Original Article

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Osteoprotegerin is up-regulated in pancreatic cancers and correlates with cancer-associated new-onset diabetes

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Summary **New-onset diabetes might help to yield biomarkers for the early diagnosis of pancreatic cancer (PaC). In this study, we computationally predicted and experimentally validated osteoprotegerin (OPG) being associated with pancreatic cancer related new-onset diabetes. We first performed a meta-analysis on microarray datasets to search for genes specifically highly expressed in PaC, and then filtered for cytokines involved in islet dysfunction. The expression of OPG in PaC and normal pancreas were validated by immunohistochemistry. Serum OPG levels in healthy controls, non-cancerous diabetes and PaC patients with or without diabetes were detected by enzyme-linked immunosorbent assay (ELISA). *In silico* assay found that OPG up-regulated in PaC tissues in comparison to normal pancreas. Immunohistochemical data further confirmed that OPG was overexpressed in PaC samples. Furthermore, increased expression of OPG in PaC tissues correlated to the occurrence of new-onset diabetes, and adversely affected the patients' overall survival in both univariate and multivariate analysis. In addition, the serum levels of OPG were significantly higher in pancreatic cancer patients with new-onset diabetes than other groups including pancreatic patients without diabetes, new-onset type 2 diabetes and healthy controls. In conclusion, there is a close association between OPG and pancreatic cancer related new-onset diabetes, and OPG might serve as a potential biomarker for the early diagnosis of pancreatic cancer from populations with new-onset diabetes.**

Keywords: Pancreatic cancer, diabetes, OPG, immunohistochemistry, serum

1. Introduction

Pancreatic cancer (PaC) ranks as the fourth or fifth leading cause of cancer death in the world (*1*). At diagnosis, more than 85% of patients with PaC are at an advanced stage and unresectable, with an extremely poor survival. Therefore, there is an urgent need to identify novel early biomarkers for PaC.

New-onset diabetes pancreatic cancer (PaC-DM) frequently occurs within about 2 years prior to cancer diagnosis (*2*). Therefore, new-onset diabetes may be a useful clue to screen early PaC patients (*3-5*). However, although several putative biomarkers such as S100-A8 have been proposed, currently, it is still difficult to clinically distinguish cancerous and non-cancerous new-onset diabetes (*6*).

Accumulating evidence suggests that some circulating proteins or cytokines secreted by the tumor cells may exert a para-neoplastic effect on islets, and cause insulin resistance and β-cell dysfunction, which results in new-onset diabetes in patients with PaC (*7*).

In this study, in searching for the publicly available gene expression microarray datasets, we identified osteoprotegerin (OPG), a potential diabetes-related

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cytokine, selectively upregulated in PC tissues. We further investigated the clinical and pathological significance of OPG in a pancreatic cancer cohort, and its utility as a serum biomarker to distinguish cancerous and non-cancerous new-onset diabetes was also evaluated.

2. Materials and Methods

2.1. *Data mining*

The Oncomine database (*https://www.oncomine.org*) was used to interrogate 7 publicly available datasets for mining cytokine gene expression in human pancreatic carcinomas as described previously (*8*). We first identified a p-value below 0.05 and a fold-change of 1.5 for differently expressed genes in pancreatic cancer tissues compared to control. The gene ranks across 7 datasets were compared, and then a concept filter "cytokines" was used to identify known diabetes-related factors up-regulated in pancreatic cancer tissues at the mRNA level.

2.2. *Immunohistochemical expression of OPG in pancreatic cancer tissues*

For immunohistochemical analysis, 83 stage I-II pancreatic cancer tumors and paired normal adjacent pancreas from patients who underwent primary surgical resection between September 2004 and December 2008 were analyzed. Forty patients were determined to be in stage I, and 43 in stage II. No patients received previous radiotherapy and/or chemotherapy before surgical resections. Clinicopathological information for each subject, including gender, age, TNM classification, history of diabetes, perineural invasion status, and overall survival, was collected retrospectively and is summarized in Table 1.

Each pathological section was subjected to immunohistochemical staining with an avidin-biotin-peroxidase complex system. Briefly, after deparaffinization and rehydration, epitope retrieval was carried out in 10 mmol/L citrate buffer (pH = 6.0) for 15 min. The activity of endogenous peroxidases was blocked with 3% hydrogen peroxide for 10 min at room temperature. The sections were then incubated with anti-OPG antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) at a dilution of 1:100 overnight at 4°C, followed by incubation with an HRP-conjugated secondary antibody for 30 minutes at room temperature. 3,3'-Diaminobenzidine (DAB) was used as a chromogenic substrate. The positivity index was expressed as the percentage of moderate or strong staining cancer cells in each lesion. The cases with an index more than the median value were defined as high-OPG expression level, and the others as low-OPG expression level.

2.3. *Evaluation of serum OPG levels*

After excluding poor quality samples, we measured fasting serum OPG levels in 24 healthy subjects with normal fasting glucose levels, 23 non-cancer patients with new-onset type 2 diabetes, 20 pancreatic cancer cases with normal fasting glucose levels, and 25 pancreatic cancer patients with new-onset diabetes. All these four groups were age and sex matched. Among the patients with pancreatic cancer, 27 cases were at early-stage (I/II) and 18 were at late-stage (III/IV). The TNM stage of two pancreatic cancer groups were not significantly different. The levels of serum OPG were quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (R&D Systems Inc. Minneapolis, Minnesota, USA).

2.4. *Statistics*

The correlation between OPG expression levels and clinicopathological characteristics was analyzed using the Pearson χ^2 test. The Kaplan-Meier method was used for survival analysis in terms of OPG expression, and evaluated by the log-rank test. Multivariate analysis was performed using a Cox proportional hazard model. Statistical comparisons of serum OPG levels between groups were performed using *t* tests. Differences were considered statistically significant at *p* value less than 0.05.

3. Results

3.1. *In silico assay identified OPG up-regulated in PaC tissues in comparison with normal pancreas.*

We performed a meta-analysis on 7 gene expression microarray datasets from the Oncomine database to identify up-regulated pancreatic cancer-related genes, then filtered for cytokines with known involvement in diabetes. Among the top 20 up-regulated cytokines in pancreatic cancers, OPG has been validated to inhibit islet function using multiple lines of evidence. Therefore, OPG may be a candidate cancer-derived mediator for pancreatic cancer-related diabetes. Our *in silico* results indicated that a significant increase in OPG mRNA level was observed in pancreatic cancer samples compared to adjacent non-tumor or normal tissues in 5 (including the largest two cohorts) out of 7 databases.

3.2. *Immunohistochemical results of OPG expression*

To further evaluate the potential clinical implication of OPG in pancreatic cancer progression, we next analyzed its expression by immunohistochemistry in 83 pairs of pancreatic cancer specimens at early stages. As

seen in Figure 1, normal adjacent and normal pancreas showed weak OPG immunostaining, while prominent staining was observed in the tumor samples compared to that in the normal tissues. The positive staining is diffuse, cytoplasmic/nuclear in cancer cells, but occasionally in stromal cells.

3.3. *Association between OPG expression levels in pancreatic cancer tissues with clinicopathological characteristics and survival*

In tumor samples, high OPG expression level was more frequently observed in patients with new-onset diabetes compared with patients without new-onset diabetes (40.9% *vs*. 66.7%, $p = 0.0311$) (Table 1). Except for diabetes history, no significant association was been identified between OPG expression levels compared to other clinicopathological characteristics (Table 1). Furthermore, increased OPG adversely affected survival in univariate analysis, and remained a strong independent prognostic factor for patients with pancreatic cancers in multivariate analysis (Table 2).

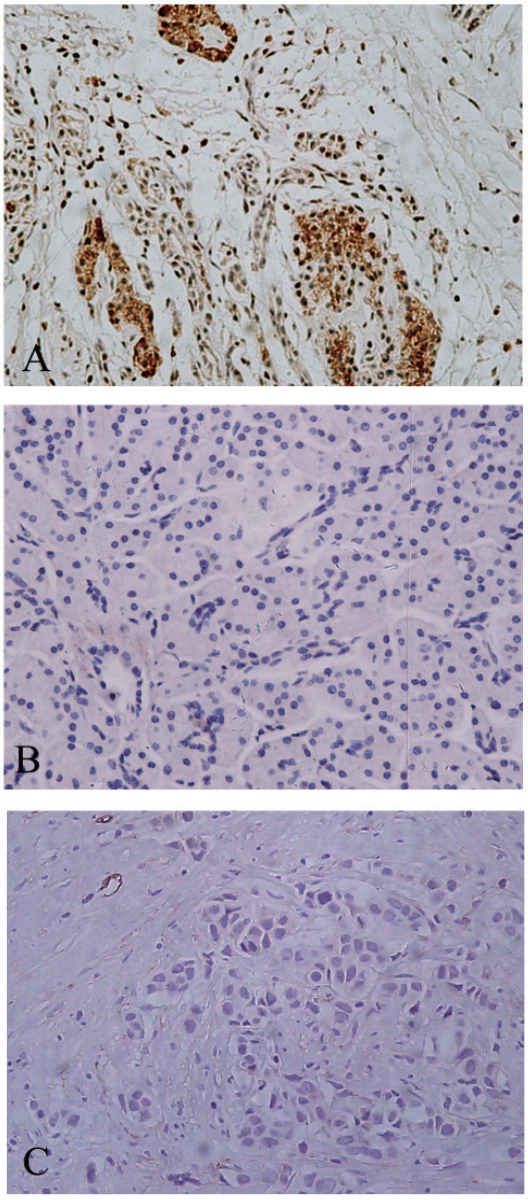

Figure 1. Representative images of osteoprotegerin immunostaining. Positive staining in pancreatic cancer tissues **(A)**; negative staining in normal adjacent pancreas tissues **(B)** and pancreatic cancer tissues **(C)**.

3.4. *Serum OPG levels in PaC-DM patients compared with other groups*

A Box-and-whisker plot of OPG serum levels in two non-cancer subject groups (with and without new-onset diabetes) and two cancer subject groups (with and without new-onset diabetes) is shown in Figure 2. *PaC-DM* patients had the highest level of serum OPG. In particular, the receiver operating characteristic curve (ROC) revealed that serum OPG had an Area under the ROC curve (AUC) of 0.737 ($P = 0.0012$) with a sensitivity of 68.0% and a specificity of 73.9% to distinguish *PaC-DM* cases from new-onset DM patients.

4. Discussion

Identification of the mediators to distinguish new-onset PaC-DM from the more common type 2 diabetes mellitus could serve as an important tool for screening for PaC at an asymptomatic time. Recent studies have

Table 1. ACorrelation of the expression of OPG expression with clinicopathological characteristics in 83 pancreatic cancers

Variable	No.	OPG Expression		*p* value
		Low ($n = 41$)	High ($n = 42$)	
Age (years)				
<65	49	20	29	0.2590
≥65	34	19	15	
Gender				
Male	52	25	27	0.9760
Female	31	14	17	
Grade				
1-2	60	31	29	0.2569
3	23	8	15	
Tumor size				
T1	5	1	4	
T2	65	33	32	0.3305
T3	13	5	8	
Lymph node metastasis				
Negative	48	24	24	0.6736
Positive	35	15	20	
TNM stage				
I	40	22	18	0.2338
II	43	17	26	
Neural invasion				
Negative	35	18	17	0.6387
Positive	48	21	27	
Diabetes history				
No	44	26	18	0.0311
New-onset	33	11	22	
Type 2	6	2	4	

Table 2. Univariate and multivariate Cox regression analysis for overall survival based on OPG expression and clinicopathological characteristics

	Univariate survival analyses			Multivariate survival analysis		
Variable	*P* value	HR	95% CI of HR	*P* value	HR	95% CI of HR
Age	0.9750	0.9911	0.5682 to 1.7288			
Gender	0.0639	0.5665	0.3115 to 1.0302			
Grade	0.0098	2.1415	1.2047 to 3.8067	0.0283	1.9344	1.0759 to 3.4780
Tumor size	0.7694	0.9111	0.4903 to 1.6930			
Lymph node metastasis	0.0133	1.9977	1.1583 to 3.4453	0.7801	1.1504	0.4323 to 3.0612
TNM stage	0.0122	2.0501	1.1725 to 3.5847	0.4240	1.5007	0.5576 to 4.0394
Neural invasion	0.0197	1.9991	1.1199 to 3.5685	0.2198	1.4894	0.7908 to 2.8051
Diabetes history	0.7464	0.9334	0.6160 to 1.4144			
OPG expression	0.0041	2.3272	1.3116 to 4.1293	0.0189	2.0314	1.1277 to 3.6591

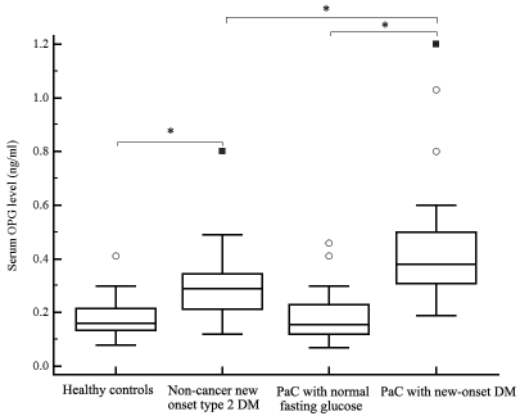

Figure 2. OPG is a potential candidate biomarker for PaC-DM. New-onset diabetes pancreatic cancer patients had the highest level of serum OPG. DM means diabetes mellitus.

found several diabetogenic secretory products of pancreatic cancer, such as adrenomedullin, macrophage migration inhibitory factor (*9-11*).

Osteoprotegerin (OPG) is a soluble decoy receptor for tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), and belongs to the tumor necrosis factor receptor superfamily (TNFRSF) (*12,13*). In this study, for the first time, we discovered several lines of evidence supporting that OPG may serve as a novel diabetogenic cytokine of pancreatic cancer. First, OPG expression was significantly increased in PaC tissues compared with normal pancreas. Second, in PaC tumors, OPG overexpression is associated with new-onset PaC-DM. Third, Patients with new-onset PaC-DM had a higher serum OPG level than those without diabetes mellitus. Toffoli *et al*. (*14*) found that OPG induces morphological alterations and reduction of islet function in mouse pancreatic islets, and islet RAS overactivity represents one possible mechanism responsible for this effect. Together with our findings, we suggest that at least in part of PaC tissues, islet cells would face high OPG stress, and thus be more susceptible to damage than normal human islets. This study also provides the rationale to consider OPG as a candidate target for new approaches to retard the development of PaC-DM.

Moreover, this study further found that over-expression of OPG predicts poor prognosis for PaC, and acts as an independent prognostic factor. These findings strongly indicated that except as a diabetogenic factor, OPG may play a critical role in PaC development and progression, and recent evidence also support that OPG acts as key modulator on a metastasis-promoting effect and resistance to TRAIL-induced apoptosis in cancers including PaC (*15*).

Serum OPG levels increased in both patients with diabetes and patients with pancreatic cancer (*16-18*). Our results are consistent with the previous findings, and further demonstrated that PaC-DM had a higher serum OPG level than the new onset common diabetes mellitus and PaC without DM. Interestingly, serum OPG levels could distinguish patients with PaC-DM from those with new-onset type 2 DM, suggesting the potential utility of OPG as a candidate serum biomarker of new-onset PaC-DM.

In conclusion, this present study indicates OPG is a candidate diabetogenic cytokine for new-onset PaC-DM, and may also serve as a novel prognostic factor for PaC and a new serum biomarker for patients with early PaC. Further experimental and clinical studies are required to investigate the exact role of OPG in PaC as well as its utility in the screening of PaC-DM among subjects with new-onset diabetes mellitus.

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