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ARTICLES

Serum Levels of Variants of Transthyretin Down-Regulation in Cholangiocarcinoma

Liyun Liu,[1,2] Jianing Wang,[1,2] Bo Liu,[3] Songwei Dai,[1,2] Xiaomin Wang,[1,2] Junzhou Chen,[3] Lingyun Huang,[1,2] Xueyuan Xiao,[1,2]* and Dacheng He[1,2]*

[1]Key Laboratory for Cell Proliferation and Regulation Biology Ministry of Education, Beijing Normal University, The People's Republic of China
[2]Universities' Confederated Institute of Proteomics, Beijing 100875, The People's Republic of China
[3]The Department of Hepatobiliary Surgery, General Hospital of the Chinese People's Liberation Army, Beijing 100853, The People's Republic of China

Abstract Background: Cholangiocarcinoma (CC) is devastating neoplasm and very few specific biomarkers could be used in clinical diagnosis. A study was taken to find novel serum biomarkers for CC. Methods: Surface enhanced laser desorption/ionization (SELDI) technology was used to analyze 427 serum samples including 56 CCs, 49 hepatobiliary diseases, 269 other cancer control, and 53 healthy individuals. The candidates were isolated and identified by SDS–PAGE, ESI/MS-MS, Western blot, and immunoprecipitation. Liver functions of CC patients were examined and enzyme-linked immunosorbent assay (ELISA) of transthyretin (TTR) and CA19-9 were further performed in some sets of serum samples. Results: 13.76, 13.88, and 14.04 k m/z peaks in sera were significantly decreased in CC compared with the control groups ($P < 0.001$). Subsequently, these three peaks were identified as native TTR and its two variants. ELISA assay indicated that TTR levels were consistent with SELDI analysis in CC compared with healthy control and benign diseases of hepatobiliary ($P < 0.001$). Liver function test levels were obviously elevated for CC patients. TTR combining with CA19-9 to differentiate CC from benign hepatobiliary diseases showed sensitivity and specificity were 98.2% and 100%, respectively. Conclusion: The levels of TTR were significantly down-regulated in sera of CC patients and may be complementary markers of CA19-9 in diagnosis for CC. J. Cell. Biochem. 104: 745–755, 2008.

Key words: CC; TTR; SELDI-TOF-MS; ELISA

Cholangiocarcinoma (CC) is a rare but devastating neoplasm that accounts for 3% of all gastrointestinal cancers and 15% of all primary liver cancers worldwide [Shaib and El-Serag, 2004]. During the last two decades, the incidence of CCs has been raised. People elder than age 65 are more likely to get CC, and men more than women. CCs are slow growing and usually hard to get diagnosed in the early stage due to the unfavorable anatomic location [Patel, 2006]. At present, although the serous tumor markers, CEA and CA 19-9, begin to be used to detect CC, the sensitivity and specificity were very poor and a diagnosis would still be made rely on the patient's signs and symptoms [Akdogan et al., 2003]. In addition, an endoscopic retrograde cholangiopancreatography, cytological approaches, and a positive PET scan could be helpful for diagnosis; however these methods were not really suitable for the early stage [Campbell et al., 1989]. Therefore, to

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Abbreviations used: TTR, transthyretin; cysTTR, cysteinylated transthyretin; glutTTR, glutathionylated transthyretin; ELISA, enzyme-linked immunosorbent assay; SELDI-TOF-MS, surface enhanced laser desorption/ionization time of flight mass spectrometry.

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*Correspondence to: Dacheng He and Xueyuan Xiao, Key Laboratory for Cell Proliferation and Regulation Biology Ministry of Education, Beijing Normal University, 19th Xin jie kou wai da jie, Beijing 100875, China.
E-mail: dhe@bnu.edu.cn; xyxiao@bnu.edu.cn

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discover specific and facilitative biomarkers for CC is critically needed.

Currently, cancer biomarkers screening becomes a hot field in serum proteomics. Serum proteins may serve as indicators of diseases and are also valuable resources for biomarker discovery. Up to now, an increasing number of cancer-related biomarkers for diagnosis, progression, and prognosis have been successfully identified by surface enhanced laser desorption/ionization (SELDI) technology [Yu et al., 2005; Munro et al., 2006; Scarlett et al., 2006; Ward et al., 2006]. However, none of biomarkers was identified due to the limitation of SELDI technology. In fact, identification of these candidates will not only assist in exploring the mechanisms of carcinogenesis, but also facilitate the development of traditional multiprotein antibody array for early detection of cancer. Recently, as a parallel effort in discovery of biomarkers, the identification of candidates and evaluation of their utility as potential biomarkers for cancers have been paid more attention.

Transthyretin (TTR) is a 55-kDa homotetrameric protein involved in transport of thyroid hormones in blood [Power et al., 2000; Fung et al., 2005], and plays a major role in retinol metabolism. It is synthesized partly from liver, partly from extrahepatic tissues such as retinal pigment epithelium (RPE), choroids plexus and islet A and B cells [Jacobsson et al., 1989]. As high abundant protein, TTR binds to the circulating RBP to prevent glomerular filtration of low molecular mass RBP [Raghu and Sivakumar, 2004; Lowenthal et al., 2005; Geho et al., 2006; Liotta and Petricoin, 2006; Menard et al., 2006]. The levels of TTR could decrease in cases of severe liver disease, malnutrition, and acute inflammation [Imanishi, 1981; Marten et al., 1996; Ritchie et al., 1999]. In addition, TTR was found to decline in the sera of patients with ovarian cancer, advanced cervical and endometrial carcinomas [Kozak et al., 2005]. In the present study, TTR is demonstrated to be a useful biomarker to discriminate CC from both benign hepatobiliary diseases and normal control group, suggesting a potential value in CC diagnosis.

MATERIALS AND METHODS

Materials

Serum samples of 56 CC (Table I), 49 benign hepatobiliary diseases were obtained from Department of Hepatobiliary Surgery, General Hospital of the Chinese People's Liberation Army. Serum samples of 146 lung cancer, 65 laryngeal carcinomas (LGC) and 58 laryngopharyngeal carcinomas (LPC), were acted as the other cancer control groups and provided by Department of Thoracic Surgery, General Hospital of Beijing Unit, PLA and Department of Otolaryngology-Head and Neck Surgery, Third Hospital of Jilin University, respectively. Sera under the study were collected between October 2005 and March 2006. After obtaining informed consent from the patient, 5 ml of blood were collected into a 10 ml vacutainer and kept at 4°C for 1 h. Each blood samples was centrifuged at 4,000 rpm for 20 min and sera were collected and kept frozen at −80°C until the analysis was carried out. Fifty-three healthy serum samples, consisting of 30 males and 23 females ranging in age 40–60 years, were obtained from a general health examination in General Hospital of the Chinese People's Liberation Army. The processing, collection, and storage protocols for normal individuals were exactly the same as aforementioned of the patients'.

TABLE I. Patients' Characteristics for Cholangiocarcinoma Serum Samples

Characteristics	No. of patients (mean age; range)
Cholangiocarcinoma	56 (59; 32–86)
Male	38
Female	18
Disease stage	
II	11
III	25
IV	20
Pathological diagnosis	
Extrahepatic biliary epithelium adenocarcinomas	25
Intrahepatic biliary epithelium adenocarcinomas	31

Protein Chip Profiling Analysis

Serum protein profiling analysis was performed on CM10 protein chips (Ciphergen, Fremont, CA) [Xiao et al., 2004]. Briefly, 20 μl 9 mol/L urea, 1% CHAPS in PBS, pH 7.4, was added to 10 μl of each serum samples. The mixture was vortexed at 4°C for 30 min and diluted 1:9 in binding buffer (100 mmol/L NaAc, pH 4.0) and 100 μl of diluted samples was applied to each spot. After binding at room temperature on a platform shaker at 2,500 rpm for 35 min, the chips were washed three times with 200 μl of washing buffer for 5 min. After air drying, 0.5 μl of saturated sinapinic acid (SPA) was applied twice to each spot. The chips were read in PCS 4,000 ProteinChip Reader (Ciphergen, Fremont, CA), Automated analytical protocol was used to control the data acquisition process. Data were collected at the high laser intensity 2,800, low laser intensity 2,800, mass range of 3,000–80,000 Da, focus mass 11,500 Da. Mass accuracy was calibrated externally using all-in-one peptide or all-in-one protein (Ciphergen Biosystem Inc.). Serum samples from patients and normal controls were run concurrently to minimize experimental variation.

1-D Gel and In-Gel Digestion Followed by ESI-MS/MS Identification

Equal quantitative serum samples from CC patients and normal individuals were run on 15% SDS–PAGE gels and then the gels were silver stained. Differentially expressed bands below 16 kDa were manually excised from gels and other steps were as described previously [Technical resource, 2005]. The tryptic peptide mixture was extracted and purified with Millipore ZIPTIP C18 column (Millipore, Bedford, MA). The resultant peptides were analyzed with Qstar Pulser I Q-TOF-MS (Applied Biosystems/MDS Sciex, Toronto, Canada) mounted with NanoESI source. The protein identification was determined by MS/MS fragment ions using Peptide Fragment Search and Sequence Tag for mass matching and protein identity. The search was performed allowing one missed cleavage and possible modifications.

Western Blot Analysis

Total amount of serum proteins were determined by Bradford assay using BSA as a standard (Sigma–Aldrich) and aliquot sera samples were separated through 15% SDS–PAGE. Western analyses were performed as described previously [Bagga et al., 2003]. Rabbit anti-TTR antibody (catalog number A0002, Dako) was used at a 1:2,000 dilution, and secondary antibodies, alkaline phosphatase conjugated goat anti-rabbit IgG (Golden Bridge Co., China), at 1:3,000 dilution.

Immunoprecipitation

25 μl of Protein G Sepharose beads (Sigma) was washed with PBS for three times. After the removal of supernatant carefully, 10 μl TTR antibody was diluted (1:20) in immunoprecipitation buffer (1 × PBS, 0.1% Triton X-100, pH 7.4), then blended with 25 μl of pre-cleared Protein G Sepharose beads for 3 h at room temperature with tumbling. Washing three times with PBS, 10 μl serum samples were used at 1:20 dilution and then added to beads with tumbling overnight at 4°C. After three washes with PBS, half of the beads were eluted with organic elution buffer (33.3% isopropanol/16.7% acetonitrile/0.1% trifluoracetic acid) [Fung et al., 2005]. Supernatant and eluate were analyzed by CM10 chips and SDS–PAGE gels. Pure TTR protein from human plasma (Sigma, P1742) was used as the positive control. The negative controls were set up by adding an equal volume of PBS buffer instead of the specific antibody or adding an equal volume of PBS buffer instead of serum samples.

Quantitative Validation by Enzyme-Linked Immunosorbent Assay

Total TTR level was quantified by using direct enzyme-linked immunosorbent assay (ELISA) with purified commercial polyclonal antibody against TTR and pure TTR protein was used as antigen for standard calibration in each assay. Serum samples were coated onto 96-well Immobilon plates overnight at 4°C in coating buffer (0.05 M carbonate buffer, pH 9.6), after washing, plates were blocked with 0.05% Tween 20/3%BSA/1 × PBS at RT for 1.5 h. Primary TTR antibody was used at a 1:2,000 dilution and secondary antibody (goat anti-rabbit IgG-HRP, Golden Bridge Co., China) was used at a 1:6,000 dilution. Detection was OPD and stopped with 3 M sulfuric acid. Plates were read in a microplate reader (Bio-Rad model 550, Bio-Rad,

Hercules, CA) at 490 nm. The same normal sera were used as positive control while primary antibody-depleted serum sample was used as negative control. All analyses were assayed in two parallels.

CA19-9 was also assayed in the same sets of serum samples by a microparticle enzyme immunoassay with a commercially available the AXSYM CA19-9 Reagent Pack on AXSYM SYSTEM Plus (ABBOTT).

Statistical Analysis

Surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) data were analyzed with Ciphergen's ProteinChip data analysis software version 3.0 (Ciphergen Biosystems). SELDI-TOF-MS and ELISA marker intensities were analyzed using statistical analysis software (SPSS software version 13.0; SPSS INC., Chicago, IL). The Mann–Whitney non-parametric two-tailed *U*-test with 95% confidence interval was used to determine *P*-values. ROC (receiver operating characteristic) analysis was used to detect the optimal cut-off points (i.e., those with the highest total accuracy) for separating CC from other tested groups.

RESULTS

13.76, 13.88, and 14.04 k m/z Serum Peaks Remarkably Decreased in Cholangiocarcinoma Patients

Four hundred and twenty-seven serum samples including 56 CC, 49 hepatobiliary diseases, 146 lung cancer, 65 LGC, and 58 LPC, as well as 53 normal healthy individuals were screened by CM10 chips. After normalization with intensity of total ion, within the mass region analyzed (3,000–50,000 m/z), it was found that three protein peaks, 13.76, 13.88, and 14.04 k m/z, were significantly down-regulated in sera of CC group than in sera of the other tested cancer groups, benign hepatobiliary disease groups and the healthy control group ($P < 0.001$) (Fig. 1). Although there was overlap in the levels of the three peaks in the tested groups, visible discrimination between sera of CC and the control groups could be achieved when a non-parametric test of Mann–Whitney *U*-test and ROC curve were employed (Table II). Therefore, it implied that these three peaks may be the potential useful biomarkers for CC diagnosis.

Identification of ~16 kDa Differential Band Between Cholangiocarcinoma and Normal Sera as Transthyretin

To identify proteins that correspond to the observed 13.76, 13.88, and 14.04 k m/z peaks on CM10 chips, pairs of CC and normal control sera were analyzed in SDS–PAGE gel and silver staining to visualize the proteins. In each case, a protein band, with an apparent molecular weight at ~16 kDa in the SDS–PAGE, was detected significant down-expression in sera of CC patients than that in normal sera (Fig. 2A). The differential expressed band at ~16 kDa could very possibly be the protein corresponding to peaks observed on chips, for the reason that migration rates for proteins with molecular masses less than 20 kDa may become more dependent on differences of structural features than of masses [Altland and Winter, 2003]. To confirm the doubt, differential expression band at about ~16 kDa was excised from the gels for trypsin digestion and identified by ESI-MS/MS. The MS/MS fragment ions were searched by Peptide Fragment Search and Sequence Tag methods. The candidate biomarker at about ~16 kDa was identified as native TTR (TTR, MW 13761.41 Da, Swiss-Prot accession no. P02766). Three tryptic fragments 819.4, 697.8, and 634.3 m/z from ~16 kDa band were all identified as native TTR and the scores of the tandem MS spectra were 661, 681, and 586 by sequence Tag methods, respectively (Fig. 2B–C). Besides, the same proteins identification procedures were repeated on multiple normal and cancer serum samples, and each time the excised protein bands were identified in accordance with native TTR.

To further confirm the identities, Western blot with TTR antibody was performed on a small set of above sera of CC and normal individuals, and only a single specific band at ~16 kDa was clearly observed on both the membrane probed with TTR antibody. Furthermore, this band in the former was over-expression than that in the latter (Fig. 2D). Moreover, when pure TTR protein was used as positive control, the specific band at ~16 kDa was also observed by Western blot with TTR antibody (data not shown). Thus, it strongly suggested that a differential expressed band at ~16 kDa should be TTR.

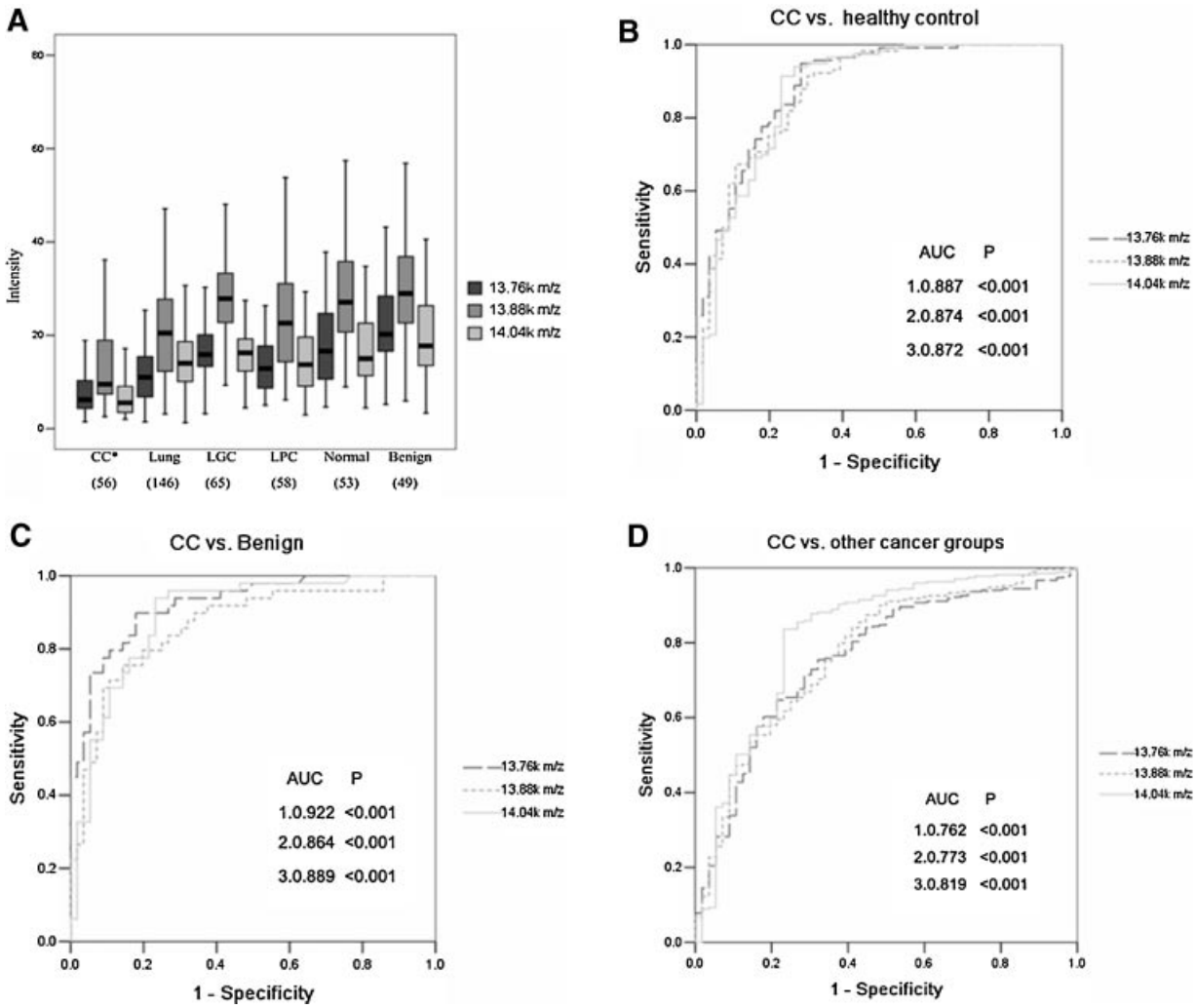

Fig. 1. **A**: A whisker plot of intensity values obtained using the SELDI chromatographic assay for 13.76, 13.88, and 14.04 k m/z protein peaks across samples from cholangiocarcinoma (CC), lung cancer (Lung), laryngeal carcinomas (LGC), and laryngopharyngeal carcinomas (LPC) as well as age-matched normal controls (Normal) and benign hepatobiliary disease groups (Benign). *$P < 0.001$; using 2-group *t*-test (Mann–Whitney test) comparing these three peaks for CC versus the other tested cancer groups, benign hepatobiliary disease groups, and the healthy control group. **B**: ROC curves between CC and age-matched healthy controls. **C**: ROC curves between CC and benign hepatobiliary disease groups. **D**: ROC curves between CC and the other tested cancer groups (including lung cancer, LGC, LPC). AUC is area under the curve, *P* estimated by Mann–Whitney *U*-test for two-group comparison, 1, 13.76 k m/z protein peak; 2, 13.88 k m/z protein peak; 3, 14.04 k m/z protein peak.

TTR is Validated Corresponding to the Observed 13.76, 13.88, and 14.04 k m/z Peaks on CM10 Chips

Considering that the band at ~16 kDa may represent another protein or may be not a single protein, immunoprecipitation with commercial TTR antibody was performed, in order to ensure that 13.76, 13.88, and 14.04 k m/z peaks on chips were actually corresponding to the identified protein TTR by ESI-MS/MS. The supernatant and eluate resulted from the immunoprecipitation of CC and normal individual sera with TTR antibody were analyzed by SELDI-TOF-MS and 1-D gel, respectively. It was not surprising to find that the selective removal of three peaks at 13.76, 13.88, and 14.04 k m/z were observed in CC patients and normal individuals sera treated with TTR antibody. It was noteworthy that the intensities of three peaks were spontaneously decreased or depleted, whereas the intensities of other peaks in the same samples did not change (Fig. 3A). Pure TTR protein was also analyzed by SELDI-TOF-MS on CM10 chip. It was exciting to notice that three peaks at 13.76, 13.88, and 14.04 k m/z were observed.

TABLE II. Comparison of Sensitivities and Specificities of 13.76, 13.88, and 14.04 k m/z Protein Peaks* in Cholangiocarcinoma Versus Benign Hepatobiliary Diseases and Healthy Control

	Normal vs. cholangiocarcinoma			Benign vs. cholangiocarcinoma		
Biomarkers (k m/z)	Cut-off point (peak intensity)	Sensitivity (%)	Specificity (%)	Cut-off point (peak intensity)	Sensitivity (%)	Specificity (%)
13.76	9.5	73.2 (41/56)	84.9 (45/53)	9.7	73.2 (41/56)	91.8 (45/49)
13.88	17.6	73.2 (41/56)	84.9 (45/53)	17.9	73.2 (41/56)	83.7 (41/49)
14.04	9.1	78.6 (44/56)	90.6 (48/53)	9.8	76.8 (43/56)	93.8 (46/49)

*The 13.76, 13.88, and 14.04 k m/z intensities value greater than or equal to the cut-off value is considered the "positive group" to compute the true positive ratio (sensitivity) and the false positive ratio (1-specificity) for each cut-off value of the peaks.

On the other hand, an example of 1-D SDS–PAGE gel showed that only one band at ~16 kDa could be clearly observed in the serum sample immunoprecipitated with TTR antibody (Fig. 3B). It has been reported that native TTR in serum is more likely to be posttranslationally modified in the form of S-cysteinylation and S-glutathionylation [Amareth et al., 2003]. Therefore, we postulated that 13.88 and 14.04 k m/z peaks may be two variants of TTR according to molecular weight and previous reports [Gericke et al., 2005]. To confirm this hypothesis, some serum samples were treated with DTT to destroy disulfide bonds and then analyzed on CM10 chips. It was found that the intensities of 13.90 and 14.07 k m/z peaks were lowered, even disappeared, whereas the intensity of 13.78 k m/z peak almost was not changed (data not shown). Thus, the combined analysis using immunodepletion, SDS–PAGE and Western blot strongly suggested that 13.78, 13.90, and 14.07 k m/z peaks observed with SELDI-TOF-MS should be native TTR and two variants of TTR.

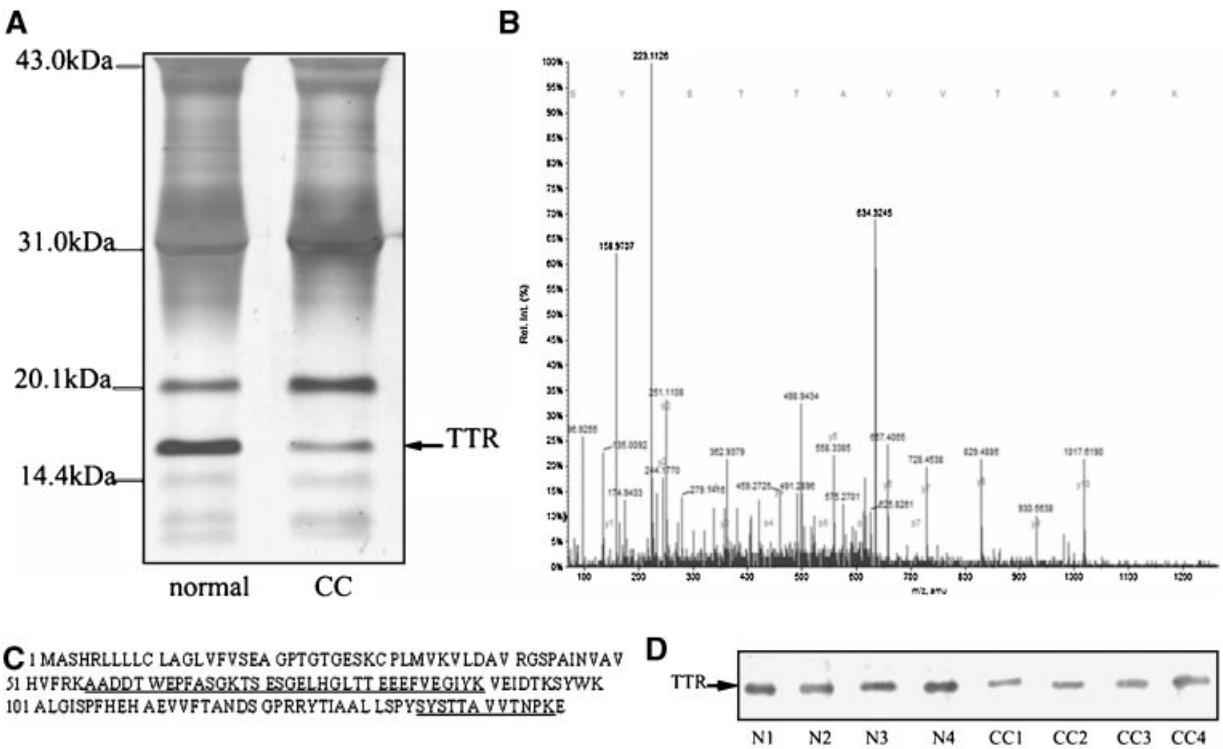

Fig. 2. **A**: SDS–PAGE gel (15%) with silver staining for normal and CC sera. The differential expression band between two groups is at about ~16 kDa by the black arrows labeled. **B**: The tandem MS spectra of peptide m/z 634.3, in which sequence is confirmed from the labeled b- and y-ions; **C**: Amino acid sequences of TTR, in which three matched peptide sequences are underlined. **D**: The level of TTR is detected in sera of four normal individuals and CC patients by Western blot. N 1~4 represents sera of normal individuals, CC 1~4 represents sera of CC patients. Band of TTR is shown in ''bold'' with black arrows.

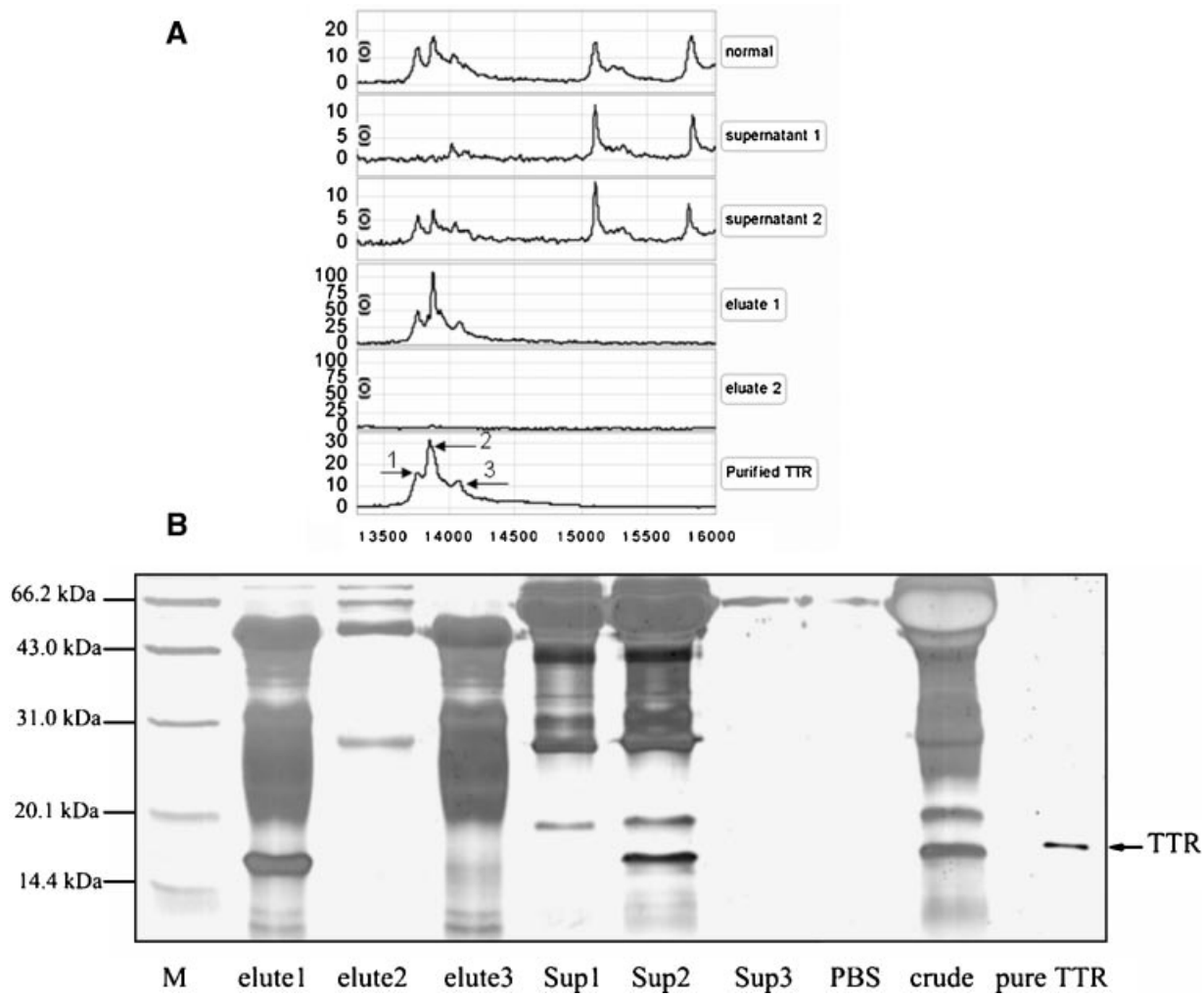

Fig. 3. **A**: CM10 protein profilings show the successful depletion of TTRs peaks in one of the normal serum samples immunoprecipitated with TTR antibody. From **top** to **bottom** expressed the untreated normal serum; supernatant of the serum with TTR antibody; supernatant of the serum without TTR antibody; eluate of precipitate of the serum with TTR antibody; eluate of precipitate of the serum without TTR antibody; purified TTR. Black arrows identify depleted normal serum markers no. 1, 2, and 3 represent native TTR and its two variants, respectively. **B**: Immunoprecipitation results of one of the normal sera are shown by 1-D gels. M is protein marker; elute1 is eluate of precipitate of the normal serum with TTR antibody; elute2 is eluate of precipitate of the normal serum without TTR antibody; elute3 is eluate of precipitate of TTR antibody without serum, Sup1 is supernatant of the normal serum with TTR antibody; Sup2 is supernatant of the normal serum without TTR antibody; Sup3 is supernatant of TTR antibody without serum; PBS as negative control; crude is the same normal serum sample as positive control; pure TTR is purified protein of TTR. Band of TTR is shown in ''bold'' with black arrows.

Serous Levels of TTR was Significantly Decreased in Cholangiocarcinoma Patients

Now that the discovery of biomarkers by SELDI-TOF-MS mainly depends on peak intensity, the value may be variable due to laser energy and detector sensitivity. In addition, easy operation and reproduction is an important factor for clinic usage. Therefore, quantitative analysis of TTR in part of the same sets sera including 56 CC, 49 benign hepatobiliary diseases, and 37 normal individuals was further validated by ELISA assay. Results from ELISA were consisted with data of chip arrays. The average of total TTR unit (μg/ml) for CC group (71.2 ± 26.1) was significantly lower than that in normal controls group (110.2 ± 15.6, $P < 0.001$) or benign hepatobiliary diseases group (110.9 ± 12.9, $P < 0.001$) (Fig. 4). Serum TTR levels between 100 and ~400 μg/ml constitute the normal reference range. Of the 56 patients with CC, 49 (87.5%) patients had a concentration below 100 μg/ml Using a TTR (100 μg/ml) as the optimal cut-off

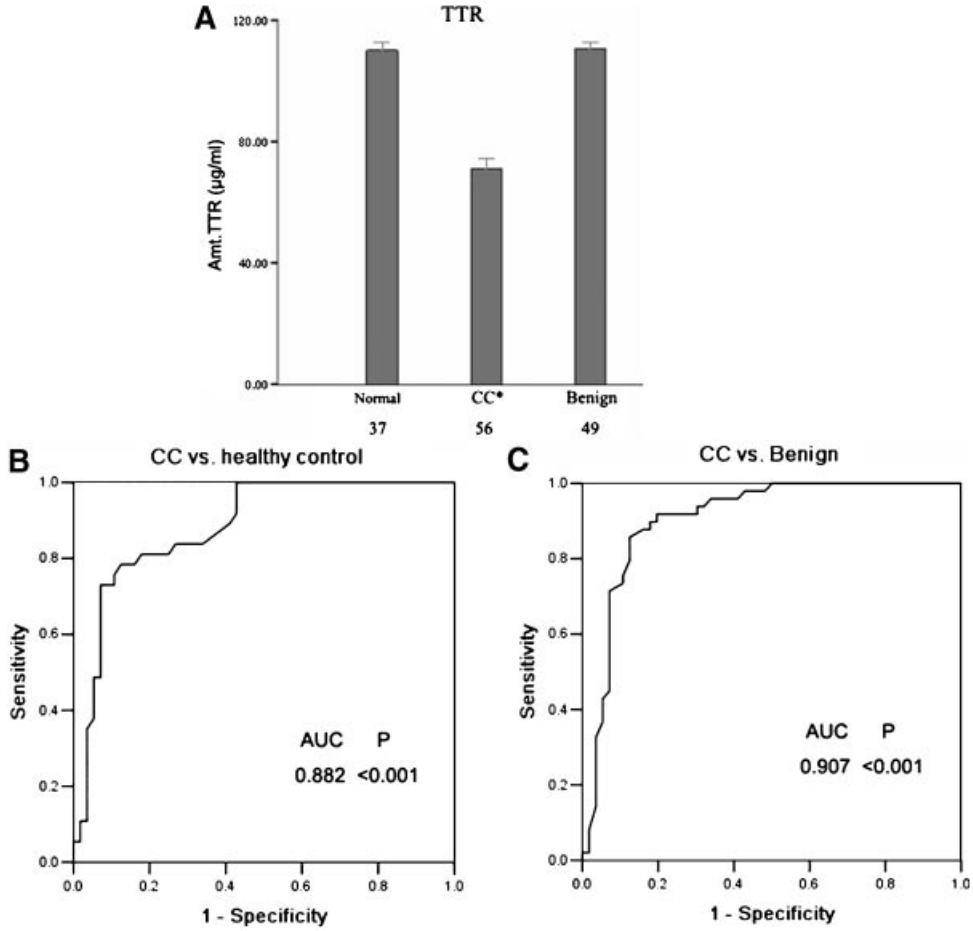

Fig. 4. **A**: Histogram of intensity values obtained using ELISA assay for serum TTR concentrations from CC, age-matched healthy controls (Normal), and benign hepatobiliary disease groups (Benign). $^*P < 0.001$; using 2-group *t*-test (Mann–Whitney test) comparing serum TTR concentrations for CC versus benign hepatobiliary disease and healthy control group. **B**: ROC curves between CC and age-matched healthy controls. **C**: ROC curves between CC and benign hepatobiliary disease groups. AUC is area under the curve, *P* estimated by Mann–Whitney *U*-test for two-group comparison.

points, The specificity was 85.7%, when assessed as the benign hepatobiliary diseases. The specificity was 78.4%, when compared with healthy individuals group (Table III). It demonstrated that using ELISA assay to measure the sum of all forms of TTRs could also discriminate CC patients from benign hepatobiliary diseases group and normal individuals. Although the contents of TTR were detected lower in III–IV stage than in II stage and higher in well-differentiated CC than in moderately and poorly differentiated, no statistical significances were obtained ($P > 0.05$) (Table IV).

TABLE III. Comparison of Serum TTR and CA19-9 Levels in Patients With Cholangiocarcinoma Versus Benign Hepatobiliary Diseases and Normal Individuals

	Benign vs. cholangiocarcinoma			Normal vs. cholangiocarcinoma		
	TTR	CA19-9	TTR and CA19-9	TTR	CA19-9	TTR and CA19-9
Sensitivity (true positive)	87.5% (49/56)	85.7% (48/56)	98.2% (55/56)	87.5% (49/56)	85.7% (48/56)	98.2% (55/56)
Specificity (true negative)	85.7% (42/49)	100% (49/49)	100% (49/49)	78.4% (29/37)	100% (37/37)	100% (37/37)
Accuracy	86.7% (91/105)	92.4% (97/105)	99.0% (104/105)	83.9% (78/93)	91.4% (85/93)	98.9% (92/93)
False positive rate	14.3% (7/49)	0 (0/49)	0 (0/49)	21.6% (8/37)	0 (0/37)	0 (0/37)

TABLE IV. Relation Between Serum TTR Levels and Clinicopathologic Features of Tumors in 56 Patients With Cholangiocarcinoma (Mean ± SD)

	No. of patients	TTR (μg/ml)
Well-differentiated[a]	10	76.6 ± 32.5
Moderately differentiated	24	70.1 ± 23.7
Poorly differentiated	15	74.3 ± 26.6
II Stage	11	78.6 ± 32.9
III Stage	25	69.0 ± 23.6
IV Stage	20	69.9 ± 25.8

[a]Seven serum samples intangible differentiation.

CA19-9 and General Serum Biochemistry was Significantly Increased in Cholangiocarcinoma Patients

The concentration of CA19-9 in the same sets sera including 56 CC, 49 benign hepatobiliary diseases, and 37 normal individuals was assayed by a microparticle enzyme immunoassay. The median CA19-9 concentration was significantly higher in the CC group (769.9 ± 1131.4 U/ml; range 1.61–7586 U/ml) than those in healthy individuals group (9.6 ± 6.3, $P < 0.001$) or the benign hepatobiliary diseases group (7.7 ± 1.4, $P < 0.001$). Furthermore, liver function test levels including alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltranspeptidase, and bilirubin were also obviously elevated for CC patients ($P < 0.001$; data in supplement).

DISCUSSION

SELDI-TOF-MS, as a powerful and sensitive tool to screen protein profiling, not only offer a discriminatory power for separating any given set of cases and control samples based on their peak intensity, but also provide high dimensional information of proteins, like posttranslational proteins. Therefore, it is becoming more and more useful in discovery of cancer-related biomarker. Recently, pathologists seem desirable to know the identity of biomarkers in the pattern in order to understand their significance in disease pathogenesis. Identification of individual differentially expressed proteins with potential diagnostic value is essential to the development of routine clinical analysis, as well as helpful to explore the mechanisms of carcinogenesis. In the present study, 13.76, 13.88, and 14.04 k m/z protein peaks were the most significant peaks with highest discriminatory power for CC from hepatobiliary diseases and normal group on CM10 protein chip analysis. For the reason that SELDI technology was limited in supporting direct identification on chip, 1-D gel and ESI-MS/MS technologies were together used to separate the candidates and finally identified TTR as a potential biomarker for CC. It was also found that 13.88 and 14.04 k m/z peaks were two variants of TTR and the location of TTR band in SDS–PAGE was migrated due to its posttranslational modification.

Native TTR is a homotetramer in blood that is mainly synthesized by liver, choroids plexus of brain, and pancreatic islet A and B cells. Under physiological conditions, TTR functions as a carrier for both thyroxine (T4) and retinol (vitamin A) [Jacobsson et al., 1989; Power et al., 2000; Raghu and Sivakumar, 2004; Fung et al., 2005; Lowenthal et al., 2005; Geho et al., 2006; Liotta and Petricoin, 2006; Menard et al., 2006]. Genetic differences, such as L55P point mutations in TTR produce an altered primary sequence in the protein. These variant proteins cause the eventual formation of insoluble protein fibrils (amyloid) in various tissue and organs. The deposited amyloid eventually leads to organ dysfunction and ultimately death when major organs such as heart are involved. The mechanisms in which soluble proteins self-assemble into a fibrillary structure are unknown. On the other hand, some variants of TTR exist in sera due to modifications on one readily accessible cystein that really forms adducts with other molecules, including cysteine, cysteinylglycine and glutathione within the molecule and could be detected based on their mass shift [Power et al., 2000; Ferlini et al., 1992]. Typically, native TTR only accounts for 5∼15% of total TTR circulating in plasma. The other 85∼95% is posttranslationally modified in the forms of S-sulfonation and S-thiolation [Altland and Winter, 2003]. Importantly, it has been reported that the levels of TTR could decrease in cases of severe liver disease, malnutrition, and acute inflammation [Imanishi, 1981; Marten et al., 1996; Ritchie et al., 1999]. In addition, TTR was found to decline in the sera of patients with ovarian cancer, advanced cervical, and endometrial carcinomas [Kozak et al., 2005], the mechanisms are unknown. In this study, we successfully identified native TTR and its two variants

(they are most likely cysteinylated transthyretin (cysTTR) and glutTTR according to their molecular weight and related reports [Lim et al., 2003; Bergen et al., 2004; Gericke et al., 2005]) decreased in CC by Western blot and SELDI-TOF-MS according to their molecular weight and comparison with pure TTR. Based on these results, it inferred that combination of variants of TTR may be specially associated with certain given diseases and this specific biomarker pattern may be very useful for cancer diagnosis. The advantage of SELDI technology in discovery of novel cancer-related biomarkers should be paid more attention.

TTR as a potential biomarker can discriminate CC from benign hepatobiliary diseases and normal group, especially, when the CA19-9 values are <37 U/ml, TTR may prove a useful biomarker for contributing to the CA19-9 test by complementing this marker in the range where CA19-9 fails to detect cancer. Moreover, the concentration of TTR in sera of benign hepatobiliary disease groups belongs to the range of normal groups, even slightly increases than normal individuals, so it will help for distinguishing CC patients from benign hepatobiliary disease groups. Therefore, these results suggested that it may foreshow the apparent risk when TTR began to decline and CA19-9 still raised in patients of benign hepatobiliary disease [Minato et al., 1996; Horsmans et al., 1997; Lin et al., 1997; Bjornsson et al., 1999; Shaib and El-Serag, 2004]. Although the increment of CA19-9 in benign diseases is usually not significant increased, it is not easy to differentiate CC patients of the slight increment of CA19-9 from benign hepatobiliary disease. Importantly, in the present study, the diagnostic positive rate of CC was proved from 87.5 to 98.2% when TTR was combined with CA19-9 usage (Table IV).

Changing in the levels of TTR in serum samples of patients is mainly due to liver activity, which could be converted to the synthesis of acute-phase response proteins and result in a dramatic drop in TTR [Ingenbleek and Young, 1994; Schreiber, 2002; Bagga et al., 2003]. In this study, the level of TTR was significantly decreased in sera of CC compared with sera of benign hepatobiliary diseases and normal control group, and liver function test levels, including alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyltranspeptidase, and bilirubin, were also obviously elevated for CC patients. However, TTRs did not expressed in CC and benign hepatobiliary disease tissue by immunohistochemistry (data not shown), we consider the reasons of TTRs decrease in CC sera are liver dysfunction, inflammatory, or malnutrition, the precious mechanism has not been known until now and needs to be further studied.

In conclusion, this study used SELDI-TOF-MS to discover three biomarker panels (m/z 13.76, 13.88, and 14.04 k) that could effectively distinguish CC from benign biliary disease and normal individuals, and identified these three potential biomarkers of CC as native TTR and its two variants which could improve the diagnostic positive rate of CC when combining with CA19-9. This study supports the continued investigation of protein array technology in diagnostic cancer research.

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