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Dysbindin as a novel biomarker for pancreatic ductal adenocarcinoma identified by proteomic profiling

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Pancreatic adenocarcinoma (PDAC) is known to have a poor prognosis partly because of lack of effective biomarkers. In the test set, we investigated dysbindin (DTNBP1) as a potential biomarker for PDAC by comparing preoperative and postoperative serum mass spectrometry (MS) proteomic profilings. Of the included 50 PDAC patients, 42 (positivity of 84.0%) detected a lower MS peak in postoperative serums than preoperative ones which was then identified as dysbindin. In the verification set, receiver operating characteristics (ROC) were used to assess diagnostic efficiency. 550 participants were included in the verification set [250 with PDAC, 80 with benign biliary obstruction (BBO), 70 with chronic pancreatitis (CP) and 150 healthy donors (HD)]. Dysbindin was increased in PDAC patient sera than in all controls. ROC curves revealed the optimum diagnostic cutoff for dysbindin was 699.16 pg/ml [area under curve (AUC) 0.849 (95% CI 0.812–0.885), sensitivity 81.9% and specificity 84.7%]. Raised concentration of dysbindin in sera could differentiate PDAC from BBO, CP and HD. Moreover, dysbindin maintained its diagnostic accuracy for PDAC patients who were CA19-9 negative [AUC 0.875 (95% CI 0.804–0.945), sensitivity 83.0%, specificity 89.0%] and for patients with benign biliary obstruction [AUC 0.849 (95% CI 0.803–0.894), sensitivity 82.3%, specificity 84.0%].Our discovery of dysbindin may complement measurement of CA19-9 in the diagnosis of PDAC and help to discriminate PDAC from other pancreatic diseases or begin biliary obstruction.

Key words: dysbindin, CA19-9, pancreatic ductal adenocarcinoma, ROC, biomarker

Abbreviation: ANT: adjacent noncancerous tissue;; AUC: area under curve; BBO: benign biliary obstruction; CP: chronic pancreatitis; HD: healthy donors; PDAC: pancreatic ductal adenocarcinoma; ROC: receiver operating characteristics

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Pancreatic ductal adenocarcinoma (PDAC) is lethal and its incidence has increased in the past decade.[1]The fourth leading cause of cancer-related deaths in the US, PDAC has the lowest five-year survival rate among all solid cancers (estimated 0.4–4% survival).[2]Due to late-detection of PDAC and late-onset of positive symptoms, <8% patients are diagnosed at the localized stage.[3–5] Thus, identifying early biomarkers at a surgically resectable stage is needed for detection of PDAC.

With newly emerging proteomic methods such as mass spectrometry, liquid chromatography, free flow electrophoresis and gel electrophoresis, combinations of these techniques have been used to identify biomarkers based on serum proteomics.[6,7]CA125,[8] CA242,[9] osteopontin[10] and macrophage inhibitory cytokine 1 (MIC-1)[11] are only some of the biomarkers that tend to be potentially effective. However, carbohydrate antigen 19–9 (CA 19–9) is still currently the only serum biomarker which has been widely used for detection of PDAC; however, this approach is frustrating in regard to both specificity and sensitivity.[12–14] It is reported that 40% of chronic pancreatitis (CP) patients and 70% benign biliary obstruction (BBO) patients have elevated CA19-9 levels,[15–17] making it difficult to discriminate PDAC from CP or BBO.

What's new?

A new biomarker could help diagnose pancreatic cancer early, giving patients a better shot at survival. Since patients experience few symptoms, pancreatic cancer is rarely detected while it is still localized, and has a terrible survival rate. Biomarkers help with diagnosis, but the most commonly used biomarker, CA19-9, shows up frequently in other gastrointestinal diseases, making it less than ideal. This study compared samples from cancer patients before and after surgery, and identified dysbindin as a reliable marker. Dysbindin showed up more in pancreatic cancer than either healthy controls or other gastrointestinal diseases, suggesting it could complement CA19-9 well.

Hence, the efficiency of CA19-9 reduced but we can complement the specificity and sensitivity of CA19-9 in combination with other biomarkers.[18–22]

Many studies focused on investigating biomarkers between PDAC patients and healthy donors. To our knowledge, an important characteristic of biomarker is to be strictly correlated with the disease. Thus, we developed a strategy that compared sera samples from preoperative and postoperative PDAC patients, with affinity chromatography (AFC), reversed-phase liquid chromatography (RPLC) and matrix-assisted-lasers desorption/ionization time of flight mass spectrometry (MALDI-MS) to identify a potential biomarker which may be strictly correlated with PDAC (Fig. 1). With this approach, we identified dysbindin as a potential PDAC biomarker. This biomarker candidate was then validated in clinical serum samples to assess the diagnostic efficiency and compared with CA19-9.

Material and Methods

Materials

An LC-10A (Shimadzu, Japan) including two pumps, a gradient elution system, a Rheodyne 7725 manual sample injector, and an SPD-10AVP UV detector was used. The reversed-phase packings (YMC-ODS, 10 μm, 30 nm) were purchased from the Great Euro-Asia Science & Technology Development (Beijing, China) and packed in a stainless steel column (150×4.6 mm^2 i.d.). This was used for desalting and simultaneous separation of the eluted components from the concanavalin A (Con A) affinity column with a 30 min gradient elution time with acetonitrile and water as the mobile phase.

Preparation of affinity packings column

Affinity packings were improved according to our previous work.[23]Silica gel was treated with 6 mol•L^{-1} of hydrochloric acid and then dried under vacuum. Two milliliter of γ-glycidochloropropyl methyl trimethoxy silane was slowly added to 100 mL of acetate buffer solution (pH 4.7) in a three-necked flask containing 2.0 g treated silica gel. The solution was incubated overnight at 110°C to ensure reaction completion, and then the filtered product was hydrolyzed with 200 mL of 0.1 mol•L^{-1} sulfuric acid at 60°C for 5 hr. The silica was activated by reacting with CDI in a dried dioxane solution and incubated at 60°C overnight.

Two hundred milligrams of Con A was added into a sodium bicarbonate buffer solution (pH 7.5) including 0.2 mol•L^{-1}of α-Me-D-Man and stirred for 0.5 hr, followed by the addition of the activated silica (2.0 g). The reaction was allowed to proceed with stirring for 12 hr at room temperature, after which the immobilized Con-A adsorbent was recovered by centrifugation and washed with 0.1 mol•L^{-1} Tris-HCl buffer (pH 7.5) containing 0.2 mol•L^{-1} NaCl. The adsorbent was then packed into a stainless steel column (50 mm 4.6 mm i.d.) using the previously mentioned Tris buffer and a high-pressure pump from Shimadzu (Kyoto, Japan). The column was washed with a Tris buffer (containing 0.2 mol•L^{-1} NaCl, 1 m mol•L^{-1} $CaCl^2$, 1 m mol•L^{-1} $MnCl^2$ and 0.1 mol•L^{-1} Tris buffer, pH 7.5) to reach equilibrium.

Reversed-phase liquid chromatography

The mobile phase for RPLC consisted of solution A (100% H_2O, 0.1% TFA) and solution B (100% CH_3CN, 0.1% TFA). The sample was directly injected into a RPLC column equilibrated with mobile phase A, and then eluted with a linear gradient from 100% of solution A to 100% of solution B for 30 min with the delay for 10 min. The flow rate was 1.0 mL•min^{-1} and the detection wavelength was 280 nm. The fractions were collected and lyophilized for MALDI-MS analysis.

Mass spectrometry analysis

MALDI-MS (Kratos Analytical Company of Shimadzu Biotech, Manchester, UK) was used as follows: 1 μL of the mixture (1:1, V/V) of the sample solution and the matrix was spotted onto the wells of the MALDI sample plate and air-dried. The proteins and peptides were analyzed in the linear ion-mode with CHCA as a matrix. External calibration was achieved using a standard peptide mixture and a protein mixture from Sigma.

Study population

Consecutive patients with PDAC ($n = 250$) were recruited from Xijing Hospital, Fourth Military Medical University, from December, 2010, to August, 2012. Patients with benign biliary obstruction (BBO, $n = 80$) or chronic pancreatitis (CP, $n = 70$) and healthy donors (HD, $n = 150$) were also recruited.

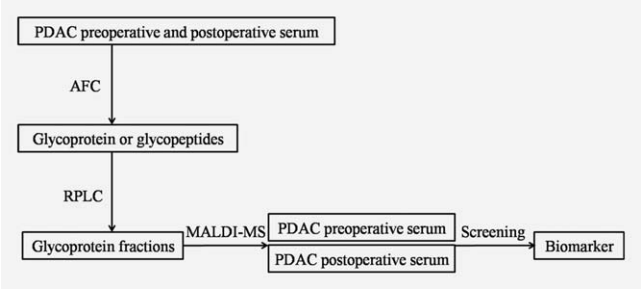

Figure 1. Scheme for discovering novel PDAC biomarkers. AFC: affinity chromatography; MALDI-MS: Matrix assisted lasers desorption/ionization time of flight mass spectrometer; PDAC: pancreatic ductal adenocarcinoma; RPLC: reverse phase liquid chromatography.

PDAC was defined by ultrasound, CT, or MRI and serum biochemistry (CA19-9) and was confirmed by pathology after surgery. Tumor stage was based on American Joint Committee on Cancer (AJCC) system. Benign biliary obstruction (due to gall stone) was based on ultrasound, CT and pathology[24] and the mean concentration of total bilirubin was 64.2 μmol/L (range from 19.2 to 143.2 μmol/L). Chronic pancreatitis was defined based on typical clinical findings; ultrasound, CT and MRI indicating pancreatic calcifications or stones; pathological findings; exocrine functional insufficiency.[25]

The four groups were matched for age and gender as far as possible. Data collection was carried out by two independent researchers (GX and LX). Approval for the study was obtained from the Internal Review and the Ethics Boards of the Xijing Hospital of the Fourth Military Medical University.

Sera samples

Sera samples were obtained and processed according to the standardized protocol. Briefly, venous blood was drawn into gel-coated serum tubes, clotted at room temperature for 1 hr and centrifuged at 3,000*g* for 10 min. Thereafter, aliquots of samples were immediately stored at −130°C until use. All sera samples were labeled with a unique identifier to protect patient confidentiality. All samples were collected and used with informed consent according to committees' regulations.

Testing of blood and tissue samples

Dysbindin in the sera of participants were measured by a sandwich enzyme-linked ELISA using a commercially available ELISA kit (Abcam, UK), as described by the manufacturer. The level of CA199 was determined by the EILSA kits from R&D Systems and Alpha Diagnostic (TX).

To further compare the expression of dysbindin in serum and tissues, parallel expression profiles were analyzed at mRNA, protein and secreted protein level in PDAC tissues and adjacent noncancerous tissues and in serum samples. 14 samples of PDAC patients and two healthy donors were randomly chosen and investigated at Xijing Hospital. The tissues samples were obtained immediately after surgical resection and were frozen in liquid nitrogen and stored at −130°C.

Quantitative real-time reverse transcription PCR (qRT-PCR) was performed with a Bio-Rad instrument (Roche Diagnostics, Switzerland) with the SYBR Premix Ex Taq TM (TaKaRa, Japan) according to the manufacturer's instruction. All reactions were performed in a 25 μL reaction volume in triplicate. Primers for dysbindin and GAPDH were obtained from Sangon Biotech. After an initial denaturation at 95°C for 30 sec, 40 cycles of PCR amplification were performed at 95°C for 5 sec and 60°C for 30 sec. The primer sequences were as follows: dysbindin, sense (5'-GTACCTGTCCA CTGGCTAC-3') and antisense (5'-CCTCCTGGTCCGATA TGTC-3'); GAPDH, sense (5'-CTGACTTCAACAGCGAC ACC-3') and antisense (5'-TGCTGTAGCCAAATTCGTTG-3').[26] The relative amount of target gene mRNA was normalized to house-keeping gene GAPDH and the specificity was verified by melt curve analysis.

Proteins extracted either from tissues or cells were resolved in SDS-PAGE, transferred to PVDF membranes and incubated with anti-dysbindin (1:2,000; Abcam, UK). Peroxidase-conjugated anti-mouse or rabbit antibodies were used as secondary antibodies, and the final band was visualized by enhanced chemiluminescence assay (ECL, Thermo, Shanghai).

Statistical analysis

Immumohistochemical scores and serum levels of target proteins between groups were tested using either the Wilcoxon test or Kruskal–Wallis test. Statistical analysis was performed using the Student two-tailed *t test* or as indicated. Data are presented as means ± standard deviation (SD) or ± standard errors of the mean (SEM). Differences were considered statistically significant when $p < 0.05$. Survival curves were plotted by the Kaplan–Meier method and compared by using the log-rank test.

Results

Separation of proteins and peptides in human sera with 2DLC

As well known, the sample preparation is a bottle-neck problem in proteomics. The presence of the high abundance proteins in the sample can seriously interfere with the detection of hundreds of low abundance proteins. Therefore, the reduction in the sample complexity (*e.g.*, to deplete high abundance proteins and to replete low abundance proteins) of serum samples is essential prior to any analyses aimed at determining proteins present in small quantities, which potentially include protein biomarkers. Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. Because the high abundance proteins in human serum, such as human serum albumin (HSA) and globulins have larger molecule weights (> 60 KD), SEC is

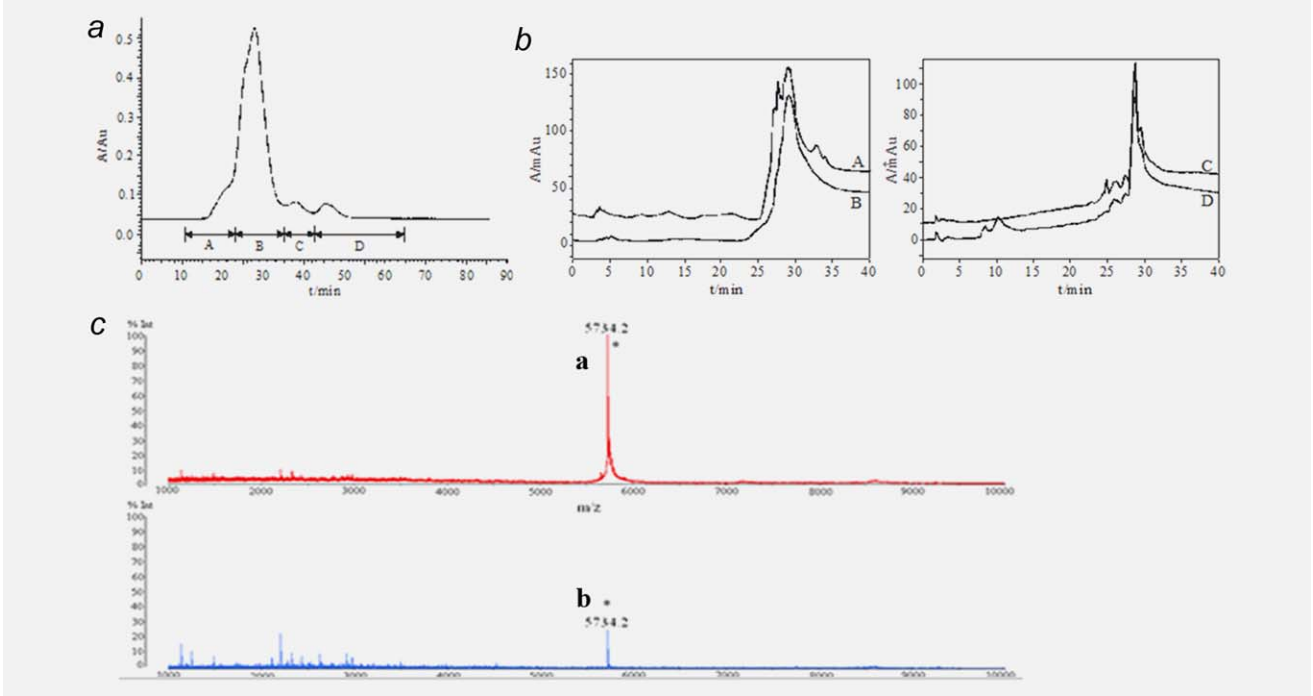

Figure 2. Screening for candidates of PDAC. (*a*) Chromatogram of human serum denatured by urea on a SEC column. Mobile phase: 0.020 mol/L KH2PO4 + 0.10 mol/L NaCl, pH = 7.0; Flow rate: 0.5 mL/min, Sample size: 300 μL, Detection wavelength: 280 nm. (*b*) Chromatograms of SEC fractions separated by RPLC. Mobile phase A: H2O + 0.1% TFA, mobile phase B: CH3OH + 0.1% TFA; linear gradient elution: 100% A ~100% B, 30 min, flow rate: 1.0 mL/min, detection wavelength: 280 nm. A~D: Fraction A~D collected from SEC in Figure 1*a*. (*c*) MALDI-TOF MS analysis of serum fractions eluted from the Con A affinity column. a. preoperative serum from PDAC patient; b. postoperative serum from PDAC patient. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the best choice to eliminate these high abundance proteins. Therefore, the offline 2DLC (SEC-RPLC) coupled with MALDI-TOF MS was used to screen the biomarker of pancreatic cancer in serum.

First, the pretreated human serum was isolated by SEC, and the chromatogram was shown in Figure 2a. From Figure 2, it was observed that four peaks could be obtained according to the protein size. The four fractions (Fraction A-D) were collected and offline separated again with RPLC in second dimension separation, respectively (as shown in Fig. 2b). All collected fractions were detected by MALDI-TOF MS.

Human pancreatic cancer biomarker

Sera from preoperative and postoperative (5 days after surgery) patients with PDAC were separated with offline SEC-RPLC 2DLC and analyzed by MALDI-TOF MS. Among all detected m/z peaks, one was significantly distinct with respect to m/z between PDAC preoperative and postoperative samples. The two groups differed after comprehensive protein identification in Figure 2c (a and b). An obviously higher intensity peak (m/z 5,734.2) appeared in sera of preoperative patients with PDAC, whereas a lower peak was observed in postoperative samples. Thus, this may indicate that this peak indicates a potential biomarker strictly associated with PDAC.

To validate the biomarker for PDAC, preoperative and postoperative sera from 50 PDAC patients were assessed as previously described. The same peak with an m/z 5,734.2 was observed in 42 of 50 PDAC patients. Biomarker intensity was greater in preoperative samples and decreased after five days after surgery. Statistical analysis indicated that the positivity rate of the biomarker was 84.0%. Thus, this may be a suitable biomarker for pancreatic cancer. After peptide sequencing, a 44 amino acid peptide was matched to the N-terminal of dysbindin, a protein implicated in schizophrenia. Further characterization of the novel candidate was then performed.

Validation of the potential biomarker in PDAC patients

550 participants were recruited in the verification set to validate the diagnostic accuracy of dysbindin. The clinicopathological characteristics of pancreatic ductal adenocarcinoma, chronic pancreatitis, benign biliary obstruction and healthy donors were presented in Table 1. The four cohorts were matched for age and gender.

We performed Elisa to qualify the levels of dysbindin in the sera. Dysbindin was dramatically increased in PDAC ($1{,}097.0 \pm 30.3$ pg/ml, $n = 250$) in comparison with CP (641.5 ± 35.8 pg/ml, $p < 0.0001$, $n = 70$), BBO (576.5 ± 24.2 pg/ml, $p < 0.0001$, $n = 80$) and HDs (580.2 ± 10.5 pg/ml, $p < 0.0001$, $n = 150$). Meanwhile, values showed no statistical significance between the three control groups (Fig. 3a). Moreover, dysbindin in preoperative serums of PDAC patients was higher in contrast with ones in 5-day postoperative

Table 1. Clinical characteristics of respective discovery and verification sets populations

	Discovery Set ($n = 50$)	Verification Set ($n = 550$)			
Characteristics	Experimental group PDAC-pre/post	Experimental group PDAC-pre/post	Control group BBO	CP	HD
No. of patients	50	250	80	70	150
Gender (male/female)	27/23	156/94	66/34	62/38	56/44
Age (years) Median (range)	66 (35–76)	67 (29–75)	47 (22–59)	40 (19–65)	56 (31–78)
CA19-9 (KU/L) Median (range)	131.5(62.1–154.9)	168.9(23.5–3,989.5)	70.1 (41.7–766.9)	130.5 (51.7–667.9)	26.4 (9–187)
Tumor size (mm) Median (range)	30(29.2–35)	28 (15–42)	45 (23–79)	NA	NA
Pathologic differentiation (well: moderate/poor)	10/31/9	16/234	NA	NA	NA
Clinical stage (I/II/III/IV)	7/22/16/5	34/115/50/51	NA	NA	NA

BBO: pancreatic benign disease; CP: chronic pancreatitis; HD: healthy donors; NA: not available; PDAC: pancreatic ductal adenocarcinoma

serums ($1{,}097.0 \pm 30.3$ *vs.* 766.2 ± 20.2 pg/ml, $p < 0.0001$, $n = 250$ pairs), which was consistent with the results of RPLC (Figs. 3c and 3d). These results preliminarily showed that dysbindin level in serum was associated with pancreatic ductal adenocarcinoma.

Comparative diagnostic efficiency of serum dysbindin and CA19-9 for PDAC

As expected, the mean concentration of CA19-9 was increased in PDAC patients compared with that in healthy donors ($p < 0.0001$); meanwhile, significant increases was also detected in patients with benign biliary obstruction ($p < 0.001$) and chronic pancreatitis ($p < 0.001$, Fig. 3b), which indicated high false positivity of CA19-9 in discriminating PDAC, BBO and CP.[17] Additionally, more than half of PDAC patients were positive for dysbindin than for CA19-9 [209 (83.6%) *vs.* 202 (80.8%) of 250 patients, Fig. 3f].

We used receiver operating characteristics (ROC) to assess the diagnostic accuracy of dysbindin and compare the efficiency with CA19-9. Based on ROC curves compared between PDAC and noncancerous controls, the optimal diagnostic cutoff for dysbindin was 699.16 pg/ml (AUC 0.848, 95% CI 0.820–0.875, sensitivity 69.1%, specificity 90.6%; Fig. 3e). The optimal cutoff value for CA19-9 was 37.8 U/ml (AUC 0.830, 0.802–0.858, sensitivity 59.4%, specificity of 87.4%, Fig. 3e) which was similar to the recommended clinical cutoff of 37U/ml.

In the assessment of diagnostic efficiency, serum dysbindin had better AUC, specificity and sensitivity than did CA19-9 in PDAC patients in contrast with benign biliary obstruction and chronic pancreatitis (Fig. 3).

Diagnostic efficiency of dysbindin in subgroups of PDAC and BBO

It is reported that 10% of PDAC patients are CA19-9-negative, making it difficult to detect subgroups of PDAC. In our study, the level of dysbindin in CA19-9-negative patients and CA19-9-positive patients showed no statistical significance ($p = 0.2275$, Fig. 4a). ROC curves concerning in CA19-9-negative patients versus three control groups indicated that AUC of dysbindin was 0.875 (95% CI: 0.804–0.945) with a sensitivity of 83.0% and a specificity of 89.0% (Fig. 4b). In the discrimination of CA19-9-negative PDAC from the well-known risk group of PDAC (chronic pancreatitis), the AUC of dysbindin was 0.820 (95% CI: 0.738–0.903) with a sensitivity of 78.7% and a specificity of 73.2% (Fig. 4c). Moreover, we also assessed the diagnostic efficiency of dysbindin in CA19-9-positive PDAC group. As shown in Figures 4d and 4e, dysbindin revealed a good diagnostic value in differentiating PDAC and noncancerous groups. In addition, CA19-9-negative patients with PDAC had high proportion of dysbindin detection rate (36 (75.0%) of 48). Similar results were also observed in CA19-9-positive patients [173 (85.6%) of 202]. Thus, the ROC curves for dysbindin revealed that a diagnosis of dysbindin not correlated with CA19-9 status.

It is also well known that CA19-9 is limited in patients with PDAC by increased false positivity in terms of benign biliary obstruction[7] (10–70%).In this study, serum CA19-9 was detected with a positivity of 76.3% in benign biliary obstruction Group (61 of 80 BBO patients). However, serum dysbindin was only with a positivity of 15.0% (12 of 80 BBO patients) in BBO group, suggesting a better efficiency of dysbindin regardless of obstructive jaundice (Fig. 4f). The ROC curve showed that in comparison of PDAC and BBO, AUC of dysbindin was 0.849 (95% CI: 0.803–0.894) with a sensitivity of 82.3% and a specificity of 84.0%, whereas AUC of CA19-9 was 0.589 (95% CI= 0.526–0.653) with a sensitivity of 53.0% and a specificity of 54.3% (Fig. 4g). These results indicated that dysbindin had a better efficiency than CA19-9 in discriminating PDAC and BBO. All the data of ROC curves were presented in Table 2.

Parallel expression analysis of dysbindin

Although dysbindin is high in sera of PDAC patients, it is reasonable to wonder whether the ELISA values are in accordance with dysbindin expression level in PDAC tumors. Thus we investigated the expression of dysbindin in 16

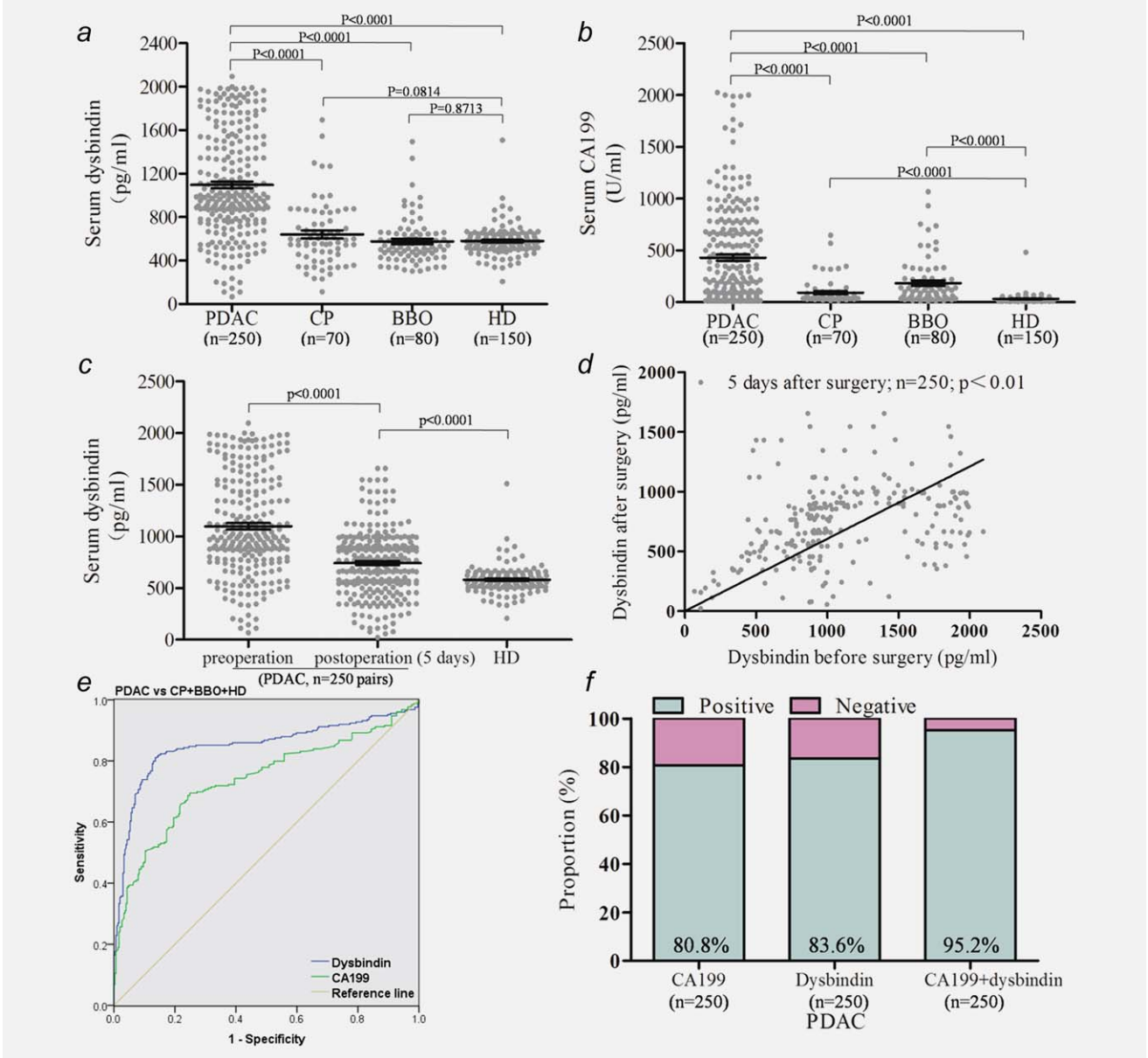

Figure 3. Results for serum dysbindin and CA199 in the diagnosis of PDAC. (*a*) Dysbindin for verification set. (*b*) CA199 for verification set. (*c*) Serum dysbindin in PDAC patients before and 5-day after surgery and healthy donors. t. Black horizontal lines are means, and error bars are SEs. (*d*) Scatter plot of serum dysbindin concentrations in serum samples before and 5-day after surgery from the same patient with PDAC. (*e*) ROC curve for dysbindin and CA199 for patients with PDAC versus all controls. (*f*) The rate of positive results for dysbindin, CA199 or both in patients with PDAC. AUC: areas under the curves; BBO: benign biliary obstruction; 95% CI = 95% confidence interval; CP: chronic pancreatitis; HD: healthy donors; PDAC: pancreatic ductal adenocarcinoma; ROC = receiver operating characteristics. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PDAC tissues and adjacent noncancerous tissues (ANT) at mRNA, protein and secreted protein level. Dysbindin mRNA can be detected at lower level in ANT but overexpressed in 15 of 16 PDAC tissues (Fig. 5c). Additionally, dysbindin expression in PDAC tissues was greater than that in ANT (Figs. 5a and 5b). According to the cutoff value at 11 defined by ROC curves, dysbindin was positive in 14 of 16 PDAC patients by serum ELISA (Fig. 5d).

Discussion

CA19-9 is currently the most commonly used biomarker for PDAC. However, CA19-9 may not be reliable because it is also increased in other gastrointestinal diseases such as benign biliary obstruction and pancreatitis.[27,28] In addition, 5–10% of humans cannot produce CA19-9 due to Lewis $a^{-}b^{-}$ genotype,[29] and poorly differentiated PC appears to produce less CA 19–9 than moderately or well differentiated

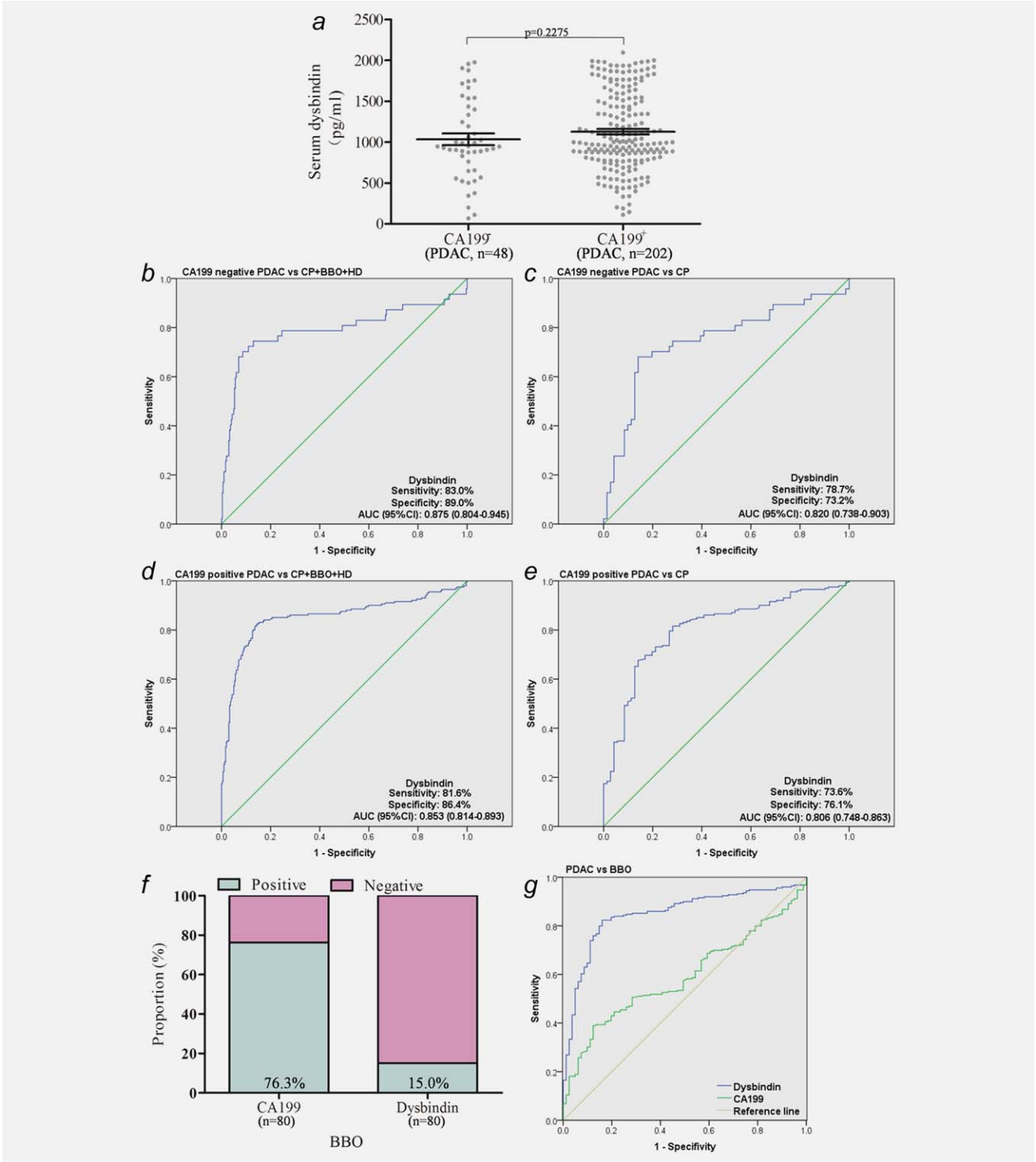

Figure 4. Diagnostic efficiency of serum dysbindin in subgroups of PDAC. (*a*) The concentrations of dysbindin in CA19-9-negative and CA19-9-positive PDAC groups. (*b*) ROC curve of dysbindin in CA19-9 PDAC negative patients versus noncancerous subjects. (*c*) ROC curve of dysbindin in CA19-9 negative PDAC patients versus CP subjects. (*d*) ROC curve of dysbindin in CA19-9 positive PDAC patients versus noncancerous subjects. (*e*) ROC curve of dysbindin in CA19-9 positive PDAC patients versus CP subjects. (*f*) Proportions of positive results for dysbindin and CA19-9 in patients with BBO. (*g*) ROC curve of dysbindin in PDAC patients versus BBO subjects. AUC: areas under the curves; BBO: benign biliary obstruction; CA19-9^{-}: patients with negative CA19-9 (serum CA199 $\leq$ 37U/ml); CA19-9^{+}: patients with positive CA19-9 (serum CA19-9 $>$ 37U/ml); 95% CI = 95% confidence interval; CP: chronic pancreatitis; HD: healthy donors; PDAC: pancreatic ductal adenocarcinoma; ROC = receiver operating characteristics. [Color figure can be viewed in the online issue, which is available at wileyonline library.com.]

Table 2. Results of ROC curves for serum dysbindin and CA199 in the diagnosis of PDAC

Variables	AUC	95%CI	Sensitivity	Specificity
PDAC *vs.* BBO, CP and HD				
Dysbindin	0.849	0.812-0.885	81.9%	84.7%
CA199	0.740	0.696-0.783	76.3%	52.5%
PDAC *vs.* CP				
Dysbindin	0.802	0.747-0.857	73.9%	78.9%
CA199	0.689	0.632-0.746	66.3%	73.2%
PDAC *vs.* BBO				
Dysbindin	0.849	0.803-0.894	82.3%	84.0%
CA199	0.589	0.526-0.653	53.8%	49.4%
CA199 negative (Dysbindin)				
PDAC *vs.* BBO, CP and HD	0.875	0.804-0.945	83.0%	89.0%
PDAC *vs.* CP	0.820	0.738-0.903	78.7%	73.2%
CA199 positive (Dysbindin)				
PDAC *vs.* BBO, CP and HD	0.853	0.814-0.893	81.6%	86.4%
PDAC *vs.* CP	0.806	0.748-0.863	73.6%	76.1%

AUC: area under curve; BBO: pancreatic benign biliary obstruction; CP: chronic pancreatitis; HD: healthy donors; PDAC: pancreatic ductal adenocarcinoma; ROC: receiver operating characteristics.

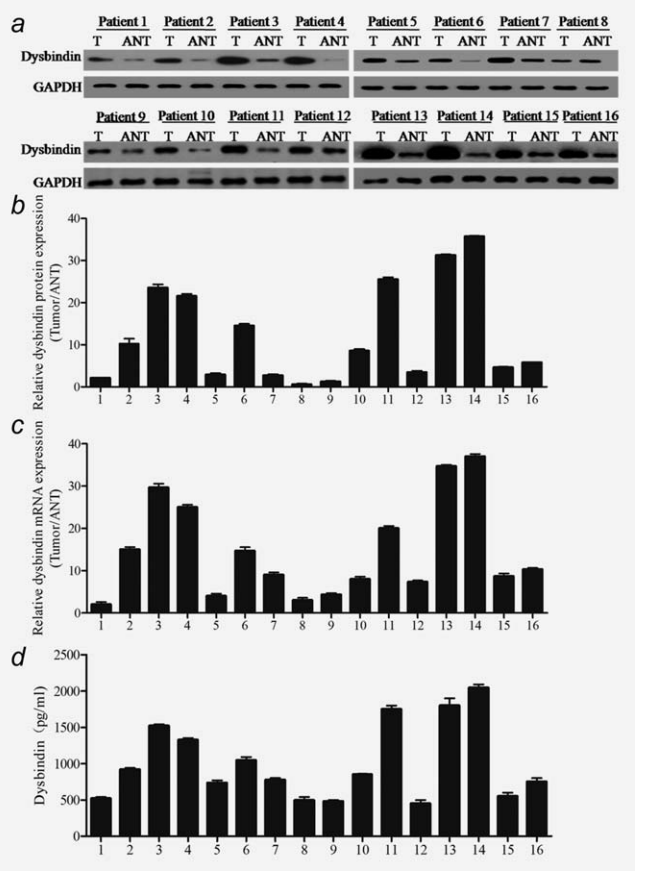

Figure 5. Corresponding expression profiles of dysbindin in PDAC tissues and sera. (*a* and *b*) Western blot of PDAC tissues and matched adjacent noncancerous tissues, and corresponding optical density ratios. (*c*) The mRNAs of dysbindin in tumors and adjacent noncancerous tissues were parallel analyzed by qRT-PCR. (*d*) The serum dysbindin level was measured by ELISA. All the experiments were performed in triplicate. ANT: adjacent noncancerous tissue; ELISA: enzyme linked immunosorbent assay; PDAC: pancreatic ductal adenocarcinoma; qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction.

tumors.[30] Thus, an effective biomarker to complement CA19-9 is needed.

In this study, we have shown that serum dysbindin level was significant higher in PDAC compared with the three controls. Meanwhile, level of dysbindin in postoperative sera was obviously lower than that of preoperative sera, indicating a strong relationship between dysbindin and PDAC. In addition, dysbindin also had better diagnostic value than that of CA19-9, especially for patients with negative CA19-9. For BBO patients who are with elevated CA19-9, dysbindin remained its efficiency. Thus, dysbindin could be a potential biomarker for PDAC and the remarkable decrease in serum dysbindin concentration after surgery indicates that it is correlated with PDAC tumor burden and may be a useful biomarker for the assessment of therapeutic response. However, to further illuminate this potential role, more patients with longer follow-up are needed.

Dysbindin, encoded by the human dystrobrevin-binding protein (DTNBP1), was first identified as a putative schizophrenia susceptibility gene.[31] Decreased dysbindin mRNA and protein in the frontal cortex and hippocampal formation have been documented in patients with schizophrenia compared to controls.[32,33] Several studies indicate that dysbindin is associated with brain functions such as intelligence, memory and cognition.[34,35] At the cellular level, dysbindin is located pre and postsynaptically in the central nervous system and its chief functions reside there.[35]

To our knowledge, few reports exist to describe the role of dysbindin in human cancers. Dysbindin is overexpressed in many cancerous tissues,[36] but its overexpression has not been reported in PDAC. Dysbindin overexpression in cancer may contribute to cell proliferation, because its occurrence has been noted in mouse embryonic germinal cells and is known to promote phosphorylation of the PI3/Akt signaling pathway, which can stimulate cell proliferation *via* multiple other pathways.[37–40] Moreover, the DTNBP1promotercontains a binding site for transcription factor (E2F1) which regulates cell proliferation.[37] However, this conclusion has not been verified in cancer cells. For the first time, we suggest that dysbindin is overexpressed in PDAC lesions compared with adjacent noncancerous tissues and in cancer cell lines compared with ductal epithelial cells. Patient characteristics indicate a significant correlation between dysbindin expression and tumor size, tumor differentiation and clinical stage. Also, dysbindin promotes human PDAC cell proliferation. Thus, dysbindin has a role in the progression of PDAC.

In summary, we have investigated dysbindin as a novel biomarker for discriminating PDAC and noncancerous controls. The diagnostic efficiency of dysbindin showed better performance than CA19-9 especially in differentiating PDAC and benign biliary obstruction. These findings suggest that dysbindin is an effective biomarker and can compliment measurement of CA19-9 in the diagnosis of PDAC, helping to distinguish PDAC from other pancreatic diseases. However, the number of patients included in this study is still no enough; we will consider carrying our further investigations to provide more accurate assess of dysbindin for PDAC.

References

1. Costello E, Neoptolemos JP. Pancreatic cancer in 2010: new insights forearly intervention and detection. *Nat Rev Gastroenterol Hepatol* 2011;8: 71–3.
2. Ryan DP, Hong TS, Bardeesy N. Pancreatic adenocarcinoma. *N Engl J Med* 2014;371:1039–49.
3. Okano K, Suzuki Y. Strategies for early detection of resectable pancreatic cancer. *World J Gastroenterol* 2014;20:11230–40.
4. Martin KJ, Fournier MV, Reddy GP, et al. A need for basic research onfluid-based early detection biomarkers. *Cancer Res* 2010;70:5203–6.
5. Locker GY, Hamilton S, Harris J, et al. Asco: ASCO 2006 update ofrecommendations for the use of tumor markers in gastrointestinalcancer. *J Clin Oncol* 2006;24:5313–27.
6. Marx V. Proteomics: an atlas of expression. *Nature* 2014;509:645–9.
7. Nie S, Lo A, Wu J, et al. Glycoprotein biomarker panel for pancreatic cancer discovered by quantitative proteomics analysis. *J Proteome Res* 2014; 13:1873–84.
8. Haglund C. Tumour marker antigen CA125 inpancreatic cancer: a comparison with CA19-9and CEA. *Br J cancer* 1986;54:897–901.
9. Li X, Guo X, Li H, et al. Serum carbohydrate antigen 242 expression exerts crucial function in the diagnosis of pancreatic cancer. *Tumour Biol* 2014;35:5281–6.
10. Koopmann J, Fedarko NS, Jain A, et al. Evaluation of osteopontin as biomarker for pancreaticadenocarcinoma. *Cancer Epidemiol BiomarkersPrev* 2004;13:487–91.
11. Wang X, Li Y, Tian H, et al. Macrophage inhibitory cytokine 1 (MIC-1/GDF15) as a novel diagnostic serum biomarker in pancreatic ductal adenocarcinoma. *BMC Cancer* 2014;14:578
12. Katz MH, Savides TJ, Moossa AR, et al. An evidence-basedapproach to the diagnosis and staging of pancreatic cancer. *Pancreatology* 2005; 5:576–90.
13. Ballehaninna UK, Chamberlain RS. Serum CA 19-9 as a Biomarker for Pancreatic Cancer-A Comprehensive Review. *Indian J Surg Oncol* 2011;2:88–100.
14. Winter JM, Yeo CJ, Brody JR. Diagnostic, prognostic, and predictivebiomarkers in pancreatic cancer. *J Surg Oncol* 2013;107:15–22.
15. Leung TK, Lee CM, Wang FC. Difficulty with diagnosis of malignant pancreatic neoplasms coexisting with chronic pancreatitis. *World J Gastroenterol* 2005;11:5075–8.
16. Singh S, Tang SJ, Sreenarasimhaiah J, et al. The clinical utilityand limitations of serum carbohydrate antigen (CA19-9) as a diagnostictool for pancreatic cancer and cholangiocarcinoma. *Dig Dis Sci* 2011;56:2491–6.
17. Hamada S, Shimosegawa T. Biomarkers of pancreatic cancer. *Pancreatology* 2011;11:14–9.
18. Wingren C, Sandstrom A, Segersvard R, et al. Identification of serum biomarker signatures associatedwith pancreatic cancer. *Cancer Res* 2012;72:2481–90.
19. Park HD, Kang ES, Kim JW, et al. Serum CA19-9, cathepsin D, andmatrix metalloproteinase-7 as a diagnostic panel for pancreatic ductaladenocarcinoma. *Proteomics* 2012;12:3590–7.
20. Pan S, Chen R, Crispin DA, et al. Protein alterations associated with pancreaticcancer and chronic pancreatitis found in human plasma using globalquantitative proteomics profiling. *JProteomeRes* 2011;10:2359–76.
21. Brand RE, Nolen BM, Zeh HJ, et al. Serum biomarker panels for thedetection of pancreatic cancer. *Clin Cancer Res* 2011;17:805–16.
22. Liu F, Du F, Chen X. Multiple tumor marker protein chip detection system in diagnosis of pancreatic cancer. *World J Surg Oncol* 2014;12: 333.
23. Chen G, Bai Q, Geng X. Preparation of a concanavalin A immobilized affinity column and its application in the structural analysis of ribonuclease B. *Chin J Chromatogr* 2006;24:425–31.
24. Singh A, Gelrud A, Agarwal B. Biliary strictures: diagnostic considerations and approach. *Gastroenterol Rep (Oxf)* 2015;3:22–31.
25. Liao Z, Jin G, Cai D, et al. Guidelines: diagnosis and therapy for chronic pancreatitis. *J Interv Gastroenterol* 2013;3:133–6.
26. Huang H, Regan KM, Wang F, et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci USA* 2005;102:1649–54.
27. Akdogan M, Sasmaz N, Kayhan B, et al. Extraordinarily elevated CA19-9 in benign conditions: a case report and reviewof the literature. *Tumori* 2001;87:337–9.
28. Wang YF, Feng FL, Zhao XH, et al. Combined detection tumor markers for diagnosis and prognosis of gallbladder cancer. *World J Gastroenterol* 2014;20:4085–92.
29. Tempero MA, Uchida E, Takasaki H, et al. Relationship of carbohydrate antigen 19-9 and Lewis antigens in pancreaticcancer. *Cancer Res* 1987;47: 5501–3.
30. Chang YT, Wu CC, Shyr YM, et al. Secretome-based identification of ULBP2 as a novel serum marker for pancreatic cancer detection. *PLoS One* 2011;6:e20029.
31. Straub RE, Jiang Y, MacLean CJ, et al. Genetic variation in the 6p22.3 gene DTNBP1, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. *Am J Hum Genet* 2002; 71:337–48.
32. Talbot K, Eidem WL, Tinsley CL, et al. Dysbindin-1 is reduced in intrinsic, glutamatergic terminals of the hippocampal formation in schizophrenia. *J Clin Invest* 2004;113:1353–63.
33. Weickert CS, Straub RE, McClintock BW, et al. Human dysbindin (DTNBP1) gene expression in normal brain and in schizophrenic prefrontal cortex and midbrain. *Arch Gen Psychiatry* 2004;61: 544–55.
34. Corvin A, Donohoe G, Nangle JM, et al. A dysbindin risk haplotype associated with less severe manic-type symptoms in psychosis. *Neurosci Lett* 2008;431:146–9.
35. Donohoe G, Morris DW, Clarke S, et al. Variance in neurocognitive performance is associated with dysbindin-1 in schizophrenia: a preliminary study. *Neuropsychologia* 2007;45:454–8.
36. Sillitoe RV, Benson MA, Blake DJ, et al. Abnormal dysbindin expression in cerebellar mossy fiber synapses in the mdx mouse model of Duchenne muscular dystrophy. *J Neurosci* 2003;23: 6576–85.
37. Higo M, Uzawa K, Kouzu Y, et al. Identification of candidate radioresistant genes in human squamous cell carcinoma cells through gene expression analysis using DNA microarrays. *Oncol Rep* 2005;14:1293–8.
38. Javitt DC, Kantrowitz JT. Handbook of Neurochemistry and Molecular Neurobiology-Schizophrenia, 3rd ed. Springer Science+Business Media, 2009. 191.
39. Numakawa T, Yagasaki Y, Ishimoto T, et al. Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia. *Hum Mol Genet* 2004;13:2699–78.
40. Manning BD, Cantley LC. AKT/PKB signaling: Navigation downstream. *Cell* 2007;129:1261–74.