慢性膵炎における膵石形成機序の解明

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はしがき

膵石は慢性膵炎の特徴的な病態であり、また膵管内圧の上昇とそれに伴う膵腺房細胞の破壊をひきおこす。そこで、膵石形成機序を解明することは、慢性膵炎の病態生理を明らかにするとともに、膵石形成抑制や膵石溶解などの治療法への応用が期待できる。膵液中に分泌されているPancreatic stone protein(PSP)は、５種類のisoformとして膵液に分泌されているが、その中でPSP-S2,S3,S4,S5の４種のisoformはin vitro での炭酸カルシウム析出を抑制する。膵石の主成分が、炭酸カルシウムであることからPSP S2-5の分泌低下が膵石形成を促進するのではないかと推察されているが、PSP S2-5にたいする特異抗体が得られていないために、一定の見解は得られていない現状である。本研究ではPSP S2-5の特異抗体を作成し、膵液中のPSP-S2-5の分泌低下 が慢性膵炎における膵石の形成に関与することを明らかにした。

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研究発表

（１）学会誌等

○Kazushi Yamadera, Kaori Wada, Manabu Goto, Kazunori Yokoyama, Yukari Morita, Yohei Kitano, Isao Makino. Quantification of Human Lithostathine S2-5 Forms Using the Antibody to the N-terminal Peptide Region.

Pancreas（投稿中）

（２）口頭発表

○K.Yamadera, K.Arai, I.Makino

The development of a specific antibody to the N-terminal undecapeptide of the lithostathine

American Pancreatic Association、平成６年１１月３日

○山寺一司、荒井謙一、横山和典、後藤学、牧野勲

ヒト膵液中の活性型lithostathine(LS)の検出

第81回日本消化器病学会総会、平成７年５月９日

○山寺一司、横山和典、後藤学、牧野勲

Dot ELISAによるヒト膵液中の活性型lithostathine(LS)の測定

第37回日本消化器病学会大会、平成７年１１月５日

○ K.Yamadera, K.Kitano, M.Goto, K.Wada, D.Koike, I.Malino

Quantification of lithostathine in human pancreatic juice by a specific antibody to the N-terminal undecapeptide

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○山寺一司、北野陽平、和田佳織利、後藤学、横山和典、牧野勲

慢性膵炎における膵液中の活性型Lithostathine(LS)濃度

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Quantification of Human Lithostathine S2-5 Forms Using the Antibody to the N-terminal Peptide Region

Kazushi Yamadera, Kaori Wada, Manabu Goto, Kazunori Yokoyama, Yukari Morita, Yohei Kitano, Isao Makino

（*Pancreas*　投稿中）

Summary

Lithostathine S2-5 inhibits in vitro crystal growth of $CaCO_3$. We developed an antibody against the peptide region responsible for inhibitory effect to determine whether lithostathine S2-5 levels are different in the pancreatic juice of patients with or without chronic pancreatitis. The antibody against the synthetic peptide of N-terminal end of lithostathine S2-5 detected lithostathine S2-5, but not lithostathine S1 or lithostathine extracted from pancreatic calculi. Lithostathine S2-5 was detected in samples of pancreatic juice protein by immunoblotting using the specific antibody. The concentration of lithostathine S2-5 was compared between control and chronic pancreatitis groups. The mean concentrations of lithostathine S2-5 were significantly($p=0.002$) lower in chronic pancreatitis, 16.3 μg/mg of total protein, than in the control, 47.1 μg/mg of total protein. Decreased concentration of lithostathine S2-5 seems to increase the risk of stone formation in the ducts during the course of chronic pancreatitis because of insufficient inhibition of $CaCO_3$ crystal growth.

Key words: Lithostathine, Chronic pancreatitis, Pancreatic juice.

Introduction

Lithostathine was originally isolated from pancreatic stones(1), and was later shown to be a protein secreted from the acinar cell along with other pancreatic enzymes(2). Lithostathine is a physiological pancreatic juice protein that inhibits stone formation(3). However, various levels of lithostathine have been found in pancreatic juice of patients with chronic pancreatitis. In some studies lithostathine concentrations were low in chronic pancreatitis(3,4), but in other studies it was normal(5,6). Therefore, the role of lithostathine in the pathogenesis of stone formation in chronic pancreatitis is uncertain.

Lithostathine S2-5, which is a glycoprotein consisting of 144 amino acids, is the secretory form of lithostathine(7,8). The 11 amino acids of the N-terminal end of lithostathine S2-5 are in the region of the protein responsible for its inhibitory effect on in vitro $CaCO_3$ crystal growth(9). Lithostathine S1 is the protein remaining after removal of the 11 amino acids of the N-terminal end from lithostathine S2-5. The antibodies that were used to detect lithostathine S2-5 in previous studies recognized both lithostathine S1 and S2-5(4,6).

To clarify the role of lithostathine during the course of chronic pancreatitis it would seem to be more logical to assess lithostathine S2-5 levels in pancreatic juice. The aim of the present work was to use a specific antibody against N-terminal end of S2-5 to determine whether the lithostathine S2-5 levels are lower in the pancreatic juice of patients with chronic pancreatitis.

Methods

Pure Pancreatic Juice

On separate days from ERP, samples of pancreatic juice were collected by endoscopic cannulation of main pancreatic duct after a bolus intravenous injection of secretin, 100 IU, for 10 min with the first sample from the first 2-min, the second from the next 3-min, and the third from the last 5-min(10). Pancreatic juice was collected into tubes each containing 0.1 ml of trypsin inhibitors(3000 U/ml trasylol, 50 mM benzamide, 1 mg/ml soybean trypsin inhibitor, 10 mM phenylmethylsulfonyl fluoride, 5 mM FOY305) immersed in ice, and immediately frozen in liquid nitrogen and kept in -70 ^{o}C until use.

Pancreatic juice collected during the first 2-min was not used for lithostathine assay because of the possibility of activated proteolytic enzymes. To determine the presence of proteolytic activation, 10 μg protein of each of the second and the third samples was analyzed by SDS-PAGE with 10 to 20% SDS gradient slab gel(Daiichi Pure Chemicals, Tokyo, Japan) followed by Coomassie blue staining. Proteolytic activation was confirmed by absence of protein band identical to trypsin. Only samples devoid of proteolytic activation were used for lithostathine assay. Protein concentration was measured by bicinchoninic acid(BCA) protein assay reagent(Pierce, Rockford, IL, USA).

Production of Specific Antibody Against the N-terminal End of Lithostathine S2-5

The 11 amino acids of N-terminal end of lithostathine S2-5, previously determined as 5-oxoPro-Glu-Ala-Gln-Thr-Glu-Leu-Pro-Gln-Ala-Arg (8), was synthesized chemically, but without the glycan reside on the threonine(Nova biochem, Switzerland). Synthetic peptide was coupled to keyhole limpet hemocyanin(KLH) or bovine serum albumin(BSA) by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide(11). Antiserum against synthetic peptide/KLH conjugate was produced in two rabbits according to standard protocol of immunization to produce antipeptide antiserum(11). Indirect enzyme-linked immunosorbent assay, which was performed by synthetic peptide/BSA conjugate as antigen, was used to determine antipeptide antibody titer(11).

Immunoblotting of Lithostathine

Immunoblotting analyses of lithostathine S2-5 were performed using the modification of the method previously described(12). After electroblotting, membranes(Immobilon-P, Millipore, MA, USA) were blocked with 0.05% Tween20-PBS(T-PBS) containing 5% skim milk for 12 h at room temperature. The membranes were then incubated either with rabbit anti-synthetic N-terminal end of lithostathine S2-5 polyclonal antibody diluted 1 to 2,500 or with commercial mouse anti-pancreatic stone protein monoclonal antibody(Immunotech S.A., Marseille, France) diluted 1 to 750 with T-PBS for 1 h at room temperature. After washing membranes with T-PBS, they were incubated either with anti-rabbit Ig-digoxigenin(Boehringer Mannheim Biochemica, Mannheim, Germany) after using rabbit polyclonal antibody or with anti-mouse Ig-digoxigenin(Boehringer Mannheim Biochemica) after using mouse monoclonal antibody for 30 min at room temperature. The bands on the membrane were detected on the X-ray film by using DIG luminescent detection kit(Boehringer Mannheim Biochemica) according to the instructions.

Purified human lithostathine S2-5 and lithostathine S1 were kindly provided by Dr. Dagorn(INSERM, Marseille, France). Pancreatic stone protein, which is lithostathine extracted from pancreatic stone, was purified from pancreatic calculi obtained from patients with chronic calcifying pancreatitis using the HPLC method previously described(13).

Lithostathine Assay in Pancreatic Juice

Lithostathine S2-5 was detected in 10 μg samples of pancreatic juice protein by SDS-PAGE separation followed by immunoblotting using anti-synthetic N-terminal end of lithostathine S2-5 polyclonal antibody. The density of the bands were determined by scanner and densitometric analysis software, NIH image. A standard curve, which was determined by the density of the bands from different amounts of purified lithostathine S2-

5 in the gel, was used to calculate the amount of lithostathine S2-5 in each sample. After lithostathine S2-5 concentrations were measured in each of the second 3-min and the third 5-min fractions of pancreatic juice, the higher value was used as lithostathine S2-5 concentration for each individual subject. Lithostathine S2-5 levels were expressed as μg per mg of total pancreatic protein because of large variations in the protein concentration of samples collected from different subjects. Lithostathine concentrations expressed as μg per ml were also estimated.

Patients

The diagnosis of chronic pancreatitis was based on clinical and endoscopic retrograde pancreatography(ERP) findings. The grading of pancreatogram change was Cambridge III in all patients with chronic pancreatitis(14). The six chronic pancreatitis patients included three women and three men with mean age 56.7 years(range 50-67 years). The etiology of chronic pancreatitis was alcoholic in three patients and idiopathic in three. Two patients of alcoholic had visual pancreatic calcifications on plain films of the abdomen. No patients had steatorrhoea. The control group included six women and two men with mean age 51.4 years(range 27-66 years). Subjects in control group were nonalcoholic patients without pancreatic disease, all showing normal ERP findings. The diagnosis of subjects was biliary stones in six patients and adenomyomatosis of the gallbladder in two.

Statistical Analysis

Lithostathine S2-5 concentration from control and chronic pancreatitis patients was compared using a Student's t test for unpaired data. P values <0.05 were considered statistically significant.

Results

Specific Detection of Lithostathine S2-5 by Using the Antibody Against the Synthetic N-terminal End

By using immunoblotting, we compared the immunological specificity of the polyclonal antibody against synthetic N-terminal end of lithostathine S2-5 with the specificity of the monoclonal antibody against pancreatic stone protein(Figure 1). Monoclonal antibody detected each purification of lithostathine S1 and pancreatic stone protein as a single band with molecular weight of 13,500, and purified lithostathine S2-5 as a different molecular weight range of 15,500 to 19,000. Also, protein bands corresponding to the molecular weight of purified lithostathine S2-5 were detected in pancreatic juice of two control subjects with the monoclonal antibody. In contrast, the antibody against the synthetic N-terminal end of lithostathine S2-5 detected purified lithostathine S2-5, but not lithostathine S1 or pancreatic stone protein. In each pancreatic juice obtained from control subjects this polyclonal antibody detected proteins bands with identical molecular weights to those of purified lithostathine S2-5. These findings demonstrated that the polyclonal antibody specifically recognized the N-terminal end of lithostathine S2-5.

Lithostathine S2-5 Level in Pancreatic Juice

In the lithostathine assay, the density increased in a dose dependent manner when 0.1 to 1.0 μg purified lithostathine S2-5 was applied into the gel(Figure 2). Similarly, the density also increased in a dose dependent fashion when 0.01 to 0.1 μg purified lithostathine S2-5 was applied into the gel(data was not shown). The bands corresponding to the molecular weight of purified lithostathine S2-5 were specifically detected when pancreatic juice protein was examined(Figure 3). Lithostathine S2-5 concentrations were measured in the individual gels with a standard curve obtained from 0.1, 0.5 and 1.0 μg purified lithostathine S2-5 applied to lanes different from juice samples. When lithostathine S2-5 concentrations were found to be less than 0.1 μg, a second application of samples was performed using the same standards of 0.01, 0.05 and 0.1 μg.

Intraassay and interassay coefficients of variation of this assay were 6.5 and 13.5%, respectively.

The values of lithostathine S2-5 levels expressed as μg per mg of total protein were 47.1±13.2 in the control and 16.3±16.2 in chronic pancreatitis patients(mean ±SD, Figure 4). Lithostathine S2-5 levels in pancreatic juice were significantly lower in chronic pancreatitis than in controls($p=0.002$). Among patients with chronic pancreatitis, there was no difference of lithostathine S2-5 concentrations between alcoholic pancreatitis and idiopathic pancreatitis. The lithostathine S2-5 levels of two patients with chronic pancreatitis were within the range of the control group. Lithostathine S2-5 concentrations expressed as μg per ml were 63.8±47.1 in the control and 13.3±15.3 in chronic pancreatitis patients(mean±SD, $p=0.04$), similar to the results expressed as μg per mg of protein.

Discussion

The conflicting results of lithostathine levels in previous investigations may be due to the use of different antibodies which recognized different regions of lithostathine. In all previous studies that measured the concentration of lithostathine in pancreatic juice by immunological method(3,4,5,6), the antibodies used recognized regions of lithostathine other than the N-terminal peptide region(4,6). Therefore, both lithostathine S2-5 and lithostathine S1 might have been measured in those studies, resulting in different findings of lithostathine concentration. In present study, we developed and used an antibody which distinguished lithostathine S2-5 from lithostathine S1.

Also, inconsistent results might have been due to the proteolytic activation in pancreatic juice samples which was induced by inadequate collection or storage. Because lithostathine S2-5 is extremely sensitive to trypsin(15,16), it is important to examine the presence of tryptic activation in pancreatic juice protein samples. Trypsin activity in the samples could not be measured in our assay because of adding trypsin inhibitors to the juice. As the cleavage of the 11 amino acids of N-terminal end of lithostathine S2-5 is dependent on trypsin and not chymotrypsin(15), measuring chymotorypsin activity may not be sufficient to detect transformation of lithostathine S2-5 to lithostathine S1. In present study we used SDS-PAGE analysis instead of trypsin or chymotrypsin activity to detect proteolytic changes in pancreatic juice. We used both the second sample from the second 3-min and the third sample from the third 5-min, and took the higher value as the lithostathine S2-5 concentration for an individual subject.

Our mean value of 47 μg/mg of total protein lithostathine S2-5 is in agreement with the value of 17(16) or 35(17) obtained by HPLC. Lithostathine S2-5 is approximately 5% of pancreatic juice protein in human, a smaller proportion than the 14% of previous findings using different estimation(18). Lithostathine S2-5 level decreases approximately 35% of physiological levels in chronic pancreatitis. This finding agrees with previous findings that messenger RNA level of lithostathine was three times lower in pancreas of chronic calcifying pancreatitis than in control(19). Specific reduction of messenger RNA

level seems to cause concentrations of lithostathine in chronic pancreatitis.

Because lithostathine is a secretory protein responsible for inhibition of $CaCO_3$ crystal growth in pancreatic juice, which is physiologically supersaturated with calcium and bicarbonate ion(20), the decreased concentration of lithostathine S2-5 probably promotes stone formation in chronic pancreatitis. However, it is difficult to conclude that stone formation in chronic pancreatitis is completely dependent on decreased levels of lithostathine S2-5. In the present study one patient with chronic calcifying pancreatitis showed relatively high amounts of lithostathine S2-5. Moreover, recent findings demonstrate that a low level of secretory lithostathine was observed in alcoholic patients who did not have chronic pancreatitis(16). Thus, although lithostathine S2-5 must be a important factor for stone formation, decreased lithostathine S2-5 concentration may not be sufficient by itself to account for stone formation during the course of chronic pancreatitis.

Finally, in the present work we determined the specific concentration of lithostathine in pancreatic juice of patients with chronic pancreatitis by using the antibody against the peptide region responsible for the inhibitory effect on in vitro $CaCO_3$ crystal growth. The weak inhibition of $CaCO_3$ crystal growth secondary to low levels of lithostathine may explain, in part, the development of stone formation in the pancreatic juice of patients with chronic pancreatitis.

Acknowledgments

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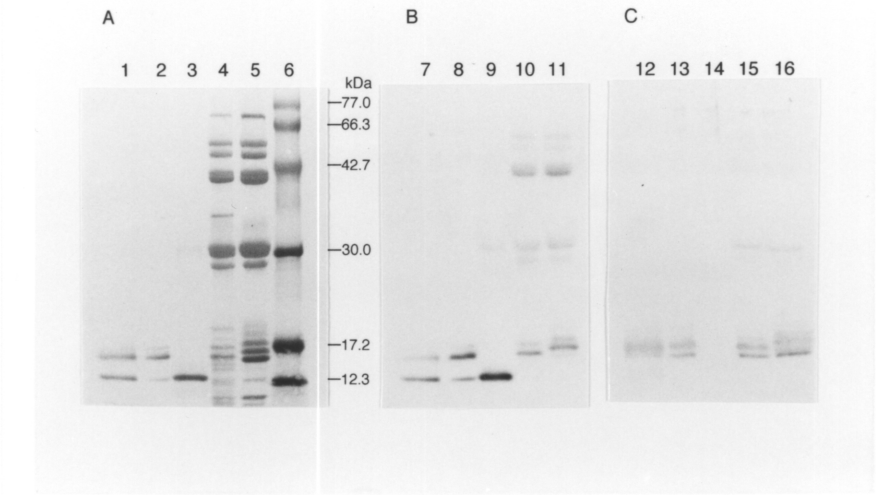
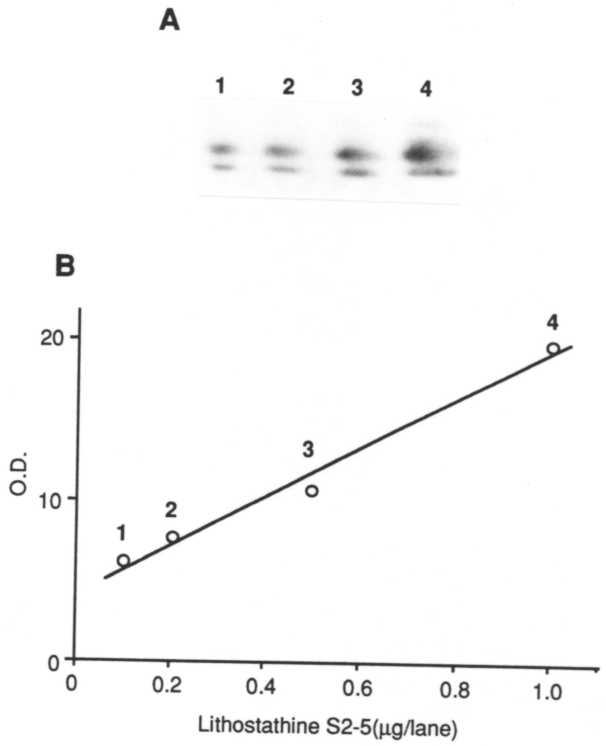
Figure 1. Determination of the specificity of the anti-synthetic peptide of N-terminal end of lithostathine S2-5 antibody.



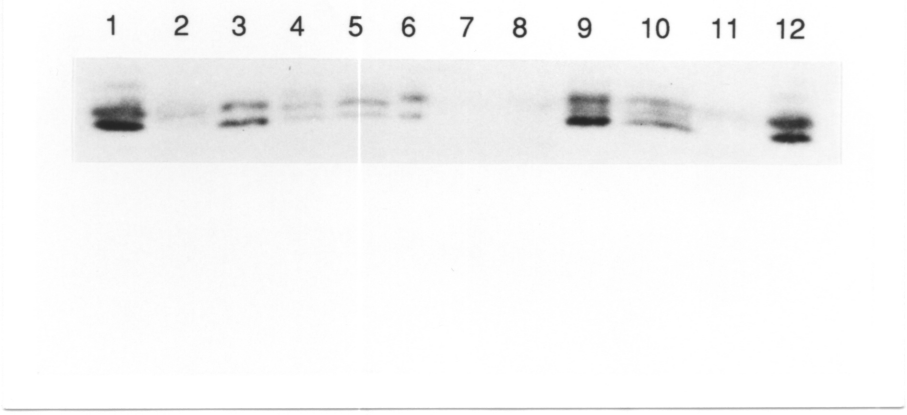
(A)SDS-PAGE followed by protein staining with Coomassie blue. (B)Immunoblotting analysis with commercial monoclonal antibody against the pancreatic stone protein. (C)Immunoblotting analysis with polyclonal antibody against the synthetic N-terminal end of lithostathine S2-5. Lane 1, 7 and 12, purified human lithostathine S1(2.3 μg). Lane 2, 8 and 13, purified human lithostathine S2-5(0.5 μg). Lane 3, 9 and 14, pancreatic stone protein(2.5 μg). Lane 4, 10 and 15, human pancreatic juice of control subject(15 μg), case 1; lane 5, 11 and 16, human pancreatic juice of control subject(15 μg), case 2. Lane 6, molecular weight markers. In lane 1, the trace contamination of lithostathine S2-5 was found in purified lithostathine S1. In lane 7, each band of lithostathine S1 and contamination of lithostathine S2-5 as shown lane 1 was detected in purified lithostathine S1. In lane 12, detected bands are contamination of lithostathine S2-5 as shown in lane 1, but not lithostathine S1.

Figure 2. Representative standard curve of lithostathine assay.



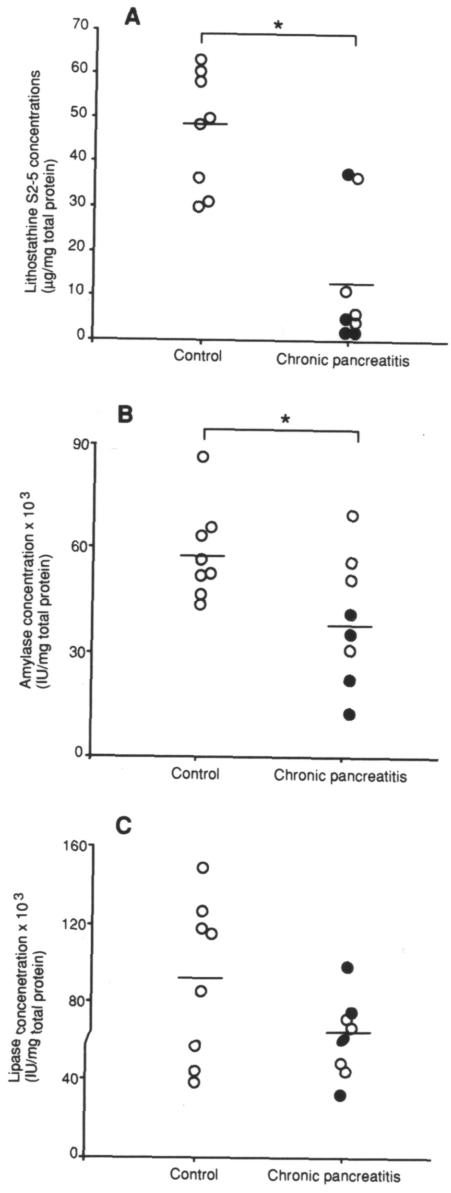
(A)SDS-PAGE separation of different amounts of purified lithostathine S2-5 followed by immunoblotting with antibody against synthetic N-terminal end of lithostathine S2-5. Lane 1, 0.1 μg; lane 2, 0.2 μg; lane 3, 0.5 μg; lane 4, 1.0 μg. (B)The density of the bands on the X-ray film. Each number of circle is corresponding with that of lane in (A)

Figure 3. Quantification of lithostathine in pancreatic juice from control and patients with chronic pancreatitis by immunoblotting analysis.



10 μg of pancreatic juice protein was analyzed by SDS-PAGE followed by immunoblotting with antibody to N-terminal end of lithostathine S2-5. Purified lithostathine S2-5 was applied in lane 1(1 μg), lane 6(0.1 μg) and lane 12(0.5 μg). Pancreatic juice from control was applied in lane 9 and 10. Pancreatic juice from patient with chronic pancreatitis was applied in lane 2, 3, 4, 5, 7, 8 and 11.

Figure 4. Concentrations of lithostathine S2-5 in pancreatic juice from two groups of control and patients with chronic pancreatitis.



Patients with chronic calcifying pancreatitis are shown by closed circle(●). Concentrations of lithostathine S2-5 were lower in chronic pancreatitis group than in the control($p=0.002$).