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A Prospective Proteomic-Based Study for Identifying Potential Biomarkers for the Diagnosis of Cholangiocarcinoma

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Abstract

Background Cholangiocarcinoma (CCA) is becoming a common fatal hepatic tumor. Early detection of CCA is hampered by the absence of a sufficiently accurate and noninvasive diagnostic test. Proteomic analysis would be a powerful tool to identify potential biomarkers of this cancer.

Aims This study aims to identify new protein markers that are specific for CCA using proteomic approaches and to evaluate the performance of S100 calcium-binding protein A9 (S100A9) and chaperonin-containing TCR1, subunit 3 (CCTγ) as diagnostic markers for screening test of CCA.

Methods Two-dimensional differential gel electrophoresis (2-D DIGE) coupled with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry were used to analyze and screen biomarker candidates in the proteomes of five human CCA samples and five healthy control samples. Subsequently, two potential biomarkers, S100A9 and CCTγ, were chosen for validation and analysis by immunohistochemical methods using CCA tissue microarrays.

Results Twenty protein spots were significantly elevated and five protein spots were downregulated in all patients ($p < 0.05$). The positive rate was significantly higher in patients with CCA (48 ± 35 %) compared with the normal liver control group (5 ± 10 %, $p < 0.001$), the hepatocellular carcinoma group (15 ± 20 %, $p < 0.001$), and the cirrhosis group (12 ± 16 %, $p < 0.001$). A greater proportion of patients with CCA were positive for CCTγ (72 ± 18 %) compared with the normal liver control group (43 ± 22 %, $p < 0.001$), the hepatocellular carcinoma group (45 ± 20 %, $p < 0.001$), and the cirrhosis group (39 ± 25 %, $p < 0.001$).

Conclusions Combined comparative proteomic analysis using 2-D DIGE and MALDI-TOF is an effective method for identifying differentially expressed proteins in CCA tissues. The expression of S100A9 and CCTγ showed promise as novel diagnostic markers for CCA.

Keywords Liver cancer · Cholangiocarcinoma · 2-D DIGE · Proteomics · S100 A9 · CCTγ

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Introduction

Cholangiocarcinoma (CCA) is a malignant tumor that originates from the bile duct epithelium. Among primary liver tumors, CCA accounts for an estimated 15 % of primary liver cancers worldwide[1] and is becoming a common type of fatal hepatic tumor. CCA is responsible for more than 60 % of liver tumors in parts of Southeast Asia, especially in northeastern Thailand. Tumor stage, resectability, and survival are all closely correlated in patients with bile duct carcinoma, suggesting that early detection strategies may improve the prognosis of patients with this disease.[2,3] However, early detection of CCA is hampered by the absence of a sufficiently accurate and noninvasive diagnostic

test.[4] Carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) are used as markers for early-stage biliary carcinoma in patients. CEA is nonspecific and can be elevated in the setting of other gastrointestinal or gynecologic malignancies or other bile duct pathologies, such as cholangitis and hepatolithiasis.[5] The sensitivity and specificity of CA19-9 are 53–89 and 80.5–86.0 %, respectively.[6,7] Several biliary markers, including MMP-9, MMP-7, Mac-2-binding protein, and the transcriptional repressor Slug, have shown promise in the diagnosis of CCA.[7–10]

In recent years, proteomic analysis has been considered a powerful tool for investigating cancer mechanisms.[11] Combined comparative proteomic analysis by two-dimensional differential gel electrophoresis (2-D DIGE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is an effective method for identifying differentially expressed proteins.[12,13]

In this study, we identified several proteins that were elevated or downregulated in CCA compared with normal liver using a 2-D DIGE approach. Some of these proteins may be biomarkers for early diagnosis, prognosis, or monitoring in the therapy of CCA. Two of these proteins, chaperonin-containing TCP1 subunit 3 (γ) (CCTγ) and S100 calcium-binding protein A9 (S100A9), were measured using tissue microarrays to evaluate their performance as diagnostic markers for CCA.

Materials and Methods

Patients and Samples

The study design was approved by the independent ethics committee of Ruijin Hospital, and the procedure was described in detail to all patients before admission and informed consent was obtained. The human liver tissue samples used for 2D-DIGE were obtained from seven surgical patients (mean age 62 years) in Ruijin Hospital who had a clinical diagnosis of CCA between January 2008 and June 2009. These patients included four intrahepatic cholangiocarcinoma cases and three hilar cholangiocarcinoma cases. Two cases that were hepatoma by pathologic diagnosis were excluded from this study. Human liver tissue samples from five patients (mean age 57 years) with a pathologic diagnosis of cholangitis between January 2009 and June 2009 were used as controls. The specimens were quickly removed during surgery and stored immediately in liquid nitrogen until later experiments.

For immunohistochemistry, formalin-fixed, paraffin-embedded biliary carcinoma tissue samples and control samples were obtained from the surgical pathology archives of the Department of Pathology at the Ruijin Hospital of Shanghai Jiao Tong University. Forty CCA patients (26 men and 14 women) aged 41–80 years (mean, 62.5±11 years) underwent liver resection at the Ruijin Hospital of Shanghai

Jiao Tong University (China) from 2007 to 2009. Tissue samples were collected from patients with intrahepatic biliary cystadenocarcinoma ($n=5$), patients with intrahepatic cholangiocarcinoma ($n=17$), patients with hilar cholangiocarcinoma ($n=14$), and patients with hepatobiliary cystadenoma ($n=4$), and 30 patients with hepatocellular carcinoma, 30 patients with cirrhosis, and 10 normal liver tissues were collected as control group.

2-D DIGE and Image Analysis

This study was followed the standard methods of two-dimensional differential gel electrophoresis proteomics technology.[14] These tissues were cut into 3 mm^3 pieces and digested with 1 ml DIGE lysis buffer. The digested samples were homogenized with Dounce Tissue Grinders, vortexed, sonicated at 4 °C, and placed on ice. Extracts were centrifuged at 14,000×g for 1 h, and the supernatants were collected. A pooled internal standard to control for gel-to-gel variation was prepared by mixing an equal amount of all tumoral and non-tumoral samples that had been diluted to 5 μg/μl. A total of 50 μg of tumoral and non-tumoral protein extracts with a pH of 8.9–9.0 were minimally labeled with cyanine fluorochrome 3 (Cy3) or Cy5, and the pooled internal standard was labeled with Cy2.

Three Cy-labeled protein samples (tumoral, non-tumoral, and internal standard) were mixed. The mixtures were added to an equal volume of sample buffer. All of the samples were mixed, and the volume was adjusted to 250 μl with rehydration buffer. The first dimension was performed on an Ettan IPGphor Isoelectric Focusing System (GE Amersham) using 13 cm immobilized pH gradient strips with a pH of 3–10. After isoelectric focusing, the gel strips were then equilibrated in equilibration buffer. The equilibrated strips were loaded on the top of a 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Electrophoresis was performed using a Hoefer SE 600 unit (GE, Amersham Biosciences). After 2D-DIGE, the gels were scanned with an UMax PowerLook 2100XL (GE, Amersham Biosciences). The excitation and emission wavelengths for Cy2, Cy3, and Cy5 are 488/520, 532/580, and 633/670 nm, respectively.

MS

After scanning, the gels were stained using the Coomassie Blue Staining method. The protein spots were cut from gels destained by washing in MilliQ distilled water. After trypsin digestion and evaporation, the MALDI-TOF/MALDI-TOF-TOF analyses (Bruker Daltonics, Bremen Germany) were performed to acquire mass spectra for all the peptides. Tryptic digests were prepared in an AnchorChip sample plate (Bruker Daltonics) according to the manufacturer's protocol. MS data were acquired with an N_2 laser at a sampling rate of 25 Hz. The tryptic peptide mass maps were transferred with the MS

BioToolsTM program (Bruker Daltonics) using MASCOT software (Matrix Science). Then, the National Center for Biotechnology Information (NCBI) non-redundant database was searched with human as the taxonomy.

Proteins were unambiguously identified through their peptide mass fingerprinting (PMF) and MS/MS ion search using MASCOT to interrogate the NCBI Inr 20090820 (9511482 sequences; 3251602805 residues). The ion score is $-10 \times \text{Log}(P)$, where P is the probability that the observed match is a random event. Individual ions scores greater than a certain number indicate identity or extensive homology ($p < 0.05$). The protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits. Protein scores >66 are considered statistically significant ($p < 0.05$) under the parameters described previously.

S100A9 and CCTγ Tissue Microarrays

Tissue microarrays were constructed as described previously.[15] For the immunohistochemical analysis, the tissue microarrays were deparaffinized in xylene and graded ethanols, washed with three times with PBS, and boiled in citrate buffer (antigen retrieval solution; DAKO, Carpinteria, CA). Endogenous peroxidases were blocked by incubation and incubation overnight with mouse antihuman monoclonal antibody. After washing, biotin and a streptavidin–horseradish peroxidase conjugate were applied. The peroxidase reaction was visualized by applying diaminobenzidine tetrahydrochloride (DAB) containing hydrogen peroxide (Liquid DAB Substrate Chromogen System; DAKO); sections were subsequently developed by washing by water, counterstained with hematoxylin, dehydrated, and mounted.

Immunohistochemical staining intensity and area were assessed by two observers and were expressed as the staining intensity of positive carcinoma. Staining intensity was scored as negative, *1, 1, 2, and 3, which represent negative, weakly positive, mildly positive, moderately positive, or strongly positive. The staining area was scored as negative (without staining), *1 (weak yellow staining), 1 (light brown staining), 2 (yellow or brown staining), or 3 (dark yellow or dark brown staining). The positive rate of the microarrays was calculated as the percentage of positively stained carcinoma cells among all cells.

Statistical Analysis

All statistical analyses were performed using SPSS 13.0 software (SPSS, Chicago, USA). After 2D-DIGE, three images corresponding to three samples labeled with three different Cy dyes were generated in one gel. A t test was performed for every matched spot set, comparing the average and SD of protein abundance for a given spot. Pixel values from images of a small area of fluorescent-stained gels were converted into

3-D representations to illustrate the differential quantification between the two groups.

Differences between S100A9 and CCTγ expression in tumoral tissues and control group were analyzed using a paired Student's t test. An receiver operating characteristic (ROC) curve was generated by plotting the sensitivity against 1-specificity, and the area under the curve with 95 % confidence intervals (CI) was calculated. The optimal cutoff points for S100A9 and CCTγ were selected based on the ROC curve analysis. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated using a 2×2 table of the collected data.

Results

Analysis of Differentially Expressed Proteins

Tissue samples collected from five patients with CCA and five normal controls were run on 2-D DIGE to elucidate changes in protein expression between tumors and normal controls (Fig. 1). The average ratio and p for a given spot between patients and normal controls was calculated, and these values are shown in Table 1. Using the biological variation analysis module of the DeCyder software, 25 differentially expressed proteins were selected. Twenty proteins were significantly elevated in all patient groups (with average ratios from −1.60 to −4.47), while five proteins were significantly downregulated in patients (with ratios
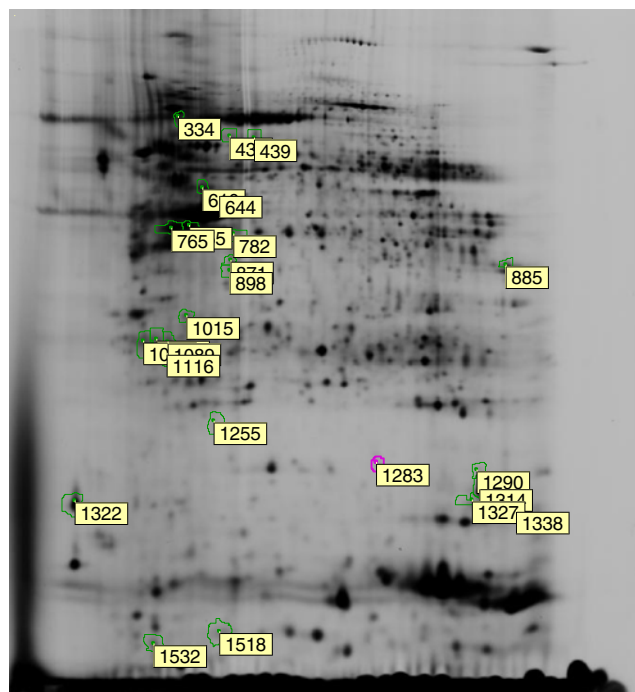
Fig. 1 Representative 2D gel images indicating the differential spots

Table 1 Differentially expressed proteins, as determined by Student's *t* test between the normal control and tumor groups

| Pos.	Protein spots	*p* value	Average ratio
1	765	0.0054	−4.47
2	1532	0.0060	−4.46
3	755	0.0012	−3.66
4	885	0.0056	−3.55
5	1314	0.0023	−3.33
6	1283	0.0095	−3.27
7	1518	0.0020	−3.21
8	1015	0.00069	−2.65
9	1089	0.0042	−2.62
10	1290	0.0091	−2.47
11	644	0.0094	−2.46
12	1327	0.0043	−2.31
13	1116	0.0042	−1.97
14	1338	0.0097	−1.92
15	1322	0.0047	−1.89
16	1083	0.0013	−1.82
17	1082	0.0093	−1.78
18	871	0.0081	−1.69
19	898	0.0070	−1.69
20	610	0.0014	−1.60
21	439	0.0030	1.35
22	1255	0.0033	1.49
23	431	0.0020	1.83
24	334	0.0056	1.90
25	782	0.0085	2.16

from 1.35 to 2.16), and the statistical variance of the tumor versus the normal spot volume ratios was within the 95 % confidence level (Student's *t* test; $p < 0.05$) (Table 1).

Identification of Differentially Expressed Proteins

Sixteen protein spots were selected for tryptic digestion and MALDI-TOF or MALDI-TOF/TOF analysis. Fortunately, 13 out of 16 proteins were unambiguously identified (Fig. 2 is the Mascot score schematic diagram of two of all the identified proteins). Three proteins (644, 898, and 1327) were identified by PMF. The other ten proteins were searched by MS/MS ion search. Eleven protein spots were upregulated and two protein spots were downregulated in patients (Table 2).

S100A9 and CCTγ Immunohistochemistry

S100A9 and CCTγ expression was measured by immuno-histochemistry in tissue samples from 40 patients with CCA, 30 patients with hepatocellular carcinoma, 30 patients with cirrhosis, and 10 normal liver tissues. S100A9 was detected
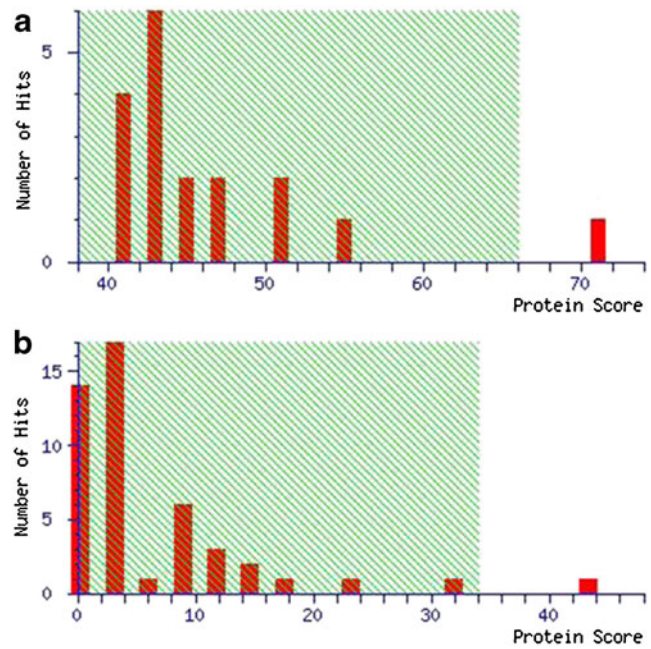
Fig. 2 Mascot score schematic diagram: *x*-axis as Mascot score (protein score), the *y*-axis is the number of matching. Score that exceeds the shaded area obtained positive identification ($p < 0.05$). **a** The matching and sequence coverage of protein no. 644 and protein ID gi|55960506 in database. **b** The matching and sequence coverage of protein no. 1518 and protein ID gi|4506773 in database

in 37 of 40 tumor tissue samples (92.5 %). Staining scores of *1–1 (weak–mildly positive), 1 (mildly positive), 1–2 (mild–moderately positive), and 2 (moderately positive) were found in 2, 23, 9, and 3 tumor tissue samples, respectively. Positive S100A9 staining (>5 % of carcinoma cells stained) was observed in the cytoplasm of carcinoma cells (Fig. 3a). S100A9 was expressed only in three out of ten normal tissue samples (Fig. 3b), 14 out of 30 hepatocellular carcinoma samples (Fig. 3c), and 12 out of 30 cirrhosis samples (Fig. 3d). The positive rate was significantly higher in patients with CCA (48±35 %) compared to the normal liver control group (5±10 %, $p < 0.001$), the hepatocellular carcinoma group (15±20 %, $p < 0.001$), and the cirrhosis group (12±16 %, $p < 0.001$).

CCTγ was also detected in 37 of 40 tumor tissue samples (92.5 %). Staining scores of *1–1 (weak–mildly positive), 1 (mildly positive), 1–2 (mild–moderately positive), and 2 (moderately positive) were found in 3, 14, 14, and 6 tumor tissue samples, respectively. Positive CCTγ staining (>10 % of carcinoma cells stained) was observed in the cytoplasm of carcinoma cells (Fig. 4a). CCTγ had low levels of expression in normal tissue samples (Fig. 4b), hepatocellular carcinoma tissue samples (Fig. 4c), and cirrhosis samples (Fig. 4d). Staining scores of 1 and 1–2 were found in eight and two normal tissue samples, respectively, and the positive rate was significantly higher in patients with CCA (72±18 %) compared to the normal liver control group (43±

Table 2 Differentially expressed proteins identified by MALDI-TOF after 2-D DIGE

Spot no.	Protein ID	Name	Mr	PI	Sequence coverage (%)	Score	Peptide matches	Matched sequence
334	gi|28592	Serum albumin (*Homo sapiens*)	71316	6.05	3	65	2	K.YLYEIAR.R K.KVPQVSTPTLVEVSR.N
610	gi|3420181	WDR1 protein (*Homo sapiens*)	58593	6.41	5	38	2	K.VFASLPQVER.G R.LATGSDDNCAAFFEGPPFK.F
644	gi|55960506	Chaperonin-containing TCP1, subunit 3 (γ) (*Homo sapiens*)	58505	6.46	22	71	9	R.TQDEEVGDGTTTVVISAYRK.A R.VEKIPGGIIEDSCVLR.G K.IPGGIIEDSCVLR.G K.GISDLAQHYLMR.A R.IVSRPEELREDDVGTGAGLLEIK.K K.AMTGVEQWPYR.A R.AVAQALEVIPR.T R.TLIQNCGASTIR.L K.TAVETAVLLLR.I
782	gi|4501901	Aminoacylase 1 (*Homo sapiens*)	46084	5.77	5	39	2	K.GPEEEHPSVTLFR.Q K.VVNSILAFR.E
871	gi|5031777	Isocitrate dehydrogenase 3 (NAD+) alpha precursor (*Homo sapiens*)	40022	6.47	8	108	3	K.APIQWEER.N K.TPYTDVNIVTIR.E R.IAEFAFEYAR.N
885	gi|1575000	2,4-Dienoyl-CoA reductase	36096	9.50	6	45	1	K.VAGHPNIVINNAAGNFISPTER.L
898	gi|62896849	*N*-acetylglucosamine kinase variant (*Homo sapiens*)	37697	5.81	31	120	11	R.KAGVDPLVPLR.S K.AGVDPLVPLR.S R.SLGLSLSGGDQEDAGR.I K.QAMFHYFQVPDR.L R.LGILTHLYR.D R.FAGFCRK.I R.KIAEGAQQGDPLSR.Y K.IAEGAQQGDPLSR.Y K.EGFLLALTQGR.E R.EIQAQNFFSSFTLMK.L R.HSSALGGASLGAR.H
1082	gi|4757768	Rho GDP dissociation inhibitor (GDI) alpha (*Homo sapiens*)	23250	5.02	17	119	3	K.YIQHTYR.K K.IDKTDYMVGSYGPR.A R.AEEYEFLTPVEEAPK.G
1089	gi|539605	GTP-binding protein G25K —human (fragments)	6419	6.34	23	42	1	R.IDKTDYMVGSYGPR.-
1314	gi|1421609	Chain A, X-ray structure of Nm23 human nucleoside diphosphate kinase B complexed with GDP at 2 Å resolution	17290	8.55	20	45	3	R.TFIAIKPDGVQR.G K.DRPFFPGLVK.Y R.GDFCIQVGR.N
1322	gi|226449	Cytochrome b5	15218	4.82	49	242	5	K.YYTLEEIQK.H K.STWLILHHK.V K.FLEEHPGGEEVLR.E R.EQAGGDATENFEDVGHSTDAR.E K.TFIIGELHPDDRPK.L
1327	Mixture 1, gi|5453541 + gi|1421609	Anterior gradient 2 homolog (*Homo sapiens*) chain A, X-ray structure of Nm23 human nucleoside diphosphate	20024	9.03	26	68	5	K.LPQTLSR.G K.HLSPDGQYVPR.I R.IMFVDPSLTVR.A R.LYAYEPADTALLLDNMK.K

Table 2 (continued)

Spot no.	Protein ID	Name	Mr	PI	Sequence coverage (%)	Score	Peptide matches	Matched sequence
		kinase B complexed with GDP at 2 Å resolution	17270	8.55	38	67	5	R.LYAYEPADTALLLDNMKK.A R.TFIAIKPDGVQR.G K.FLRASEEHLK.Q K.DRPFFPGLVK.Y R.VMLGETNPADSKPGTIR.G R.GDFCIQVGR.N
1518	gi|4506773	S100 calcium-binding protein A9 (*Homo sapiens*)	13291	5.71	29	43	2	K.LGHPDTLNQGEFK.E K.MHEGDEGPGHHHKPGLGEGTP.-

22 %, $p<0.001$), the hepatocellular carcinoma group (45± 20 %, $p<0.001$), and the cirrhosis group (39±25 %, $p<0.001$).

An ROC curve analysis (Fig. 5) was used to calculate an area under the curve of 0.894 (95 % CI=0.825–0.962) for S100A9 and 0.860 (95 % CI=0.779–0.942) for CCTγ. When the cutoff value of S100A9 was set at 4 %, the sensitivity and specificity for the diagnosis of CCA were 92.6 and 75 %, respectively. When the cutoff value for CCTγ was set at 62.5 %, the sensitivity and specificity for the diagnosis of CCA were 80.8 and 77.8 %, respectively.

Discussion

Detecting new biomarkers for early-stage biliary carcinoma in patients may improve the prognosis of patients with CCA. Due to the low incidence of CCA, a retrospective study based on liver tissue is necessary for further research.

In this study, the analysis of the 2-D DIGE gels showed a large number of differentially expressed proteins in CCA. Some of these proteins are predicted to play roles in tumorigenesis or tumor growth[16–18] and may be potential biomarkers in early screening test. S100A9 and CCTγ were detected by immunohistochemistry in 50 tissue samples, and the expression was significantly elevated in patients with a diagnosis of CCA when compared to control groups. It is shown that the sensitivity and specificity of S100A9 were 92.6 and 75 % when using a cutoff value of 4 %. The sensitivity and the specificity of CCTγ, however, were 80.8 and 77.8 %, respectively, when using a cutoff value of 4 %. The diagnostic accuracy of S100A9 and CCTγ as biomarkers compared favorably with the performance of other protein markers for patients with biliary tract carcinoma (specifically, CEA, CA125, CA19–9, and fibronectin), which have reported sensitivities of 58–78 % and specificities of 33–84 %. This study demonstrated that there is a significant difference in S100A9

Fig. 3 a Immunoperoxidase stains of a CCA case that is positive for S100A9, ×400 (the arrow points out a positive cell). **b** Immunoperoxidase stains of a non-CCA case that is negative for S100A9, ×400. **c** Immunoperoxidase stain of a case of hepatocellular carcinoma that is negative for S100A9, ×400. **d** Immunoperoxidase stain of case of cirrhosis that is negative for S100A9, ×400
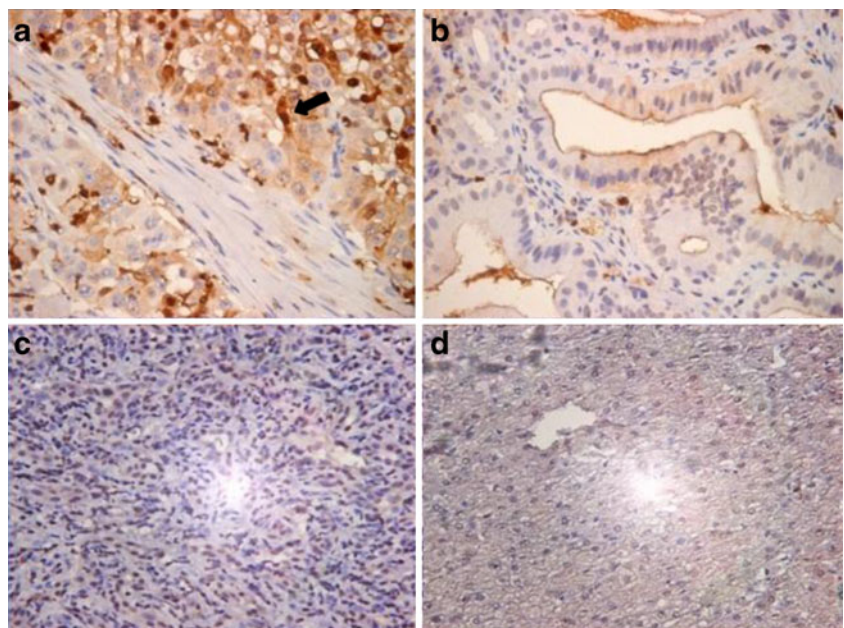
Fig. 4 **a** Immunoperoxidase stains of a CCA case that is positive for CCTγ, ×200 (the arrow points out a positive cell). **b** Immunoperoxidase stains of a non-CCA case that is negative for CCTγ, ×200. **c** Immunoperoxidase stain of a case of hepatocellular carcinoma that is negative for CCTγ, ×400. **d** Immunoperoxidase stain of case of cirrhosis that is negative for CCTγ, ×400
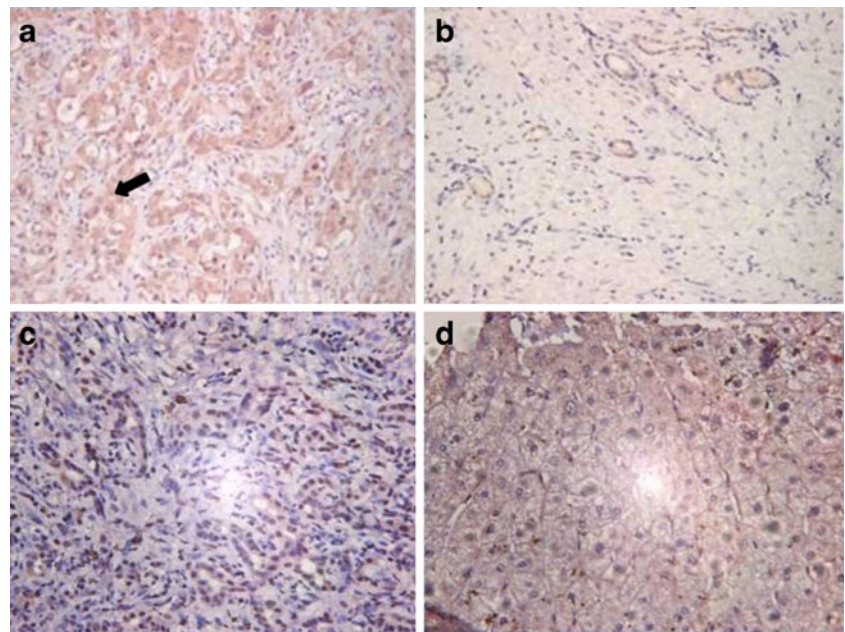
and CCTγ expression between CCA patients and control patients.

The S100 family of proteins includes a group of small acidic proteins with common EF-hand calcium-binding motifs. S100 proteins regulate a variety of cellular processes, including cellular proliferation, differentiation, motility, secretion, membrane permeability, protein synthesis, and extracellular signal transduction. S100A8 and S100A9 are two members of the S100 protein family. S100A8 and S100A9 are key effectors/amplifiers of inflammation, with a wide range of activities, including cytokine induction. Subsequently, it has emerged as an important proinflammatory mediator in acute and chronic inflammation. More recently, increased S100A8 and S100A9 levels were also detected in various human cancers, such as skin, colorectal, breast, prostate, and lung.[19–22] Numerous studies demonstrate that the serum level of S100A9 is significantly elevated in many types of cancers, including breast cancer, esophageal cancer, and lung cancer.[23,24] A recent report suggested the calcium-binding protein S100P is

upregulated in CCA. S100P was frequently expressed in the CCA tissues but not in the normal bile duct. The brushing cytology samples from the CCA cases revealed increased expression of S100P compared with the benign biliary strictures. The relative expression level of S100P could identify CCA at a higher sensitivity than classical cytology, and the combination of the S100P expression level and cytology yielded a sensitivity of 90.0 %, with a specificity of 92.0 %.[25]

The chaperonin-containing t-complex polypeptide 1 (CCT), also called TRiC or c-cpn, mediates protein folding in the cytosol. CCT is a member of the chaperonin family, which includes mitochondrial Hsp60, bacterial GroEL, plastid Rubisco subunit-binding protein, and archaea group II chaperonins.[26,27] CCT shows a double-torus-like structure with eightfold rotational symmetry, and the complete complex is assembled from 16 subunits. The chaperonins are key molecular complexes that are essential in protein folding to produce stable and functionally competent protein conformations.[28,29] One member of the chaperonin group of proteins is TCP1

Fig. 5 Receiver operating characteristic (ROC) curve analyses of S100A9 and CCTγ for the diagnosis of cholangiocarcinoma. The diagnostic accuracy of each biomarker, in terms of its sensitivity and specificity, were presented by ROC curve analysis. **a** and **b** correspond to S100A9 and CCTγ. Only the area under the curve (AUC) of the ROC is significantly higher than a chance value (0.5)
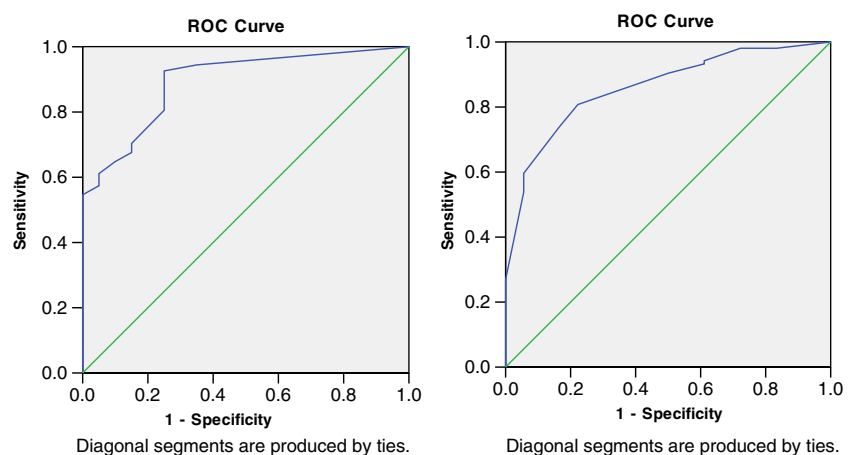
(chaperonin-containing t-complex polypeptide 1 or CCT). Increased expression of cytosolic chaperonin CCT in human hepatocellular and colonic carcinoma has been reported.[18]

Though proteomic approaches in CCA are currently at an early stage, further research could be conducted to early diagnose or evaluate the prognosis. It is believed that varied protein expression of different tumor location, size, lymphatic involvement, degree of fibrosis, specific histology, etc. would also be found in further research based on this study. Further experiments are being conducted to contrast the expression of S100A9 and CCTγ between CCA and HCC patients based on bile or serum, which could be helpful in early diagnosis of CCA.

In conclusion, two potential biomarkers, S100A9 and CCTγ, were found by proteomic screening and confirmed by immunohistochemistry. Our research suggests that these two proteins have the potential to be used as new tumor markers for early detection of CCA. Further studies involving a larger patient population and serum S100A9 and CCTγ levels are needed to provide more information regarding the diagnostic accuracy of S100A9 and CCTγ.

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