male	White; Non-Hispanic
PDACCS00#169	38	PDAC	IV	28mm by 25mm	49		Female	Asian; Non-Hispanic
PDACCS00#149	39	PDAC	Metastatic to liver=Stage IV		87		Female	White; Non-Hispanic
PDACCS00#189	40	PDAC	IA	3.5x3.2 cm	68		Female	Asian; Non-Hispanic

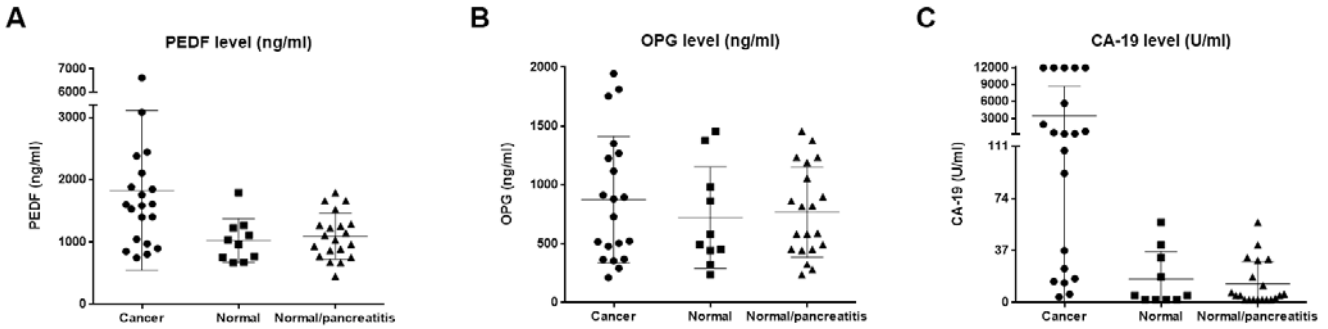

Figure 2: Comparison of protein expression levels of PEDF, OPG and CA-19 in human serum samples. (A) Expression of PEDF which is significantly increased in cancer patients compared to either normal or patients diagnosed with pancreatitis. (B) Serum levels of OPG. Protein levels did not show significant discrimination among the three groups. (C) Serum levels of CA-19. CA19, as a control marker, showed significant increase similar PEDF in cancer patients. All measurement was performed using ELISA.

Table 2: Analysis of the expression levels of PEDF and CA-19.

	Thresholds	PEDE		CA-19		PEDF or CA-19	
		>1300ng/ml	%	>37U/ml	%	>1300 or >37	%
Sensivity	Cancer	13/20	65%	14/20	70%	16/20	80%
Specificity	Normal	1/10	90%	2/10	80%	3/10	70%
	Normal+Pancreatitis	3/20	85%	2/20	80%	5/20	75%

Next, we analyzed the protein level of PEDF and OPG in blood samples collected from patients with normal pancreas, pancreatitis, and PDAC. Table 1 shows the clinical information about the patients used in the study. 20 Patients had PDAC including eleven patients with stage 4, one with stage 3, two with stage 2, five with stage 1, and one patient with non-identified stage (Table 1).

Measurement of the protein levels of PEDF and OPG showed a significant increase in the level of PEDF in PDAC patients compared to normal or normal plus pancreatitis patients (Figure 2A). In cancer patients we can see two populations, one with high level of PEDF compared to normal patients and another one with a small number of patients showing levels of PEDF like the healthy group. The same pattern was observed when measuring the levels of CA-19 in these patients (Figure 2C). Differently from PEDF, OPG did not show any significant difference between PDAC patients and healthy patients (Figure 2B). Statistical analysis indicated that a threshold of 1330ng/ml of PEDF will allow the PED measurement to induce a sensitivity of 65% (13 out of 20 patients) and a specificity of 90% (9 out of 10 patients) (Table 2). CA-19 showed a sensitivity of 70% and specificity of 80% (Table 2). When including the pancreatitis patients in the control (non-cancer) group, the specificity of PEDF was at 85% and the specificity of CA-19 at 80% (Table 2). Importantly, by considering higher cutoff value for either PEDF or CA-19 as a marker for PDAC, we found that the sensitivity increased to 80% (Figure 2B). To examine whether PEDF/CA-19 can predict early stages of cancer, we compared the molecular markers from stage-I and II patients. Interestingly, we noted improved sensitivity among the PDAC patients with stage I and II; when using either CA-19 or PEDF as a diagnostic marker, sensitivity increased from 60% (3 out of 5 patients) when using CA-19 to 80% (4 out of 5 patients). These observations suggest that PEDF, along with conventional marker CA-19, may be a useful marker to monitor progression of cancer as well as response to therapies in certain situations.

Conclusion

The data indicate that PEDF is a promising marker for PDAC. We predict that PEDF and CA-19-9 can be used to enhance detection of cancer progression. Limitation of the study is the small number of patients included in the study. A larger cohort is needed to determine statistical significance of the study. Nonetheless, we believe our pilot study results can be guidance for further studies aimed at early diagnosis and to monitor therapeutic responses.

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