The Ubiquitin-Proteasome Pathway Mediates Gelsolin Protein Downregulation in Pancreatic Cancer

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A well-known observation with respect to cancer biology is that transformed cells display a disturbed cytoskeleton. The underlying mechanisms, however, remain only partly understood. In an effort to identify possible mechanisms, we compared the proteome of pancreatic cancer with matched normal pancreas and observed diminished protein levels of gelsolin—an actin filament severing and capping protein of crucial importance for maintaining cytoskeletal integrity—in pancreatic cancer. Additionally, pancreatic ductal adenocarcinomas displayed substantially decreased levels of gelsolin as judged by Western blot and immunohistochemical analyses of tissue micoarrays, when compared with cancerous and untransformed tissue from the same patients ($P < 0.05$). Importantly, no marked downregulation of gelsolin mRNA was observed ($P > 0.05$), suggesting that post-transcriptional mechanisms mediate low gelsolin protein levels. In apparent agreement, high activity ubiquitin-proteasome pathway in both patient samples and the BxPC-3 pancreatic cancer cell line was detected, and inhibition of the 26s proteasome system quickly restored gelsolin protein levels in the latter cell line. The status of ubiquitinated gelsolin is related to lymph node metastasis of pancreatic cancer. In conclusion, gelsolin levels are actively downregulated in pancreatic cancer and enhanced targeting of gelsolin to the ubiquitin-proteasome pathway is an important contributing factor for this effect.

Online address: http://www.molmed.org
doi: 10.2119/2008-00020.Ni

INTRODUCTION

Pancreatic cancer is one of the most virulent malignances with an overall five-year survival rate of only 3–5% and a median survival time after diagnosis of < 6 months (1). Despite this immense clinical problem, pancreatic cancer biology remains poorly understood in comparison with other cancers. For instance, although it has been recognized for almost 40 years that transformed cells display a disturbed actin cytoskeletal structure, the mechanisms mediating disturbed actin filament organization in pancreatic cancer remain largely obscure.

Among the mechanisms that mediate deviant cytoskeletal structure in cancer cells is an aberrant expression of gelsolin. Gelsolin is a Ca^{2+}- and polyphosphoinositide 4, 5-bisphosphate (PIP_2)-regulated actin filament severing and capping protein (2). Gelsolin was first isolated from rabbit lung macrophages as a modulator of the cytoplasmic actin gel-sol transformation (3), and is generally considered one of the most important regulators of the actin cytoskeleton (4). Thus, gelsolin is an obvious candidate for explaining altered cytoskeletal reorganization in pancreatic cancer. In support for such a notion, diminished expression and even silencing of the gelsolin gene has been documented in many types of human cancers, including bladder, breast, lung, prostate, gastric, and ovarian cancers (5–10). Strikingly, forced expression of gelsolin can cause cancer cell morphological reversion and greatly reduce colony-forming ability *in vitro*, as well as tumorigenicity *in vivo* in various tumor types (11). However, downregulation of gelsolin protein levels in pancreatic cancer has not been demonstrated convincingly, nor have the molecular mechanisms that could mediate such downregulation been identified.

In the present study, we provide convincing evidence that gelsolin protein levels become severely downregulated in pancreatic cancer. This downregulation differs from most other tumor types because the downregulation is not mirrored at the mRNA level, apparently excluding pretranslational mechanisms. In apparent agreement, we see that gelsolin is specifically routed into the ubiquitination-

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Submitted February 15, 2008; Accepted for publication June 6, 2008; Epub (www.molmed.org) ahead of print June 11, 2008.

proteasome system and that inhibition of this system, at least *in vitro*, is sufficient to restore gelsolin protein levels. Thus, downregulation of gelsolin is an intrinsic part of the pancreatic cancerous process which, unique to this type of cancer, seems to be partly mediated by ubiquitin-proteasome machinery.

MATERIALS AND METHODS

Patients and Specimens

Fresh tissue samples of 11 pancreatic ductal adenocarcinomas and their corresponding distant normal counterparts were obtained at the time of resection with informed consent from Cancer Institute and Hospital (CIH), Chinese Academy of Medical Sciences (CAMS), and Peking Union Medical College (PUMC) between November 2001 and March 2003. The samples were snap-frozen in liquid nitrogen before storage at –80° C. None of them received preoperative radiotherapy or chemotherapy. Formalin-fixed paraffin-embedded tissue blocks containing pancreatic cancer and normal pancreatic tissue were collected from the archives of the Departments of Surgery at CIH, CAMS, and PUMC between January 1991 and August 2002, and subjected to tissue microarray construction. The relevant ethical committee approved of the investigations performed.

Cell Culture and Drug Treatment

Human pancreatic adenocarcinoma cell line BxPC-3 was obtained from American Type Culture Collection (Manassas, VA, USA). BxPC-3 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U mL^{-1} penicillin, and 100 μg/mL streptomycin at 37° C in 5% CO_2. Specific proteasome inhibitor lactacystin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO). Cells were seeded on 100 mm plates. After 70% to 90% confluent, cells were treated with 10 μM lactacystin in serum-free medium for the indicated times. Cells were treated with 0.1% DMSO as a vehicle control.

Antibody Microarray Detection Procedure and Data Analysis

Antibody microarrays (BD Clontech 380, Cat. K1847-1, Lot. 2070683) were obtained from BD Biosciences Clontech (Palo Alto, CA, USA), and sample preparation and processing procedures were performed as described in the antibody microarray user manual. Briefly, to reduce the influence of biological heterogeneity among tumor samples derived from different patients, 200 mg from each of seven pancreatic ductal adenocarcinoma samples were pooled together and pulverized using liquid nitrogen. An equivalent pool was made from matched normal pancreas. Total protein was extracted from 200 mg of pooled cancer sample or pooled normal pancreas using extraction/labeling buffer, and then mixed with Cy3 or Cy5 (Amersham Biosciences, Piscataway, NJ, USA) separately. To prevent skewing of the array results, cancer sample (C) and normal sample (N) were each split into two equal portions. Each portion was then labeled with either Cy3 or Cy5 to produce four samples: C-Cy3, C-Cy5, N-Cy3, and N-Cy5. There are two same slides in the kit. C-Cy5 (40 μg) and N-Cy3 (40 μg) were mixed and added to slide 1, and N-Cy5 (40 μg) and C-Cy3 (40 μg) were mixed and added to slide 2. Following 30 min incubation at room temperature, the slides were washed, dried, and scanned using the GenePix 4000B Microaray Scanner (Axon Instruments, Union City, CA, USA). There are 768 spots in this antibody microarray. Each antibody is arrayed by duplicate spots side-by-side on the slide. Besides the antibody capture-dependent spots, there are four paired spots pre-labeled with Cy3 and Cy5 bovine serum albumin (BSA) located near the outermost corners of the printed area that serve as orientation markers. Two paired BSA spots, not labeled with fluorophore, serve as negative controls. Therefore, this slide contains 378 different antibodies.

Primary data collection and analysis were carried out using the GenePix Pro 4.0 (Axon Instruments) software package. The antibody spot was excluded from further analysis if it had a signal: background ratio of < 1.5 in both channels. For each spot, the median of ratios was used in subsequent analysis. The data were imported into the BD Clontech Ab Microarray Analysis Workbook. The relative fluorescence units of C-Cy5/N-Cy3 from slide 1 (ratio 1) and N-Cy5/C-Cy3 from slide 2 (ratio 2) were calculated and an internally normalized ratio (INR) was obtained by computing the square root of ratio 1/ratio 2. This value represents the abundance of an antigen in pancreatic cancer relative to that of normal pancreas. The value of INR > 1.5 or < 0.7 was considered as a significantly different protein expression level between the two samples.

Measurement of Gelsolin mRNA Expression by Semiquantitative Reverse Transcriptase-PCR

Total RNA was isolated from pancreatic cancer specimens (n = 9) and their corresponding distant normal counterparts and cell line BxPC-3 using RNeasy MinElute cleanup kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instruction. RNA quality was assessed on agarose gel electrophoresis and spectrophotometric analysis. Reverse transcription (RT) reactions were performed on 5 μg of total RNA using SuperScript First-Strand synthesis for RT-PCR II kit (Invitrogen, Carlsbad, CA, USA) at 42° C for 80 min, and 0.5–1 μg aliquots of the cDNA then were subjected to RT-PCR. The specific gelsolin primer sequences were used as follows: 5′-CCACTGCGTCGCGGGG-3′ (sense) and 5′-GGCAGCCAGCTCAGCCATG-3′ (antisense). The PCR step was performed using Taq DNA polymerase (Invitrogen). As an internal control, GAPDH was amplified to ensure cDNA quality and quantity for each RT-PCR reaction. Band intensity was quantified using Quantity One 4.4.1 software (Bio-Rad Laboratories Inc, Hercules, CA, USA).

Western Blot

Total tissues (n = 11) and cell lysates were prepared in extraction buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton-100, 0.1% SDS, 1 mM EDTA, 1 mM AEBSF, 20 μg/mL aprotinin, and 20 μg/mL leupeptin. Equal amounts of total protein (10 μg) were separated by 10% SDS-PAGE and transferred to Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking with 5% nonfat dry milk in PBS with 0.1% Tween-20, membranes were probed with mouse anti-gelsolin monoclonal antibody (1:1000 dilution in PBS; BD Biosciences Pharmingen, San Diego, CA, USA), followed by subsequent incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:3000 dilution in PBS, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Visualization of the protein bands was performed by the enhanced chemiluminescence kit (Santa Cruz Biotechnology). Parallel Western blot was probed with an anti-α-tubulin monoclonal antibody (Santa Cruz Biotechnology) as a loading control. Band intensity was quantified using Quantity One 4.4.1 software (Bio-Rad).

Immunohistochemistry

Pancreatic cancer tissue microarray was constructed as previously described (12), with a total of 256 tissue spots consisting of 32 pancreatic ductal adenocarcinomas, 6 mucinous adenocarcinomas, 4 acinar cell carcinomas, 7 islet cell carcinomas, 8 carcinomas of the ampulla of Vater, and 38 normal pancreatic tissues. The streptavidin-peroxidase method was used for the immunostaining of gelsolin. Briefly, after deparaffinization in xylene and rehydration in grade ethanol, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min. Tissue sections then were heated at 100° C in 10 mM pH 6.0 citrate buffer for 10 min to retrieve antigens. After incubation with mouse anti-gelsolin monoclonal antibody, gelsolin immunostaining was detected by adding biotinylated anti-mouse secondary antibody and streptavidin-horseradish peroxidase (Zymed Laboratories, South San Francisco, CA, USA). 3, 3′-Diaminobenzidine was used as a chromogen, and hematoxylin was used for counterstaining.

Immunoprecipitation

Total tissue and cell extracts were lysed with lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 1 mM sodium orthovanadate) containing protease inhibitors 1 mM PMSF, 1 mM AEBSF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. After centrifugation, the clarified supernatant was collected, and protein concentration was determined by Bradford method. Whole cell lysate containing

Table 1. Differentially expressed proteins in pancreatic ductal adenocarcinoma compared with normal pancreas detected by antibody microarray.

Upregulated in pancreatic cancer		Downregulated in pancreatic cancer	
Name	Function	Name	Function
UbcH6 (ubiquitin-conjugating enzyme H6)	E2 ubiquitin-conjugating enzyme	DCC (deleted in colorectal carcinoma)	Tumor suppressor gene
GABA b R2 (γ-aminobutyric acid type B receptor, subunit 2)	G-protein-coupled receptor 51	HPV-16 L1 (human papillomavirus type 16 capsid protein L1)	Human papillomavirus type 16 capsid protein L1
Plakophilin 2a	Diverse roles in desmosomes and β-catenin signaling	RACK1 (receptor for activated C kinase 1)	Receptor of activated PKC
Inhibitor 2	Inhibit the activity of protein phosphatase 1	Gelsolin	Actin filament regulatory protein
Nestin	Intermediate filament protein	Rabaptin-5	Effector of the small GTPase Rab 5
ShcC	Shc family protein, regulation of Ras pathway	DBP2 (DEAD box protein 2)	RNA helicase involved in pre-mRNA splicing
PRK2 (protein-kinase C-related kinase 2)	The Serine/threonine family of protein kinase, PKC subfamily	IKKα/1 (inhibitor of nuclear factor kappa-B kinase α subunit)	Serine/threonine kinase, active NF-kα
Neurogenin 3	Transcription factor, mediate neuronal differentiation	c-Cbl	Proto-oncogene, E3 ubiquitin-protein ligase
STAT 3 (signal transducer and activator of transcription 3)	Transcription activator	FXR2 (fragile × mental retardation syndrome related protein 2)	RNA binding protein
NHE-3 (Na^+/H^+ exchanger 3)	Regulation of Na^+/H^+ homeostasis	SRP54 (signal recognition particle 54 kDa protein)	Route proteins from the cytosol to the ER

10 μg of protein from each sample was prepared for Western blot analysis using anti-gelsolin antibody. For gelsolin immunoprecipitation analysis, 1 ml of cell lysate corresponding to 0.5 mg of total cellular protein first was precleared by incubating with protein G-agarose beads (Roche Diagnostics, Mannheim, Germany) at 4° C for 1 h. The collected supernatant was then incubated at 4° C with 1.5 μg of mouse anti-gelsolin monoclonal antibody or nonimmune mouse IgG (Zhongshan Biotechnology, Beijing, China) for overnight with rotation. The immune complex was precipitated by incubation with 30 μL of protein G-agarose for 3 h at 4° C. The agarose beads were pelleted by centrifugation and washed three times with lysis buffer. The beads were suspended in 2×Laemmli sample buffer and boiled for 10 min. Protein G-agarose beads were removed from the complex by centrifugation at 10,000*g* for 5 min. The supernatant was loaded onto 10% SDS-PAGE for Western blot analysis with monoclonal antibody to gelsolin and ubiquitin (Santa Cruz Biotechnology).

Statistical Analysis

Statistical analysis was performed using the SPSS 11.5 software package (SPSS, Chicago, IL, USA). The Student *t* test was used to determine the statistical significance of Western blot or RT-PCR band intensity. Values were expressed as mean ± SD of at least three experiments. The chi-square test was used to assess the difference of gelsolin expression among different groups scored by immunohistochemistry. The correlation between gelsolin and each variable was assessed with the Spearman Rank correlation test. *P* value of less than 0.05 was considered statistically significant.

RESULTS

Proteomic Chip Analysis

In an effort to obtain insight into the molecular mechanisms that govern aberrant cytoskeletal structure in pancreatic cancer, we compared the holo-proteome of pancreatic cancer tissue to normal tissue of the same patients on antibody capture-based proteomic chips. As this methodology is strongly biased toward highly expressed proteins, it is particularly useful to identify differential expression of cytoskeletal components, which is the most abundant class of proteins in the cellular proteome. Importantly, we did observe significant differences in the protein levels of some cytoskeletal components (Table 1), but gelsolin especially was strongly downregulated in pancreatic cancer. As gelsolin is an obvious candidate to regulate the aberrant cytoskeletal structure, further experiments were initiated to establish whether reduced gelsolin expression is a bona-fide candidate mechanism for explaining disturbed cytoskeletal organization in pancreatic cancer.

The Expression of Gelsolin mRNA and Protein in Pancreatic Cancer Tissues and Cell Line

A supportive role for reduced gelsolin levels in pancreatic cancer includes observations that downregulation of gelsolin mRNA, via various genetic mechanisms, is an important characteristic of many tumor types. Whether gelsolin mRNA also is downregulated in ductal pancreatic cancer had not been investigated, prompting studies into whether the observations made using the antibody capture chips can be confirmed using conventional technology. To detect the alterations of gelsolin mRNA and protein expression, the total RNA and protein were extracted from the pancreatic cancer cell line, pancreatic ductal adenocarcinoma tissues, and their corresponding distant normal pancreatic tissues. Gelsolin mRNA was determined by RT-PCR method with cytoplasm gelsolin gene full-length specific primers (from 96 bp to 2,360 bp). Amplification products were analyzed by 1% agarose gel electrophoresis. Gelsolin mRNA can be detected in these extracts. No significant difference was observed between nine matched pancreatic cancer tissues and normal pancreas ($P > 0.05$). Representative electrophoretic analyses of the RT-PCR products of gelsolin and control GAPDH are shown in Figure 1A and 1B.

Importantly, however, gelsolin protein levels are diminished substantially in ductal pancreatic cancer. Protein extracts from pancreatic tissues and cell cultures were prepared for Western blot analysis using monoclonal anti-gelsolin antibody. This antibody can detect specific bands migrating at 90 kDa. Western blot revealed that 73% (8/11) of pancreatic

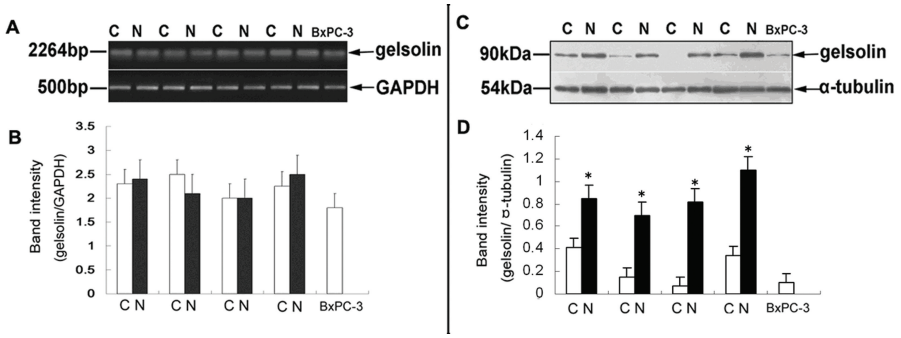

Figure 1. The expression of gelsolin mRNA and protein in pancreatic cancer. (A,C) Representative RT-PCR gel and Western blot. (B,D) Band intensity was quantified using Quantity One software. The expression of GAPDH and α-tubulin was examined as an internal control. Results are expressed as the mean ± SD of three separate experiments. **P* < 0.05, compared with their corresponding cancer samples. C, pancreatic ductal adenocarcinoma tissues; N, corresponding distant normal pancreatic tissues; BxPC-3, pancreatic cancer cell line.

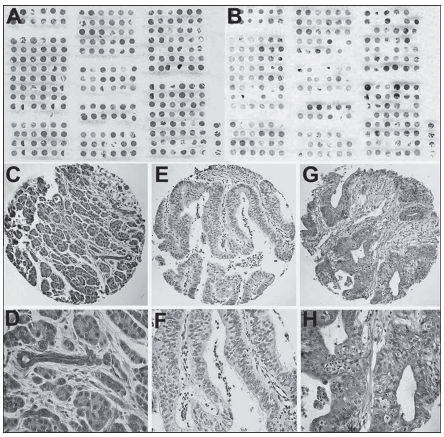

Figure 2. Immunohistochemical analysis of gelsolin expression on pancreatic cancer tissue microarray. (A,B) Overview of the H&E staining and gelsolin immunohistochemical staining on pancreatic cancer tissue microarray. (C) (200×) and (D) (400×) The gelsolin expression in normal pancreas. (E) (200×) and (F) (400×) The gelsolin expression in pancreatic ductal adenocarcinoma without lymph node metastasis. (G) (200×) and (H) (400×) The gelsolin expression in pancreatic ductal adenocarcinoma with lymph node metastasis.

ductal adenocarcinoma tissues showed extremely low or undetectable expressions of gelsolin compared with matched normal pancreatic tissues ($P < 0.05$). There was a weak gelsolin expression in pancreatic cancer cell line BxPC-3 (Figure 1C,1D). Thus downregulation of gelsolin is a feature of pancreatic cancer, but this effect seems to involve a non-classical post-transcriptional effect.

The Correlation of Gelsolin Expression with Clinicopathologic Factors of Pancreatic Cancer

Downregulation of gelsolin protein was confirmed by immunohistochemistry. We carried out immunohistochemical studies for gelsolin on the paraffin-embedded pancreatic cancer tissue microarray (Figure 2). In the normal pancreas, there were diffuse strong cytoplasmic immunostaining for gelsolin in duct cells and acinar cells, but occasionally nuclei appeared to stain. The positive expression rate of gelsolin in normal pancreas was 71% (27/38). In contrast, gelsolin expression in pancreatic adenocarcinoma was reduced significantly ($\chi^2 = 14.41$, $P < 0.0001$), and only 29% (12/42) pancreatic adenocarcinomas were scored as positive for gelsolin expression. There were no obvious differences among ductal adenocarcinoma (31%, 10/32), mucinous adenocarcinoma (17%, 1/6), acinar cell carcinoma (25%, 1/4), and islet cell carcinoma (43%, 3/7) ($P = 0.758$). However, it was found that the positive expression rate of gelsolin in pancreatic ductal adenocarcinomas with lymph node metastasis (71.4%, 5/7) increased markedly ($\chi^2 = 6.28$, $P = 0.02$) compared with negative lymph node types (20%, 5/25). There were no statistically significant correlations between gelsolin expression and histological differentiation of pancreatic ductal adenocarcinoma ($\chi^2 = 0.283$, $P = 0.868$). Further experiments were initiated to investigate the molecular mechanisms mediating downregulation of gelsolin expression in the absence of reduced mRNA levels.

Effect of Proteasome Inhibitor Lactacystin on the Gelsolin Expression in Pancreatic Cancer Cells

An obvious explanation for reduced gelsolin levels in pancreatic cancer despite equal levels of gelsolin mRNA is that gelsolin is targeted efficiently for degradation to proteasome in pancreatic cancer. To test this possibility directly, we employed the BxPC-3 pancreatic cancer cell line as model for *in vivo* pancreatic cancer that is accessible to pharmacologic manipulation by proteasome inhibitors. Importantly, we observed that when the BxPC-3 cell line was treated with specific 26s proteasome inhibitor lactacystin (10 μM), gelsolin expression quickly increased as compared with DMSO vehicle control ($P < 0.05$), and markedly increased gelsolin levels being evident from 12 h of treatment onwards ($P < 0.01$) (Figure 3). Thus it seems that the proteasome is indeed an important factor in pancreatic cancer cells regulating stability of gelsolin proteins.

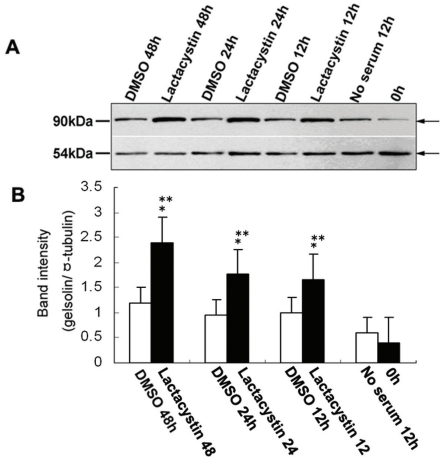

Figure 3. Effect of proteasome inhibitor lactacystin enhances the gelsolin expression. BxPC-3 cells were treated with proteasome inhibitor lactacystin, DMSO or serum-free vehicle control for 12 h, 24 h, or 48 h. (A) After incubation, lysates were prepared and an equal amount of protein (10 μg) from each lysate was analyzed by Western blot for gelsolin. Arrow at 90 kDA indicates gelsolin. Arrow at 54 kDA indicates α-tubulin. (B) Band intensity was quantified by Quantity One software. α-Tubulin was examined as an internal control. Results are expressed as the mean ± SD of three separate experiments. *$P < 0.05$, compared with DMSO controls of each group treated with lactacystin. **$P < 0.01$, compared with 0 h control of each group treated with lactacystin.

Gelsolin Protein Undergoes Ubiquitination in Pancreatic Cancer

Degradation by the proteasome is thought to be triggered by the polyubiquitination of a target protein. To determine whether the proteasome-mediated degradation of gelsolin is accompanied by increased levels of poly-ubiquitinated gelsolin, we investigated the ubiquitination status of gelsolin in extracts of pancreatic cancer cell BxPC-3 treated in the absence or presence of lactacystin (10 μM, 12 h treatment). Upon subsequent immunoprecipitation with an anti-gelsolin antibody, followed by Western blot using monoclonal ubiquitin antibody and gelsolin antibody, polyubiquitinated gelsolin is clearly visible in BxPC-3 cell ex-

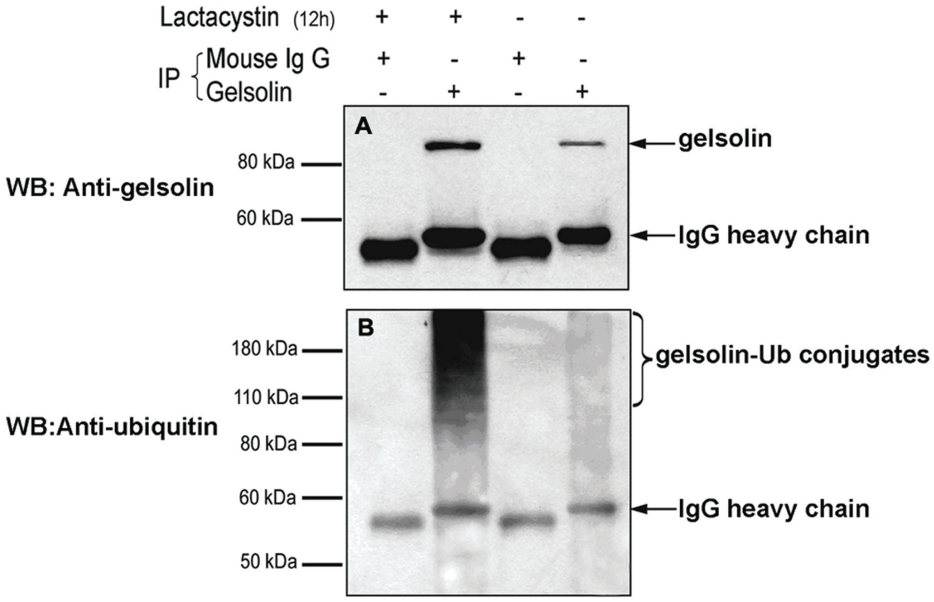

Figure 4. Detection of gelsolin-ubiquitin conjugates. BxPC-3 cells were exposed to lactacystin or DMSO vehicle for 12 h. Equal amounts of whole cell lysates were subjected to immunoprecipitation with anti-gelsolin antibody or nonimmune mouse IgG, followed by Western blot (WB) with anti-gelsolin antibody to detect unmodified gelsolin (A) or with anti-ubiquitin antibody to detect gelsolin-ubiquitin conjugates (B).

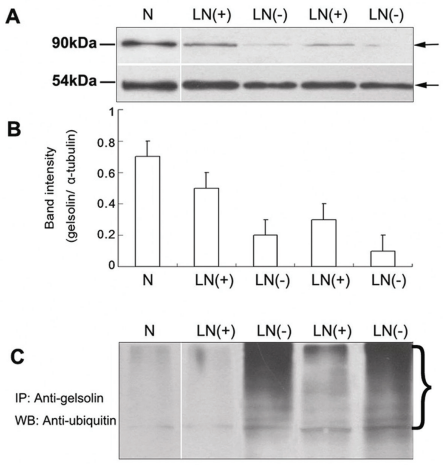

Figure 5. Correlation of gelsolin protein levels with pancreatic cancer lymph node metastasis. (A) Representative Western blot analysis of pancreatic cancer tissues with and without lymph node (LN) metastasis. Arrow at 90 kDA indicates gelsolin. Arrow at 54 kDA indicates α-tubulin. (B) Band intensity was quantified using Quantity One software. α-Tubulin serves as an internal control. (C) Gelsolin protein in the pancreatic cancer tissues with and without lymph node metastasis is immunoprecipitated (IP) by anti-gelsolin antibody, and then Western blot (WB) analyzes the ubiquitin-tagged gelsolin with anti-ubiquitin antibody. Bracket indicates gelsolin-Ub conjugates.

tracts (Figure 4). Thus, the proteasome-dependent degradation of gelsolin in this pancreatic cancer cell is accompanied by a high level of ubiquitin ligase activity toward gelsolin in an experimental system. Subsequently, experiments were initiated to ascertain whether a similar mechanism is operative in pancreatic cancer *in vivo*.

Immunohistochemical results show that gelsolin expression in pancreatic cancer with lymph node metastasis is higher than that observed in negative lymph node metastasis (see above). In apparent agreement, Western blot analysis of fresh pancreatic cancer tissue with and without lymph node metastasis shows re-expression of gelsolin in the advanced lymph node positive phase of the disease. Levels of gelsolin protein, not mRNA, in pancreatic cancer with lymph node metastasis ($n = 3$) are higher than those in negative lymph node metastasis ($n = 8$) ($P < 0.05$) (Figure 5A,5B), corroborating the immunohistochemical results. Strikingly, when we immunoprecipitate gelsolin protein of the pancreatic cancer tissues with and without lymph node metastasis by anti-gelsolin antibody, and analyze its ubiquitin status, we find downregulation of ubiquitin ligase activity toward gelsolin in pancreatic cancers with lymph node metastasis conditions. (Figure 5C). This provides further support for the notion that gelsolin protein levels are controlled by the extent of ubiquitin ligase activity toward gelsolin in pancreatic cancer.

DISCUSSION

One of the most fundamental characteristics of malignant and transformed cells is the aberrant organization of the cytoskeleton (13). The mechanisms which mediate transformation-associated cytoskeletal organization are only partly understood (14). Here we present evidence based on proteomic comparison, immunohistochemistry, and classical biochemistry that diminished expression of gelsolin through a transcription-independent ubiquitination-dependent mechanism is involved. Gelsolin is one of the most important actin structure-regulating proteins, and its expression in almost all eukaryotic cells is testimony to its fundamental importance in maintaining an organized actin cytoskeleton (15,16). The physiological functions of the 90kDa gelsolin protein are regulated by the intracellular Ca^{2+} concentration and by its binding to diphosphoinositides, both of which serve to activate its actin-severing potential, capping the barbed ends, and promoting nucleation of polymerization (17). In many cancers, gelsolin downregulation has been identified as a factor mediating aberrant cytoskeletal organization, substantially reduced levels, for instance, in 77.8% (14/18) of bladder cancers (5), 70% (21/30) of breast cancers (6), 55%

(48/68) of non-small cell lung cancers (NSCLC) (7), 68.1% (15/22) of gastric cancers (9), and 85% (64/75) of ovarian cancers (10). In this study, pancreatic cancer also is added to the growing list of malignancies displaying reduced gelsolin protein levels, gelsolin expression being diminished in 71% (30/42) of the cases of pancreatic cancer as compared with matched control tissue.

Nevertheless, the mechanisms mediating diminished gelsolin expression in different cancer cells are not very clear. It has been reported that there were no major mutations, gross rearrangements, or deletions in the coding sequence of the gelsolin gene in human breast cancer and NSCLC cells (6,7). Recently, Noske *et al*. found inactivation of gelsolin might be mediated by epigenetic modification (methylation and histone deacetylation) in human ovarian carcinoma (10). We observed, however, gelsolin protein, but not mRNA, was downregulated in pancreatic cancer. Thus, an alternative nonclassical mechanism might mediate this downregulation. The high activity of ubiquitin-proteasome machinery toward gelsolin observed in the present study seems to provide an appealing explanation for the gelsolin degradation. To test this hypothesis, we use specific proteasome inhibitor lactacystin to inhibit the ubiquitin-proteasome pathway in the pancreatic cancer cell line. It was found that a proteasome inhibitor could increase the levels of gelsolin protein. Immunoprecipitation results showed that gelsolin conjugated with ubiquitin in pancreatic cancer cells. The ubiquitin-conjugated forms of gelsolin protein were increased after treatment with proteasome inhibitor lactacystin. Taken together, these results indicate that gelsolin protein might be ubiquitinated in pancreatic cancer and degraded by the ubiquitin-proteasome pathway.

Importantly, later in the transformation sequence, gelsolin expression increases again, as suggested by our observation that lymph node positive pancreatic cancers react stronger toward anti-gelsolin antibodies as compared with lymph node negative pancreatic cancers. This result is consistent with an earlier study of Thomson *et al*. (18), who already reported increased gelsolin expression in lymph node positive cancers and would suggest that the expression of the putative gelsolin-specific ubiquitin ligase active in the earlier phases of the transformation process is lost at later stages. In this respect, pancreatic cancer would resemble gastric cancer, where, in earlier stages of the disease, Smad4-specific ubiquitin ligases seem implicated in disease progression, but where their expression is lost at later stages of the disease, possibly as a consequence of the increased genetic instability at these later stages (19). In apparent agreement, Thompson *et al*. reported that high levels of nuclear gelsolin were associated with reduced patient survival times, which was an independent prognostic parameter for pancreatic cancer (18). Thus, reemergence of gelsolin expression would be associated with the most advanced aggressive stages of the disease.

In earlier stages of pancreatic cancer, loss of gelsolin expression may well be important. It was found that transfection of the human bladder cancer cell line UMUC-2 using exogenous full-length gelsolin cDNA inhibited cell growth, reduced the colony-forming ability and the tumorigenicity *in vivo*. The cell cycle of bladder cancer cells was arrested or delayed at the G2/M phase after these cells were transduced with recombinant adenovirus encoding wild-type gelsolin (Ad-GSN) *in vitro*. In the orthotopic bladder cancer model, the mass of the tumor was approximately 90% less in Ad-GSN treated animals than in controls.[11] Thus, for increasing the size of the transformed compartment, lower gelsolin expression may well be an important factor. At later stages, re-expression of gelsolin may help the tumor with forming metastases. In human pancreatic adenocarcinoma cell lines, RNA interference was used to reduce gelsolin protein levels with two gelsolin-targeting small interfering RNAs (siRNAs), GSN 1 and GSN 2. In translocation assays using Boyden-style chambers, GSN 1 and GSN 2 siRNAs significantly reduced cell motility. In wound-healing assays, both GSN 1 and GSN 2 siRNAs significantly impaired the ability of pancreatic cancer cells to migrate into the wounded monolayer (18). These results demonstrated that gelsolin might contribute to the motility of pancreatic cancer cells.

In conclusion, our results show significantly lower levels of gelsolin protein in the early stages of human pancreatic adenocarcinoma. This reduction of gelsolin protein seems dependent on the ubiquitin-proteasome-dependent degradation of gelsolin in the pancreatic cancer cell, defining a novel pathway for such downregulation in cancer. Study of the correlation between proteasome-mediated gelsolin degradation and pancreatic cancer progression should have important clinical significance in the diagnosis, treatment, and prognosis of human pancreatic and other cancers.

ACKNOWLEDGMENTS

We thank Dr. Bing Guo for the sample collection. This work was supported by the 10th Five-year National Key Technologies R&D Program (Grant 2004BA703B11) and the National Natural Science Foundation of China (Grant 30500582).

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