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Differential expression of up-regulated cofilin-1 and down-regulated cofilin-2 characteristic of pancreatic cancer tissues

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Abstract. Pancreatic cancer (PC) is one of the most deadly malignant tumors. The aim of this study was to identify potential biomarkers for PC. Using two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry, the proteomic profiles of pancreatic cancerous and non-cancerous tissues from ten patients with PC were compared. One of the numerous spots that showed stronger intensity in cancerous compared to non-cancerous tissues was identified as non-muscle cofilin (cofilin-1). This up-regulation was validated by Western blot analysis. It is noteworthy that Western blot analysis showed significantly lower expression of muscle cofilin (cofilin-2) in pancreatic cancerous tissues compared to non-cancerous tissues. This is the first time that cofilin isoforms (cofilin-1/2) have been identified to be differentially expressed in pancreatic cancerous tissues. Therefore, cofilin isoforms may serve as candidates for clinically useful biomarkers or therapeutic targets for PC.

Introduction

Pancreatic cancer (PC) is a malignant tumor which is associated with an extremely unfavorable prognosis. Because of delayed diagnosis and the lack of response to various therapies, few patients with PC survive for more than 5 years; due to rapid aggressiveness, most cases are diagnosed after metastatic spread (1). Therefore, it is critical to discover more sensitive biomarkers for the diagnosis of patients with PC, and the biological mechanisms involved in the extreme aggressiveness of PC should be clarified.

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Proteomics has been widely applied to discover candidate biomarkers in various types of cancers. Using proteomic differential display analysis, research groups have identified biomarkers such as PEA-15 in human malignant pleural mesothelioma cell lines, heat-shock proteins (Hsp27, Hsp70, GRP78) in hepatocellular carcinoma, numerous candidate proteins in colorectal cancer and α-1-antitrypsin isoforms as a possible serum biomarker in pancreatic cancer (2-6). Proteomic technology that combines two-dimensional gel electrophoresis (2-DE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) has high throughput and accuracy, and these technologies are considered as useful tools to comprehensively analyze proteins.

Using these techniques, we found that cofilin-1 displayed differential expression on 2-DE gels between pancreatic cancerous and non-cancerous tissue samples. Western blotting demonstrated that cofilin-1 was significantly up-regulated in cancerous tissues compare to non-cancerous tissues; but opposingly cofilin-2 expression was significantly diminished in cancerous tissues. The findings of the present study incite an interest in cofilin and focus on the relationship between cofilin isoforms and cancer progression.

Materials and methods

Sample preparation. Twenty-four pairs of pancreatic non-cancerous and cancerous tissues were collected from 24 patients diagnosed with PC who underwent surgical resection of the pancreas at the Department of Surgery II, Yamaguchi University Hospital. None of the patients received any pre-operative therapy. Written informed consent was obtained from all patients before surgery. The study protocol was approved by the Institutional Review Board for Human Research of the Yamaguchi University School of Medicine.

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 μg/ml leupeptin and 10 μg/ml aprotinin) on ice (5). After centrifugation (21,500 x g, 30 min, 4˚C), the supernatants were used as samples. Ten pairs from the samples were used for 2-DE, and 24 pairs were used for Western blotting.

Two-dimensional electrophoresis. Isoelectric focusing (IEF) was performed in an IPGphor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized linear pH gradient, pH 3.0-10.0 linear gradient IPG strips (Bio-Rad, Hercules, CA, USA) at 50 μA/strip. Samples were mixed with 200 μl of rehydration buffer [8 M urea, 2% CHAPS, 0.01% bromophenol blue, 1.2% Destreak reagent (GE Healthcare, Uppsala, Sweden)] and 0.5% IPG buffer (GE Healthcare) and loaded into the IPGphor strip holder (GE Healthcare). Eighty micrograms of protein was loaded for each 2-DE. The IEF was performed overnight according to the following program: rehydration for 10 h (0 V); 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 (7).

The IPG strips were 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 buffer 2 (6 M urea, 0.5 M Tris-HCl pH 8.8, 30% glycerol, 2% SDS, 2.5% iodoacetoamide) for another 10 min. The IPG strips were then transferred onto the gels, run at 200 V; SDS-PAGE was performed on a precast-polyacrylamide gel with a linear concentration gradient of 5-20% (Bio-Rad) (8,9). Each sample was repeated three times to ensure protein pattern reproducibility.

Fluorescence staining and image analysis. After washing with Milli-Q water 3 times, the SDS-PAGE gels were incubated with 40% ethanol and 10% acetic acid for 2.5 h. After fixation, the gel were then stained with a fluorescent gel staining, Flamingo™ Fluorescent Gel Stain (Bio-Rad) overnight (10). The stained gels were rinsed with Milli-Q water 5 times on a shaker for 5 min. The gels were scanned by using the ProXpress 2-D Proteomic Imaging System (PerkinElmer, Waltham, MA, USA) and then analyzed using Progenesis Samespots software (Nonlinear, Newcastle upon Tyne, UK). Subsequently, the gels were stained with SeePico™ (Benebiosis, Seoul, Korea) overnight (11). The protein spots showing different intensities were picked up from the gels and analyzed with LC-MS/MS.

Liquid chromatography tandem mass spectrometry analysis. The gel pieces were digested with trypsin and lyophilized overnight with the use of Labconco Lyph-lock 1L Model 77400 (Labconco, Kansas City, MO, USA). Lyophilized samples were dissolved in 15 μl of 0.1% formic acid, and then analyzed using the LC-MS/MS system. Peptide sequencing of the identified protein spot was performed using LC-MS/MS with a Spectrum Mill MS Proteomics Workbench (Agilent Technology, Palo Alto, CA, USA).

Western blotting. Samples were separated by electrophoresis with SDS-PAGE gels and then transfered onto PVDF membranes at 90 mA for 78 min. The PVDF membranes were blocked overnight with TBS containing 5% milk at 4˚C (12). The membranes were incubated with the primary antibody against cofilin-1 (anti-CFL1 mouse monoclonal antibody, 1:1,000; Sigma, St. Louis, MO, USA) or cofilin-2 (anti-CFL2 goat polyclonal antibody, 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight at 4˚C. The membranes were further incubated with the secondary antibody conjugated with horseradish peroxidase (1:10,000) for 1 h at room temperature. The membranes were then reacted with a chemiluminescent reagent (ImmunoStar Long Detection; Wako, Osaka, Japan) and scanned using the Image Reader LAS-1000 Pro (FujiFilm Corporation, Tokyo, Japan).

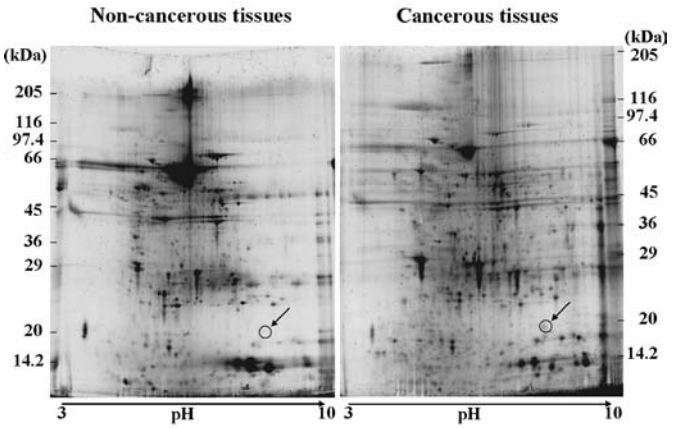

Figure 1. Two-dimensional gel electrophoresis image of pancreatic cancerous and paired non-cancerous tissues stained with Flamingo™ Gel Staining and scanned using the ProXpress 2-D Proteomic Imaging system. Proteins were separated on pH 3.0-10.0 linear, immobilized pH gradient strips and then by 12.5% SDS-PAGE. The spot (indicated by a circle and an arrow) exhibited a stronger intensity on the gel from the cancerous tissue compared to the non-cancerous tissue.

Results

2-DE in the pancreatic non-cancerous and cancerous tissues. 2-DE gels were stained with a fluorescent gel staining and analyzed using Progenesis Samespots software. At least 300 protein spots were matched on each 2-DE gel. A spot of ~20 kDa in mass with an isoelectric point (pI) 8.0 showed stronger intensity in the pancreatic cancerous tissues than in the non-cancerous tissues (Figs. 1 and 2A). The quantification information is summarized in Table I.

Protein identification by LC-MS/MS. Cofilin-1 was identified as the up-regulated protein spot on the 2-DE gels. The peptide sequence of cofilin-1 was identified as (K) LGGSAVISLEGKPL(-) by MS and MS/MS spectra of trypsin-digested gels. MS/MS data for this protein are summarized in Table I.

Western blot analysis of cofilin isoforms (cofilin-1/2). Cofilin-1 on the 2-DE gels was identified by 2-D gel electrophoresis and Western blotting (Fig. 2B). Twenty-four pairs of cancerous and non-cancerous tissues were analyzed by Western blotting using the anti-cofilin-1 (Fig. 3A) or the anti-cofilin-2 antibody (Fig. 4A). The different intensities of the bands between the cancerous and non-cancerous tissues were quantified by the Student's t-test. The expression of cofilin-1 was increased in the pancreatic cancerous tissues when compared to the non-cancerous tissues (22/24; 91.6%), (Fig. 3A). The intensities of the bands of cofilin-1 in the cancerous and non-cancerous tissue samples were 116.3 and 31.7 ($p<0.001$), respectively (Fig. 3B). The intensities of the bands of cofilin-2 in the cancerous and non-cancerous tissues samples were 67.4 and 9.1 ($p<0.001$), respectively (Fig. 4B). The expression of cofilin-2 was

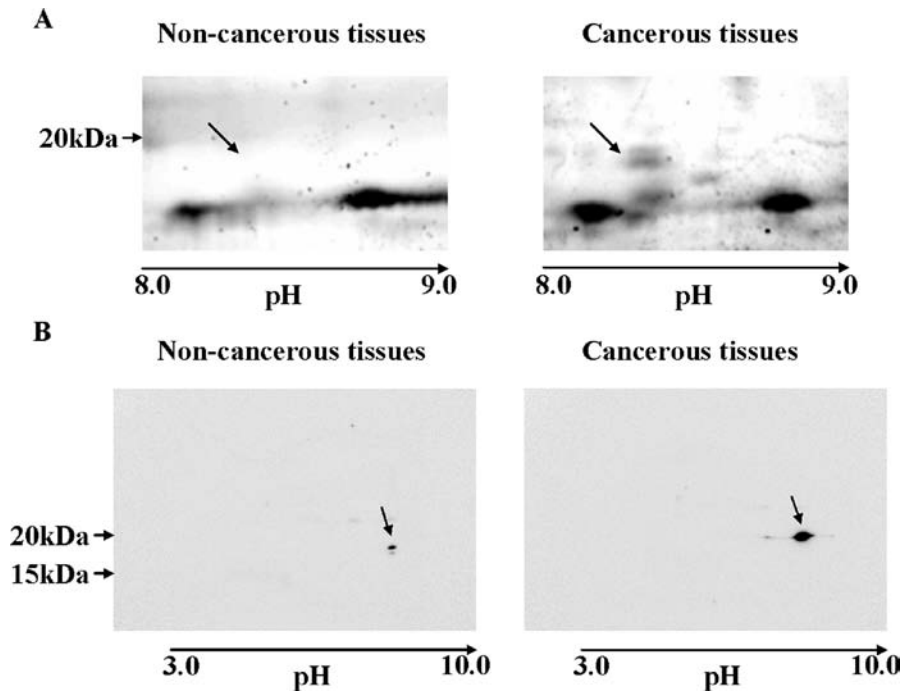

Figure 2. Up-regulated expression of the spot as determined by two-dimensional gel electrophoresis (2-DE) and 2-D Western blotting between cancerous and paired non-cancerous tissues. (A) Differential intensity of the spot between the cancerous tissue compared to the paired non-cancerous tissue as shown on 2-DE gels. (B) Localization of the spot (cofilin-1) was displayed on PDVF membranes by 2-D Western blotting using the anti-cofilin-1 antibody.

Table I. Identification and intensity of the up-regulated protein spot in the cancerous tissues.

Protein	Accession no.	pI/Mr[a]	Peptide[b]	Intensity[c]	Frequency	p-value
Cofilin-1	P23528	8.22/18502.6	(K)LGGSAVISLEGKPL(-)	1.8	8/10	0.005

[a]Theoretical pI and molecular weight (Da) from the protein database. [b]Peptide sequencing of cofilin-1 was identified by LC-MS/MS analysis. [c]Ratio of spot intensity in cancerous to non-cancerous tissues on 2-DE gels.

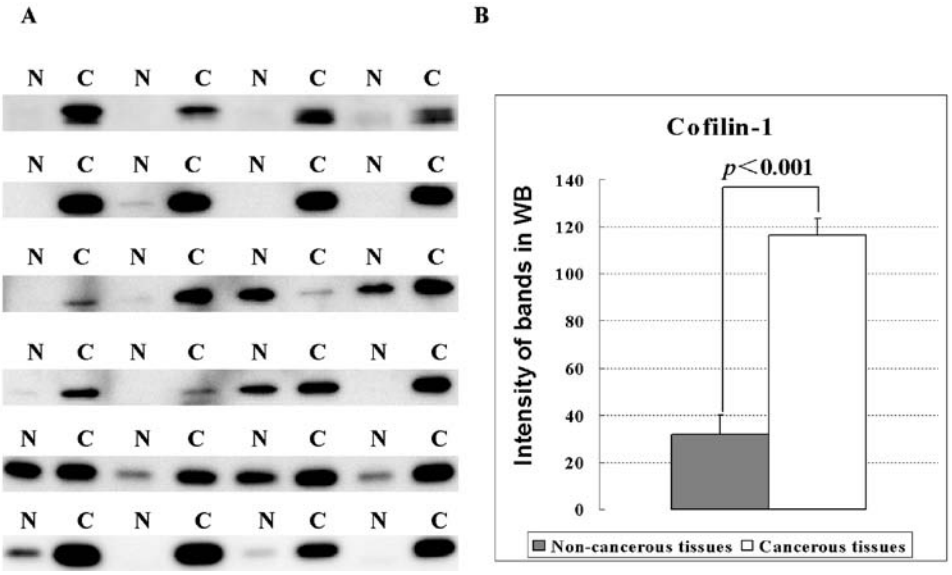

Figure 3. Western blot analysis of cofilin-1 was carried out in pancreatic cancerous and paired non-cancerous tissues. (A) Pancreatic cancerous (C) and paired non-cancerous (N) tissues from 24 PC patients were used, and the anti-cofilin-1 antibody was applied. The expression of cofilin-1 was increased in the pancreatic cancerous tissues compared to that in the paired non-cancerous tissues (91.67%). (B) Comparison of the intensity of the bands between the cancerous and non-cancerous tissues by the Student's t-test (n=24, p<0.001). The relative standard errors (SE) of cancerous and non-cancerous tissue samples were 8.112 and 6.852, respectively.

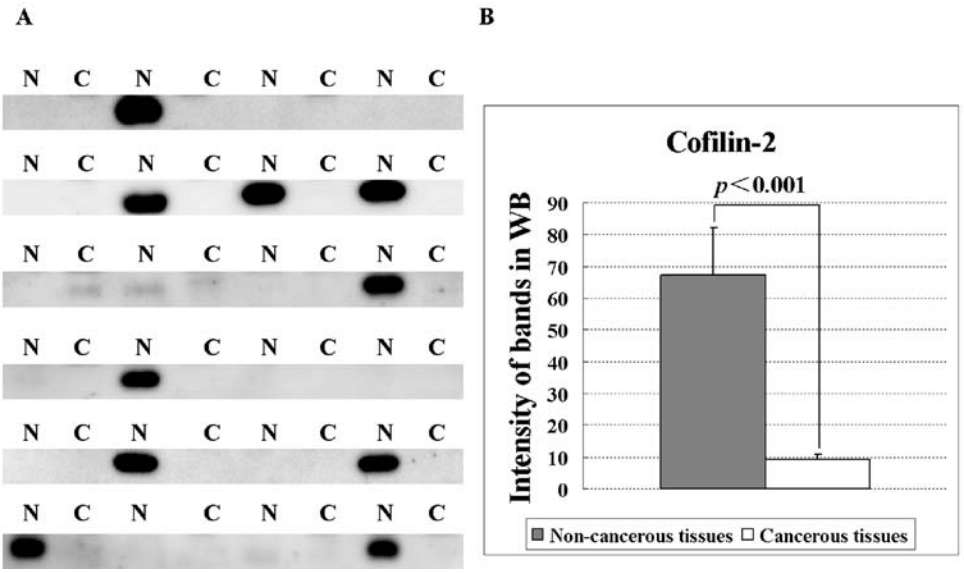

Figure 4. Western blot analysis of cofilin-2 was carried out in pancreatic cancerous and paired non-cancerous tissues. (A) Pancreatic cancerous (C) and paired non-cancerous (N) tissues from 24 PC patients were used, and the anti-cofilin-2 antibody was applied. The expression of cofilin-2 was barely detectable in the pancreatic non-cancerous tissues (58.3%) and in the cancerous tissues (100%). (B) Comparison of the intensity of the bands between cancerous and non-cancerous tissues by the Student's t-test (n=24, p<0.001). The relative standard errors (SE) of cancerous and non-cancerous tissue samples were 14.749 and 1.758, respectively.

detectable in the non-cancerous tissue samples (10/24; 41.7%), but was not in the cancerous tissues (0/24) (Fig. 4A).

Discussion

LIM domain kinase 1 (LIMK1) and cofilin are important regulators of actin cytoskeleton, and up-regulated actin cytoskeleton enhances tumor cell migration and invasion (13). The LIMK1-mediated cofilin pathway is directly related with mammary tumor invasion and migration (14). Cofilin was found to regulate cell protrusion and motility through the spatial interaction of lamellipodium and lamella (15). Epidermal growth factor (EGF)-induced phosphatidylinositol 4,5-bisphosphate (PIP2) was found to regulate membrane translocation of cofilin in carcinoma cells (16). In the present study, non-muscle cofilin (cofilin-1) was up-regulated in pancreatic cancerous tissues compared with non-cancerous tissues as determined by proteomic profiling. Notably, we demonstrated that expression of the muscle cofilin (cofilin-2), an isoform of cofilin-1, was completely disrupted in pancreatic cancerous tissues by Western blotting.

Cofilin-1, a small ubiquitous protein (~18.5 kDa), regulates actin dynamics through its ability to bind and sever actin filaments during cell migration (17). Cofilin-1 plays roles in cell proliferation, phagocytosis, chemotactic movement and macropinocytosis (18,19). It is generally regarded as an accessory to tumor cell invasion and motility (13). Inhibition of cofilin-1 activity in carcinoma cells reduces cell motility and invasion (20). Cofilin-1 expression was found to be up-regulated in many types of tumor cells, such as invasive mammary tumor cells (21), human glioblastoma cells (22) and the C6 rat glioblastoma cell line (23). In mammals, actin-depolymerizing factor (ADF)/cofilins are a family of monomeric and filamentous actin binding proteins, consisting of three members, cofilin-1, cofilin-2 and ADF (17,24). Cytoskeletal dynamics and cell motility in mammalian cells require ADF and cofilin-1 activity (20). Thus, cofilin-1 may be involved in motility and invasion of tumor cells in PC.

However, little is known about cofilin-2 in humans. Cofilin-2 accumulates at substrate adhesion sites where cofilin-1 is almost completely excluded (25); a significant increase in cofilin-2 expression was noted during the aggregation stage of cell development under conditions of starvation in *Dictyostelium* cells. However, cofilin-1 exhibited an opposing and concomitant action (25).

These findings indicate that cofilin-1 and cofilin-2 may play different roles in the dynamic reorganization of the actin architecture respectively, and cell development may involve the participation of cofilin-2 under conditions of starvation (25). Cofilin-2 expression in human skeletal muscle and the heart is different from its expression in various tissues in post-transcriptional splicing of mRNA (26). Albeit cofilin-2 gene transcript was detected in the human pancreas; we demonstrated that this protein was barely expressed in pancreatic cancerous tissues (100%). Notably, not all non-cancerous tissue samples exhibited a high level of cofilin-2 expression; approximately 41.7% of the non-cancerous tissues showed obviously detectable cofilin-2 expression. The mechanism by which this occurs is not clearly understood. Presumably cofilin-2 exists in non-cancerous tissues only under conditions of starvation at levels required to maintain cell development (25) or survival. Starvation-induced autophagy is a mechanism which promotes cell survival (27). However, it has yet to be revealed whether starvation-induced cell autophagy is related to cofilin-2 expression. However, the expression of cofilin-2 may not be required by well-nourished non-cancerous tissues in PC. Therefore, cofilin-2 may be related to the struggle between the body and tumors under conditions of starvation. Whether cofilin-2 is involved in resistance to starvation by a

self-regulating protective mechanism in non-cancerous tissue cells during PC progression must be clarified by further study.

The present study suggests that cofilin isoforms (cofilin-1/2) play essential roles in the destabilization of the actin cytoskeleton in PC progression involving different pathways. In order to clarify whether cofilin isoforms dysregulated in PC lead to rapid tumor spread, further investigations concerning their expression levels in PC cell lines and the determination of the effects of the cofilin pathways on invasion, proliferation or autophagy of these cells must be carried out. In addition, reports have demonstrated that cofilin expression is related to treatment resistance in PC cell lines (28,29). Therefore, our study indicates that cofilin isoforms may be useful clinical biomarkers or effective targets for controlling PC progression.

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