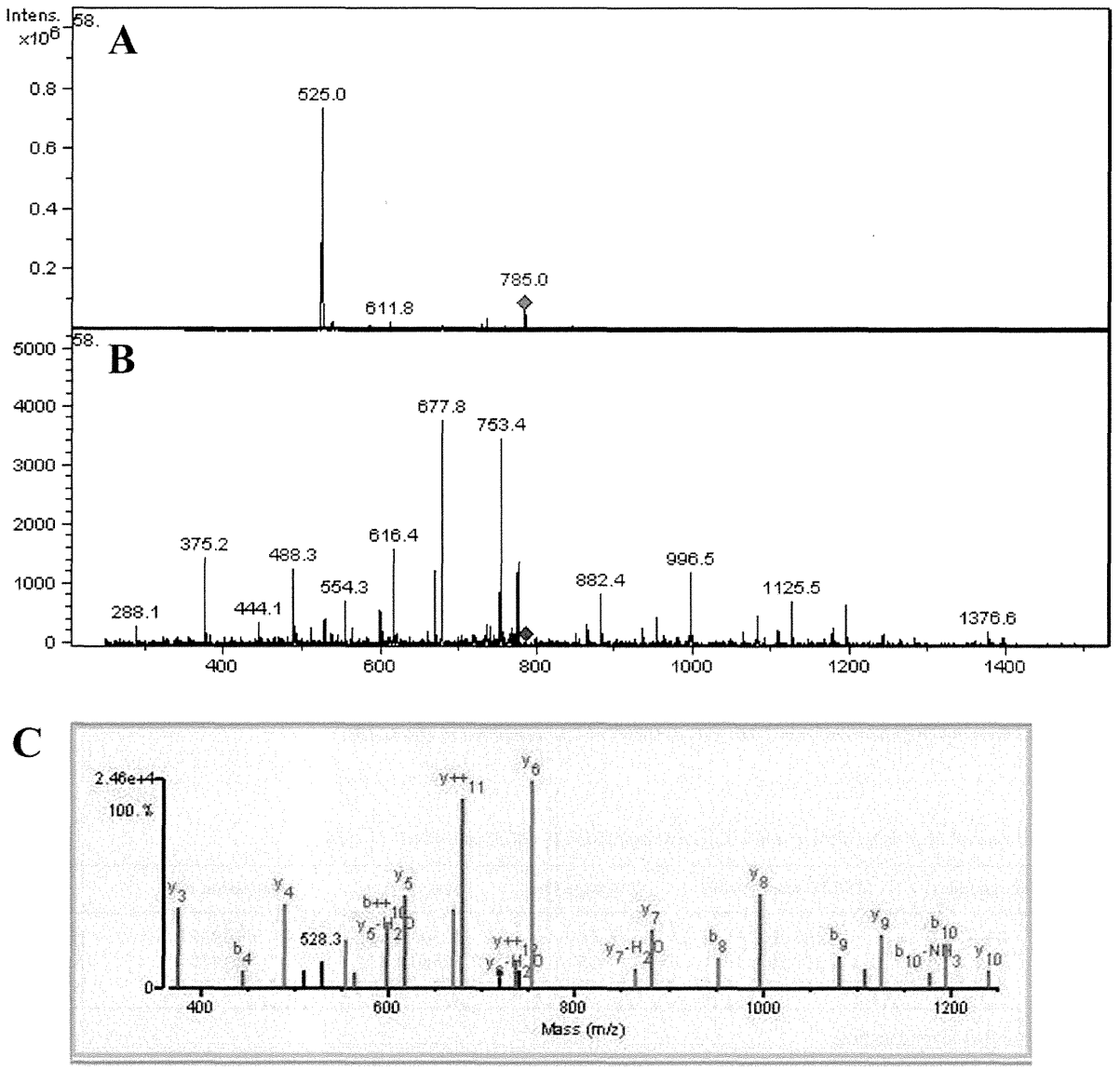

Figure 2. *Mass spectrometry (MS) and MS/MS spectra of trypsin-digested spot 1, shown in Figure 1. A: MS spectra of trypsin-digested spots; nucleophosmin precursor ion m/z is 785.0. B and C: MS/MS spectrum of a precursor ion with m/z 785.0 marked by a diamond in (A). The MS/MS spectrum identifies the partial tryptic peptide [K]VDNDENEHQLSLR[T] from nucleophosmin, processed with a spectrum Mill workbench.*

among different species, with a molecular weight of 35 to 40 kDa. In the human and rat, NPM exists at least with two isoforms, NPM1 and NPM1.2 (B23.1 and B23.2, respectively), which are generated from a single gene *via* alternate splicing (15). However, in mice, only NPM1 exists.

NPM is a non-ribosomal nucleolar phosphoprotein, implicated in cancer, which shuttles continuously between the nucleus and the cytoplasm (16). NPM is a multifunctional protein that is indispensable for various cellular processes including centrosome duplication, ribosome biogenesis, cell-cycle progression, apoptosis, transformation and genomic stability (17-20). Importantly, NPM displays several interrelated nucleolar functions that contribute to cell growth. Therefore, many researchers have focused their attention on these NPM activities and numerous attempts have been made to elucidate its role in cancer cells. Although the role of NPM in oncogenesis has been an object of study for a long time, this is still controversial because its precise role is complex.

NPM protein levels are generally correlated with mitogen-induced cell proliferation, and NPM fusion proteins are found in several malignancies (21). NPM would be expected to be a

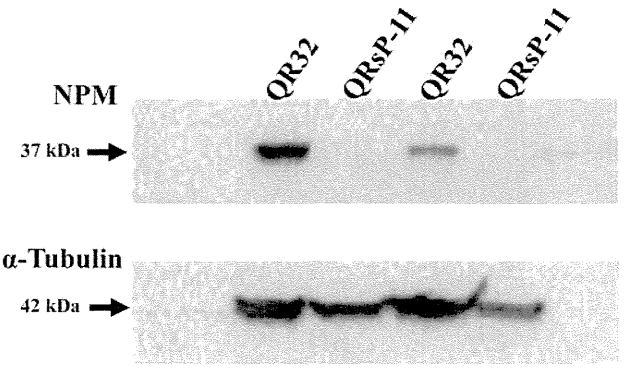

Figure 3. *Western blot analysis of nucleophosmin (NPM). Figure shows the band of NPM (37 kDa) and α-tubulin (42 kDa), as a loading control, in QR-32 and QRsP-11 cells.*

protein with proto-oncogenic activity, and in fact the NPM gene is a direct transcriptional target of the *c-MYC* proto-oncogene transcription factor (22). Moreover, NPM protein levels are elevated in dividing cells and cancer cells (23), but tend to decrease when cells differentiate and stop growing (18). Therefore, we focused our attention on NPM protein levels to possibly elucidate tumorigenesis in the present study.

From many reports, NPM has been reported to be up-regulated, mutated, re-arranged and deleted in cancer cells. Many studies have reported that NPM overexpression is related to carcinogenesis and tumor progression in prostate (24), gastric (25), colon (23), ovarian (26), hepatocellular (27) and urinary bladder carcinomas (28). Grisendi *et al.* reported that NPM was frequently up-regulated in carcinomas and was involved in chromosome translocation in hematological malignancies, where it forms fusion proteins with different partners (16). Overexpression of NPM has also been reported to suppress p53-mediated growth arrest and apoptosis, following exposure of cells to UV radiation or hypoxia (29, 30).

On the other hand, there are opposing reports for the studies described above. Li *et al.* reported that NPM protected cells from apoptotic cell death induced by diverse stresses through a mechanism involving inhibition of the p53 tumor-suppressor protein (31). They pointed-out that NPM might stabilize p53, a major regulator of apoptosis, or promote its degradation during nucleolar stress (31, 32). Our finding in this study supports their theory. There are conflicting reports on NPM levels (gain or loss of expression) and the effect of NPM on p53 activity.

The potent activity of p53 as inducer of cellular apoptosis and cell-cycle arrest demands tight control of its function. The major mode for provoking p53 action is DNA damage, which leads to the interaction between murine double minute 2 homolog (MDM2) and p53, increased p53 stability and activity (33). p53 activity is also induced by cellular oncogenes, such as *RAS* and *MYC* (34), and viral proteins such as viral cyclin (K cyclin) expressed by Kaposi's sarcoma-associated herpesvirus (35). *RAS* and *MYC* increase the levels of alternative reading frame (ARF), which binds MDM2 and translocates it to the nucleoli, relieving the negative regulation of p53 by MDM2 (36-38).

The stress response pathway that correlates with p53, p14 alternate reading frame (ARF) and MDM2 plays a central role in mediating cellular responses to oncogene activation, and genomic instability, and induces various forms of DNA damage. Recent studies pointed out that invalidation of the pathway results in many kinds of cancer, and may be crucial for tumor progression. One of the triggers of cell-cycle arrest and apoptosis induced by DNA damage is p53. It works as a transcription factor that serves as a point of convergence for various stress signals, such as DNA damage, oncogene activation and hypoxia-induced factor, all of which are common features of cancer cells. Activation of the p53 pathway centers on regulation of p53 protein stability and function (38). MDM2 is a p53 antagonist consisting of a nucleoplasmic and nucleolar really interesting new gene (RING)-finger protein that targets p53 degradation and inhibits p53 transcriptional activity through the proteasome (39). MDM2 also interacts with other tumor suppressor proteins, such as p14ARF, retinoblastoma protein, and promyelocytic leukemia protein (40-45). MDM2 expression is induced by p53 and establishes a feedback mechanism that prevents the protein from accumulating under normal conditions (46).

Several reports demonstrated that NPM actually stabilized p53 through direct interaction with the tumor suppressor and indirect action by inhibition of MDM2 (32, 47). Therefore, we hypothesized that the malignant alteration of tumor might be closely related to the specific ability of NPM in influencing the stabilization of p53 interacting with MDM2.

NPM is known to translocate between the nucleolus and nucleoplasm in response to cytotoxic drugs and genotoxic stress, such as inhibition of RNA polymerase I, DNA intercalating agents, and UV damage (48-50). NPM and MDM2 interact or co-localize increasingly, consequently to divergent cellular stresses, such as UV damage, proteasome inhibition, and expression of apoptosis-inducing viral proteins. Colombo *et al.* reported that high levels of ectopic NPM were seen to activate p53 and trigger p53-mediated growth arrest and cellular senescence in normal mouse embryo fibroblasts (MEFs), while promoting a slight stimulation of growth in *p53*-null MEFs (47). They pointed out that the effect of NPM in suppressing the early stages of tumorigenesis may be related to its role in maintaining genome stability and ensuring DNA integrity (47). Kurki *et al.* reported that p53 stabilization was augmented by rapid UVC-induced nucleoplasmic translocation of NPM and an increase in nucleoplasmic p53–NPM and MDM2–NPM

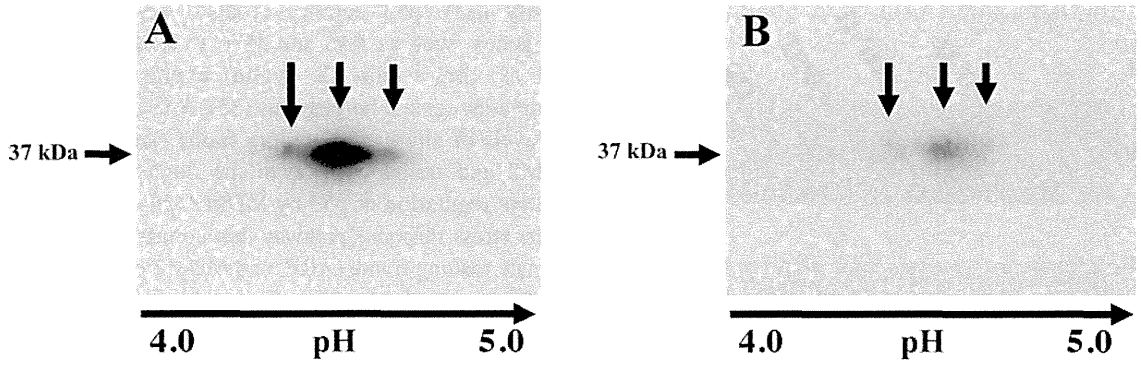

Figure 4. *Two-dimensional western blot analysis of nucleophosmin. Three spots of nucleophosmin can be seen in samples from QR-32 (A) and QRsP-11 (B) cells.*

complexes (32). They pointed-out that the dynamic re-organization of the complexes allows for early p53 stabilization and possibly assists in its modifications, such as sumoylation, commencing the subsequent stabilization effects. High levels of p53 competed for the interaction of NPM and MDM2 and depletion of NPM increased p53–MDM2 complex formation (32).

NPM can bind to p53 and regulate its stability and activity (47). Moreover, Kurki *et al.* reported that NPM tended to form complexes with MDM2 and may be unable to bind to and dissociate pre-formed p53–MDM2 complexes (32). They also indicated that the interaction of NPM with MDM2 was independent of p53 and is reduced *in vitro* by high levels of p53 (32). This suggests competition between p53 and MDM2 for binding sites on NPM.

The multiple interactions of NPM with cellular proteins suggest that it can act as a platform for protein interactions, affect their stabilization, or allow protein modifications to take place. Therefore, NPM could stabilize the complex formation between p53, MDM2, and other proteins needed for post-translational modifications in response to cellular stimulation. NPM might affect p53 stabilization through inhibition of its negative regulator, MDM2. Therefore, the depletion of NPM might cause the regressive tumor cells QR-32 to acquire progressive malignancy.

In this study, we performed 2-DE and MS to identify intracellular proteins expressed differentially in QR-32 and QRsP-11 cells, and we identified the intracellular protein NPM, whose expression differed between these two clones. The expression of NPM was down-regulated in the progressive tumor cells compared to the regressive tumor cells. It is possible that the tumor cells may acquire the malignant phenotypes in the course of progression *via* the dual function in regulation of p53 activity: by inhibiting p53 degradation through NPM interactions with MDM2, and by assembling complexes that are needed for p53 modifications, augmenting its activation and stabilization. Reduction of NPM might cause the stabilization of p53 and result in tumor progression.

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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, α-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 pancreatic 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 μg/ml leupeptin, and 10 μg/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 μA/strip. Protein (80 μg) 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[a]	79	Male	III	Moderately differentiated
2[a]	67	Male	III	Moderately differentiated
3[a]	54	Male	III	Moderately differentiated
4[a]	75	Male	IVb	Poorly differentiated
5[a]	71	Female	III	Well differentiated
6[a]	58	Male	IVb	Mucinous carcinoma
7[a]	70	Female	III	Moderately differentiated
8[a]	64	Male	III	Moderately differentiated
9[a]	61	Male	IVa	Moderately differentiated
10[a]	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

[a] indicates samples were used in 2-DE analysis.

for each 2-DE. Samples were mixed with 200 μl 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.[a]	pI[b]	Mr (Da)[b]	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	α-enolase
6	P04406	8.57	6053.4	1.70	7/10	Glyceraldehyde 3-phosphate dehydrogenase

[a]Accession number derived from the protein database. [b]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 μg/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 μl 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 μm, 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 μl/min for primary flow, otherwise 300 μl/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). 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), α-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 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).

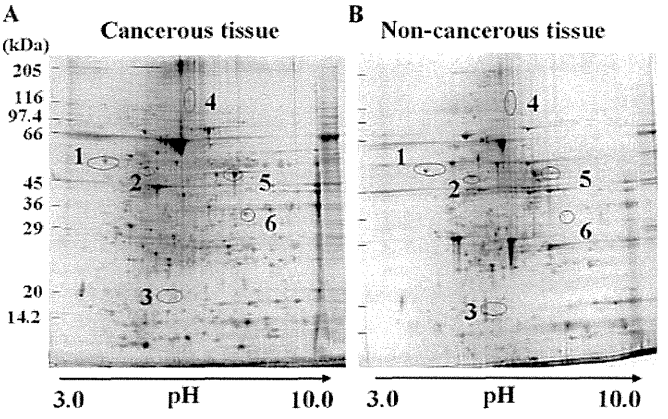

Figure 1. Two-dimensional gel electrophoresis images of pancreatic cancerous and non-cancerous tissues stained with Flamingo™ fluorescent gel stain. Proteins were separated on pH 3-10 linear, immobilized pH gradient strips followed by 5-20% SDS-PAGE. Six spots showed enhanced intensity on gels of cancerous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

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).

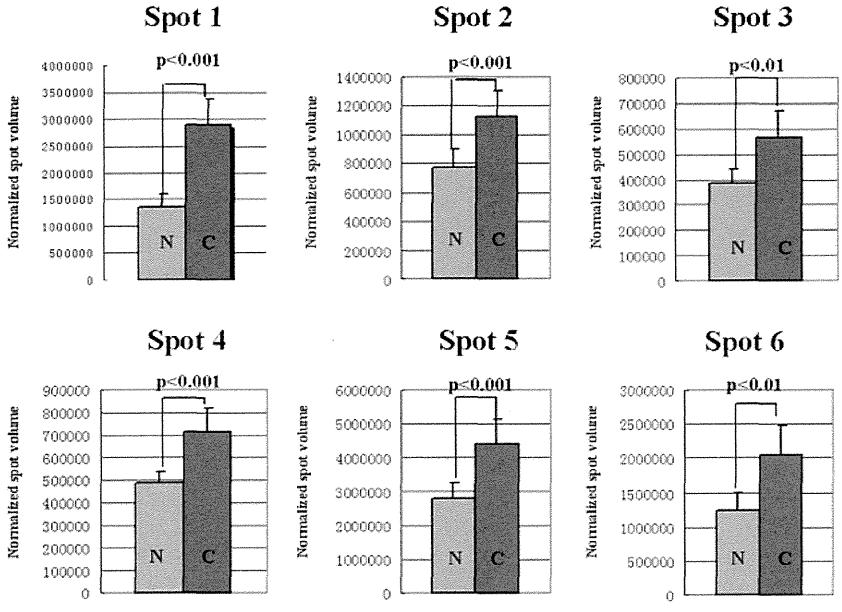

Figure 2. Enhanced protein expressions of carlreticulin (spot 1), glutathione synthetase (spot 2), stathmin (spot 3), vinculin (spot 4), α-enolase (spot 5) and glyceraldehyde-3-phosphate dehydrogenase (spot 6) in pancreatic cancerous tissues. The graphs show the normalized intensity of each spot in cancerous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

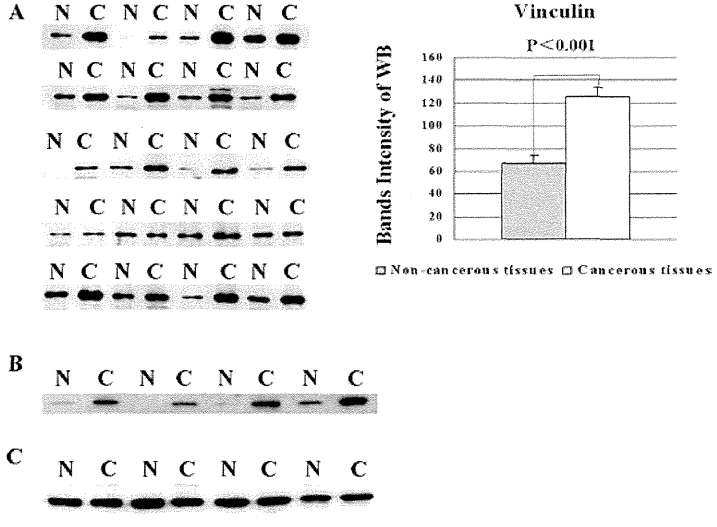

Figure 3. Western-blot analyses of vinculin and α-enolase in pancreatic cancerous and non-cancerous tissues. (A) Tissues from 20 patients with pancreatic cancerous and paired non-cancerous tissues were used for western blotting with anti-vinculin antibody. The expression of vinculin was increased in 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 α-enolase (B) and actin (C) were confirmed by western blotting respectively; the intensity of each band of α-enolase was stronger in cancerous tissues than in non-cancerous tissues. N, non-cancerous tissues; C, cancerous tissues.

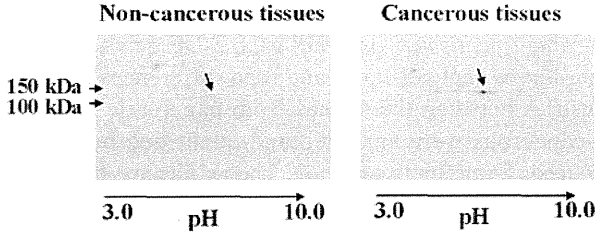

Figure 4. Two-dimensional (2-D) western blotting of vinculin in pancreatic cancerous tissues. Two-dimensional western blotting was performed on a pair of samples on pH 3-10 linear, which confirmed the locations of vinculin on PVDF membranes. The upregulated spot of vinculin was observed in cancerous 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), α-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 α-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 α-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, α-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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Glyoxalase I (GLO1) is Up-regulated in Pancreatic Cancerous Tissues Compared With Related Non-cancerous Tissues

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Abstract. *Background: Glyoxalase I (GLO1), an enzyme involved in the detoxification of methylglyoxal in glycolysis pathway, was found to be frequently overexpressed in various types of cancer. Recent studies showed that GLO1 is related to proliferation and apoptosis in human cancer cells. However, expression of GLO1 in pancreatic cancer (PC) has not been precisely defined. Since PC is one of the most malignant types of cancer, we investigated the level of GLO1 in tissues from patients with PC. Methods: We examined the expression of GLO1 in tumor from patients with PC and adjacent normal tissue by Western blotting. Results: Western blotting demonstrated that GLO1 was significantly overexpressed in pancreatic cancerous tissues compared with adjacent non-cancerous tissues (n=20, p<0.05). Conclusion: GLO1 could be a clinically useful target in therapy of PC.*

Glyoxalase I (GLO1) is a ubiquitous enzyme in all mammalian cells that plays a role in the detoxification of methylglyoxal, tissue maturation and cell death; glyoxalase components, including GLO1 and GLO2, reduce glutathione and transform electrophilic reactive α-oxoaldehydes including methylglyoxal into the corresponding non-cytotoxic α-hydroxy acids (1). Overexpression of GLO1 has been reported in various tumor tissues and cells, including colon, breast, prostate, lung, stomach, ovary, brain and renal cancer (2-5). Moreover, GLO1 was found to be frequently overexpressed in antitumor agent-resistant human leukemia cells, and the overexpression of GLO1 enhances resistance to antitumor agents such as etoposide and adriamycin (6). A recent study showed that GLO1 was related to proliferation and apoptosis in human malignant melanoma (7). Pancreatic cancer (PC) is one of the most malignant types of cancer, and the median survival period is less than 12 months, with an overall 5-year survival rate of less than 5% (8). The mechanisms of rapid spread and high chemotherapy resistance in PC are not completely clear. However, expression of GLO1 in pancreatic cancerous tissues has not been defined. In this study, we investigated the expression of GLO1 in cancerous tissues compared with paired non-cancerous tissues from 20 patients with PC by western blotting.

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Key Words: Western blotting, pancreatic cancer, glyoxalase I.

Materials and Methods

Tissues. Twenty pairs of non-cancerous and cancerous pancreatic tissues were collected from 20 patients with pancreatic cancer (Table I), and resected pancreas at the Department of Surgery II, Yamaguchi University Hospital. None of the patients had received any preoperative therapy. Written informed consent was obtained from all patients before surgery. The study protocol was approved by the Institutional Review Board for human use of the Yamaguchi University School of Medicine.

Sample preparation. Tissues were homogenized in lysis buffer [1% NP-40, 1 mM sodium vanadate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM NaF, 10 mM EDTA, 50 mM Tris, 165 mM NaCl, 10 μg/ml leupeptin, and 10 μg/ml aprotinin] on ice (9-11). Supernatants were incubated for 1 h at 4oC and stored at –80°C until use (12-14). Protein concentration was determined by the Lowry method.

Western blotting. Proteins of samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transfered onto polyvinylidene fluoride (PVDF) membranes at 90 mA for 78 min. The membrances were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk at room temperature for 1 h (15). Membranes were incubated with primary antibody against GLO1 (anti-glyoxalase I mouse polyclonal antibody, diluted 1:1000; Abnova, Taipei, Taiwan) or anti-extracellular regulated protein kinases (ERK1/2) (anti-ERK1/2 rabbit polyclonal antibody, diluted 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight, and then incubated with the secondary antibody conjugated with horse

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Table I. *Clinicopathological parameters of patients with pancreatic cancer included in this study.*

No.	Age (year)	Gender	TNM stage	Tumor grade[a]
1	54	Male	IVa	Moderately differentiated
2	57	Male	IVb	Moderately differentiated
3	54	Male	III	Moderately differentiated
4	74	Female	IVa	Poorly differentiated
5	72	Male	III	Moderately differentiated
6	72	Male	IVa	Well differentiated
7	76	Female	IVa	Moderately differentiated
8	73	Female	III	Papillary carcinoma
9	53	Male	IVb	Well differentiated
10	69	Female	III	Moderately differentiated
11	79	Male	IVb	Mucinous carcinoma
12	34	Male	IVb	Acinor carcinoma
13	71	Female	III	Moderately differentiated
14	67	Female	IVa	Moderately differentiated
15	68	Male	III	Moderately differentiated
16	60	Male	IVb	Moderately differentiated
17	67	Male	IVa	Well differentiated
18	60	Female	III	Moderately differentiated
19	48	Female	IVa	Moderately differentiated
20	73	Male	IVa	Moderately differentiated

[a]Tumor was graded according to the degree of histologic differentiation, as follows: Well differentiation, 5% or less of a nonsquamous or nonmorular solid growth pattern; Moderate differentiation, 6% to 50% of a nonsquamous or nonmorular solid growth pattern; Poor differentiation, more than 50% of a nonsquamous or nonmorular solid growth pattern.

radish peroxidase (1:10,000) for 1 h at room temperature after washing three times with TBS containing Tween-20 and once with TBS. Membranes were then treated with a chemiluminescent reagent (ImmunoStar Long Detection; Wako, Osaka, Japan) and proteins detected by using Image Reader LAS-1000 Pro (Fujifilm Corporation, Tokyo, Japan) (16).

Statistical analysis. Statistical significance was calculated by Student's t-test.

Results

Western blot analysis of GLO1 in tumor from patients with PC and adjacent normal tissue. Twenty pairs of pancreatic cancerous and non-cancerous tissues were analyzed by western blotting with primary antibody against GLO1 and ERK1/2. The protein expression levels were elevated significantly (n=20, $p<0.05$) in cancerous tissues compared with paired non-cancerous tissues (75%) (Figure 1A). The different intensities of the bands between cancerous and non-cancerous tissues were analyzed by Students *t*-test. The intensity of the bands in non-cancerous and cancerous tissue samples was 853.9 and 1598.5 units, respectively (Figure 1B).

Discussion

Overexpression of GLO1 in various types of cancer have been reported (2-5). Research showed that GLO1 may play an important role in malignant transformation, tumor progression, cancer cell survival and resistance to chemotherapeutic agents (6, 17-20). Recent studies indicate that GLO1 may be a useful molecular target for cancer chemotherapy, and pharmacological inhibitors of GLO1 have shown anticancer activity (21, 22). The mechanism of multidrug resistance (MDR) and tumor progression associated with GLO1 overexpression is not fully understood, but it may be linked to increased formation of methylglyoxal by anticancer drugs and related toxicity (23). Overexpression of GLO1 inhibited methylglyoxal-induced tumor growth arrest and toxicity; silencing of GLO1 in cancer cells with high rates of glycolysis and methylglyoxal formation leads to a high level of accumulation of methylglyoxal and cytotoxicity (24). Methylglyoxal induces apoptosis and indirectly stimulates the release of cytochrome *c* from mitochondria and subsequent apoptosis (25-27). Activation of c-Jun *N*-terminal protein kinase 1 (JNK1) and p38 mitogen-activated protein kinases (p38MAPK) may also be involved in GLO1-associated MDR in lung cancer cells (5).

Recently, *GLO1* gene was found to exhibit altered expression in cases of liver metastasis, but not in lymph node metastasis of PC cells (28). This indicates a possible role of GLO1 in PC progression. However, the differences in GLO1 expression between pancreatic cancerous and related non-cancerous tissues have not been defined, although these may be necessary for understanding the mechanisms of rapid spread and chemotherapy resistance of PC (1, 29). In this study, we investigated the expression of GLO1 in tissues from patients with PC. The results indicate that GLO1 was significantly overexpressed (n=20, $p<0.05$) in pancreatic cancerous tissues compared with related non-cancerous tissues (75%). The intensity of GLO1 was more than 1.8-fold increased in PC tissues. These results suggest that GLO1 may play a role in tumor progression and resistance to chemotherapeutic agents of PC. Our study also indicates that GLO1 could be a new clinically useful target for therapy of PC.

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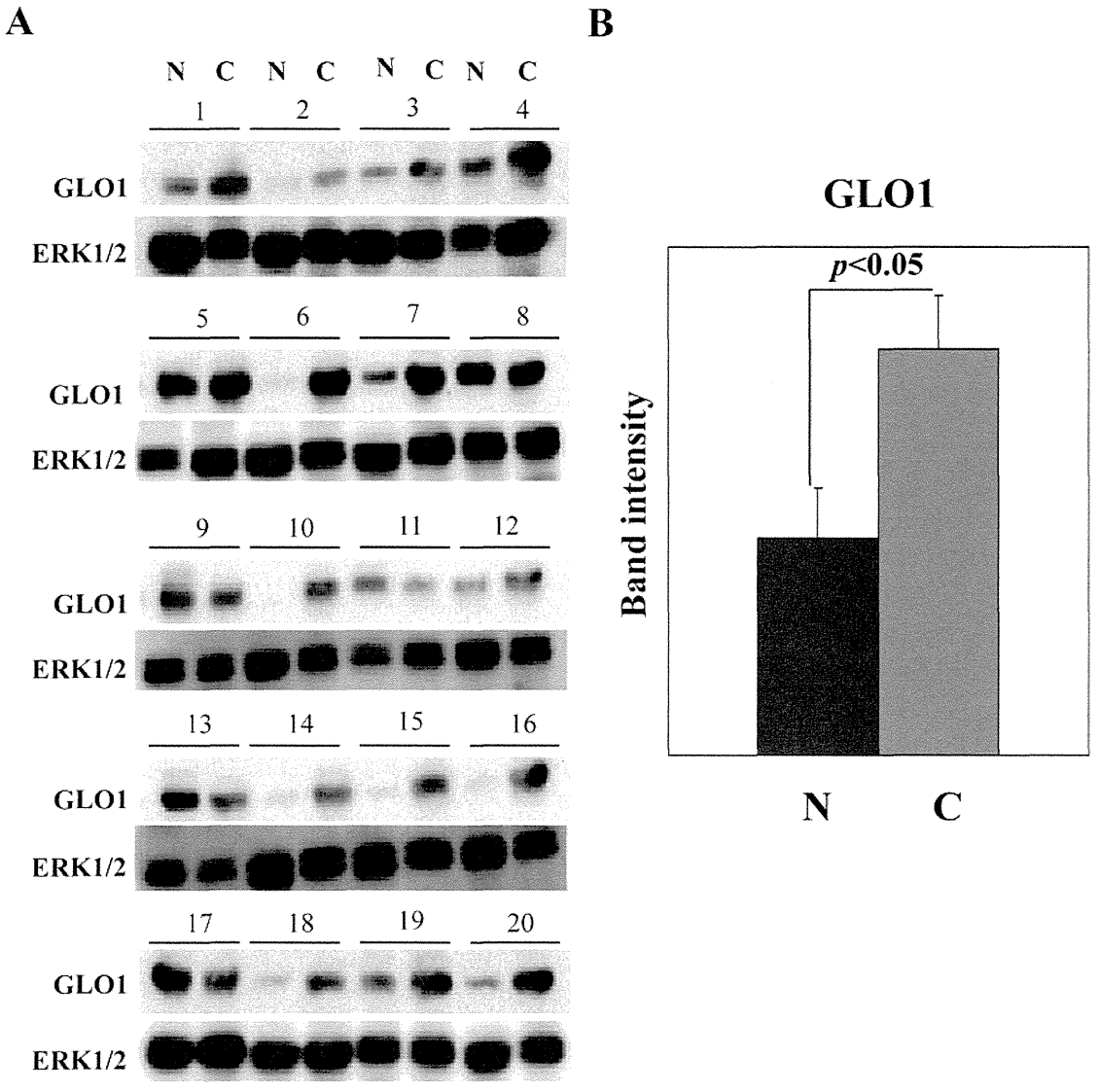

Figure 1. *Western blot analysis of glyoxalase I (GLO1) in PC. A: Tissues from 20 patients with pancreatic cancer (C) and paired non-cancerous tissues (N) were used for western blotting with anti-GLO1 and anti-extracellular regulated protein kinases (ERK1/2) antibody. The expression of GLO1 was confirmed to be increased in pancreatic cancerous tissues (75%). Each patient number (1-20) is the same as that in Table I. B: Comparison of the intensity of the bands between cancerous and non-cancerous tissues by Students t-test (n=20, p<0.05). The relative standard errors (SE) of cancerous and non-cancerous tissues samples were 198.2 and 213.3 units, respectively.*

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Review

Heat-shock Protein 27 Plays the Key Role in Gemcitabine-Resistance of Pancreatic Cancer Cells

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Abstract. *Pancreatic cancer is one of the most fatal types of cancer in developed countries. Most patients have locally advanced or metastatic cancerous lesion when they are diagnosed because of the progressive, invasive and metastatic capacity of this disease to liver, lymph nodes and distant organs during early stages. Although the only curative therapy is complete surgical resection, the disease has usually already progressed by the time of diagnosis, and the majority of patients have metastatic disease. Therefore, palliative chemotherapy remains the only therapy for these patients with progressive disease. Gemcitabine has been used for pancreatic cancer as the most effective anticancer drug. However, there are many cases resistant to gemcitabine. Thus, a better understanding of the molecular mechanisms of resistance to gemcitabine is essential to allow it to be used more effectively. Our previous proteomic studies demonstrated that the expression of heat-shock protein 27 (HSP27) was increased in gemcitabine-resistant pancreatic cancer cells and this might play a role in determining the sensitivity of pancreatic cancer to gemcitabine. Increased HSP27 expression in tumor specimens was related to higher resistance to gemcitabine and shorter survival period in patients of pancreatic cancer. Furthermore, it was shown that treatment strategies combining the HSP inhibitor KNK437 or interferon-γ with gemcitabine were effective for gemcitabine-resistant pancreatic cancer cells in vitro. Furthermore, combined therapy of gemcitabine with interferon-γ (IFN-γ) of gemcitabine-resistant pancreatic cancer-bearing nude mice showed synergistic therapeutic effects on gemcitabine-resistant pancreatic cancer bearers. In this review, we summarize the current understanding of HSP27 and the roles that it plays in gemcitabine resistance.*

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Pancreatic cancer is very aggressive and difficult to diagnose at an early stage, and to treat with effective therapies. Therefore the prognosis of pancreatic cancer patients is still very poor (1, 2). When diagnosed as having pancreatic cancer, most patients have locally advanced or metastatic cancerous lesions and these who survive more than five years are very few. At this point, the surgical resection of all of the tumor tissues is the only curative therapy. However, the number of patients who can be treated by complete surgical resection is very limited. Thus patients with progressive pancreatic cancer have no choice but to depend on palliative chemotherapy.

Gemcitabine (2'-deoxy-2'-difluorodeoxycytidine: Gemzar) is a deoxycytidine analog with structural and metabolic similarities to cytarabine. Gemcitabine is currently the drug of choice to treat patients with advanced pancreatic cancer. The use of gemcitabine is expected to prolong survival of patients with advanced pancreatic cancer. However, intrinsic or acquired resistance of pancreatic cancer impacts the therapeutic effect of gemcitabine (3). Since it is important to understand the molecular mechanisms of resistance to gemcitabine, we have carried out proteomic analysis of gemcitabine-resistant pancreatic cancer cells.

Proteomics is a useful tool to identify proteins which are differently expressed, or have different post-translational modifications or functions. Proteomic differential display is a powerful method to analyze protein expression or carry out post-translational modification profiling of two or more groups. For the quantitative comparison of protein expression among samples, two-dimensional gel electrophoresis (2-DE)

has been commonly used. For characterizing the sequence of the spots, mass spectrometry (MS) has been commonly used. The technique of 2-DE is able to separate proteins according to both their charge in isoelectoric focusing (IEF) gels and their weight in sodium dodecyl sulfate (SDS) gels. Two-DE has unique advantages for examining the expressions of hundreds of proteins simultaneously and for examining post-translational modifications of the protein spots. After in-gel trypsin digestion of the spots, digested peptides are ionized from the sample by matrix-assisted laser desorption/ionization (MALDI) or by electrospray ionization directly from the samples. In MS, ionized peptides are separated on the basis of their m/z and analyzed. For protein identification, peptide-mass fingerprinting and peptide sequencing are usually used. Peptide-mass fingerprinting is usually used for MALDI–time-of-flight (MALDI-TOF) MS and peptide sequencing is used for tandem mass spectrometers (MS/MS).

By using these proteomic technologies our previous studies identified heat-shock protein 27 (HSP27) as a key molecule playing an important role in gemcitabine resistance. We investigated protein expression in gemcitabine-resistant and -sensitive human pancreatic adenocarcinoma cell lines by proteomics. Two-DE showed proteins up-regulated and down-regulated in gemcitabine-resistant cell lines compared with gemcitabine-sensitive cell lines, and these were identified by LC-MS/MS. Three isoform spots of HSP27 on 2-DE were found to be increased in the resistant cell lines compared with sensitive cell lines. The knock-down analysis for HSP27 in KLM1-R pancreatic cancer cells restored sensitivity to gemcitabine, and increased HSP27 expression in tumor specimens was related to higher resistance to gemcitabine in patients with pancreatic cancer (4, 5). Further experiments showed the treatment of KLM1-R cells with interferon-γ (IFN-γ) or the HSP inhibitor KNK-437 down-regulated expression of HSP27 and increased the cytotoxic effect of gemcitabine on gemcitabine-resistant KLM1-R cells (6, 7). The up-regulated isoforms were identified as phosphorylated HSP27 in gemcitabine-resistant pancreatic cancer cells. This suggested that the phosphorylation of HSP27 plays an important role in gemcitabine resistance (8).

Identification of Gemcitabine-resistance-related Protein by Proteomic Differential Display

To identify the gemcitabine-resistance-related protein proteomic differential display by using 2-DE and MS was performed. The protein spots whose expression was different between gemcitabine-resistant and -sensitive pancreatic cancer cells were selected by means of commercial software for 2-DE image analysis. Many kinds of software have been used by researchers. In our study Progenesis PG240 and Progenesis SameSpot (Nonlinear Dynamics Ltd. Newcastle upon Tyne, UK) were used. Both software packages decide spot positions on the gels and measure spot intensities automatically. After statistical analysis for the selected spots, the candidate proteins which may play important roles in gemcitabine-resistance can be obtained. By proteomic differential display analysis, some proteins, including HSP27, were identified. From the candidate proteins, the expression of HSP27 in gemcitabine-resistant and -sensitive cells was investigated, and the results showed up-regulation of HSP27 in gemcitabine-resistant cells compared to gemcitabine-sensitive cells, not only in those with acquired gemcitabine resistance, but also in those which were intrinsically resistant (4).

HSP27 and Drug Resistance

Many reports about HSP27 being an important protein in cancer cell drug resistance have been published. Garrido *et al.* showed that HSP27 inhibited cytochrome *c*-dependent activation of procaspase-9 and prevented etoposide-induced apoptosis (9). Hansen *et al.* reported that HSP27 overexpression inhibited doxorubicin-induced apoptosis in human breast cancer cells by altering the expression of topoisomerase II (10). Richards *et al.* showed that the HSP27-overexpressing human testicular tumor cells were more resistant to cisplatin and doxorubicin, and this was associated with modest increases (17-30%) in population doubling times and a small reduction in the number of S-phase cells (11). Why does HSP27 induce chemo resistance in cancer cells? The cause of HSP27-induced chemo resistance seems to be less the specific inhibition of various anticancer drugs and more the common inhibition of apoptosis. Anti-apoptotic pathways are induced by HSP27, and these pathways lead to cancer cells being resistant to apoptosis. How does HSP27 induce resistance to apoptosis in cancer cells? It was reported that HSP27 protects the cells from apoptosis by associating with death-associated protein 6 (DAXX), truncated BH3 interacting domain death agonist (tBid), Bcl2 associated X-protein (Bax), cytochrome *c*, I-kappa-B kinase (IKK), caspase-3 and others (12, 13). The stress signals which are caused by chemotherapy activate c-Jun N-terminal kinase (JNK). This activation induces the activation of procaspase-9. In turn, active caspase-9 activates procaspase-3. Active caspase-3 catalyzes death substrates, and induces the cells to undergo apoptosis. HSP27 might be one of the inhibitors of this pathway. HSP27 was also reported to interact with Ak-thymoma (Akt), and to increase its stability (14). For chemotherapies which aim at the induction of apoptosis in cancer cells, it is very important to control such factors concerning HSP27.

Effect of HSP27 Inhibition on Chemotherapy

In considering the success of chemotherapy for pancreatic cancer patients, it seems crucial important to control HSP27

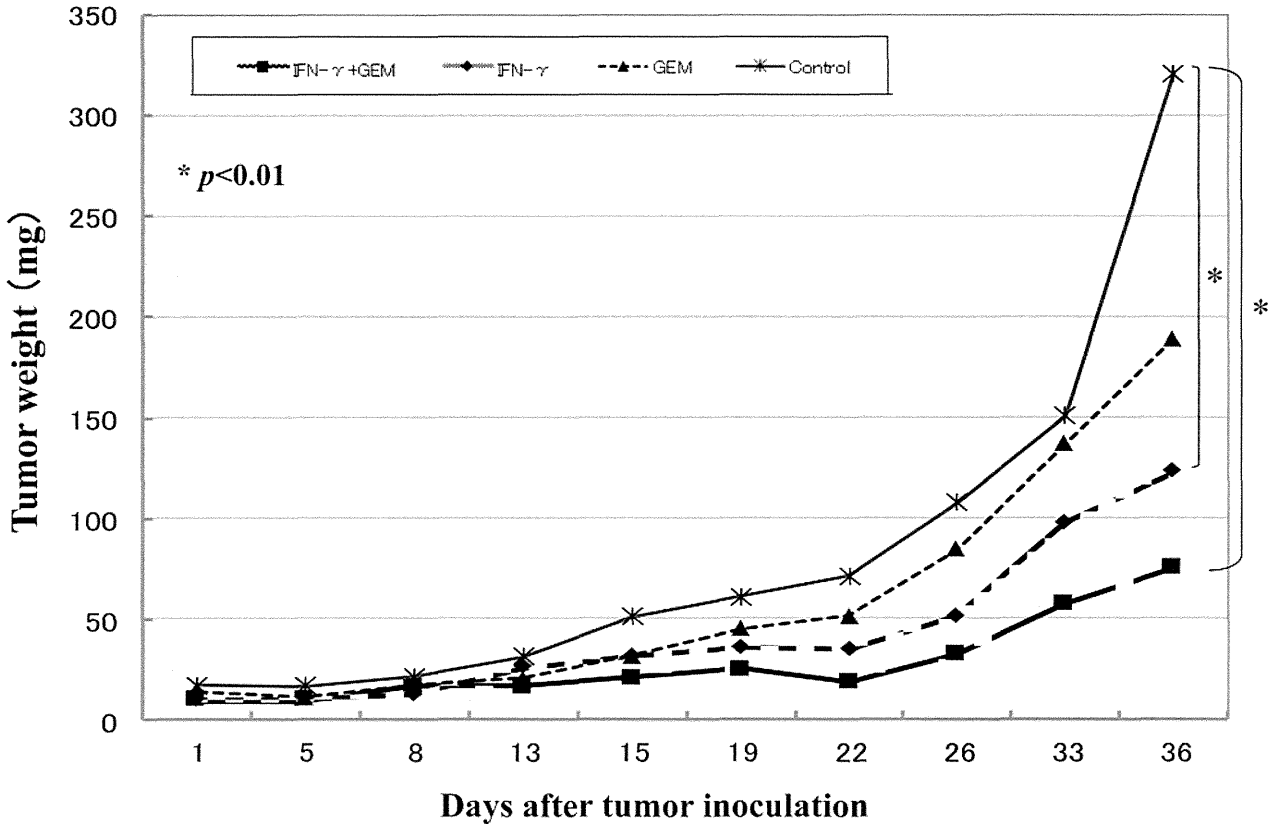

Figure 1. *The growth curves of gemcitabine(GEM)-resistant pancreatic cancer PK59 cells in nude mice treated with or without GEM and/or interferon-γ (IFN-γ). GEM-resistant PK59 cells (1×10^7) were transplanted subcutaneously in nude mice on the day 0, and they were treated with or without IFN-γ and/or GEM. IFN-γ ($1\times105U$) was injected intratumorally into mice twice a week. GEM (80 mg/kg) was injected intraperitoneally once a week. The tumor diameters were measured twice a week. Each group had 5 mice. The graphs show the average tumor weight of the 5 mice from each group.*

expression. Our previous studies on the *in vitro* synergistic effects of gemcitabine and HSP27 inhibitors (siRNA for HSP27, IFN-γ, KNK-437) showed that down-regulation of HSP27 changed the gemcitabine-resistant pancreatic cancer cells to being gemcitabine sensitive. Recently, we performed combined therapy of gemcitabine with IFN-γ for gemcitabine-resistant pancreatic cancer-bearing nude mice in vivo. Gemcitabine-resistant PK59 cells (1×10^7) were transplanted subcutaneously in nude mice on day 0. One group of mice was then treated 1 had 5 mice treated with PBS only; another was treated intraperitoneally with gemcitabine at 80 mg/kg once per week only; another was treated intratumorally with IFN-γ at 1×10^5U twice a week only; and the final group was treated with both gemcitabine and IFN-γ. The tumor diameters were measured twice a week. Figure 1 shows the growth curves of gemcitabine-resistant PK59 cells in nude mice treated with or without gemcitabine and/or IFN-γ. Although the treatment with IFN-γ only had a good suppressive effect on the tumor growth, the combined treatment of gemcitabine with IFN-γ showed significant synergistic effect on the growth curve of gemcitabine-resistant PK59 pancreatic cancer cells. This shows that the combined therapy of GEM and HSP27 suppressor can be expected to be effective chemotherapy of gemcitabine-resistant pancreatic cancer cells.

Some agents and proteins which down-regulate HSP27 have been reported. Tumor necrosis factor-α and IFN-γ are cytokines, and they were reported to down-regulate HSP27 (15, 16). KNK-437 is a benzylidene lactam compound, and this reagent has shown cytotoxic activity towards gemcitabine-resistant cells treated with GEM synergistically (7). Quercetin is one of the most widely distributed bioflavonoids. This flavonoid was reported to inhibit the expression of HSP27 in tumor cells (17). Tanshinone IIA is a phenanthrene quinine extracted from the roots of *Salvia miltiorrhiza* Bunge. This reagent down-regulates expression of HSP27 in cancer cells (18). Triptolide is a diterpene triepoxide from the plant *Triptergium wilfordii*. Westerheide *et al.* showed triptolide abrogated the transactivation function of heat-shock transcription factor HSF1, so it is expected to

use them for combinatorial therapy with gemcitabine in order to down-regulate expression of HSP27, and up-regulate the sensitivity to gemcitabine of pancreatic cancer cells (19).

In order to down-regulate HSP27 expression, we need to try the combined therapy of gemcitabine with HSP27. KRIBB3 {[5-(5-ethyl-2-hydroxy-4-methoxyphenyl)-4-(4-methoxyphenyl) isoxazole]} is a synthetic agent. This agent inhibits HSP27 phosphorylation (20). Since phosphoryration of HSP27 is increased in gemcitabine-resistant cells, inhibition of phosphorylation by using KRIBB3 may be useful for combined therapy with gemcitabine for gemcitabine-resistant pancreatic cancer cells.

Besides the role of HSP27 in drug resistance, increased levels of HSP27 in cancer tissues including gastric, head and neck, renal and prostate cancer have been reported (21-24). What is the role of increased HSP27 expression in cancer tissues? Anti-apoptosis activity of HSP27 is necessary for cancer cells suffering from stress including anticancer drugs, oxidative stress and irradiation. Cancer cells are defended from apoptosis induced by stress by up-regulation of HSP27. HSP27 also has a role in the progression of cancer. Song *et al.* showed increased HSP27 expression in metastatic hepatocellular carcinoma tissues (25). Cancer cells overexpressing HSP27 had increased metastatic capacity (26, 27). On the other hand, HSP27 depletion induces the cells to undergo apoptosis and down-regulates tumor progression in prostate cancer cells (28). Not only mature cancer cells, but also cancer stem cells were reported to show increased HSP27 expression. Wei *et al.* reported up-regulation and phosphorylation of HSP27 in breast cancer stem cells, and silencing of HSP27 in these cells reduced cancer stem cell-like features, including the epithelial–mesenchymal transition (29).

These reported features of HSP27 show that the control of HSP27 is very important in the treatment of cancer cells, not only from the view of gemcitabine-resistance, but also with regard to cancer progression and cancer stem cell-like features.

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