Expression of glycolytic enzymes is increased in pancreatic cancerous tissues as evidenced by proteomic profiling by two-dimensional electrophoresis and liquid chromatography-mass spectrometry/mass spectrometry

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Abstract. The prognosis of patients with pancreatic cancer is very poor because of late diagnosis and the lack of response to various therapies. We tried to identify proteins that might be available for early diagnosis and effective therapies by proteomic profiling of pancreatic cancer tissues. Pancreatic cancerous and paired non-cancerous tissues obtained from surgical resections or autopsies of 10 patients were analyzed by two-dimensional gel electrophoresis. The differential display showed 11 spots whose expression was increased in cancerous tissues compared with the paired non-cancerous tissues. The liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) system identified the spots as α-enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triosephosphate isomerase, transgelin, calmodulin, superoxide dismutase(Mn) mitochondrial precursor, glutathione S-transferase P, cyclophilin A, protein disulfide isomerase A3 precursor, and apolipoprotein A-I precursor. Two of the 11 spots were detected as GAPDH. We noticed that 4 of 11 spots were enzymes involved in glycolytic pathway. Increased glycolysis in cancer cells has been regarded as the effect of intratumoral hypoxia and is possibly associated with tumor invasion, metastasis or resistance to therapies. These glycolytic proteins and transgelin, were confirmed by Western blotting and immunohistochemistry.

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Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TPI, triosephosphate isomerase; 2-DE, two-dimensional gel electrophoresis; MnSOD, superoxide dismutase(Mn) mitochondrial precursor; PDI-A3, protein disulfide isomerase A3 precursor; GST-p, glutathione S-transferase P

Key words: pancreatic adenocarcinoma, two-dimensional gel electrophoresis, α-enolase, glyceraldehyde 3-phosphatase dehydrogenase, triosphosphate isomerase, transgelin

Introduction

Molecular diagnostics and thrapeutics for human malignancies have been developed recently. However, the prognosis of patients with pancreatic cancer is still very poor because of its aggressiveness and lack of early diagnosis and effective therapies (1). Surgical resection is the only curative therapy, but the disease has usually already progressed by the time of diagnosis. Therefore, novel diagnostic tools for pancreatic cancer have to be developed and the biological characteristics giving rise to aggressiveness of this disease should be clarified.

Recent intensive studies have identified genetic abnormalities frequently expressed in human malignancies, including pancreatic cancer (2-4). Following gene analysis, proteomic studies have been performed to find proteins as candidates for new diagnostic markers and therapeutic targets (5,6).

Two-dimensional gel electrophoresis (2-DE) is regarded as a useful method to analyze proteins comprehensively. Using this method, we tried to identify proteins overexpressed in pancreatic cancerous tissues and to detect protein factors that might be available for diagnosis and more effective therapies.

Materials and methods

Tissue samples. We examined 10 pairs of cancerous and corresponding non-cancerous pancreas tissues obtained from patients who were diagnosed with pancreatic adenocarcinoma and underwent surgical resection or autopsy at Yamaguchi University Hospital between 2001 and 2004. The authors received informed consent for all patients, including 7 males and 3 females whose mean age at collection was 65 years (range, 51-79 years). None of the patients had received chemotherapy or radiation therapy prior to cancer resection.

Histologically, 7 were classified as moderately differentiated tubular adenocarcinoma, 1 as well differentiated, 1 as poorly differentiated, and 1 as mucinous carcinoma. According to the TMN classification (7), 1 was classified as stage II, 6 as III, 1 as IVA, and 2 as IVB (Table I).

Table I. Summary of all cases of pancreatic cancer.

No.	Age	Sex	Histology	T	N	M	Stage
1	71	F	Tubular (moderate)	3	0	0	II
2	75	M	Tubular (moderate)	4	1	0	IVA
3	54	M	Tubular (moderate)	2	1	0	III
4	67	F	Tubular (moderate)	2	1	0	III
5	79	M	Mucinous	4	1	1	IVB
6	51	F	Tubular (well)	3	1	0	III
7	61	M	Tubular (poor)	4	1	1	IVB
8	64	M	Tubular (moderate)	3	1	0	III
9	70	M	Tubular (moderate)	2	1	0	III
10	58	M	Tubular (moderate)	3	1	0	III

M, male; F, female; tubular, tubular adenocarcinoma; well, well differentiated; moderate, moderately differentiated; poor, poorly differentiated type; mucinous, mucinous carcinoma. Tumor staging was performed according to the TMN classification.

Sample preparation. Resected pancreas tissues were immediately frozen in liquid nitrogen and stored at -80˚C until use. They were disrupted and homogenized in lysis buffer (1% NP-40, 1 mM sodium vanadate, 1 mM PMSF, 50 mM Tris-HCl, 10 mM NaF, 10 mM EDTA, 165 mM NaCl, 10 μg/ ml leupeptin, 10 μg/ml aprotinin) using a microtube mixer with a teflon tip at 4˚C for 2 h. The lysate was separated by centrifugation at 15,000 x g for 30 min. The supernatant was stored at -80˚C until use.

Two-dimensional gel electrophoresis (2-DE). Three hundred micrograms of protein from the supernatant was applied to immobiline dry strips (pH 3.0-10.0, 7 cm; Amersham Bioscience, NJ) in a total volume of 125 μl containing 8 M urea, 2% CHAPS, 0.5% IPG buffer (Amersham Bioscience) and 2.8 mg/ml dithiothreitol (DTT). After dehydration for 14 h, proteins were separated by isoelectrofocusing (IEF) at 20˚C using 50 μA/strips with the following linear voltage increases: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2 h. The strips were then equilibrated twice in 50 mM Tris-HCl containing 6 M urea, 30% glycerol and 2% sodium dodecyl sulfate (SDS) for 15 min. DTT was then added, followed by iodoacetamide. The second dimension was performed on SDS-polyacrylamide gels (2-D homogeneous 12.5%; Amersham Bioscience) in two steps: 600 V, 20 mA for 30 min and 600 V, 50 mA for 70 min in a multiphor horizontal electrophoresis unit (Amersham Pharmacia Biotechnology). Separated protein spots were stained on the gel with 30% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue R-250 (CBB) overnight. The gel was destained with 30% methanol and 10% acetic acid for 30 min, and then with 7% acetic acid until the background of the spots turned clear.

Image analysis. The protein spots on the gel were recorded using an Agfa ARCUS 1200™ image scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and analyzed using Image Master 2D Platinum ver. 5.0 (Amersham Bioscience). Spots stained at different intensities between cancerous and non-cancerous tissues were excised from the gels and identified by LC-MS/MS (LC-MSD XCT, Agilent).

In-gel digestion. The CBB dye was removed by rising the gel twice in 60% methanol containing 50 mM ammonium bicarbonate and 5 mM DTT for 15 min each time, and twice in 50% acetonitrile containing 50 mM ammonium bicarbonate and 5 mM DTT for 7 min each time. The gel piece was dehydrated in 100% acetonitrile, and then rehydrated with an in-gel digestion reagent containing 10 μg/ml sequencing grade trypsin (Promega, Madison, WI) in 30% acetonitrile with 50 mM ammonium bicarbonate and 5 mM DTT. The in-gel digestion was performed overnight at 30˚C.

Amino acid sequencing by LC-MS/MS. Lyophilized samples were dissolved in 20 μl of 0.1% formic acid and centrifuged at 15,000 x g for 5 min. Peptide sequencing of identified protein spots was performed using the LC-MS/MS system with a Spectrum Mill MS Proteoics Workbench (Agilent Technologies, Santa Clara, CA).

Statistical analysis. Expression levels of the protein spots were quantified by analyzing the intensity of each spot with Image Master (Amersham Bioscience). The differences in expression between cancerous tissues and non-cancerous tissues were analyzed by Student's t-test.

Western blot analysis. Protein samples of 15 μg were separated by SDS-PAGE and 100 μg were separated by 2-DE. Fractionated proteins were transferred electrophoretically onto a PVDF membrane and blocked with TBS containing 5% skim milk. Primary antibodies used were anti-enolase goat polyclonal antibody (1:200), anti-GAPDH polyclonal antibody (1:200), anti-triosephosphate isomerase polyclonal antibody (1:200) and anti-transgelin goat polyclonal antibody (1:250) (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were incubated for 1 h, washed four times with TBS containing 0.05% Tween-20, incubated for 1 h with a secondary antibody (1:2,000), and developed with a chemiluminescence reagent (ECL; Amersham Bioscience).

Immunohistochemical analysis. Immunohistochemical analysis was performed using ABC Kit (Vector Laboratories, Burlingame, CA) on the same samples as tissue specimens described in *Tissue samples*. The primary antibodies were the same as those used for Western blot analysis and used at dilutions of 1:200 or 1:250.

Results

Detection of protein spots on 2-DE gels. At least 190 protein spots were matched on each 2-DE gel. The differential

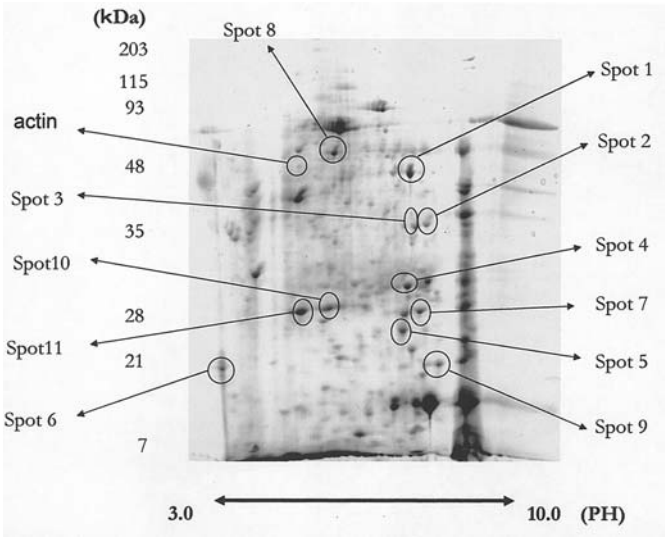

Figure 1. Protein patterns obtained by 2-DE of cancerous tissues and paired non-cancerous tissues of patients with pancreatic adenocarcinoma. Proteins were separated on pH 3-10 linear, immobilized pH gradient strips and then by 12.5% SDS-PAGE. Gels were stained with CBB R-250. Eleven spots were up-regulated in cancerous tissues and numbered from 1 to 11.

expression of paired cancerous and non-cancerous tissues was visually compared. Eleven spots were up-regulated in cancerous tissues in at least 4 of the 10 samples by ≥2-fold higher intensity (Figs. 1 and 2).

Identification of proteins. The LC-MS/MS system identified these up-regulated protein spots as α-enolase (spot 1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spots 2 and 3), triosephosphate isomerase (spot 4), transgelin (spot 5), calmodulin (spot 6), MnSOD (spot 7), PDI-A3 (spot 8), cyclophilin A (spot 9), GST-P (spot 10), and apolipoprotein A-I precursor (spot 11). In Table II, information about the eleven spots is summarized.

Expression profiles of proteins. In all 10 paired samples, the intensities of the 11 protein spots were analyzed and quantified using Image Master (Table III). The expression of four proteins, α-enolase, GAPDH, triosephosphate isomerase and transgelin were confirmed by 2-D immunoblot analysis (Fig. 3). The intensity of each spot was increased in cancerous tissues. In 2-D immunoblot analysis, α-enolase, GAPDH and TPI were observed as multiple spots with slightly different isoelectric points or molecular weights. These spots may be explained by post translational modifications of the proteins. We can also detect some of these spots on 2-DE gels stained by CBB.

The expression of α-enolase, GAPDH, TPI and transgelin was also confirmed by immunohistochemistry. α-enolase and GAPDH were predominantly expressed in cancer cells. α-enolase was also detected in islet cells. While weak staining was detected in normal epithelial cells, acinar cells and in stromal cells. TPI was detected predominantly in cancer cells and also detected in normal epithelial cells. TPI was barely detectable in acinar cells, stromal cells and islet cells. Transgelin was mainly expressed in stromal cells but not in cancer cells or normal epithelial cells. We found that transgelin expression was much stronger in stromal cells around cancer cells than in those around normal epithelial cells (Fig. 4).

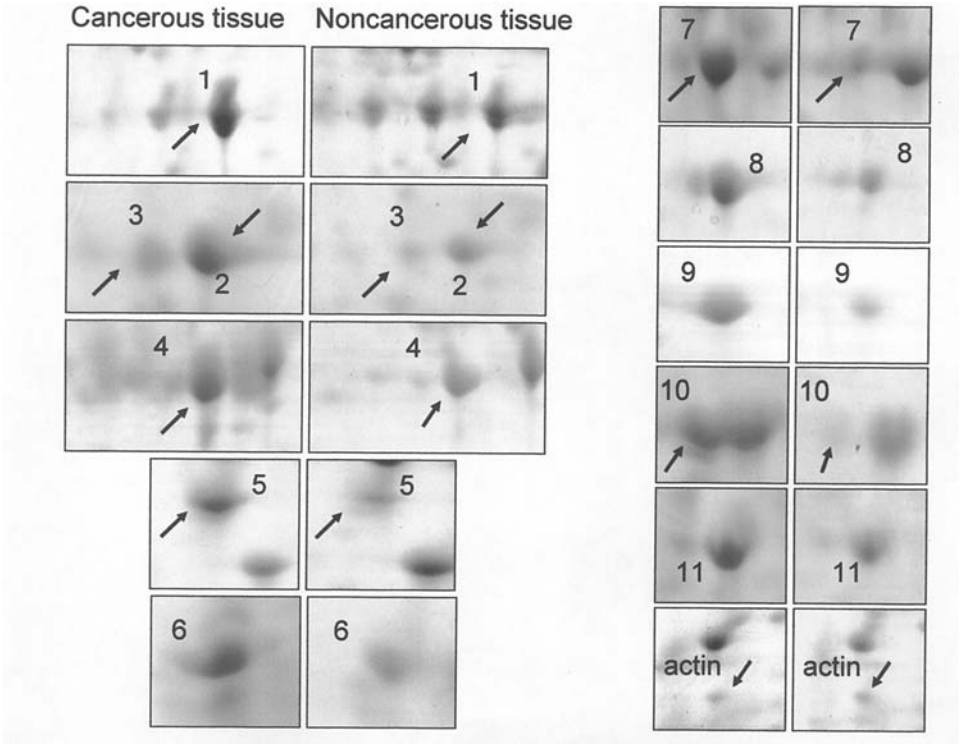

Figure 2. Comparison of 2-DE patterns between cancerous tissues and non-cancerous tissues. Eleven spots up-regulated in cancerous tissues are shown. α-enolase (1), two spots of GAPDH (2, 3), triosephosphate isomerase (4), transgelin (5), calmodulin (6), MnSOD (7), protein disulfide isomerase A3 (8), cyclophilin A (9), GST-P (10), apolipoprotein A-I (11). Each spot number is the same as those in Fig. 1.

Table II. Up-regulated proteins in pancreatic cancerous tissues.

Spot	Frequency	Mass Mr/pl	Accession no.	Protein
1	9/10	47037/6.99	P06733	α-enolase
2	8/10	35922/8.58	P04406	Glyceraldehyde 3-phosphate dehydrogenase, liver type
3	8/10	35922/8.58	P04406	Glyceraldehyde 3-phosphate dehydrogenase, liver type
4	7/10	26538/6.51	P60175	Triosephosphate isomerase
5	6/10	22479/8.88	Q01955	Transgelin
6	6/10	16706/4.09	P02593	Calmodulin
7	5/10	24722/8.34	P04179	Superoxide dismutase(Mn), mitochondrial precursor
8	6/10	56782/5.99	P30101	Protein disulfide isomerase A3 precursor
9	5/10	17881/7.82	P05092	Peptydyl-prolyl cis-trans isomerase (cyclophilin A)
10	4/10	23224/5.44	P09211	Glutatione S-transferase P
11	4/10	30778/5.56	P02647	Apolipoprotein A-I precursor

Table III. The intensities of the up-regulated protein spots in cancerous tissues.

Protein	Spot intensity (average ± SD)		Ratio of spot intensity	p-value
	Cancerous tissue	Non-cancerous tissue		
α enolase	0.30±0.24	0.12±0.11	2.46	0.0058
GAPDH	0.19±0.15	0.07±0.05	2.95	0.0145
GAPDH	0.11±0.04	0.04±0.04	3.91	0.0131
TPI	0.38±0.27	0.17±0.11	2.2	0.0449
Transgelin	0.34±0.29	0.13±0.10	2.62	0.0434
Calmodulin	0.36±0.23	0.14±0.14	2.55	0.0059
MnSOD	0.34±0.03	0.17±0.004	6.36	0.0191
PDI-A3	0.43±0.09	0.09±0.004	6.34	0.0203
Cyclophilin A	0.20±0.005	0.11±0.003	2.31	0.0161
GST-P	0.33±0.02	0.15±0.003	4.20	0.0370
Apolipoprotein A-I	0.55±0.07	0.21±0.007	2.66	0.0175

Discussion

In this study, we detected 11 protein spots whose expression increased in human pancreatic adenocarcinoma tissues. Interestingly, four of them, α-enolase, two spots of GAPDH, and triosephosphate isomerase, were enzymes involved in the glycolytic pathway. It is known that increased rates of glucose uptake and glycolysis are generally found in tumor cells (8). Hypoxia inducible factor (HIF-1), which is expressed under hypoxic conditions with tumor proliferation, has been shown to activate transcription of genes encoding vascular endothelial growth factor, glucose transporters, and glycolytic enzymes, including α-enolase and GAPDH (9,10). Up-regulation of the three enzymes might be caused by hypoxia and increased activity of glycolysis.

α-enolase has been reported to be up-regulated in several cancer cell lines in previous proteomic studies using 2-DE (11,12). α-enolase is found in the cytoplasm of most cells and works as a glycolytic enzyme. Previous studies have reported its ability to function as a heat shock protein (13), cell surface receptor for plasminogen (14), and as a Myc-binding protein that negatively regulates transcription of the c-*myc* oncogene

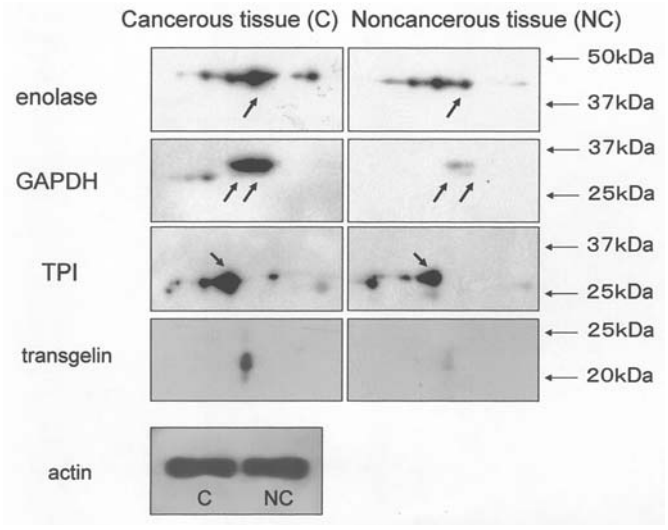

Figure 3. 2-D immunoblot analysis of up-regulated proteins in pancreatic cancerous tissues. We used three pairs of samples for each 2-D immunoblot analysis of α-enolase, GAPDH, triosephosphate isomerase and transgelin. Protein expression of all 4 increased in cancerous tissues.

in the nucleus (15,16). Enolase has three isoenzymes: α-enolase is found in a variety of tissues including liver, ß-enolase in muscle tissues and γ-enolase in neurons and neuroendocrine tissues (17). Enolase exists in heterodimetric forms such as αα, αß, ßß, αß and γγ (18). Neuron-specific enolase (NSE, αγ and γγ isoforms) in serum of small cell lung carcinoma and neuroblastoma is known to be a useful marker for monitoring the progression of disease and response to treatment (19,20). Gerbitz *et al* showed that γ-enolase was increased in the plasma of patients suffering from small cell lung carcinoma, while most patients with squamous cell carcinoma of the lung or prostatic cancer exhibited normal γ-enolase and high concentrations of α-enolase (21). Oskam *et al* studied expression of enolase isoenzymes in rat medullary thyroid carcinomas and reported that the αγ and γγ isoenzyme levels were relatively high in well-differentiated rat tumors, whereas the majority of enolase isoenzymes were αα in undifferentiated and anaplastic tumors (22). Takashima *et al* reported a relation between the expression of α-enolase and differentiation of hepatocellular carcinoma (23). In heart and skeletal muscles of the rat, the αα isoenzyme predominates in the fetus; however, this isoenzyme is replaced by the αß and ßß types as development progresses (24-26). These results might suggest that the expressed form of enolase is associated not only with tissue specificity of the three isoenzymes, but also with the degree of cell differentiation. Further experiments will be required to determine whether α-enolase increases in serum of patients with pancreatic adenocarcinoma and to clarify the relationship between the expression of enolase and cell differentiation.

GAPDH has been known as a housekeeping protein whose gene expression remains constant in spite of various cellular conditions. In recent years, however, that view has changed since GAPDH has proved to have diverse cellular functions, including nuclear RNA export, DNA replication, DNA repair, exocytotic membrane fusion, cytoskeletal organization, and phosphotransferase activity (27). Indeed, increased expression levels of GAPDH mRNA were reported in many malignant tumors (28-30), including pancreatic adenocarcinoma (31). Some of these reports showed that GAPDH expression levels were associated with hypoxic conditions (32,33) and the

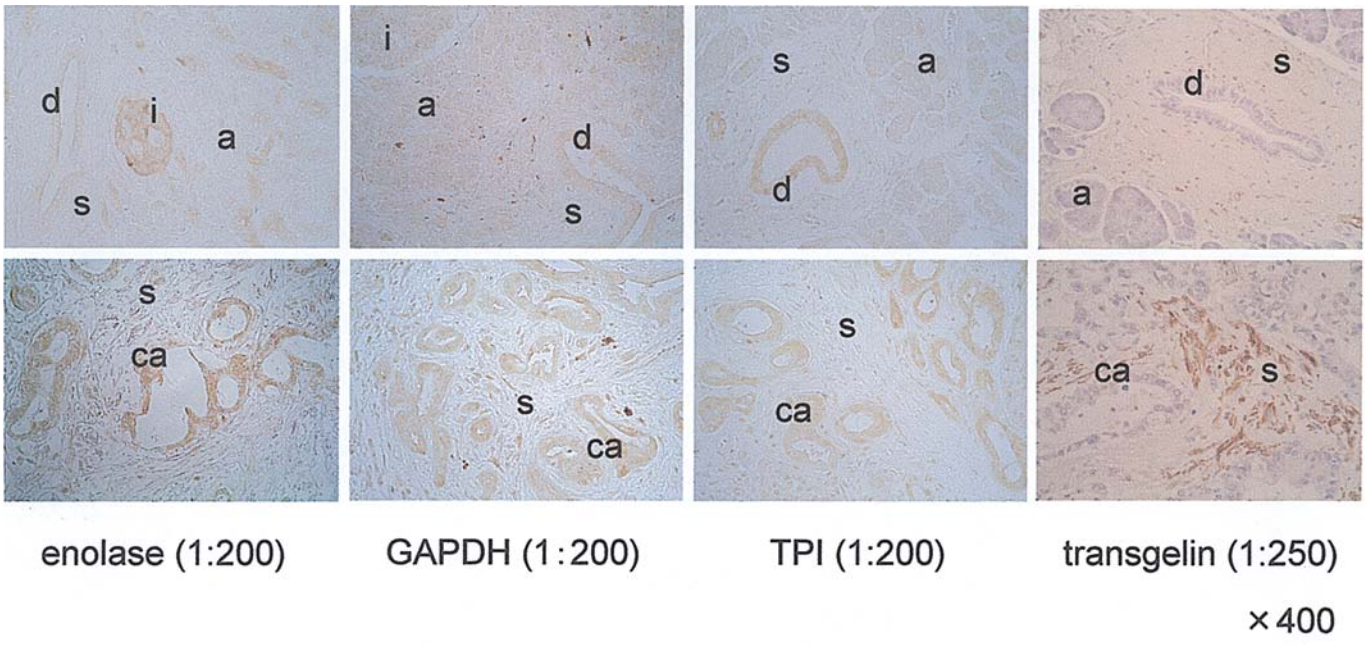

Figure 4. Immunohistochemistry staining of α-enolase, GAPDH, triosephosphate isomerase (TPI) and transgelin. α-enolase and GADPH were predominantly expressed in tumor cells. α-enolase was also detected in islet cells, while weak staining was found in acinar cells and in stromal cells. TPI was detected predominantly in cancer cells and also detected in normal epithelial cells. TPI was barely detectable in acinar cells, stromal cells and islet cells. Transgelin was mainly expressed in stromal cells and its expression was much stronger in stromal cells around cancer cells than in normal epithelial cells. We did not detect transgelin expression in cancer cells or in normal epithelial cells. d, non-cancerous pancreatic ductal cell; ca, cancerous pancreatic ductal cell; a, acinar cell; i, islet cell; s, stromal cell.

aggressiveness of tumors (34,35). Epner and Coffey have analyzed GAPDH expression in normal and malignant human prostate tissues and reported that GAPDH is abundant in the nucleus in normal prostate basal cells, but in cytoplasm of prostate cancer cells (36). They also found five forms of GAPDH with isoelectric points in metastatic rat prostate cancer cell lines, but one to four forms in non-metastatic cell lines, suggesting that each of them has a unique function. In our study, we detected two forms of GAPDH overexpressed in cancerous tissues. Further examination is necessary to analyze their functions and localization.

Triosephosphate isomerase (TPI) has been reported to be up-regulated in lung adenocarcinoma (37), melanoma (38), and squamous metaplasia and carcinoma of the bladder (39). The mRNA and protein expression of TPI has also been shown to be increased by hypoxia (40), but the mechanism is unknown. Recent studies identified a mutated form of TPI known as HLA-DR4-restricted melanoma antigen, which may become a target for immunotherapies for cancer (41,42).

Transgelin, which is also identical to SM22-α, is reported as predominantly expressed in smooth muscle cells and fibroblasts. It was shown to play a role in cell transformation and shape change by binding actin and gelling it (43,44). Tansgelin expression is observed to be lost in human breast and colon tumor samples. Down-regulation of it may associate with oncogenic Ras (45). In renal cell carcinoma (RCC), *in situ* hybridization revealed that transgelin is not expressed in the malignant cells but in mesenchymal cells of the tumor stroma (46). This report corresponds to the result of immunohistochemistry of transgelin in this study. Gene expression of transgelin is found to be up-regulated in the cell lines from metastatic lesions of RCC (47). Recent study identified the expression of this protein as down-regulated in microdissected cells of Pan IN-2 grades, precursor lesions of pancreatic ductal adenocarcinoma (48).

Ott *et al* and Kellner *et al* reported proteomic study using epithelial cell preparation procedure by epithelial cell surface antibody Ber-Ep4 (49,50). Shekouh *et al* showed that the protein profiles of undissected normal or malignant pancreas differed from those of normal or malignant pancreatic ductal epithelia dissected by laser capture microdissection (51). But they also noted that the undissected and dissected tumor samples showed similar profiles. Our data indicate that particularly non-malignant samples may include many proteins that are expressed in cells other than ductal cells. In this study, we reported only on increased proteins in cancerous tissues because many of the decreased proteins may have originated from non-epithelial cells.

It is important to clarify proteins expressed specific to cancer cells although we consider the protein profile from whole tissue biopsies to have important information because recent studies have noted that invasion and tumor metastasis are closely related to interaction between cancer cells and the surrounding tissues (52). Especially, pancreatic cancer is characterized by abundant stroma cells, so the study using whole tissues including stroma cells may help to identify proteins participating in invasion and metastasis of pancreatic cancer.

Almost all of these identified proteins are expressed not only in cancerous tissues but also in non-cancerous tissues and most of them were previously reported as differentially expressed in types of human cancer other than pancreatic. So the possibility that these proteins are specific markers of pancreatic cancer is humble, but they may reflect the mechanism of the cancer spreading.

The number of patients included in this study is not sufficient to produce any conclusion. The sample size would need to be larger in heterogeneous tissue samples from a multi-gender, and multi-age cohort of humans (53). Studies should be continued to confirm these results.

In this study, 10 proteins overexpressed in pancreatic adenocarcinoma were detected. In order to clarify the role of these proteins in pathogenesis and to estimate whether these proteins are useful for developing new diagnostic markers or therapies, further study is needed. Although it is difficult to obtain samples of early pancreatic cancer, it is particularly important to clarify if these proteins are expressed in the early stage of pancreatic cancer.

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