

# Proteomic differential display identifies upregulated vinculin as a possible biomarker of pancreatic cancer

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**Abstract.** Pancreatic cancer (PC) is characterized by rapid tumor spread, and very few patients with PC survive for more than 5 years. It is imperative to discover additional diagnostic biomarkers or specific therapeutic targets in order to improve the treatment of patients with PC. In search for useful biomarkers, we analyzed ten pairs of non-cancerous and cancer tissues from patients with PC by two-dimensional gel electrophoresis (2-DE). Nineteen protein spots showed differential expression on 2-DE gels between the cancer and non-cancerous tissues. Six upregulated protein spots were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as calreticulin, glutathione synthetase, stathmin, vinculin,  $\alpha$ -enolase and glyceraldehyde-3-phosphate dehydrogenase. Western blotting demonstrated that vinculin was predominantly expressed in the pancreatic cancer tissues compared with to non-cancerous tissues. Our findings indicate that vinculin may be a clinically useful biomarker of PC.

## Introduction

Prognosis of patients with pancreatic cancer (PC) is poor because of belated diagnosis and lack of effective therapies. This disease is characterized by rapid tumor spread, and the median survival is less than 12 months with an overall 5-year survival rate of <5% (1). It is imminent therefore to find more effective biomarkers for the diagnosis of patients with pan-

creatic cancer and to clarify the biological characteristics of rapid aggressiveness of PC.

In recent years, proteomics has been widely applied to the identification of candidate biomarkers and therapeutic targets in various cancers (2-5). Two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are the major proteomics techniques, which are utilized in analyzing proteins comprehensively.

The proteomics technology is an ideal option for finding biomarkers and therapeutic targets in cancer. By applying 2-DE and LC-MS/MS combined with western blotting, we found six differentially expressed proteins between pancreatic cancerous and non-cancerous tissues, and among them vinculin was identified as a potential biomarker for PC diagnosis or prognosis.

## Materials and methods

**Pancreatic tissues and sample preparation.** Thirty pairs of non-cancerous and cancerous pancreatic tissues were obtained from 30 patients (Table I) who underwent resection of pancreas with diagnosis of pancreatic cancer at the Department of Surgery II, Yamaguchi University Hospital.

None of the patients received any preoperative therapy. Written informed consent was obtained from all patients before surgery. Tissues were obtained immediately after surgery and stored at -80°C until use. The study protocol was approved by the Institutional Review Board for Human Use of the Yamaguchi University School of Medicine. The tissues were homogenized in lysis buffer (1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 10 mM NaF, 10 mM EDTA, 50 mM Tris, 165 mM NaCl, 10  $\mu$ g/ml leupeptin, and 10  $\mu$ g/ml aprotinin) on ice (5). Suspensions were incubated for 2 h at 4°C, and the supernatants were stored at -80°C until they were used as samples. Ten pairs of samples were used for 2-DE, and twenty pairs for western blotting.

**Two-dimensional gel electrophoresis (2-DE).** As the first dimension, isoelectric focusing (IEF) was conducted in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11-cm and pH 3-10 linear gradient IPG strips (Bio-Rad, Hercules, CA, USA) at 50  $\mu$ A/strip. Protein (80  $\mu$ g) was used

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**Abbreviations:** PC, pancreatic cancer; 2-DE, two-dimensional gel electrophoresis; LC-MS/MS, liquid chromatography-tandem mass spectrometry

**Key words:** two-dimensional gel electrophoresis, liquid chromatography-tandem mass spectrometry, pancreatic cancer, vinculin

Table I. Clinicopathological parameters of patients with pancreatic cancer.

No.	Age	Gender	TNM stage	Tumor grade
1 <sup>a</sup>	79	Male	III	Moderately differentiated
2 <sup>a</sup>	67	Male	III	Moderately differentiated
3 <sup>a</sup>	54	Male	III	Moderately differentiated
4 <sup>a</sup>	75	Male	IVb	Poorly differentiated
5 <sup>a</sup>	71	Female	III	Well differentiated
6 <sup>a</sup>	58	Male	IVb	Mucinous carcinoma
7 <sup>a</sup>	70	Female	III	Moderately differentiated
8 <sup>a</sup>	64	Male	III	Moderately differentiated
9 <sup>a</sup>	61	Male	IVa	Moderately differentiated
10 <sup>a</sup>	51	Female	II	Moderately differentiated
11	67	Male	IVa	Well differentiated
12	60	Female	III	Moderately differentiated
13	48	Female	IVa	Moderately differentiated
14	73	Male	IVa	Moderately differentiated
15	54	Male	IVa	Moderately differentiated
16	57	Male	IVb	Moderately differentiated
17	54	Male	III	Moderately differentiated
18	74	Female	IVa	Poorly differentiated
19	72	Male	III	Moderately differentiated
20	72	Male	IVa	Well differentiated
21	76	Female	IVa	Moderately differentiated
22	73	Female	III	Papillary carcinoma
23	53	Male	IVb	Well differentiated
24	69	Female	III	Moderately differentiated
25	79	Male	IVb	Mucinous carcinoma
26	34	Male	IVb	Acinor carcinoma
27	71	Female	III	Moderately differentiated
28	67	Female	IVa	Moderately differentiated
29	68	Male	III	Moderately differentiated
30	60	Male	IVb	Moderately differentiated

<sup>a</sup> indicates samples were used in 2-DE analysis.

for each 2-DE. Samples were mixed with 200  $\mu$ l of rehydration buffer [8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare)] and 0.5% IPG buffer, and loaded in the IPGphor strip holder. The strips were then focused by the following program: rehydration for 10 h (no voltage); 0-500 V for 4 h; 500-1,000 V for 1 h; 1,000-8,000 V for 4 h; 8,000 V for 20 min; and the final phase of 500 V from 20,000-30,000 Vh (6). After IEF, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a precast polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad) (7). The IPG strips were first equilibrated in equilibration buffer 1 (6 M urea, 0.5 M Tris-HCl, pH 8.8, 30% glycerol, 2% SDS, 2% 2-ME) for 10 min, and further in equilibration buffer 2 (6 M urea, 0.5 M Tris-HCl, pH 8.8, 30% glycerol, 2% SDS, 2.5% iodoacetamide) for 10 min. The IPG strips were then transferred onto the gels, which were run at 200 V (8). Each sample was replicated three times to ensure protein pattern reproducibility.

*Fluorescence staining.* The SDS-PAGE gels were fixed with 40% ethanol and 10% acetic acid for 2.5 h. The gels were then treated with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (Bio-Rad), for 18 h (9). The stained gels were washed with Milli-Q water 3 times, for 5 min each. These experimental procedures were carried out on a shaker.

*Image analysis and spot picking.* The gels were scanned by using the ProXpress 2-D Proteomic Imaging System (PerkinElmer, Waltham, MA, USA) and then analyzed by using the Progenesis SameSpots software (Nonlinear Dynamics, Newcastle, UK) following the user manual. After image analysis, the gels were stained with See Pico™ (Benebiosis Co., Ltd., Seoul, Korea) overnight (10). The selected protein spots that displayed different intensities were cut from the gels and subjected to mass spectrometry (MS) analysis.

*In-gel digestion.* The gel pieces were destained by rinsing three times in 60% methanol, 0.05 M ammonium bicarbonate,

Table II. Upregulated proteins in pancreatic cancerous tissues.

Spot	Accession no. <sup>a</sup>	pI <sup>b</sup>	Mr (Da) <sup>b</sup>	Spot intensity ratio	Frequency	Protein
1	P27797	4.29	48141.8	2.10	9/10	Calreticulin
2	P48637	5.67	52385.1	1.50	7/10	Glutathione synthetase
3	P16949	5.76	17302.6	1.50	5/10	Stathmin
4	P18206	5.50	123800.0	1.50	8/10	Vinculin
5	P06733	7.01	47169.2	1.60	9/10	$\alpha$ -enolase
6	P04406	8.57	6053.4	1.70	7/10	Glyceraldehyde 3-phosphate dehydrogenase

<sup>a</sup>Accession number derived from the protein database. <sup>b</sup>Theoretical pI and molecular weight (Da) from the protein database.

and 5 mM DTT for 15 min. The sample in the gel piece was reduced twice in 50% methanol, 0.05 M ammonium bicarbonate, and 5 mM DTT for 10 min. The gel pieces were dehydrated twice in 100% acetonitrile (ACN) for 30 min. Enzyme digestion was carried out with an in-gel digestion reagent containing 10  $\mu$ g/ml sequencing-grade-modified trypsin (Promega Corporation, Madison, WI, USA) in 30% ACN, 0.05 M ammonium bicarbonate, and 5 mM DTT at 30°C for 16 h. The samples were lyophilized overnight with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas, MO, USA).

**LC-MS/MS analysis.** The lyophilized samples were dissolved in 15  $\mu$ l of 0.1% formic acid, and then analyzed by using the LC-MS/MS system. Peptide sequencing of identified protein spots was carried out by using LC-MS/MS with a Spectrum Mill MS Proteomics Workbench (Agilent Technologies, Palo Alto, CA, USA). Fifteen microliters of each sample was injected and placed into separated columns (Zorbax 300SB-C18, 75  $\mu$ m, 150 mm, Agilent Technologies). The Agilent 1100 capillary pump was operated in the following conditions: solvent A, 0.1% formic acid; solvent B, ACN in 0.1% formic acid; column flow, 0.3  $\mu$ l/min for primary flow, otherwise 300  $\mu$ l/min; gradient, 0-5 min 2% B and 60 min 60% B; stop time: 60 min. Proteins were identified in the Agilent Spectrum Mill MS Proteomics Workbench against the Swiss-Prot protein database search engine (<http://kr.expasy.org/sprot/>) and MASCOT MS/MS Ions Search engine ([http://www.matrixscience.com/search\\_form\\_select.html](http://www.matrixscience.com/search_form_select.html)). Standards for induction of candidate proteins were set as follows: filter by protein score >10.0, and filter peptide by score >8 (percent scored peak intensity).

**Western blotting.** The samples were separated by electrophoresis with SDS-PAGE gels and then transferred onto PVDF membranes at 90 mA for 78 min. The membranes were blocked overnight with TBS containing 5% milk at 4°C (11). They were incubated with the primary antibody against vinculin (anti-vinculin mouse monoclonal antibody, Sigma, St. Louis, MO, USA; 1:10,000),  $\alpha$ -enolase (anti-enolase goat polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:1,000) and actin (anti-actin goat polyclonal antibody, Santa Cruz Biotechnology, Inc.; 1:200). The

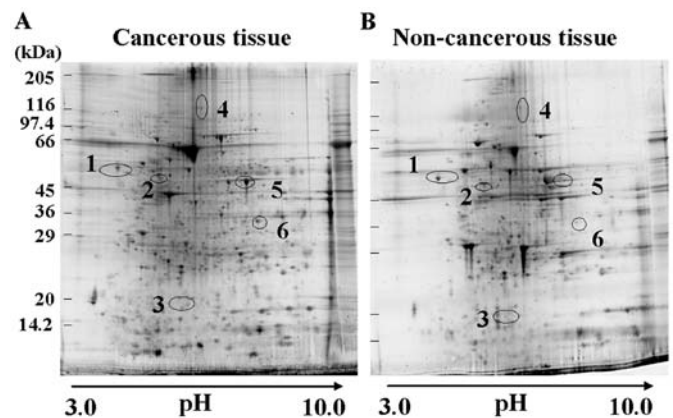


Figure 1. Two-dimensional gel electrophoresis images of pancreatic cancerous and non-cancerous tissues stained with Flamingo™ fluorescent gel stain. Proteins were separated on pH 3-10 linear, immobilized pH gradient strips followed by 5-20% SDS-PAGE. Six spots showed enhanced intensity on gels of cancerous tissues (A) compared to non-cancerous tissues (B). They were numbered as spots 1-6

membranes were incubated with the secondary antibody conjugated with horseradish peroxidase (1:10,000) for 1 h at room temperature after washing three times with TBS containing Tween-20 and once with TBS. The membranes were treated with the ImmunoStar® LD chemiluminescent reagent (Wako Pure Chemical Industries Ltd., Osaka, Japan), and protein spots were detected by using the Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan).

## Results

**Detection of protein spots in pancreatic cancerous and non-cancerous tissues on 2-DE gels.** 2-DE gels were treated with a fluorescent gel stain, and then differences in the spot intensities between the tissues from pancreatic cancer and non-cancerous were analyzed and quantified by using the Progenesis SameSpots software. The results are summarized in Table II. At least 260 protein spots were matched on each 2-DE gel. Six upregulated spots (spots 1-6) were displayed on 2-DE gel with cancerous tissues at >1.5-fold higher intensity (Fig. 1). The protein expression levels were elevated significantly ( $P < 0.05$ ) in cancerous tissues when compared to paired non-cancerous tissues (Fig. 2).

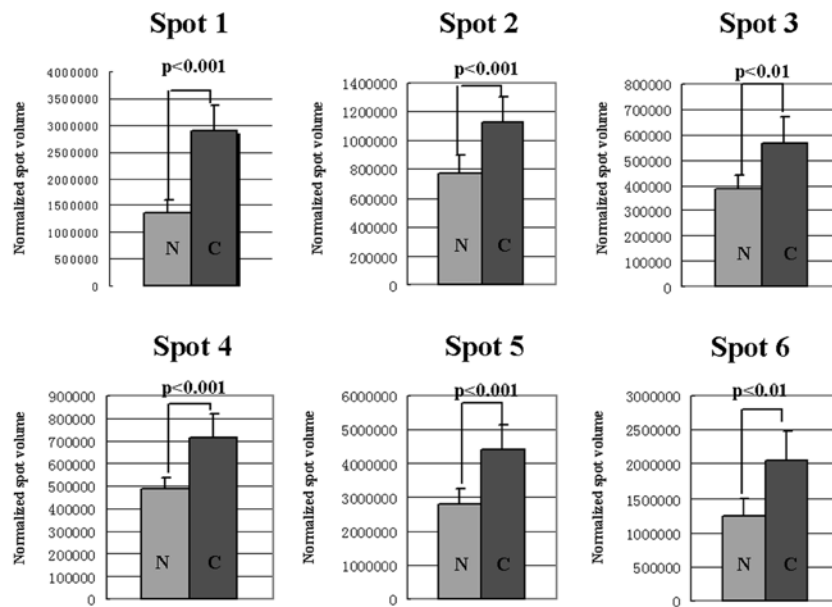


Figure 2. Enhanced protein expressions of calreticulin (spot 1), glutathione synthetase (spot 2), stathmin (spot 3), vinculin (spot 4),  $\alpha$ -enolase (spot 5) and glyceraldehyde-3-phosphate dehydrogenase (spot 6) in pancreatic cancerous tissues. The graphs show the normalized intensity of each spot in cancerous (C) compared to non-cancerous (N) tissues (n=30, P<0.05). Spot numbers are same as in Fig. 1.

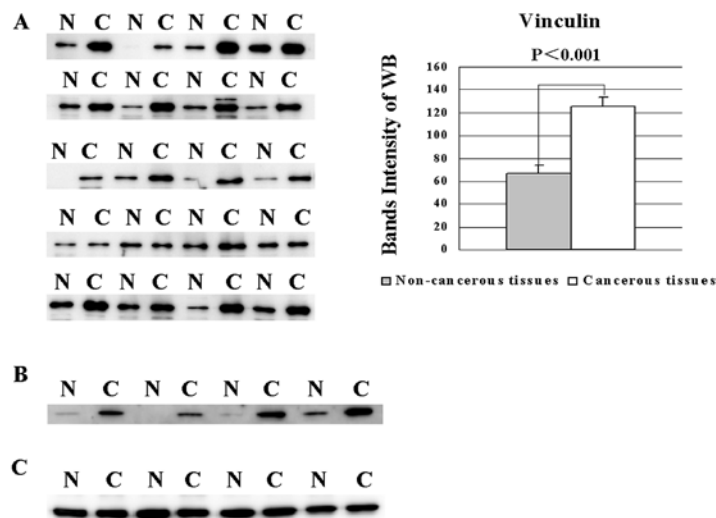


Figure 3. Western-blot analyses of vinculin and  $\alpha$ -enolase in pancreatic cancerous and non-cancerous tissues. (A) Tissues from 20 patients with pancreatic cancerous and paired non-cancerous tissues were used for western blotting with anti-vinculin antibody. The expression of vinculin was increased in pancreatic cancerous tissues (80%). Intensities of the bands were compared between cancerous and non-cancerous tissues by Student's t-test (n=20, P<0.001). The relative standard errors (SE) of cancerous and non-cancerous tissue samples were 8.438 and 7.695, respectively. Expressions of  $\alpha$ -enolase (B) and actin (C) were confirmed by western blotting respectively; the intensity of each band of  $\alpha$ -enolase was stronger in cancerous tissues than in non-cancerous tissues. N, non-cancerous tissues; C, cancerous tissues.

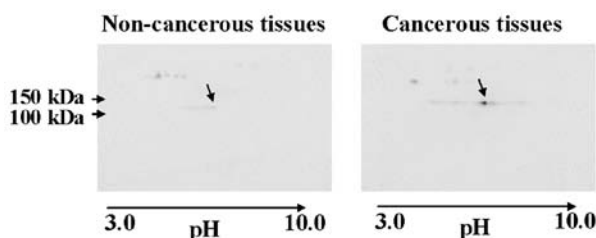


Figure 4. Two-dimensional (2-D) western blotting of vinculin in pancreatic cancerous tissues. Two-dimensional western blotting was performed on a pair of samples on pH 3-10 linear, which confirmed the locations of vinculin on PVDF membranes. The upregulated spot of vinculin was observed in cancerous tissue, compared to non-cancerous tissues.

*Identification of proteins by LC-MS/MS.* The samples were digested with trypsin and then analyzed by using LC-MS/MS system, which identified the six upregulated protein spots as calreticulin (spot 1), glutathione synthetase (spot 2), stathmin (spot 3), vinculin (spot 4),  $\alpha$ -enolase (spot 5) and glyceraldehyde-3-phosphate dehydrogenase (spot 6). The spot numbers are the same as those in Fig. 1. MS/MS data of these proteins are summarized in Table II.

*Western blot analysis of vinculin and  $\alpha$ -enolase.* There are still no reports regarding overexpression of vinculin in PC and its importance for cell adhesion and migration (12,13).

Twenty pairs of pancreatic cancerous and non-cancerous tissues were analyzed by western blotting with anti-vinculin antibody, and the different intensities of the bands between cancerous and non-cancerous tissues were analyzed by the Student's t-test (Fig. 3A). The mean intensities of the bands of cancerous and non-cancerous tissue samples were 125.2 and 66.4, respectively (Fig. 3A). Four pairs of cancerous and non-cancerous tissues were used for western blotting, to demonstrate the upregulation of  $\alpha$ -enolase (14) as a positive control in cancerous tissues, compared to non-cancerous tissues (Fig. 3B). The appearance of vinculin on the 2-DE gels was located by 2-D western blotting (Fig. 4).

## Discussion

We identified six upregulated proteins, calreticulin, glutathione synthetase, stathmin, vinculin,  $\alpha$ -enolase and glyceraldehyde-3-phosphate dehydrogenase, in pancreatic cancerous tissues, compared to non-cancerous tissues. In this study, we reported only on those increased in cancerous tissues because many of the decreased proteins may have been replaced by stromal cells. To the best of our knowledge, this is the first report suggesting that vinculin is a candidate biomarker of PC.

Vinculin is a highly conserved intracellular protein (~123.8 kDa) with an important role in the regulation of cell adhesion and migration (12,13). Bakolitsa *et al* have explained how vinculin regulates cell adhesion by their detailed protein structural analysis (15). Highly metastatic cells have been reported to lack vinculin expression (16,17). Vinculin inhibits cell metastasis when transfected back into vinculin-null cells (17). Evidence reveals that apoptosis is related to cell motility (18,19), and that vinculin regulates cell apoptosis and motility via controlling the ERK pathway (18).

Paradoxically, our study demonstrated that vinculin, which usually behaves as a potent inhibitor to the survival and motility of cells (16-18), was significantly overexpressed in pancreatic cancerous tissues. Our findings indicate that vinculin could be a useful biomarker of PC for its high specificity. Vinculin is well characterized by its intracellular connecting component within adhesion complexes (16), but its functions remain unclear. A new report suggests that vinculin is a main driver gene of the 10q22 amplification in 10q22-amplified prostate carcinomas and that overexpression of vinculin may play an enhancing role in tumor cell proliferation during prostate cancer progression (20). This may be explained by the alternative splicing of vinculin gene, resulting in the alteration of the vinculin function during prostate carcinogenesis (21). Further studies are required to clarify whether vinculin overexpression contributes to PC progression by enhancing tumor cell proliferation, and to elucidate vinculin's action in PC. Additional studies must be conducted in order to identify post-transcriptional modifications of vinculin in PC. Our data sheds light on a new facet of vinculin; its function in PC progression.

A previous report demonstrated that vinculin is related to tumor-suppressing properties (22). However, our findings revealed a different property of vinculin in PC and suggest that vinculin may play a significant role in the diagnosis or prognosis of PC.

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