東邦大学審査学位論文（博士）

Levels of soluble LR11/SorLA are highly increased in the bile of patients with biliary tract and pancreatic cancers

Doctoral dissertation

Kensuke Terai

Department of Biomolecular Science,

Toho University Graduate School of Science

Contents

1. Introduction

Biliary tract and pancreas tumors are characterized by heterogeneous clinical features and pathological etiologies [1, 2]. Recent comprehensive exome and transcriptome analyses of a large number of patients with biliary tract cancer (BTC) have shown that heterogeneous genetic backgrounds cause distinct clinical and pathological subtypes [3]. Patients with these cancers often show poor prognoses, mainly because of the difficult early-stage identification of them [1, 2]. Recent imaging technologies have overcome many of the difficulties in clinical diagnosis; however, the identification of heterogeneous cancers in patients at early stages is still not easy without any well-defined pathological indices [4, 5]. Thus, the development of novel biomarkers has been expected to support the clinical diagnosis in terms of predicting the therapeutic efficacies. To date, several biomarkers have been used to identify the malignancy in the fields of biliary tract and pancreas tumors; serum markers including carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), IL-6, mucin5AC soluble fragment of cytokeratin 19 (CYFRA21-1) and trypsinogen-2, and bile markers including CA19-9, CEA, insulin-like growth factor-1 (IGF-1), pancreatic elastase/amylase ratio, minichromosome

maintenance replication protein (Mcm5), and tumor antigen 90K-binding protein (Mac-2BP)[6-10]. Among them, CA19-9 and CEA are two such representative supportive tumor markers in the clinical setting that have contributed to increasing the efficiency of treatment. However, even in such widely accepted settings, the sensitivity and specificity of CA19-9 and CEA have been shown sometimes not to be satisfactorily high for the diagnosis or staging of biliary tract and pancreatic cancers. The sensitivity and specificity of CA19-9 and CEA levels in bile have also been shown not to be sufficient for the differentiation of malignant and benign biliary tract or pancreas-related diseases [7, 10].

LDL receptor-relative with 11 ligand-binding repeats (LR11; also known as SorLA or SORL1) is a type I membrane protein that plays a key role in the migration of immature vascular smooth muscle cells [11, 12]. Significantly, a large soluble extracellular part of LR11, sLR11, is released by proteolytic shedding and its concentration in body fluids can be quantitated [11, 13, 14]. For example, expression of the LR11 gene is extremely highly increased in immature malignant cells, and thus, sLR11 concentrations may reflect the pathological states of malignant cells [15]. Indeed, LR11 expression is increased in leukemia and lymphoma cells [15-18], and increased serum sLR11 concentrations are a

strong indicator of lymphomas [16-19]. Furthermore, sLR11 was identified as a regulator of hematological cell mobilization [20], and it was demonstrated that LR11 levels in the hematological progenitor cells are induced by hypoxic conditions in bone marrow [21]. In this context, hypoxia has been shown to be a factor that promotes tumor proliferation and migration, and to be associated with poor prognosis in BTC [22, 23] and pancreatic cancers (PC) [24, 25]. Thus, we hypothesized that sLR11, a hypoxia-induced migration inducer, could represent a possible indicator of BTC and PC progression.

In this study, we first investigated the clinical significance of sLR11 levels in bile as a novel cell-released biomarker indicative of BTC and PC, and subsequently immunohistochemically analyzed LR11 expression in surgically resected cancer tissues. Finally, the effects of hypoxia as well as proliferation on LR11 mRNA levels were examined in cultured cholangiocarcinoma and pancreatic cancer cells.

2. Materials and methods

2.1. Bile samples and patients

Bile samples for the present study were collected from residual stock samples of the clinical diagnosis of malignant biliary tract or pancreas tumors or benign biliary tract or pancreas-related diseases, which have been obtained by endoscopic nasobiliary drainage (ENBD), percutaneous transhepatic biliary drainage (PTBD), or Percutaneous transhepatic gallbladder aspiration (PTGBA) for submission to cytological examination at Toho University Sakura Medical Center from 2013 to 2015. All samples, submitted once or multiple times for each patient during treatment of their diseases at the center, were used for the measurement of sLR11, and all values were analyzed for this study. The disease category with clinical stages for each patient with the numbers of samples and the sLR11 levels are shown in Supplementary table 1. The time points for sample collection were principally depending on the routine practice for cytological examination of the patient bile in the clinic, and noninvasively performed by a definitive research protocol. The samples without residual bile sufficient for the measurement of sLR11 were excluded from the study. Thus, 84 samples from 32 patients with malignant biliary tract or pancreas

tumors and 63 samples from 40 patients with benign biliary tract or pancreas-related diseases were classified by pathological and/or clinical diagnosis (Table 1). The 32 cases of malignant tumors consisted of 16 BTC (14 extrahepatic bile duct cancer, 1 cancer of ampulla of Vater, and 1 gallbladder cancer), and 16 PC. The benign cases consisted of 34 biliary strictures (27 gallstone and/or cholecystitis, 7 choledocholithiasis) and 6 other diseases (1 pancreaticobiliary maljunction, 3 primary sclerosing cholangitis, 1 autoimmune pancreatitis, and 1 cholerrhagia disorder). Cytological diagnosis of 147 samples from 72 patients resulted in 81 ‘negative’, 29 ‘suggestive of malignancy’, and 37 ‘positive’ [26]. The study protocol was approved by the Ethics Committees of Toho University Sakura Medical Center.

2.2. Measurement of bile LR11

Bile sLR11 concentrations were determined using a revised sandwich ELISA system [19, 27] (Sekisui Medical, Ryugasaki, Japan). In brief, bile samples were diluted with the sample buffer, reacted with the capture mouse anti-LR11 monoclonal antibody M3F(ab′)$_2$ for 2 h, then incubated with a biotinylated capture rat anti-LR11 monoclonal

antibody R1 for 1 h. The LR11–antibody complex was quantified with horseradish peroxidase (HRP)-conjugated streptavidin using purified LR11 protein as a standard. The measurement range for the ELISA was from 1 ng/ml to 80.5 ng/ml. The samples of concentrations < 1 ng/ml were estimated to 0 ng/ml, and those higher than 80.5 ng/ml were diluted to the appropriate range for measurement.

2.3. Measurement of serum and bile tumor marker

Serum and bile samples were obtained before surgical treatment. Samples for analysis of serum tumor markers were obtained at one time within two weeks before or after bile sampling. All samples were immediately frozen and stored at -80 ℃ until use. CA19-9 and CEA levels were measured using ADVIA Centaur reagents for CA19-9 and CEA, respectively (Siemens Healthcare Diagnostics, NY).

2.4. Immunohistochemistry

Three to four-micrometer-thick sections of paraffin-embedded surgical specimens were prepared. The sections were deparaffinized and hydrated, subsequently

treated with the Immunosaver (Nisshin EM, Tokyo, Japan) for antigen retrieval, and finally boiled at 95 °C for 45 min. Immunohistochemical reactions were performed with an autostainer (the EnVision™ FLEX, Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked by treatment with Envision™ Flex peroxidase-blocking solution (Dako). After washing with Envision™ Flex Wash Buffer (Dako), the slides were incubated at room temperature in a moist chamber for 30 min with 25 μg/ml mouse anti-human LR11 monoclonal antibody, A2-2-3 [15]. After washing, the slides were treated with Envision™ Flex HRP (Dako) for 20 min, followed by color development in Envision™ Flex DAB+ Chromogen with Substrate Buffer (Dako). Finally, the slides were counterstained with hematoxylin.

2.5. Cell culture and hypoxia treatment

The human cholangiocarcinoma cell line HuCCT1 (JCRB0425) and the pancreatic cancer cell line SUIT-2 (JCRB1094) were purchased from Japan Health Science Research Resources Bank (Osaka, Japan), and the cells were cultured in RPMI-1640 (Gibco/ Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal

bovine serum (FBS) (Gibco/ Thermo Fisher Scientific), gentamicin (0.04 mg/ml, MSD, Tokyo) in a humidified incubator at 37 °C and 5% CO_2. Cell numbers were counted using Countess™ (Invitrogen/Thermo Fisher Scientific). For hypoxia treatment, the semi-confluent cells were cultured in a humidified multi-gas incubator (MCO-5M-PJ, Panasonic, Tokyo) with 1% O_2 and 5% CO_2 at 37 °C for 2 h.

2.6. mRNA measurement

Total RNA was prepared from cultured cells using the Maxwell® 16 LEV simply RNA Purification Kit (Promega, Madison, USA), and immediately quantified using the Quantus™ Fluorometer (Promega). For cDNA synthesis, the reverse transcription reaction was performed with the Affinity Script QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, USA). The target fragments of the cDNA samples were amplified by Applied Biosystems® StepOnePlus™ (Applied Biosystems/Thermo Fisher Scientific) using TaqMan Gene Expression Assay with TaqMan Fast Advanced Master Mix (Applied Biosystems/Thermo Fisher Scientific) with the combinations of primers and probes for LR11 (Hs00268342_mi SORL1;

https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=ABGEKeywordSearch&catID=600689), HIF1A (Hs00153153_mi HIF1A) or 18S RNA (Hs99999901_s1 18s). Samples obtained from normoxic conditions were used as calibrator to allow comparison of relative mRNA levels in the assays.

2.7. Statistical analysis

In Figs. 1 and 2, results are presented as medians ± quartile deviations with the ranges (minimum to maximum), and analyzed using the Mann-Whitney U test. In Table 2, the results are presented as mean ± standard deviations, and analyzed using the Mann-Whitney U test. In Fig. 3, the diagnostic ability of biomarkers was evaluated using receiver operating characteristic (ROC) curves, which plot true-positive rates (sensitivity) vs. false positive rates (1 - specificity) across all possible thresholds. In Table 3, we calculated the sensitivity and specificity at the cut-off values for maximum ROC–area under the curves (AUCs) as a global measure of diagnostic accuracy for each biomarker. In Fig. 6, the results are presented as mean ± standard deviations, and analyzed using the Student's *t*-test or ANOVA in combination with the Tukey test. The significance level was

set at a p-value < 0.05. All statistical analyses were performed using JMP ver. 10.0.2 (SAS Institute Inc. Cary, NC)

3. Results

3.1. Bile sLR11 levels are increased in patients with BTC or PC

We first analyzed the bile sLR11 levels in 32 patients with clinically or pathologically diagnosed cancers (16 BTC and 16 PC), in comparison to those in 40 patients with benign diseases (Table 1, also see Supplementary table 1). The median bile sLR11 levels in BTC and PC were 22.08 ng/ml and 18.90 ng/ml, respectively with both being significantly higher ($p<0.0001$) than those in benign diseases (0.00 ng/ml, Fig. 1). Although among the patients with PC, there were 2 cases with very high levels of sLR11 (over 70 ng/ml), bile sLR11 levels were not statistically different between patients with PC and BTC. These results suggested that the increased sLR11 levels in bile may represent a characteristic of cancer cells or systemic conditions in the patients with BTC or PC.

3.2. Bile sLR11 levels are increased regardless of cytological detection of malignant cells.

In order to shed light on the clinical significance of increased bile sLR11 levels in the diagnosis of patients with BTC or PC, in addition to the status of benign or

malignant disease, the levels of all 147 samples were analyzed in relation to the cytological classification of cells detected in the patients' bile (Fig. 2). Subjects were categorized by combining final status-diagnosis and cytodiagnosis into four groups: benign and negative, malignant and negative, malignant and suggestive, and, malignant and positive. The median bile sLR11 levels were 24.80 ng/ml, 16.39 ng/ml and 25.75 ng/ml for malignant and negative, malignant and suggestive, and malignant and positive, respectively, and these levels were significantly higher ($p<0.0001$) than those in benign and negative (0.00 ng/ml). These results suggest that the increased bile sLR11 levels reflect the presence of cancer cells in biliary tract or pancreas tissue, regardless of the results of cytological examination for detached malignant cells in bile.

3.3. Levels of sLR11, but not of CA19-9 or CEA, are increased during treatment of patients.

The above results prompted us to investigate the significance of bile sLR11 as a novel tumor marker in patients with BTC or PC, in comparison to the predictive power of bile levels of the established serum markers, CA19-9 and CEA, in these cancers (Table 2). The analyses of all bile samples from patients collected at multiple time points in

the course of treatment showed that bile sLR11 levels in samples from patients with BTC and PC were, as expected, significantly higher than those in samples from subjects with benign diseases. In contrast, as previously reported [10], bile CA19-9 or CEA levels did not change significantly among the three sample groups (benign diseases, BTC and PC). However, serum CA19-9 and CEA levels in patients with PC were significantly higher than those in patients with benign diseases, suggesting that sLR11 is a novel bile tumor marker that is unrelated to the classical serum biomarkers CA19-9 or CEA.

3.4. Combination of bile sLR11 with CEA or CA19-9 ameliorates the discrimination of patients with BTC and PC against those with benign diseases.

We therefore studied the combined effects of bile sLR11 with serum CEA and/or CA19-9 for the discrimination of patients with BTC or PC against patients with benign biliary tract or pancreas-related diseases. For this purpose, the thirty-six patients (26 with BTC or PC, and 10 with benign biliary tract or pancreas-related diseases), of which bile was sampled within two weeks before or after the measurement of serum CEA and CA19-9, were chosen among the study subjects (Fig. 3 and Table 3). The maximum

AUC of sLR11 for the highest sensitivity-and-specificity was 0.89 at the cut-off value 7.50 ng/ml, and thus equivalent or slightly increased to the values of 0.72 for CEA and 0.75 for CA19-9 at the cut-off values 1.90 ng/ml and 91.30 U/ml, respectively. Using the cut-off values of the three markers, combination analyses of three markers for the discrimination of patients with PC and BTC against the benign patients was performed (Fig. 4). The combination of bile sLR11 with serum CEA or serum CA19-9 ameliorated the sensitivity for discriminating the patients with PC or BTC against the benign patients from 88% (23/26) to 100 % (26/26) (Fig. 4a). On the other hand, the combination did not obviously change the specificity (from 85% (23/27) to 84 % (26/31)) (Fig. 4b). Thus, the combination of sLR11 with CA19-9 and/or CEA particularly ameliorated the discrimination sensitivity for the patients with BTC or PC against patients with benign biliary tract or pancreas-related diseases.

3.5. LR11 is highly expressed in BTC and PC.

We next investigated the localization of LR11 in tissues of patients with BTC and PC by immunohistochemistry using a specific antibody against the receptor. Staining

with a specific antibody against LR11 showed that the LR11 protein was barely detectable in epithelial cells of normal bile duct tissue (Fig. 5, panels a and b) as well as in endocrine and exocrine gland cells of normal pancreas tissue (panels c and d). The LR11 protein was specifically visualized as granular immunostaining in the cytosol, as previously reported for hematological cells [20]. In contrast, in the cancer cells of specimens from patients with BTC or PC, the strong granular staining of LR11 protein was clearly visible (compare panels e to h with panels a to d). These results indicate that LR11 is highly expressed in cancer cells, and that sLR11 levels are increased in bile of patients with BTC and PC.

3.6. In cultured cholangiocarcinoma and pancreatic cancer cells, LR11 mRNA levels are increased by induced cell proliferation and under hypoxic conditions.

Finally, we investigated the effects of cell proliferation or hypoxic stimulation, conditions known to promote progression of BTC and PC [22-25] on LR11 expression in cultured cancer cells using quantitative real-time PCR. The LR11 mRNA levels in HuCCT1 cells, a human cholangiocarcinoma cell line, progressively increased at day 6 and up to day 10 (Fig. 6a), congruent with the onset of cell proliferation (i.e., after serum

addition) (Fig. 6b). Under sustained proliferating conditions, the increased levels of LR11 transcript were maintained at day 13 and day 16. The expression levels of the transcript at semi-confluent conditions were increased nearly 80- and 700-fold, respectively, in HuCCT1 cells and SUIT-2 cells, a pancreatic cancer cell line, in comparison to that of benign smooth muscle cells (Fig. 6c). Interestingly, levels of LR11 mRNA were also increased under prolonged exposure of the HuCCT1 and SUIT-2 cells to hypoxic conditions (Fig. 6d and e), whereas the HIF-1A transcript levels in the cholangiocarcinoma cells remained relatively constant (Fig. 6f). These findings indicate that certain hepatobiliary and pancreatic cancer cells increase the expression of LR11 under conditions of rapid proliferation or hypoxia.

4. Discussion

The LDL receptor-related receptor LR11 was originally identified as a gene product expressed specifically in de-differentiated proliferating vascular smooth muscle cells, but not in mature contractile smooth muscle cells, in atherosclerosis [28]. The protein is localized in intracellular and cell membranes, released by proteinase-mediated shedding, and the released soluble form sLR11 is important for the migration of immature

proliferating cells [28]. Indeed, serum sLR11 has been identified as biomarker for various vascular diseases including arteriosclerosis [12, 29], acute coronary syndrome [30, 31] diabetic retinopathy [32]; sLR11 in cerebrospinal fluid is indicative of Alzheimer's disease [33]. Recently, LR11 gene expression has been demonstrated to be highly increased in immature malignant cells, and thus, sLR11 concentrations have been expected to potentially reflect the pathological states of malignant cells at large. In fact, LR11 expression is increased in leukemia and lymphoma cells [15-18], and increased serum sLR11 concentrations are a strong indicator for the diagnosis of patients with lymphomas [16-19]. Further supporting a role of sLR11 in malignant diseases, sLR11 is produced in bone marrow malignant cells, and induces the migration of lymphoma cells into the circulation [20]. Bone marrow LR11 expression is upregulated by hypoxic conditions in the progenitor cells [21]. These studies suggested that an increased level of sLR11 may be an indicator of tumor pathology, not only in hematological malignancies, but also in solid cancers.

In the present study, we intended to determine the clinical significance of sLR11 as a novel bile marker to strengthen the predictive power of diagnosis and/or of

therapeutic effects for BTC and PC using 147 bile samples collected from 32 patients with BTC or PC, and 40 benign biliary tract or pancreas-related diseases. The clear increase in sLR11 levels in cancer patients reflected the presence of cancer, in fact regardless of detection in bile of cells detached from tumor cells. Furthermore, considering that CA19-9 or CEA were not increased in bile, but were increased as expected [10] in the serum of the study subjects, the increase of bile sLR11 supports its role as a novel bile tumor marker. Based on these observations, we showed that the combination of sLR11 with CA19-9 and/or CEA improved particularly the discrimination sensitivity for the patients with BTC or PC against patients with benign biliary tract or pancreas-related diseases.

Finally, cell biological analyses revealed that LR11 protein was highly expressed in cancer cells, and that mRNA levels increased sharply under conditions of rapid cell proliferation or hypoxic stimulation in cultured cholangiocarcinoma or pancreatic cancer cells. Taken together, our results suggest that the increase of bile sLR11, which likely is released from the cancer cells, may reflect the characteristics of the microenvironment affecting the cellular status in patients with BTC and PC.

Although recent studies have indicated the importance of serum molecular markers in the diagnosis or follow-up of biliary tract and pancreas tumors [6, 7], biomarker(s) sufficient for early diagnosis or subsequent management have not yet been identified. Thus, cancer-cell specific markers, and particularly cell-released molecules, are candidates for serving as surrogate or companion markers for patients with BTC and PC. In this context, mechanistic studies have shown that hypoxia is a promotion factor for tumor proliferation and migration, and is associated with poor prognosis in BTC and PC [22, 23, 24, 25]. Recently, hypoxia has been shown to enhance cholangiocarcinoma invasion by the activation of hepatocyte growth factor receptor and the extracellular signal-regulated kinase signaling [34]. Based on these findings, we evaluated bile sLR11 as a hypoxia-induced migration inducer that acts as a cell-released molecule indicative of the tumor cell status in patients with BTC and PC. Indeed, our studies on the regulation of sLR11 expression in cancer cells demonstrated that LR11 mRNA levels in cultured cholangiocarcinoma and pancreatic cancer cells increase in the course of proliferation and under hypoxic conditions (Fig. 6), together with increased immunoreactive LR11 in BTC and PC (see Fig. 5), suggesting that sLR11 levels relate, at least in part, to the

proliferative and hypoxic microenvironment of cancer cells. Considering that sLR11 is also known to be released from immature hematological malignant cells [15], the levels of bile sLR11 likely reflect the pathological condition of the malignant cells of bile duct and pancreas.

Possible limitations of the present study are that the samples from subjects were collected in a single clinical facility, and that the subject number may not be fully sufficient for extensive analysis using multi-separated categorized analyses. The study was conducted in a cross-sectional research design, which cannot provide insight into any causal relationships between sLR11 and BTC and PC. Clearly, further studies using subjects with different characteristics such as disease stages, systemic, and treatment conditions are required for the elucidation of the (patho-) physiological significance, as well as the specificity and sensitivity particularly in combination with previous tumor markers, of sLR11 in biliary tract and pancreas tumors.

Nevertheless, the present study has shown that bile sLR11 levels in patients with BTC or PC are increased in comparison to those in patients with benign diseases. The bile sLR11 levels in the cancer patients were increased compared with those with benign

diseases, regardless of the presence of cancer cells in bile. The sLR11 levels in bile of cancer patients were increased, while those of CA19-9 and CEA were not. The combination of sLR11 with CA19-9 and CEA improved the discrimination sensitivity for patients with BTC or PC against patients with benign biliary tract or pancreas-related diseases. LR11 is highly expressed in the BTC and PC cells, and LR11 mRNA levels in cultured cholangiocarcinoma and pancreatic cancer cells were sharply induced in the course of proliferation and under hypoxic conditions. We conclude that bile sLR11 levels may well be a novel marker indicative of cancer cell conditions in patients with BTC or PC.

Tables and Figures

Table 1

Clinical backgrounds of study subjects

	Age (mean ± SD)	Sex	Case	Sample	Final diagnosis	Case
Malignant	71 ± 9	Male 18 Female 14	32	84	Biliary tract cancers	16
					Pancreas cancers	16
Benign	69 ± 12	Male 23 Female 17	40	63	Benign biliary strictures	34
					Other diseases	6

Malignant, malignant biliary tract or pancreas tumors; Benign, benign biliary tract or pancreas -related diseases

Table 2

Comparison of bile sLR11, bile/serum CEA and CA19-9 among patients with benign bile-tract diseases, BTC and PC.

		Benign mean± SD	BTC mean±SD	PC mean±SD
Bile	sLR11(ng/ml) (n=147)	6.18±10.60 (n=63)	27.48±17.68 * (n=43)	28.72±26.58 * (n=41)
	CA19-9(U/ml) (n=143)	63097.27±157981.12 (n=60)	1277.79±7110.77 (n=43)	54527.18±208282.42 (n=40)
	CEA(ng/ml) (n=143)	92758.56±224836.92 (n=60)	42617.06±117720.45 (n=43)	5761.81±26255.10 (n=40)
Serum	CA19-9(U/ml) (n=38)	121.24±237.85 (n=11)	148.11±183.52 (n=15)	1209.63±1625.99 * *** (n=12)
	CEA(ng/ml) (n=36)	1.56±0.95 (n=10)	2.59±2.14 (n=15)	3.51±2.34 ** (n=11)

Data are presented as the mean ± standard deviation. The statistical differences were analyzed by Mann-Whitney U test**.** Values of $p<0.05$ were evaluated significant differences.

Benign, benign biliary tract or pancreas -related diseases; BTC, biliary tract cancer; PC, pancreatic cancer.

* $p<0.01$ vs Benign,

**$p<0.05$ vs Benign,

*** $p<0.01$ vs BTC.

Table 3

Specificity and sensitivity of bile sLR11, serum CEA and serum CA19-9 for the discrimination of patients with BTC and PC against patients with benign biliary tract or pancreas -related diseases.

	Cut-off value	Sensitivity	Specificity	AUC
Bile sLR11	7.50	1.00	0.80	0.89
Serum CEA	1.90	0.73	0.80	0.72
Serum CA19-9	91.30	0.65	0.80	0.75

BTC, biliary tract cancer; PC, pancreatic cancer; AUC, area under curve.

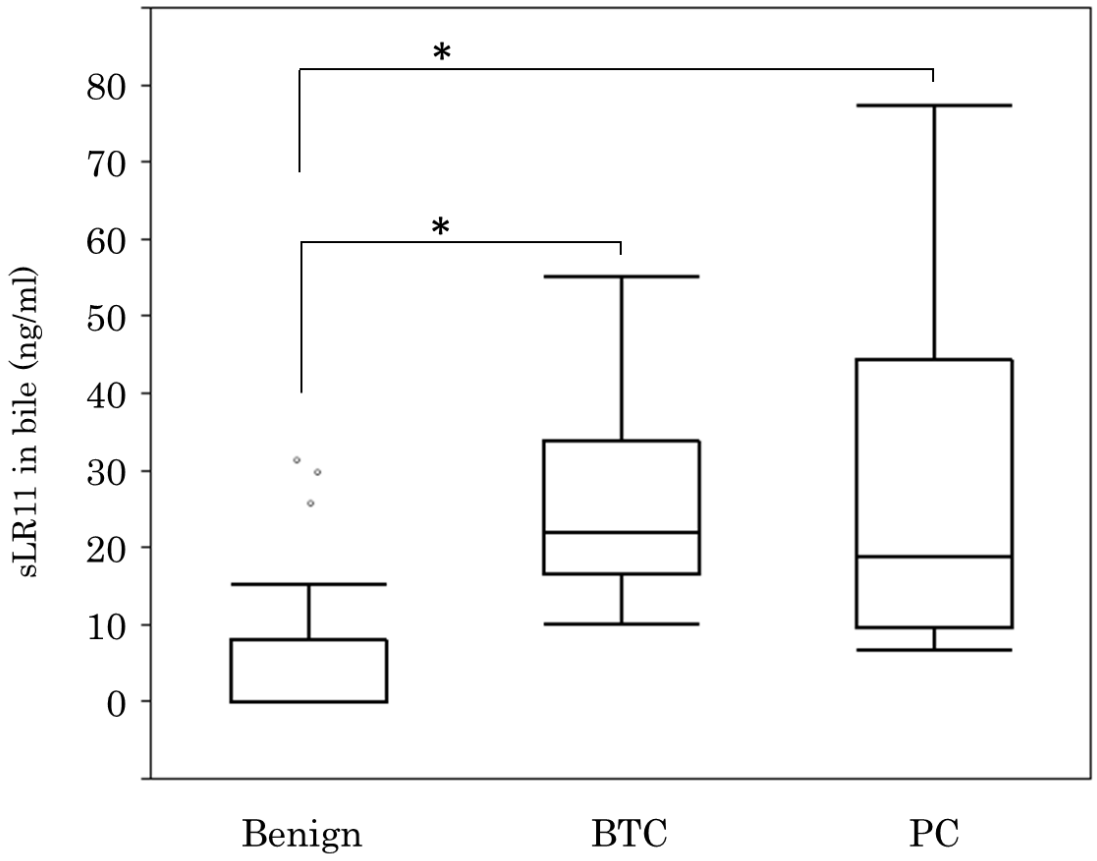

Fig. 1. Comparison of bile sLR11 levels among patients with benign diseases (Benign), BTC and PCs. When several samples of one case were measured, the mean value of these samples was used. The top of each box in the box plots indicates the 75th percentile, the bottom of each box indicates the 25th percentile, the bar inside the box >are the median values, and the whiskers extend out to the most extreme data point that is at most 1.5 times the interquartile range above the third quartile or below the first quartile. The dots indicate results from patients above this range. Values of $p < 0.05$ were evaluated as significant differences. *$p < 0.0001$.

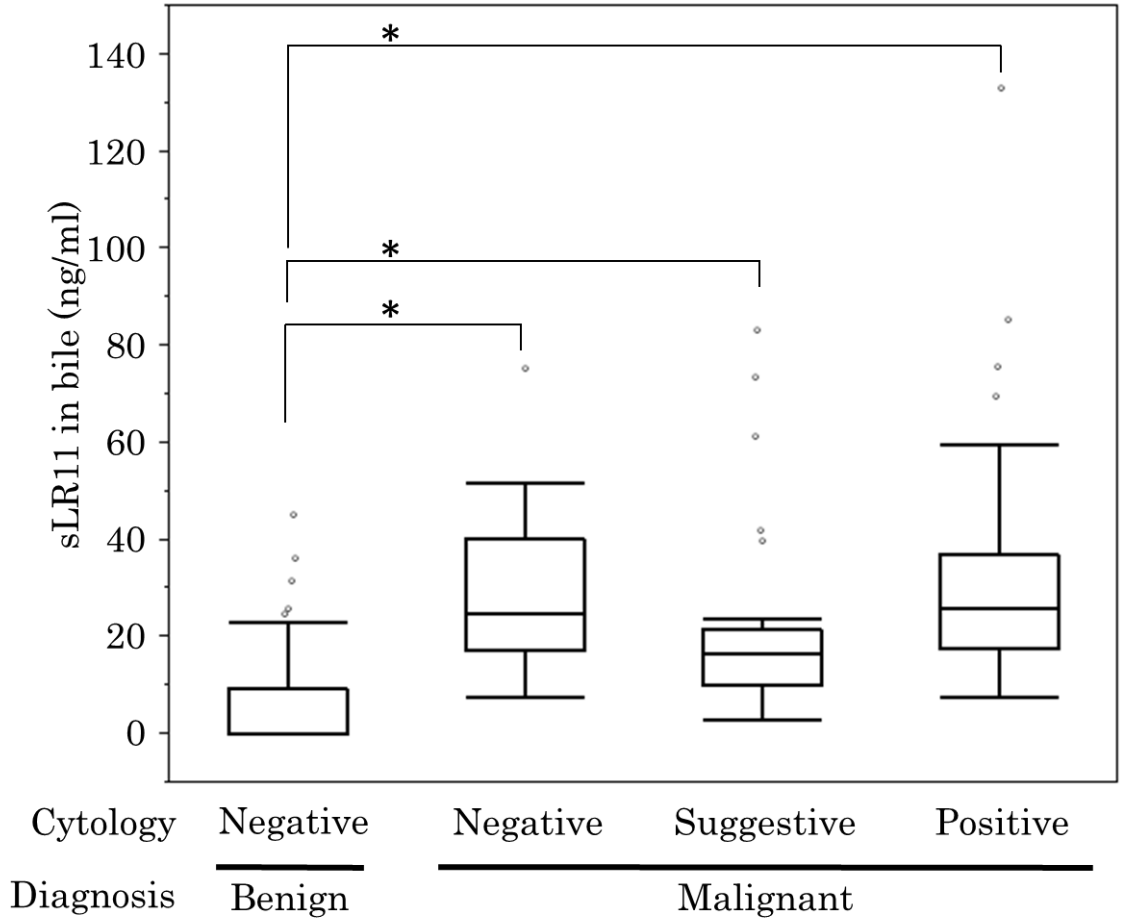

Fig. 2. Comparison of bile sLR11 levels among samples from patients classified by cytological diagnosis in combination with their final (clinical and pathological) diagnosis. The 147 bile samples were classified into four groups; cytology negative/diagnosis benign, cytology negative/diagnosis malignant, cytology suggestive/diagnosis malignant, cytology positive/diagnosis malignant. The top of each box in the box plots indicates the 75th percentile, the bottom of each box indicates the 25th percentile and the bar inside the > box >is the median (note: the median is identical with the lower edge of the box in the left hand column). The whiskers extend out to the most extreme data point that is at most 1.5 times the interquartile range above the third quartile or below the first quartile. The dots indicate results from patients above this range. Values of $p < 0.05$ were evaluated as significant differences. *$p < 0.0001$.

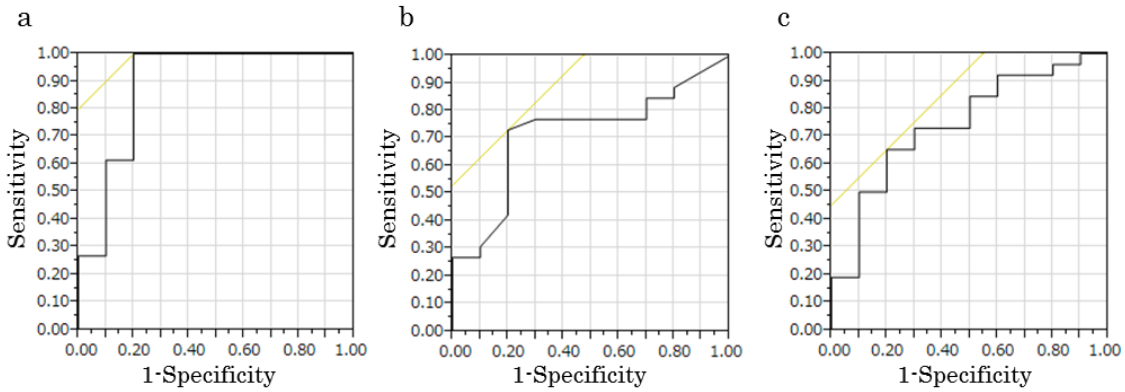

Fig. 3. Receiver operating characteristic (ROC) curves of bile sLR11 (**a**), serum CEA (**b**) and serum CA19-9 (**c**) for discrimination of patients with BTC or PC against patients with benign biliary tract or pancreas-related diseases. Thirty-six patients (26 with BTC or PC, and 10 with benign biliary tract or pancreas-related diseases), of which bile was sampled within two weeks before or after the measurement of serum CEA and CA19-9, were chosen among the study subjects.

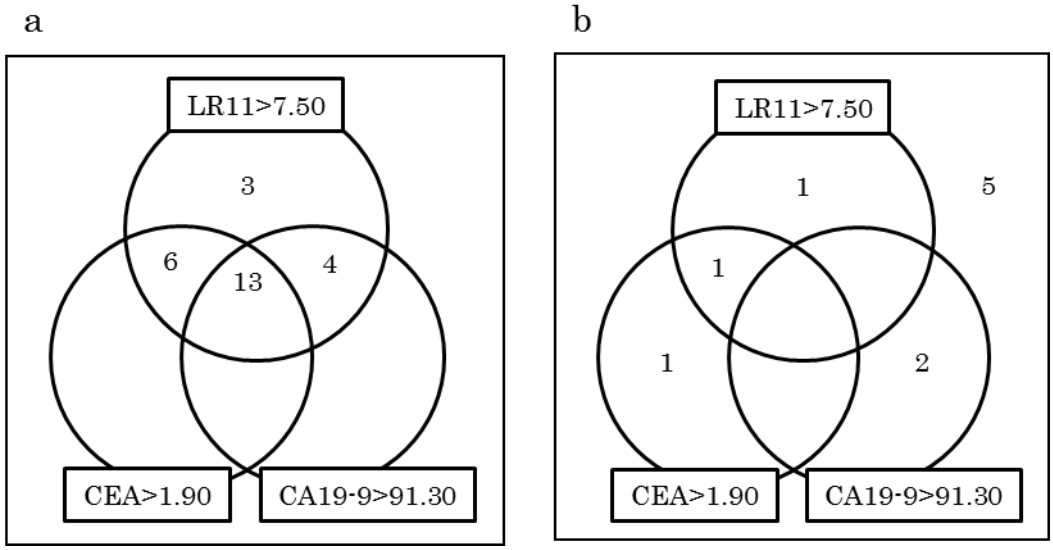

Fig. 4. Distribution of patients positive for bile sLR11, serum CEA, and/or serum CA19-9 in patients with BTC or PC (**a**) and patients with benign biliary tract or pancreas-related diseases (**b**). Thirty-six patients (26 with BTC or PC, and 10 with benign biliary tract or pancreas-related diseases), of which bile was sampled within two weeks before or after the measurement of serum CEA and CA19-9, were chosen among the study subjects.

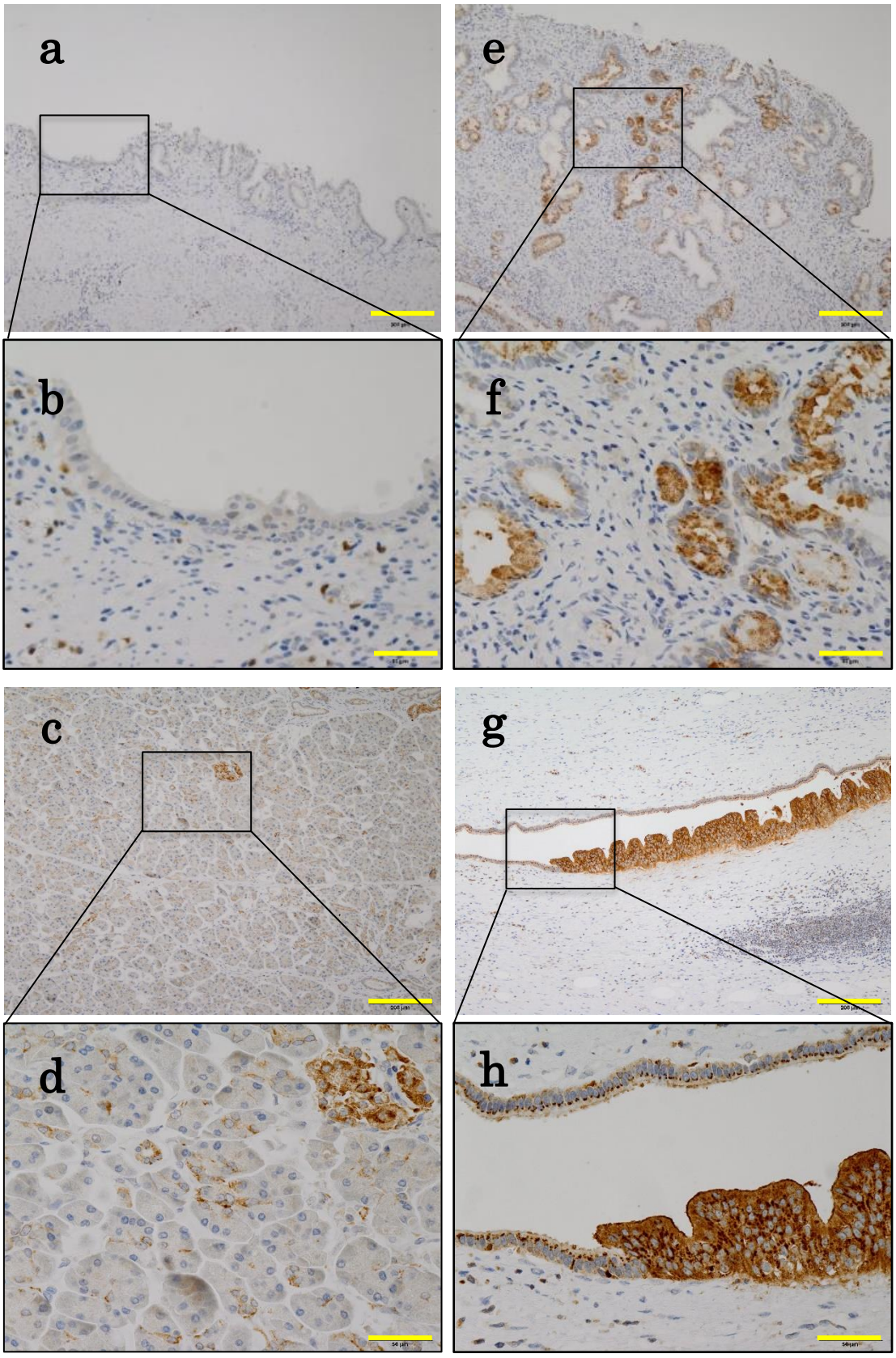

Fig. 5. Immunohistochemical analysis of LR11 in cancer tissues from patients with BTC

and PC. Sections of tissue specimens were subjected to immunohistochemistry using an antibody against LR11. A non-cancer region in a specimen from a patient with BTC, 20×(**a**) and 40×(**b**). A non-cancer region in a specimen from a patient with PC, 20×(**c**) and 40×(**d**). A cancer region in a specimen from a patient with BTC, 20×(**e**) and 40×(**f**). A cancer region in a specimen from a patient with PC, 20×(**e**) and 40×(**f**). In the cancer cells, LR11 was strongly immunostained as granular pattern in the cytosol. Scale bars; 200μm for (**a**), (**c**), (**e**) and (**g**), and 50μm for (**b**), (**d**), (**f**) and (**h**).

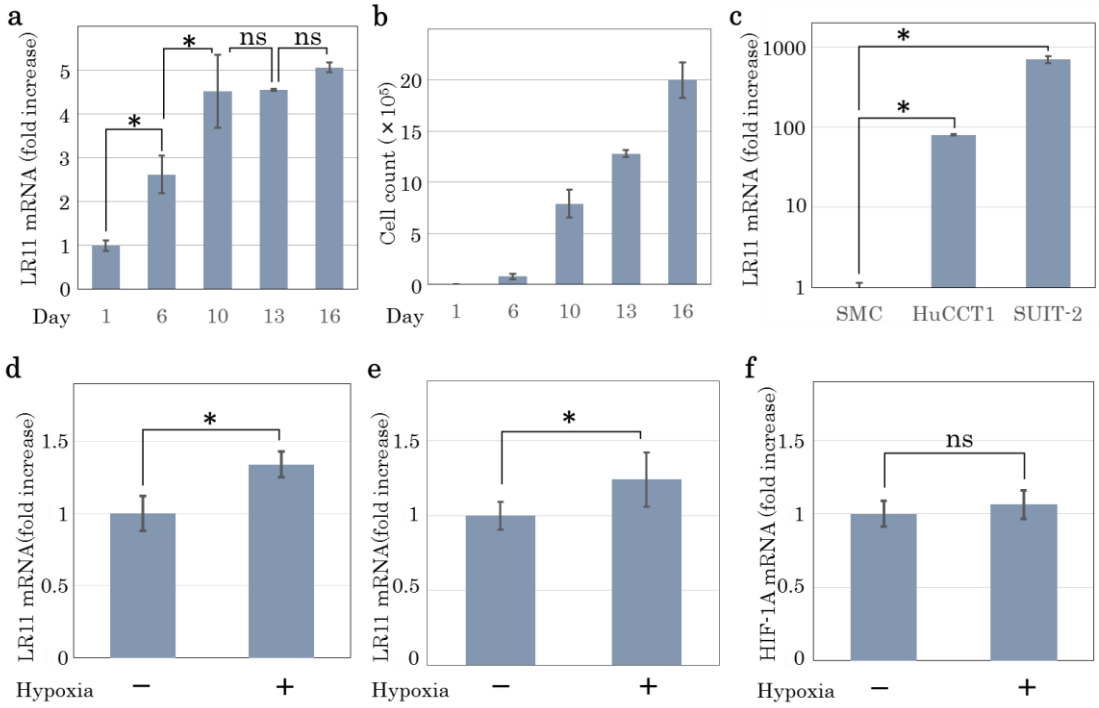

Fig. 6. The levels of LR11 mRNA in cholangiocarcinoma and pancreatic cancer cells. **(a and b)** LR11 mRNA levels (**a**) and cell numbers (**b**) 1, 6, 10, 13, and 16 days after serum addition following overnight serum depletion are shown. mRNA levels were calculated as -fold increases of the levels on day 1, and expressed as the mean ± standard deviation (n=3) for each time point. The statistical differences were analyzed by ANOVA followed by Tukey tests. *p <0.05. ns, not significant. **(c)** LR11 mRNA levels of smooth muscle cells (SMC), HuCCT1 and SUIT-2 cells at semi-confluent cell conditions are shown. mRNA levels were calculated as -fold increases of the levels of SMC, and expressed as the mean ± standard deviation (n=3) for each cell line. The statistical differences were analyzed by Student's t-test**.** *p <0.05. **(d, e and f)** LR11 (**d and e**) and HIF-1A (**f**) mRNA levels in the presence or absence of incubations under hypoxia (1% O_2) for 2h are shown in HuCCT1 (**d and f**) and SUIT-2 cells (**e**). mRNA levels were calculated as -fold increases over the levels without incubation under hypoxia, and the values represent means ± standard deviations (n=3). The statistical differences were analyzed by Student's t-test**.** *p < 0.05. ns, not significant.

Supplementary table 1

Characteristics of study cases with the sample data

Case		Stage	Sample (n)	sLR11 in bile (mean ± SD)	Case		Stage	Sample (n)	sLR11 in bile (mean ± SD)
001	Benign		1	3.98	037	BTC	pStage Ⅲ B	4	15.64 ± 5.18
002	Benign		1	1.82	038	BTC	cStage Ⅳ	3	55.21 ± 26.75
003	BTC	pStage Ⅱ B	4	47.45 ± 7.32	039	Benign		1	0.00
004	Benign		1	0.00	040	Benign		1	14.95
005	BTC	pStage Ⅱ A	1	21.78	041	PC	cStage Ⅰ B	1	45.42
006	Benign		1	0.00	042	Benign		1	31.48
007	Benign		2	0.00 , 0.00	043	BTC	pStage Ⅱ B	3	25.62 ± 4.03
008	Benign		1	0.00	044	Benign		1	0.00
009	BTC	cStage Ⅱ B	3	20.91 ± 3.22	045	BTC	cStage Ⅲ B	2	26.90 , 27.70
010	Benign		1	14.19	046	PC	pStage Ⅱ B	2	8.80 , 5.00
011	Benign		1	2.49	047	BTC	cStage Ⅳ B	2	21.98 , 21.44
012	PC	cStage Ⅳ	3	22.95 ± 8.53	048	BTC	pStage Ⅱ B	1	35.02
013	Benign		3	0.00 ± 0.00	049	PC	cStage Ⅲ	1	16.61
014	BTC	pStage Ⅰ	3	47.28 ± 28.94	050	Benign		1	25.87
015	Benign		1	0.00	051	Benign		1	0.00
016	Benign		1	0.00	052	PC	cStage Ⅲ	1	7.50
017	Benign		1	3.12	053	PC	cStage Ⅲ	2	21.60 , 133.10
018	Benign		3	0.00 ± 0.00	054	PC	pStage Ⅱ B	1	42.11
019	Benign		2	0.00 , 0.00	055	BTC	cStage Ⅱ	3	10.22 ± 0.4
020	Benign		1	0.00	056	BTC	pStage Ⅰ B	4	22.38 ± 3.5
021	PC	pStage Ⅰ B	3	21.13 ± 1.66	057	Benign		1	2.32
022	Benign		1	0.00	058	Benign		1	0.00
023	Benign		1	10.75	059	PC	pStage Ⅱ B	3	16.67 ± 8.43
024	PC	cStage Ⅰ B	5	48.19 ± 15.57	060	BTC	cStage Ⅱ	3	18.93 ± 1.39
025	Benign		1	7.91	061	Benign		2	0.00 , 0.00
026	Benign		5	29.88 ± 9.45	062	PC	cStage Ⅳ	5	25.73 ± 7.96
027	Benign		1	0.00	063	Benign		2	0.00 , 0.00
028	Benign		1	0.00	064	PC	cStage Ⅳ	3	76.27 ± 5.61
029	Benign		1	0.00	065	PC	cStage Ⅳ	2	11.81 , 2.89
030	BTC	pStage Ⅱ B	1	10.52	066	Benign		4	0.00 ± 0.00
031	Benign		4	3.08 ± 1.95	067	Benign		4	8.16 ± 13.50
032	PC	cStage Ⅳ	3	14.32 ± 4.12	068	Benign		1	12.66
033	Benign		1	0.00	069	BTC	cStage Ⅱ	3	15.81 ± 3.57
034	Benign		1	6.39	070	PC	cStage Ⅲ	3	9.60 ± 2.10
035	Benign		1	10.65	071	Benign		3	15.41 ± 5.42
036	BTC	cStage Ⅲ B	3	30.79 ± 5.55	072	PC	cStage Ⅳ	3	10.06 ± 2.78

Benign, benign biliary tract or pancreas-related diseases; BTC, biliary tract cancer; PC, pancreatic cancer

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