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# Development of a sandwich ELISA for the thrombin light chain identified by serum proteome analysis

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ABSTRACT

We previously identified novel biomarker candidates in biliary tract cancer (BTC) using serum proteome analysis. Among several candidates, we focused on thrombin light chain which is a 4204 Da peptide as the most promising biomarker for BTC. To move thrombin light chain toward potential diagnostic use, we developed an enzyme immunoassay that enables to measure serum thrombin light chain levels.

Both one monoclonal antibody specific to the N-termini and one polyclonal antibody were used to develop a sandwich ELISA for thrombin light chain. The assay was evaluated by comparing the results with those obtained by the ClinProt™ system. Serum samples were obtained from 20 patients with BTC, 20 patients with BBTDs and 20 HVs using the ClinProt™ system and ELISA.

The results of the established ELISA showed a positive correlation with the findings by ClinProt™ system (slope = 0.3386, intercept = 34.901, $r^2 = 0.9641$). The performance of the ELISA was satisfactory in terms of recovery (97.9–102.5%) and within-run (1.5–4.8%) and between-day (1.9–6.7%) reproducibility. Serum thrombin light chain levels were significantly greater in BTC (176.5 ± 47.2 ng/mL) than in BBTDs (128.6 ± 17.4 ng/mL) and HVs (127.6 ± 16.0 ng/mL) ($p < 0.001$).

The sandwich ELISA developed in this study will be useful for validation of the diagnostic significance of serum thrombin light chain levels in various cancers.

## 1. Introduction

Biliary tract cancer (BTC) is a neoplasm that accounts for 3% of all gastrointestinal cancers and 15% of all primary liver cancers. Over the last two decades, the incidence of BTC has risen, mainly due to an increase in the intrahepatic form [1,2], which has a

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particularly high incidence in Northern Thailand [3]. Surgical resection is the only curative treatment and this requires an early diagnosis. Even in cases in which surgical resection with negative histological margins is achieved, the 5-year survival rates range from 20% to 40%, the median of survival period for unresectable cases is only 6 months [4,5]. Therefore, it is important to detect and diagnose BTC early to permit multimodal treatment with surgery and chemotherapy, including molecular targeted drugs, to improve outcomes.

Proteomics using advanced analytical techniques gives a comprehensive analysis of protein levels in vivo and relationships with disease. Biomarker candidates can be detected by serum/plasma proteome analysis using mass spectrometry (MS), leading to acquisition of disease-specific diagnostic information [6,7]. However, few of these markers are currently practically useful for diagnosis and tests. We detected a 5.9 kDa αC-chain near the C-terminal fragment (FIC5.9) as a marker for drinking behavior using MS [8]. For FIC5.9-specific detection, antibodies against the N-terminal and C-terminal domains of FIC5.9 were produced and a sandwich ELISA was constructed [9]. A multicenter clinical trial showed that detection of FIC5.9 was effective for assessment of early fibrogenesis in chronic hepatic dysfunction [10].

The ClinProt™ System (Bruker Daltonics) was used to identify the thrombin light chain as a new tumor marker using serum samples from patients with BTC scheduled for surgical resection and from patients with benign biliary tract diseases (BTDs) and healthy volunteers [11]. The thrombin light chain comprises cleavage products of precursor proteins that are present in serum at a fraction of the concentrations of the corresponding full-length proteins. Neither truncation product described in the study had been reported previously as a marker for gastric cancer and hepatocellular carcinoma infected with hepatitis B virus (HCC-B), indicating that the thrombin light chain is a new candidate marker [12,13]. This marker may be the product of cleavage by one or more proteases, including plasma kallikreins, tissue kallikreins, matrix metalloproteases, or prostasin, a trypsin-like serine protease [13].

Thrombin light chain is significantly increased in patients with gastric cancer and HCC-B, but objective comparison is difficult because different measurement methods were used. Establishment of the clinical significance of marker candidates is required for the confirmation of reproducibility in large-scale validation and high-throughput screening. In this study, we have focused on an antibody against the N-terminal domain of thrombin light chain to establish a precision thrombin light chain measurement system using a sandwich ELISA. This sandwich ELISA is useful for clinical laboratory tests.

## 2. Materials and methods

### 2.1. Patient serum samples

The blood samples were obtained from 20 patients with BTC, 20 patients with benign BTDs, and age/gender-matched 20 healthy volunteers (HVs) as controls. Blood samples were obtained from all patients in the Department of General Surgery, Chiba University Hospital, who were histologically diagnosed with BTC and benign BTDs from February 2006 to April 2011. Samples were also obtained from HVs in the Kashiwado Hospital (Tables 1–3). Sample collection and processing were performed as previously reported [14]. The ethics committee for each institute approved the protocol. Written informed consent was obtained from all patients and HVs.

### 2.2. ClinProt™ system

We used weak cation exchange (WCX) magnetic beads (Bruker Daltonics, Bremen, Germany) and performed serum peptidome fractionation according to the manufacturer's protocol. A 5 μL serum sample was mixed with 10 μL of binding buffer to which 5 μL of WCX beads was added, and the solution was carefully mixed. The peptides in the serum were then allowed to bind to the WCX beads for 5 min. The tube was then placed in a magnetic bead separator (Bruker Daltonics) for separating unbound beads, and the supernatant was removed. The beads were washed three times with 100 μL of washing buffer, and the proteins as well as peptides were then eluted from the magnetic beads with 10 μL each of elution and stabilization buffer. Thereafter, 2 μL of peptide elution solution was mixed with 20 μL of alpha-cyano-4-hydroxycinnamic acid matrix (Bruker Daltonics). Then 0.8 μL of this mixture was spotted onto an AnchorChip target plate (Bruker Daltonics) and crystallized. Each sample was duplicated, and quadruplicate spotting was performed using each eluate; eight spots were developed from each sample. The mean spectra from these eight spots were used for data analyses. These procedures from bead fractionation to spotting were performed automatically using the ClinProt robot (Bruker Daltonics) under strictly controlled humidity, as we previously described [14].

The AnchorChip target plate was placed in an AutoFlex II TOF/TOF mass spectrometer (Bruker Daltonics) controlled by

**Table 1**
Clinical characteristics of patients with HVs, benign BTDs, and BTC.

| | HVs | Benign BTDs | BTC |
|---|---|---|---|
| No. patients | 20 | 20 | 20 |
| Sex (Male/Female) | 10/10 | 10/10 | 10/10 |
| Age (Mean ± SD) | 64.8 ± 6.2 | 64.2 ± 39.0 | 64.7 ± 32.1 |
| CEA (ng/mL) | 3.2 ± 3.2 | 2.7 ± 3.4 | 37.1 ± 1500.1 |
| CA19–9 (U/mL) | 16.7 ± 17.2 | 65.2 ± 543.7 | 5065.4 ± 190214.9 |

**Table 2**
Characteristics of patients with BTC.

| | BTC (n = 20) |
|---|---|
| Location | |
| Extrahepatic | 6 |
| Intarahepatic | 6 |
| Klatskin | 1 |
| Papilla vater | 1 |
| Gall bladder | 6 |
| UICC stage | |
| StageI | 4 |
| StageII | 4 |
| StageIII | 6 |
| StageIV | 6 |

Flexcontrol 2.4 software (Bruker Daltonics). The instrument was equipped with a 337 nm nitrogen laser, delayed-extraction electronics, and a 25 Hz digitizer. All acquisitions were generated by an automated method included in the instrument software and based on averaging of 1000 randomized shots. Spectra were acquired in positive linear mode in the mass range of 600–10000 Da. Peak clusters were completed using second pass peak sections (signal to noise ratio > 5). The relative peak intensities of $m/z$ between 600 and 10000 normalized to a total ion current were expressed in arbitrary units. Calibration was performed using Peptide Calibration Standard II (Bruker Daltonics). All MALDI-TOF MS spectra from $m/z$ 1000–10000 were analysed with FlexAnalysis 2.1 and ClinProtools 2.1 software (Bruker Daltonics).

### *2.3. ELISA assay*

#### *2.3.1. Immunogens for the development of anti- thrombin light chain antibody*

Synthetic peptides of 15 amino acid (Toray research center, Tokyo, Japan) corresponding to the sequences of the N- (thrombin light chain N peptide) of thrombin light chain, coupled to keyhole limpet hemocyanin (KLH) were obtained from Sigma-Aldrich Japan (Tokyo, Japan). The peptide-keyhole limpet hemocyanin conjugates were dissolved in distilled water and used as antigens for the preparation of monoclonal antibodies.

#### *2.3.2. Immunization and establishment of hybridoma cell lines*

The thrombin light chain N peptide (50 mg at 1 mg/mL in PBS buffer) was used for the immunization of BALB/c mice. Hybridoma cell lines were prepared as described [15]. The hybridoma cell line, thrombin light chain N-02, were established and antibody isotypes were determined using Mouse Monoclonal Antibody Isotyping Test Kit (AbD Serotec, Oxford, UK) following the manufacturer's instructions. To obtain pure monoclonal antibodies on a large scale, BALB/c mice were initially stimulated with 1.0 mL pristine (Sigma Aldrich Japan) and then inoculated 2 week later. Monoclonal antibodies were purified as described [15].

#### *2.3.3. Western blot analysis of anti- thrombin light chain antibodies*

To examine the specificity of the antibodies, synthetic thrombin light chain and serum sample of patient with BTC were separated by SDS-PAGE using a 15–25% gradient gel (DRC, Tokyo, Japan) in the absence of β-mercaptoethanol, and then transferred onto a PVDF membrane. To minimize nonspecific binding, the membranes were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan). After the membrane was washed three times with PBST (PBS buffer including 0.05% Tween-20), the membranes were incubated with anti- thrombin light chain N peptide antibody or anti- thrombin light chain polyclonal antibody (Bioss Inc. MA, USA) as primary antibodies for 1 h at room temperature. The membrane was washed again three times with PBST and then incubated with a secondary Rabbit anti-mouse immunoglobulins/HRP (DAKO Japan, Tokyo, Japan) for 1 h at room temperature. The reactive antibodies were visualized by staining with Pierce Western Blotting substrate (Thermo Fisher Scientific, MA, USA).

#### *2.3.4. Immobilization of antibodies to a polystyrene microtiter plate*

The anti- thrombin light chain polyclonal antibody dissolved in PBS buffer was dispensed into a 96-well polystyrene microtiter plate (Thermo Fisher Scientific) at 0.5 mg/well and incubated for 1 day at 4 °C. The plate was washed three times with PBS containing 0.05% Tween-20. The microtiter plate was coated with 20% NOF102 containing 10% sucrose for 1 day at 4 °C, and then

**Table 3**
Characteristics of patients with benign BTDs.

| | Benign BTDs (n = 20) |
|---|---|
| Cholelithiasis | 16 |
| Benigin fibrous stricture | 2 |
| Primary sclerosing cholangitis | 2 |

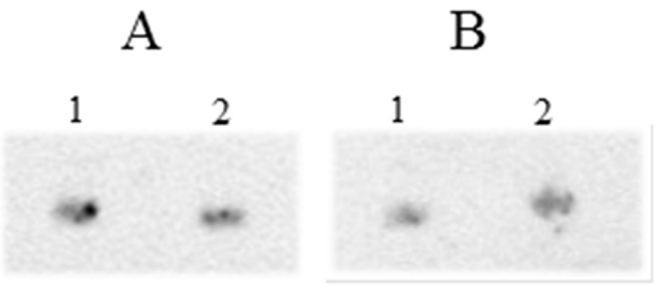


**Fig. 1.** Western blotting analysis. Immunoreactive bands were observed at thrombin light chain when synthetic and serum thrombin light chain were incubated with anti-thrombin light chain N antibody (A) or thrombin light chain polyclonal antibody (B). Lane1: synthetic thrombin light chain. Lane2: serum thrombin light chain.

dried for 7 days at 4 °C. The microtiter plate was kept at 4 °C until use.

#### *2.3.5. ELISA assay conditions*

Synthetic thrombin light chain was diluted with PBS buffer for calibration. After washing the microtiter plate with PBS buffer containing 0.05% Tween-20, 100 μL aliquots of 100-diluted serum samples were added in duplicate to wells. The plates were incubated at room temperature for 1 h and then washed three times. Anti- thrombin light chain N antibody conjugated to HRP in PBS containing 0.05% Tween-20 (100 μL) was added to each well and the plate was incubated at room temperature for 30 min. The plate was washed three times, and then 100 μL of TMB solution (Wako Pure Chemical Industries, Tokyo, Japan) was added. After incubation at room temperature for 10 min, 100 μL of stop solution was added and the absorbance at 450 nm was measured.

### *2.4. Statistical analysis*

Numerical data are presented as the mean ± standard deviation (SD). Data were analysed using SPSS version 19.0 statistical package (SPSS, Chicago, IL). Data were evaluated by linear regression analysis and correlations were assessed using Pearson correlation coefficients. For non-parametric data, the differences between two groups were analysed using the Mann–Whitney *U*-test. P-values $< 0.05$ were considered statistically significant.

## 3. Results

### *3.1. Western blot analysis*

Anti- thrombin light chain antibody (Fig. 1A) and anti- thrombin light chain N antibody (Fig. 1B) recognized synthetic thrombin light chain and serum of patient with BTC, respectively.

### *3.2. Characterization of the ELISA assay: range, dilution analysis and detection limit*

A standard curve was drawn based on the colorimetric intensity of diluted synthetic thrombin light chain to establish the relationship between the intensity and the thrombin light chain concentration (Fig. 2). The working range of the assay was 0–200 ng/mL (Fig. 2A) and the assay gave linear results from 0 to 190.4 ng/mL ($y = 1.042x + 0.191$, $r^2 = 0.9998$, $p < 0.0001$) (Fig. 2B). The detection limit was estimated by assaying the zero concentration eight times, and defined as the thrombin light chain "zero" concentration + 3 SD. The limit was found to be 1.5 ng/mL.

### *3.3. Within-run and between-run reproducibility*

The precision of the assay was determined using three concentrations (48.2, 110.3 and 210.4 ng/mL). Within-assay CVs were determined with eight replicates of each sample. Between-assay CVs were determined based on assays performed on 5 different days (two replicates of each sample per day). The within-run CV was 1.5–4.8% and the between-run CV was 1.9–6.7%.

### *3.4. Interference*

Interference was assessed in samples containing 190.4 ng/mL of thrombin light chain. Potential interference materials were added to sera at various concentrations. There was no substantial interference from hemoglobin (up to 5000 mg/L), free bilirubin (up to 207 mg/L), ditaurobilirubin (up to 204 mg/L), chyle (up to 1400 formazine turbidity units even equal 1176 mg/L as triglyceride), ascorbic acid (up to 500 mg/L) and RF (up to 500 U/L).

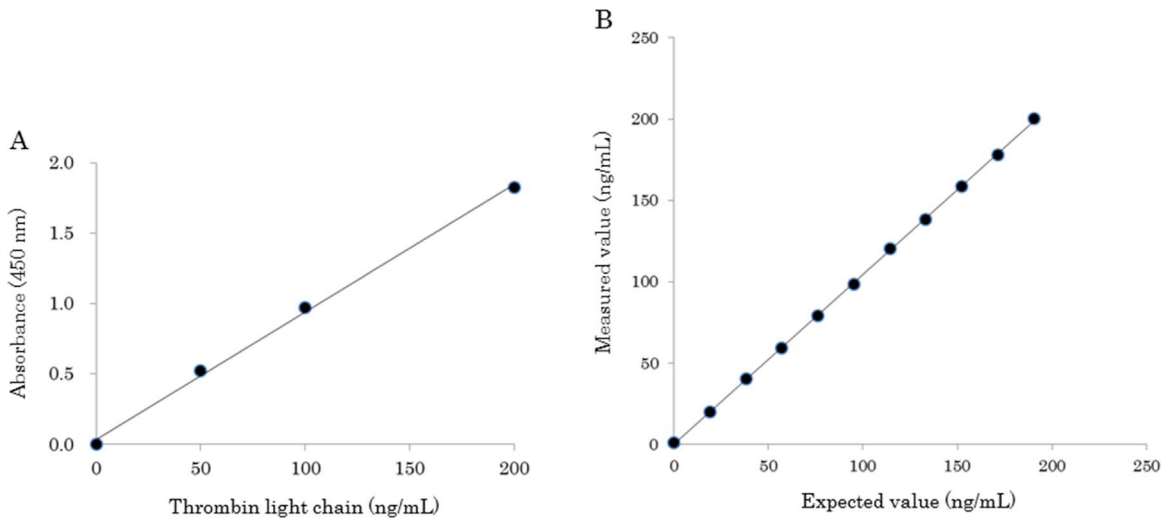


**Fig. 2.** Standard curves for thrombin light chain in the ELISA. (A) The relationship between colorimetric intensity and thrombin light chain concentration in the range of 0.0–200.0 ng/mL. Four concentrations of thrombin light chain were determined by ELISA. (B) Linearity of results from the ELISA, which fitted to an equation: $y = 1.042x + 0.1914$ ($r^2 = 0.9998$, $p < 0.0001$).

### 3.5. Recovery test

To evaluate recovery in the ELISA, three concentrations (1.25, 12.5 and 62.5 ng/mL) of synthetic thrombin light chain were added to pooled serum (190.4 ng/mL). The percentage recovery ranged from 97.9% to 102.5%.

### 3.6. Correlation between ClinProt™ System and ELISA

Thrombin light chain levels in all 60 serum samples obtained from HVs and patients with BTC and benign BTDs were determined by ClinProt™ System and ELISA. The results were significantly well-correlated, as shown in Fig. 3 (slope = 0.3386, intercept = 34.901, $r^2 = 0.9641$, $p < 0.0001$).

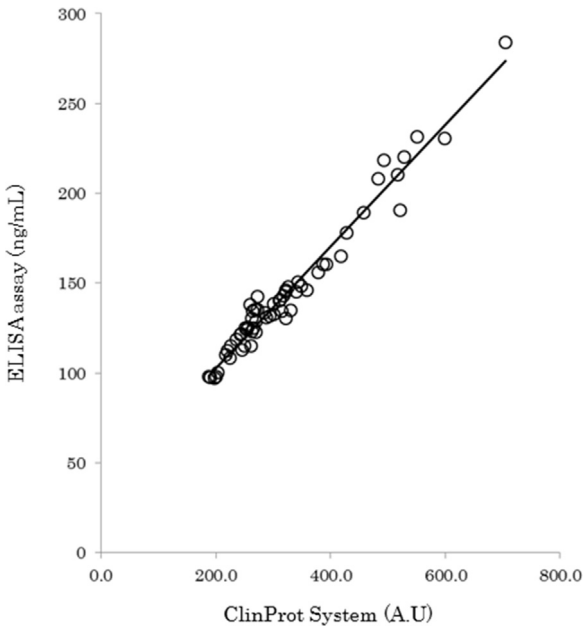


**Fig. 3.** Comparison of the results between ELISA and ClinProt System. The results between ELISA and ClinProt™ System were well-correlated (slope = 0.3386, intercept = 34.901, r2 = 0.9641, $p < 0.0001$).

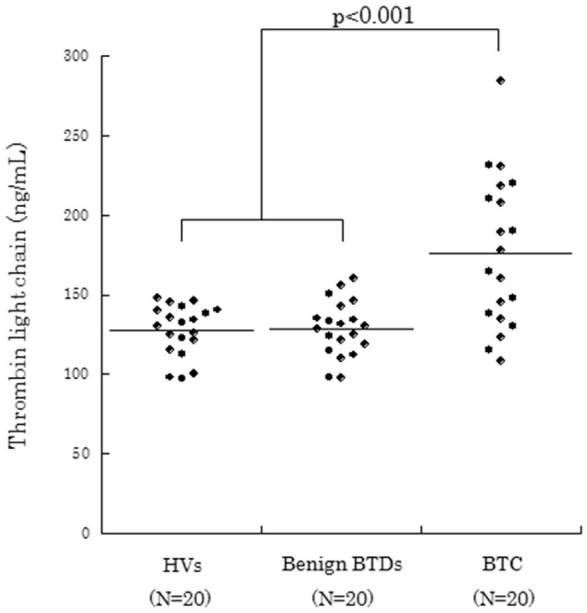


**Fig. 4.** Serum thrombin light chain levels in HVs, and in patients with benign BTDs and BTC. The levels are significantly greater in BTC (176.5 ± 47.2 ng/mL) compared with the HVs (127.6 ± 16.0 ng/mL) and benign BTDs (128.6 ± 17.4 ng/mL). Serum thrombin light chain levels in patients with BTC were significantly higher than those in HVs and in patients with benign BTDs ($P < 0.001$; Mann–Whitney *U*-test).

### *3.7. Thrombin light chain levels are increased in the sera of patients with BTC*

We measured serum thrombin light chain levels among 20 BTC patients and age-matched benign controls including 20 HVs and 20 benign BTDs patients. As shown in Fig. 4, the serum thrombin light chain levels in BTC patients (176.5 ± 47.2 ng/mL) were significantly greater than in the HVs (127.6 ± 16.0 ng/mL) and benign BTDs patients (128.6 ± 17.4 ng/mL) (BTC vs. HVs: $P < 0.001$, BTC vs. benign BTDs: $P < 0.001$; Mann–Whitney *U*-test). Importantly, there was no significant difference in serum thrombin light chain levels between HVs and benign BTDs patients.

## 4. Discussion

In this study, we have constructed that the performance of the ELISA was satisfactory in terms of recovery (97.9–102.5%) and within-run (1.5–4.8%) and between-day (1.9–6.7%) reproducibility. To the end, we have described that serum thrombin light chain level is a potential serum biomarker to distinguish BTC from benign BTDs.

Advanced techniques for proteomics analysis have resulted in identification of many new biomarkers, but the most of those are not insufficient for clinical application. The procedures for developing biomarkers include comparison of disease and control groups by semiquantitative analysis (discovery phase) and selection of marker candidates (validation phase), followed by ELISA validation of markers found by MS.

Ebert et al. found that the fibrinopeptide A concentration increased in serum of patients with gastric cancer in semiquantitative analysis using MALDI-TOF MS, and subsequently confirmed this result in a large-scale test using ELISA [16]. However, this kind of confirmation is unusual, partly because it is often difficult to produce antibodies against MS-detected marker candidates. Thus, although many marker candidates have been found by MS-based proteomics analysis, they remain unused because there is no appropriate ELISA for validation. The specificity and sensitivity of antibodies may also be low and this results in further difficulties in validation [17].

Multiple reaction monitoring (MRM) based on MS and selective reaction monitoring (SRM) can be used to detect and quantify targeted proteins [18]. However, MRM cannot be used for thrombin light chain because its molecular weight exceeds the detectable level in MRM. Therefore, we conducted ELISA using antibodies against the full length thrombin light chain and its N-terminal domain. The ELISA results showed a good correlation with those from the ClinProt™ System and accurately detected thrombin light chain in serum. Day-to-day reproducibility and repeatability were also good.

To improve treatment outcomes and prognosis of patients with BTC, early detection and resection are important. Screening in a medical check-up is necessary for early detection of cancer, but BTC is one of the most difficult cancers to detect in screening [10]. Patients with BTC often have increased hepatobiliary enzymes, but this is not found in all cases and there are no markers specific for BTC [19]. Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are tumor markers that are used for diagnosis of BTC, but their sensitivity and specificity are unsatisfactory [2]. The serum levels of thrombin light chain in healthy volunteers, patients with benign BTD, and patients with BTC were 27.6 ± 16.0, 128.6 ± 17.4 and 176.5 ± 47.2 ng/mL, respectively. There was no significant difference between healthy volunteers and patients with benign BTD, but the thrombin light chain level was

significantly elevated in patients with BTC compared to those with benign BTD ($p < 0.001$). These results suggest that thrombin light chain is a new marker for BTC that is complementary to conventional markers.

The serum level of thrombin light chain has been shown to change in patients with BTC, gastric cancer and HCC. However, the usefulness of thrombin light chain was not validated because there was no method for high-throughput measurement. The simple ELISA developed in this study is useful for establishing the diagnostic significance of serum thrombin light chain in patients with BTC. In conclusion, we have established a precise serum thrombin light chain measurement system using a sandwich ELISA. It is possible to distinguish BTC from benign BTDs using this serum biomarker. One of the critical limitations in this study is that the system has been evaluated in only a small number of patients. Further validation in an independent and prospective large cohort will be needed to establish benign BTDs as a new diagnostic marker for BTC in a clinical setting. Detection of serum thrombin light chain in patients with gastric cancer and HCC using this ELISA system may also apply in patients with other types of cancer.

## Conflict of interest

The authors declare no conflict of interest associated with this manuscript.

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## References

[1] S.A. Khan, H.C. Thomas, B.R. Davidson, S.D. Taylor-Robinson, Cholangiocarcinoma, Lancet 366 (2005) 1303–1314.
[2] T. Patel, Cholangiocarcinoma, Nat. Clin. Pract. Gastroenterol. Hepatol. 3 (2006) 33–42.
[3] Y. Shaib, H.B. El-Serag, The epidemiology of cholangiocarcinoma, Semin Liver Dis. 24 (2004) 115–125.
[4] W.R. Jarnagin, M. Shoup, Surgical management of cholangiocarcinoma, Semin Liver Dis. 24 (2004) 189–199.
[5] G.J. Gores, Cholangiocarcinoma: current concepts and insights, Hepatology 37 (2003) 961–969.
[6] G.L. Hortin, The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome, Clin. Chem. 52 (2006) 1223–1237.
[7] E.F. Petricoin, C. Belluco, R.P. Araujo, L.A. Liotta, The blood peptidome: a higher dimension of information content for cancer biomarker discovery, Nat. Rev. Cancer 6 (2006) 961–967.
[8] F. Nomura, T. Tomonaga, K. Sogawa, T. Ohashi, M. Nezu, M. Sunaga, N. Kondo, M. Iyo, H. Shimada, T. Ochiai, Identification of novel and downregulated biomarkers for alcoholism by surface enhanced laser desorption/ionization mass spectrometry, Proteomics 4 (2004) 1187–1194.
[9] K. Noda, K. Sogawa, W. Kikuchi, I. Kiyokawa, T. Miura, R. Kojima, K. Katayama, Y. Kodera, F. Nomura, Development of a sandwich ELISA for the 5.9-kDa fibrinogen alpha C chain fragment detected by serum proteome analysis, Proteom. Clin. Appl. 5 (2011) 141–146.
[10] K. Sogawa, K. Noda, H. Umemura, M. Seimiya, T. Kuga, T. Tomonaga, M. Nishimura, F. Kanai, F. Imazeki, H. Takizawa, M. Yoneda, A. Nakajima, M. Tsutsumi, O. Yokosuka, F. Nomura, Serum fibrinogen alpha C-chain 5.9 kDa fragment as a biomarker for early detection of hepatic fibrosis related to hepatitis C virus, Proteom. Clin. Appl. 7 (2013) 424–431.
[11] S. Kikkawa, K. Sogawa, M. Satoh, H. Umemura, Y. Kodera, K. Matsushita, T. Tomonaga, M. Miyazaki, O. Yokosuka, F. Nomura, Identification of a novel biomarker for biliary tract cancer using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Int. J. Proteom. (2012), http://dx.doi.org/10.1155/2012/108609.
[12] M.P. Ebert, S. Lamer, J. Meuer, P. Malfertheiner, M. Reymond, T. Buschmann, C. Röcken, V. Seibert, Identification of the thrombin light chain a as the single best mass for differentiation of gastric cancer patients from individuals with dyspepsia by proteome analysis, J. Proteome Res. 4 (2005) 586–590.
[13] F.X. Wu, Q. Wang, Z.M. Zhang, S. Huang, W.P. Yuan, J.Y. Liu, K.C. Ban, Y.N. Z hao, Identifying serological biomarkers of hepatocellular carcinoma using surface-enhanced laser desorption/ionization-time-of-flight mass spectroscopy, Cancer Lett. 279 (2009) 163–170.
[14] H. Umemura, M. Nezu, Y. Kodera, M. Satoh, A. Kimura, T. Tomonaga, F. Nomura, Effects of the time intervals between venipuncture and serum preparation for serum peptidome analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, Clin. Chim. Acta 406 (2009) 179–180.
[15] T. Ohashi, Y. Igarashi, Y. Mochizuki, T. Miura, N. Inaba, K. Katayama, T. Tomonaga, F. Nomura, Development of a novel fragments absorbed immunocapture enzyme assay system for tartrate-resistant acid phosphatase 5b, Clin. Chim. Acta 376 (2007) 205–212.
[16] M.P. Ebert, D. Niemeyer, S.O. Deininger, T. Wex, C. Knippig, J. Hoffmann, J. Sauer, W. Albrecht, P. Malfertheiner, C. Röcken, Identification and confirmation of increased fibrinopeptide a serum protein levels in gastric cancer sera by magnet bead assisted MALDI-TOF mass spectrometry, J. Proteome Res. 5 (2006) 2152–2158.
[17] K.Y. Wang, S.A. Chuang, P.C. Lin, L.S. Huang, S.H. Chen, S. Ouarda, W.H. Pan, P.Y. Lee, C.C. Lin, Y.J. Chen, Multiplexed immunoassay: quantitation and profiling of serum biomarkers using magnetic nanoprobes and MALDI-TOF MS, Anal. Chem. 80 (2008) 6159–6167.
[18] T. Fortin, A. Salvador, J.P. Charrier, C. Lenz, X. Lacoux, A. Morla, G. Choquet-Kastylevsky, J. Lemoine, Clinical quantitation of prostate-specific antigen biomarker in the low nanogram/milliliter range by conventional bore liquid chromatography-tandem mass spectrometry (multiple reaction monitoring) coupling and correlation with ELISA tests, Mol. Cell Proteom. 8 (2009) 1006–1015.
[19] K. Kimura, M. Ohto, H. Saisho, T. Unozawa, Y. Tsuchiya, M. Morita, M. Ebara, S. Matsutani, K. Okuda, Association of gallbladder carcinoma and anomalous pancreaticobiliary ductal union, Gastroenterology 89 (1985) 1258–1265.