

Role of lumican in cancer cells and adjacent stromal tissues in human pancreatic cancer

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Abstract. Lumican is a member of a small leucine-rich proteoglycan family and its overexpression has been reported in carcinoid tumor, breast, colorectal, neuroendocrine cell, uterine cervical and pancreatic cancers. The expression of lumican in stromal tissues in breast cancer is associated with a high tumor grade, a low estrogen receptor expression level and young age. Lumican expression in the cytoplasm in advanced colorectal cancer is correlated with a poor prognosis. Lumican expression was previously reported in pancreatic cancer, but the role of lumican in pancreatic cancer is still not well understood. In this study, we aimed to clarify the role of lumican in pancreatic cancer. Reverse-transcription polymerase chain reaction and Western blot analyses revealed lumican mRNA and protein expression in six pancreatic ductal adenocarcinoma cell lines (i.e. PANC-1, MIA PaCa-2, KLM-1, Capan-1, PK-1 and PK-8). On the basis of its immunoreactivity, lumican was found to be localized in islet cells of normal pancreatic tissues, but not in exocrine cells. In pancreatic cancer tissues, lumican was predominantly localized in the cytoplasm of cancer cells in 30 out of 53 (56.6%) cancer patients, whereas lumican was detected in stromal tissues in 36 out of 53 (67.9%) cancer patients. Lumican expression in pancreatic cancer cells did not correlate with clinicopathological factors, whereas lumican expression in stromal tissues correlated with the female gender, advanced stage, retroperitoneal and duodenal invasion and residual tumor ($p=0.030$, 0.038 , 0.049 , 0.049 and 0.048 , respectively). Patients with lumican-positive cancer cells tended to survive longer than those with

lumican-negative cancer cells ($p=0.286$), but patients with lumican-positive stromal tissues had shorter survival than those with lumican-negative stromal tissues ($p=0.062$). These results suggest that lumican in stromal tissues plays an important role in the growth and invasion of pancreatic cancer.

Introduction

Members of the small leucine-rich proteoglycan (SLRP) family have small core proteins of approximately 40 kD and possess 6-10 leucine-rich repeating units flanked by disulfide-bonded domains and the presence of chondroitin sulfate or keratan sulfate (1-4). Several studies have demonstrated that SLRP proteins can modulate cellular behavior, including cell migration and proliferation during embryonic development, tissue repair and tumor growth, in addition to their extracellular matrix function as regulators of tissue hydration and collagen fibrillogenesis (5-7). The SLRP family is classified into four groups and class II of the SLRP family consisting of fibromodulin, keratocan, osteomodulin, proline arginine-rich end leucine-rich repeat protein (PRELP) and lumican (8).

Human lumican has 338 amino acids, including a putative 18-residue signal peptide and its gene is located on chromosome 12q21.3-q22. The lumican core protein contains a central region of leucine-rich repeats flanked on either side by a disulfide-bonded domain and the central region of the molecule possesses four asparagine residues capable of participating in N-linked glycosylation (9,10). Lumican is the major keratan sulfate proteoglycan of the cornea and was reported to colocalize with fibrillar collagen and regulate the assembly and diameter of collagen fibers (4). Lumican is considered a key regulator of collagen fibrillogenesis, a process critical to corneal transparency. Lumican is also distributed in interstitial collagenous matrices in the skin, lungs, intervertebral disks, skeletal muscle, kidneys, intestine, brain, placenta, heart, aorta, liver and pancreas (10-15). Mice that are homozygous for null mutation in lumican show corneal opacification and skin laxity due to inhomogeneous collagen bundles (16,17).

In cancer cell proliferation, most cancer cell types exhibit various grades of fibrosis around cancer nests. Fibrous tissues adjacent to cancer cells mainly consist of collagen fibers and

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fibroblasts and are considered to affect cancer cell proliferation, migration and spread (18). The expression of SLRP family in fibrous tissues around cancer cells was reported in colon and breast cancers (19-21). In colon cancer cells, decorin, a member of the SLRP family, was reported to suppress colonic cancer cell proliferation (22,23). The decorin expression level was reported to be low in human breast cancer tissues, but lumican is overexpressed in the same tissues (20). Furthermore, a high expression level of lumican in breast cancer tissues is associated with a high tumor grade, a low estrogen receptor (ER) expression level and young age (19). Lumican mRNA is expressed specifically in breast cancer tissues, but not in normal breast tissues, suggesting that lumican is differentially expressed during breast tumor progression (19). These findings suggest that lumican in stromal tissues, adjacent to cancer cells play an important role in cancer cell growth.

In colorectal, uterine cervical and pancreatic cancers, we previously reported lumican expression in cancer cells and adjacent stromal tissues (15,24,25). However, little is known about the role of lumican in human pancreatic cancer tissues. Therefore, in this study, we examined the correlations of lumican expression in pancreatic cancer cells with clinicopathological factors and prognosis. Furthermore, we examined the correlations of lumican expression in stromal tissues with clinicopathological factors and prognosis. Here, we report that lumican protein expression in pancreatic cancer cells did not correlate with clinicopathological factors, but stromal lumican expression correlated with the female gender, advanced stage, retroperitoneal and duodenal invasion and residual tumor. Patients with lumican-positive stromal tissues have shorter survival than those with lumican-negative stromal tissues.

Materials and methods

Materials. The following were purchased: RNeasy minikit from Qiagen GmbH (Hilden, Germany); Takara RNA PCR kit (AMV) Ver. 3.0 from Takara Biotech. (Tokyo, Japan); a goat anti-rabbit IgG-HRP secondary antibody from American Qualex (San Clemente, CA, USA); IPVH membrane and Immobilon western chemiluminescent HRP substrates from Millipore (Yonezawa, Japan); M-PER Mammalian Protein Extraction reagent from Pierce (Rockford, IL, USA); Histofine Simple Stain Max PO (R) kit from Nichirei Biosciences Inc. (Tokyo, Japan); superfrosted slides with a MAS coat from Matsunami Glass Ind., Ltd. (Osaka, Japan); malinol mounting medium from Muto Chemical Co. (Tokyo, Japan); antibiotics and antimycotics from Invitrogen Co. (Carlsbad, CA, USA); Human Tissue Microarray 1 and Human Digestive Tissue Sets from Novagen (Darmstadt, Germany). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Pancreatic cancer cell lines. Pancreatic ductal adenocarcinoma cell lines (PDAC), including PANC-1, MIA PaCa-2, KLM-1, PK-1 and PK-8, were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Capan-1 was purchased from American Type Culture Collection (ATCC). The cells were grown in RPMI-1640

medium containing 10% heat-inactivated fetal bovine serum (FBS), 200 U/ml penicillin, and 200 μ g/ml kanamycin at 37°C in a humidified 5% CO₂ atmosphere. Capan-1 was grown in the same medium containing 15% FBS.

Pancreatic cancer tissues. Tissues from 53 patients with invasive PDAC were obtained for this study. These patients received treatment at Nippon Medical School Hospital (Bunkyo-ku, Tokyo, Japan) from 1995 to 2003. None of the patients received preoperative chemotherapy and radiotherapy. The patients consisted of 36 males and 17 females, whose median age was 64 years (range, 35-84 years). The clinicopathologic stage was determined according to the TNM classification system of the International Union Against Cancer (UICC) and additionally characterized on the basis of the Japanese Pancreas Society classification (Table I). The median follow-up period was 14.1 months. Paraffin-embedded specimens were prepared for immunohistochemistry as previously described (26,27). This study was carried out in accordance with the principles embodied in the Declaration of Helsinki, 1975 and the informed consent for the usage of pancreatic tissues was obtained from each patient. Normal pancreatic tissues were obtained from Human Digestive Tissue Sets and Human Tissue Microarray 1.

Reverse-transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from pancreatic cancer cell lines using the RNeasy minikit according to the manufacturer's instructions. Then, cDNA synthesis and PCR were performed using the Takara RNA PCR kit. The primer pair used for lumican corresponded to nucleotides (nts) 659-678 (5'-TGA-TCT-GCA-GTG-GCT-CAT-TC-3') and nts 1042-1064 (5'-GAA-ATA-CTC-ATC-AGG-GAT-GTT-GC-3') (406 bp, accession No. NM_002345). β -actin mRNA, as the positive control, was amplified using the following primer pairs: nts 254-273 (5'-AAG-AGA-GGC-ATC-CTC-ACC-CT-3') and nts 452-471 (5'-TAC-ATG-GCT-GGG-GTG-TTG-AA-3') (218 bp, accession No. NM_001101). PCR was carried out in a Takara PCR thermal cycler MP (Takara, Tokyo, Japan) for 2 min at 94°C, followed by 35 cycles, each consisting of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C. The authenticity of the PCR product was confirmed by the direct sequence method. Total RNA not subjected to reverse transcription was used as the negative control.

Western blot analysis. Protein extraction was performed in accordance with the protocol for the use of the M-Per Mammalian Protein Extraction reagent. Briefly, cultured pancreatic cancer cells were solubilized in the M-Per reagent with Protease Inhibitor Cocktail. Lysates were centrifuged for 10 min at 13,000 rpm to pellet cell debris. The resulting supernatants were collected and protein concentration was measured by the Bradford method. The cleared protein lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and the separated proteins were transferred to Immobilon P transfer membranes. The membranes were immersed in 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) for 60 min at room temperature (RT), then incubated for 16 h at 4°C with an anti-lumican antibody (dilution, 1:800). The

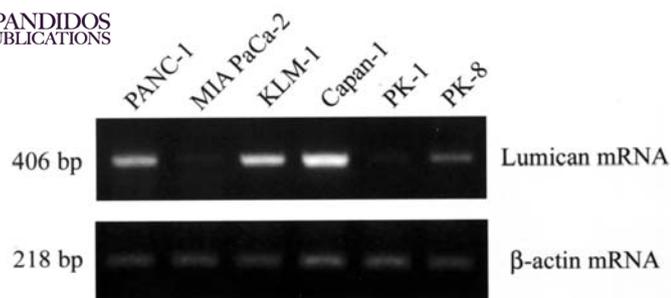


Figure 1. RT-PCR analysis of lumican mRNA in PDAC cell lines. Total RNA was extracted from PANC-1, MIA PaCa-2, KLM-1, Capan-1, PK-1 and PK-8 cells and then cDNA synthesis and PCR were performed. Lumican and β -actin mRNAs were detected as bands of 406 and 218 bp (upper and lower panels, respectively).

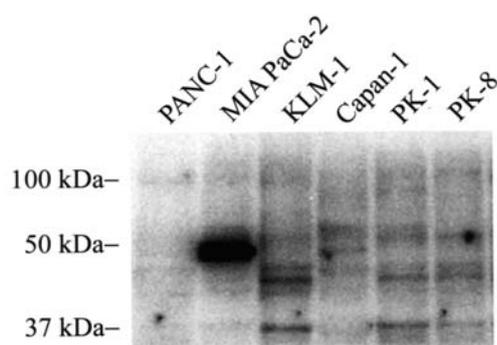


Figure 2. Western blot analysis of lumican in PDAC cell lines. In six PDAC cell lines, 37- to 100-kD lumican protein fragments are detected.

anti-lumican antibody used in this study was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids of human lumican (13). The membranes were washed and incubated with the HRP-conjugated anti-rabbit IgG antibody (dilution, 1: 4000) for 60 min. After washing, the blots were visualized using enhanced chemiluminescence and detected with a ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). To confirm the almost equal loadings of the proteins, the membranes were washed and reprobred with an anti- β -actin antibody.

Immunohistochemistry. Paraffin-embedded tissue sections (3.5 μ m) were immunostained using the Histofine Simple Stain Max PO (R) kit. After deparaffinization, endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 min and the sections were incubated with the anti-lumican antibody for 20 h at 4°C (dilution, 1:500) using PBS containing 1% bovine serum albumin (BSA). The anti-lumican antibody used was the same antibody employed in Western blot analysis. Bound antibodies were detected with Simple Stain Max PO (R) reagents using diaminobenzidine-tetrahydrochloride (DAB) as the substrate, and the sections were counterstained with Mayer's hematoxylin. Negative control studies were performed by omitting the primary antibodies. The immunohistochemical results for lumican in cancer cells were evaluated as follows:

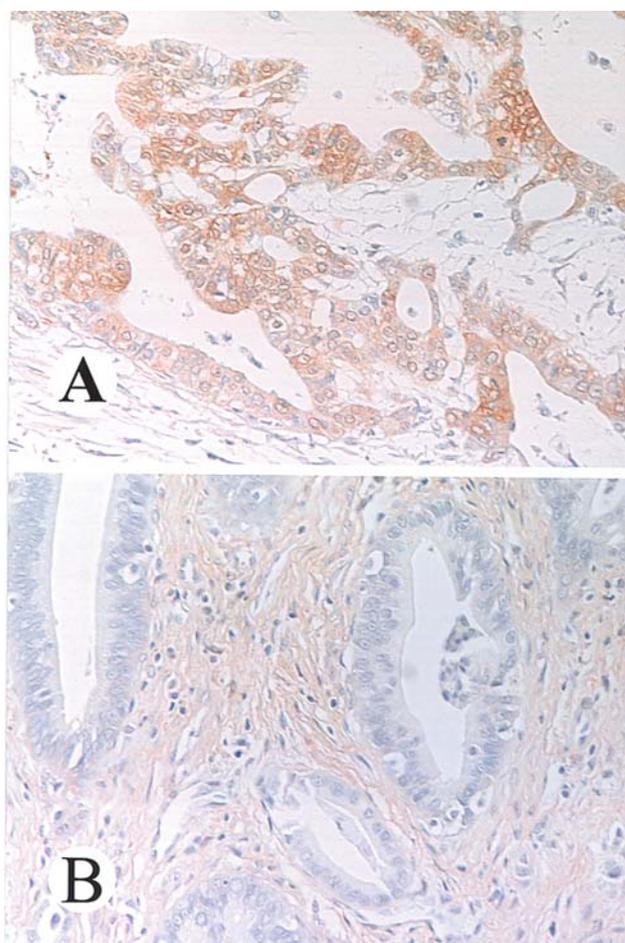


Figure 3. Characteristic staining pattern of lumican in PDAC tissues. In some patients, lumican is strongly expressed in the cytoplasm of PDAC cells, but not in adjacent stromal tissues (A). Lumican is predominantly localized in stromal tissues adjacent to cancer cells, but not in the cytoplasm of cancer cells (B). A, B: immunohistochemistry. Original magnification: A, B; X200.

when staining was noted in the cytoplasm of more than 30% of tumor cells, regardless of the intensity of staining, the cells were designated as lumican-positive. The immunohistochemical results for stromal tissues, which are adjacent to cancer cells, were also evaluated as positive or negative for lumican. Two investigators (T.I. and K.C.) separately evaluated all the specimens in a blinded manner.

Results

RT-PCR analysis of lumican mRNA in PDAC cell lines. To examine the expression of lumican mRNA in human cultured PDAC cell lines, RT-PCR analysis was performed. Lumican mRNA and β -actin mRNA, which were observed as bands of 406 bp and 218 bp, respectively, were detected in PANC-1, MIA PaCa-2, KLM-1, Capan-1, PK-1 and PK-8 cells (Fig. 1, upper and lower panels, respectively).

Western blot analysis of lumican in PDAC cell lines. To characterize lumican in PDAC cells, total protein was extracted from PANC-1, MIA PaCa-2, KLM-1, Capan-1, PK-1 and PK-8 pancreatic cancer cells. Western blot analysis

Table I. Correlation of clinicopathological factors with lumican expression in pancreatic cancer.

Variable	Lumican expression		P
	Negative (n = 23/53, 43.4%)	Positive (n = 30/53, 56.6%)	
Gender			0.823
Male	16	20	
Female	7	10	
Age mean \pm SD (range)	60.6 \pm 12.1 (35-78)	66.5 \pm 9.8 (50-84)	0.058
UICC classification			
T - primary tumor			0.765
1	1	2	
2	1	2	
3	8	7	
4	13	19	
N - Regional lymph nodes			0.472
0	7	12	
1	16	18	
M - Distant metastasis			0.848
0	22	29	
1	1	1	
G - Histological grading			0.491
1	12	19	
2	11	10	
3	0	1	
4	0	0	
Stage			0.643
I or II	5	5	
III or IV	18	25	
Other tumor characteristics			
Lymphatic invasion			0.872
Negative	2	3	
Positive	21	27	
Venous invasion			0.635
Negative	16	19	
Positive	7	11	
Nerve invasion (intrapancreatic)			0.402
Negative	6	5	
Positive	17	25	

of the proteins from these cells using an anti-lumican antibody showed that lumican was expressed in these cancer cells (Fig. 2). Several forms of lumican were reported to be localized in human tissues, which differ in the type of their glycosylated chains whose sizes range from ~37 to 100 kDa (15,24).

Immunohistochemistry of lumican in pancreatic cancer tissues. To confirm the localization of the lumican protein in

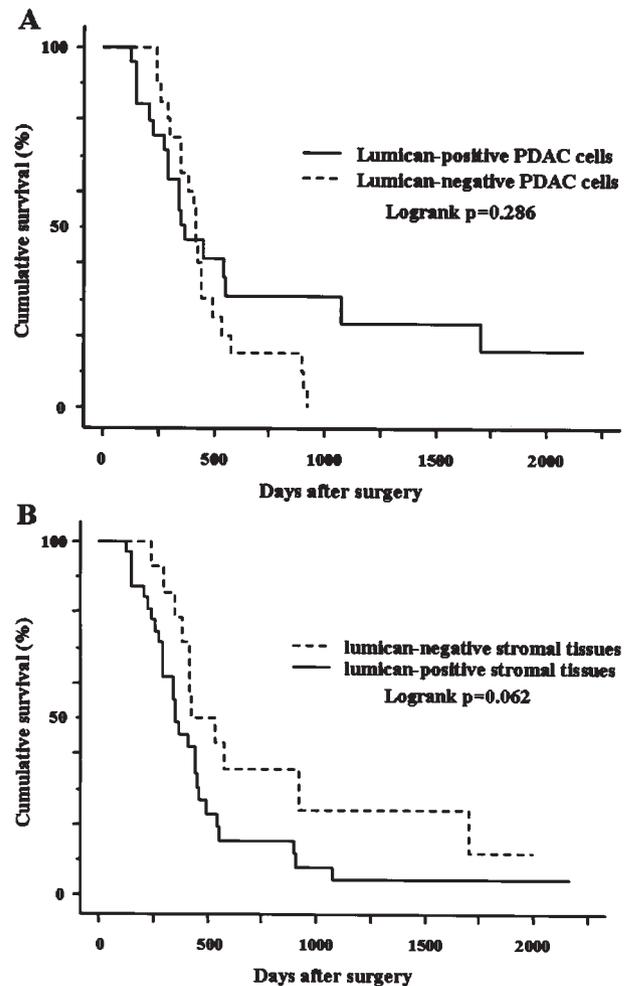


Figure 4. Curves for all patients with lumican-positive or lumican-negative cytoplasm of PDAC cells (A, $p=0.286$). Curves for all patients with lumican-positive or lumican-negative stromal tissues adjacent to PDAC cells (B, $p=0.062$).

human pancreatic cancer tissues, immunohistochemical analysis was performed. In normal pancreatic tissues, lumican was detected in alpha cells of islets, as previously reported (15). Lumican was localized in pancreatic cancer cells and adjacent stromal tissues. Lumican was expressed in the cytoplasm of pancreatic cancer cells in 30 out of 53 cancer patients (Fig. 3A, 56.6%). On the other hand, lumican was localized in stromal tissues, which is adjacent to cancer cells, in 36 out of 53 cancer patients (Fig. 3B, 67.9%).

Correlation of lumican expression in cancer cells or stromal tissues with clinicopathological factors. Lumican expression in pancreatic cancer cells did not correlate with any clinicopathological factors (Table I), whereas that in stromal tissues significantly correlate with the female gender, advanced stage, retroperitoneal and duodenal invasion and residual tumor (Table II).

Survival of patients with lumican-positive or lumican-negative pancreatic cancer cells and stromal tissues. Lumican expression in pancreatic cancer cells tended to correlate with

SPANDIDOS PUBLICATIONS Correlation of clinicopathological factors with lumican expression in pancreatic cancer.

Variable	Stromal lumican expression		P
	Negative (n = 17/53, 32.1%)	Positive (n = 36/53, 67.9%)	
Gender			0.030
Male	15	21	
Female	2	15	
Age mean + SD (range)	61.2±9.6 (45-78)	65.1±11.7 (35-84)	0.222
UICC classification			
T - primary tumor			0.068
1	0	3	
2	3	0	
3	7	8	
4	7	25	
N - Regional lymph nodes			0.242
0	8	11	
1	9	25	
M - Distant metastasis			0.322
0	17	34	
1	0	2	
G - Histological grading			0.912
1	10	21	
2	7	14	
3	0	1	
4	0	0	
Stage			0.038
I or II	6	4	
III or IV	11	32	
Other tumor characteristics			
Retroperitoneal invasion: RP			0.049
Negative	10	8	
Positive	7	28	
Duodenal invasion: DU			0.049
Negative	12	15	
Positive	5	21	
Residual tumor: R			0.048
0	11	13	
1	6	22	
2	0	1	

longer survival (Fig. 4A, $p=0.286$). In contrast, lumican expression in stromal tissues tended to correlate with shorter survival (Fig. 4B, $p=0.061$).

Discussion

We previously reported lumican expression in MIA PaCa-2 and PK-8 cells (15). In addition to the cancer cell lines, four PDAC cells also expressed lumican mRNA and protein.

Different molecular weights of lumican in the cancer cell lines were detected, indicating that pancreatic cancer possesses many types of glycosylated side chain, as previously described (15). Five colorectal cancer cell lines and three uterine cervical cancer cell lines were also reported to express lumican mRNA and protein (24,25). These cancer cell lines express lumican, but their original cells, including pancreatic duct cells, colorectal epithelial cells and uterine cervical epithelial cells, do not express lumican. A recent study has shown that lumican is expressed and secreted by melanoma cell lines, but not by normal melanocytes (28). The role of newly synthesized lumican in cancer cell lines is still not clearly elucidated. Lumican-transfected B16F1 mouse melanoma cells are characterized by a strong suppression of their anchorage-independent proliferation and capacity to invade the extracellular matrix gel *in vitro* (29). After subcutaneous injections of transfected B16F1 cells to syngenic mice, lumican expression significantly suppresses subcutaneous tumor formation *in vivo*, with a concomitant decrease in cyclin D1 expression level. Moreover, lumican induces or enhances the apoptosis of B16F1 cells. Our previous report showed that a decreased expression level of lumican in HEK 293 cells induces the growth of the cells (30). Neuroendocrine tumors possess numerous neuroendocrine granules and are classified into carcinoid tumors and neuroendocrine carcinoma (NEC) (31-33). Carcinoid tumors are slow-growing and not highly metastatic, whereas NECs are aggressive, easily metastasize to distant organs and have a poor prognosis. The lumican expression level was higher in the cytoplasm of carcinoid tumors than in that of NECs, but the expression level of lumican in stromal tissues was not significantly different from that in the stromal tissues of NECs (34). Lumican expression in the cytoplasm of pancreatic cancer cells was detected in 56.6% of cancer patients and it did not correlate with clinicopathological factors, including tumor size and local and distant metastases. Furthermore, patients with lumican expressed in pancreatic cancer cells tended to survive longer. These lines of evidence suggest that the ectopic expression or overexpression of lumican in epithelial cells, including cancer cells does not correlate with aggressive cell growth or plays a role in growth inhibition.

In breast cancer tissues, lumican mRNA is overexpressed in fibroblasts adjacent to cancer cells, but not in cancer cells (19,20). An enhanced lumican deposition was observed in the collagenous stroma of tumors, particularly at the margins of invasive tumors. A high expression level of stromal lumican is associated with a high pathological tumor grade, a low estrogen receptor level in cancer tissues and young age (19). On the other hand, a high expression level of lumican in breast cancer was reported to be associated with a slow progression and a better prognosis (35). In this study, lumican expression in stromal tissues correlated with the female gender, advanced stage, retroperitoneal and duodenal invasion and residual tumor and tended to correlate with a poor prognosis. Fibrosis of various degrees is observed in stromal tissues close to cancer cells. Collagen fibers are the main component of fibrosis and lumican was reported to regulate the assembly and diameter of collagen fibers (4). A recent study showed that SLRPs, including lumican interacting at the surface of either type I or type II collagen fibrils, can decrease the susceptibility

of collagen fibers to both MMP-1 and MMP-13 cleavage (36). Lumican in stromal tissues, adjacent to cancer cells may modulate the characteristics of collagen fibers and induce the invasion activity of pancreatic cancer cells.

In pancreatic cancer, the lumican expression level in stromal tissues was significantly higher in female patients. In breast cancer tissues, a higher expression level of lumican in stromal tissue correlated with a lower ER expression level in cancer cells (19). During pregnancy, ER mRNA and lumican mRNA expression levels in the cartilage are significantly high in multiparous rabbits compared with those in the control cartilage (37). ER mRNA expression level does not change during pregnancy in primigravida rabbits, whereas lumican mRNA expression level decreases. These findings may indicate the correlation between lumican expression in stromal tissues and female hormones. To elucidate the relationship between lumican and estrogen or progesterone, further study of female-hormone-related cancer cells, including breast cancer and endometrial cancer cells is required.

In summary, lumican expression in stromal tissues correlated with the female gender, advanced stage, retro-peritoneal and duodenal invasion and residual tumor in pancreatic cancer. Furthermore, stromal lumican expression in pancreatic cancer tended to correlate with a shorter survival. These results suggest that lumican in stromal tissues plays an important role in pancreatic cancer cell growth and invasion.

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