Original Research

Serum $\alpha_1\beta$-glycoprotein and afamin ratio as potential diagnostic and prognostic markers in cholangiocarcinoma

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Abstract

Cholangiocarcinoma (CCA) affects the intra- and extrahepatic bile ducts and is commonly burdened by a late presentation and resulting high mortality rate. Accordingly, finding non-invasive biomarkers with adequate diagnostic/prognostic values is a priority in high-risk populations. In this study, we analyzed proteomes of serum samples from six CCA cases and ten healthy subjects using two-dimensional polyacrylamide gel electrophoresis to identify CCA-associated spots. Thirty-six CCA-associated proteins found in sera were identified by mass spectrometry. α1β-Glycoprotein (A1BG) and afamin (AFM) were detected consistently at different degrees in CCA sera compared with controls and were validated for their diagnostic and prognostic potential in a larger cohort of 64 patients with CCA, 4 with benign biliary diseases and 20 healthy subjects and compared between pre- and postsurgery serum samples from 26 CCA patients to ascertain a prognostic correlation. A single blot test developed to assess the serum A1BG/AFM ratio could detect CCA cases with 87.5% specificity, 84.4% sensitivity and the levels were significantly higher in CCA compared with controls. A high level of postoperative serum A1BG/AFM ratio was associated with worse outcomes and the infiltration of resection margins. The A1BG/AFM ratio may constitute a novel non-invasive candidate marker to diagnose CCA and its outcomes with high specificity and sensitivity. Prospective studies are awaited to demonstrate the clinical value of this observation.

Keywords: tumor maker, biomarker, serum, proteomic, bile duct

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Introduction

Cholangiocarcinoma (CCA) is a highly aggressive cancer of the bile duct epithelium and accounts for 10–15% of liver malignancies worldwide,[1] albeit with non-uniform incidence rates in different areas. The highest incidence of CCA is found in Khon Kaen, a province in the northeast of Thailand, with a yearly rate currently estimated as 96 cases per 100,000 in men.[2] At present, surgical intervention is the treatment with the potential to cure CCA,[3] but most cases are diagnosed at advanced stages when surgery is no longer a feasible option. For this reason, finding an early and non-invasive marker that allows a sensitive diagnosis and characterizes patients with a more aggressive disease remains a challenge that needs to be met.

Protein-based markers are ideal candidates for their easy accessibility in routine conditions and 2D polyacrylamide gel electrophoresis (2-DE) coupled with mass spectrometry (MS) is a powerful tool to discover biomarkers, as well illustrated in primary hepatocellular, lung, prostate and bladder cancers.[4–7]

We analyzed serum proteomes of CCA cases and healthy subjects and we first identified two low-abundance differentially expressed proteins, afamin (AFM) and α1β-glycoprotein (A1BG). The AFM/A1BG ratio was then associated with the diagnosis and prognosis of CCA and we suggest that this marker can be used for the differential diagnosis of CCA and to predict the prognosis following surgical resection.

Materials and methods

Serum and tissues

Serum samples and paraffin-embedded tissues were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine,

Khon Kaen University, Thailand. Presurgery serum samples were obtained from 64 patients with histologically proven CCA, of which 58 were intra-hepatic CCA and six were hilar CCA; 27 females and 37 males with mean age 53 ± 7.7 y. Postsurgery serum samples ($N = 31$) were obtained from 26 patients at regular follow-up appointments following resection. Control serum samples were obtained from 20 healthy subjects who underwent annual health checkups and from 4 patients with benign biliary diseases (2 with common bile duct stone, 1 each with liver cirrhosis and liver Fasiola calcification); 10 females and 14 males with mean age 49 ± 6.8 y. Tumor staging was performed according to the American Joint Committee on Cancer criteria.[8] Informed consent was obtained from each subject and the study protocol was approved by the Ethics Committee for Human Research, Khon Kaen University and with the Helsinki Declaration of 1975, as revised in 1983.

All blood samples were collected and sera were separated after centrifugation at $3000 \times g$ for 15 min at 4°C and subsequently at $15{,}000 \times g$ for 15 min at 4°C to remove platelets and particulate matter. Serum samples were stored at −80°C until use.

Serum protein depletion and 2-DE

Albumin and immunoglobulins in the serum were removed using a HiTrap Blue affinity column and a HiTrap Protein G-HP column (GE Healthcare, Uppsala, Sweden). The concentration of protein in the treated serum was determined according to the method of Lowry *et al.*[9] using bovine serum albumin as a standard. Treated serum samples were diluted in rehydration buffer (USB Corporation, Cleveland, OH, USA), 2% IPG buffer pH 4–7, 40 mmol/L dithiothreitol and applied to a 18-cm, pH 4–7 linear IPG strip for the first dimension. The isoelectrofocusing was performed according to the standard protocol of the manufacturer. The second dimension electrophoresis was performed on a 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE).[10] The gel was stained with colloidal Coomassie Brilliant Blue (CBB) G-250 (Sigma-Aldrich, St Louis, MO, USA). Images were collected and analyzed using ImageMaster 2D Platinum version 7.0 software (GE Healthcare) and relative differences in terms of protein contents of the spots from healthy and CCA sera were considered as statistically different with P values <0.01. Three 2-DE gels from each CCA case and two gels from each healthy control were analyzed.

Identification of serum proteins

Candidate protein spots were isolated and in-gel digested with trypsin (Promega, Madison, WI, USA) according to the standard protocol. The digested peptide mixtures were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using the Waters SYNAPT^{TM} HDMS^{TM} system (Waters Corporation, Milford, MA, USA). The 1D-nano LC was carried out with a Waters nanoACQUITY UPLC system. The samples were electrosprayed into a mass spectrometer (Synapt HDMS; Waters Corporation) for MS/MS analysis of peptides. The spectral data were generated in a micromass file support for MS/MS Ion search using the MASCOT engine program. The *Homo sapiens* subset of sequences in the NCBI non-redundant protein sequence database was utilized for MASCOT searches. The following search parameters were used in all MS/MS Ion searches: peptide mass tolerance of ± 1.2 Da; tolerance of one missed trypsin cleavage; a maximum error tolerance of ± 0.6 Da in the MS/MS data. Protein hits with the highest score and accurate molecular mass and isoelectric point (pI) values according to the 2D-gel appearance were used for spot identifications.

Western blotting

Serum proteins (20 μg) were loaded onto a microplate of a 12% SDS-polyacrylamide gel and transferred onto a poly(vinylidene difluoride) membrane at 10 V for three hours.[11] Membranes were incubated in a blocking buffer (3% bovine serum albumin, 0.3% Tween 20 in phosphate-buffered saline [PBS], pH 7.2) overnight at 4°C and then washed with 0.3% Tween 20 in PBS, pH 7.2 for three minutes and incubated with a cocktail of primary antibody including 1:1000 AFM (Abnova, Taipei, Taiwan) and 1:20000 A1BG (Abcam, Cambridge, MA, USA) for one hour at room temperature. After washing, the membrane was incubated with secondary antibody conjugated with horseradish peroxidase (HRP) for one hour. The antigen–antibody complex was detected by enhanced chemiluminescence using ECL Prime reagents and visualized by ImageQuant LAS 4000 mini capture device (GE Healthcare). The intensity of each targeted band captured at three minutes was quantified using the ImageQuant TL software program (GE Healthcare).

Immunohistochemistry of AFM and A1BG

Detection of AFM and A1BG in CCA tissue sections by indirect immunoperoxidase method was performed according to the standard protocol. Liver sections were incubated with 5 μg/mL of anti-AFM antibody or 5 μg/mL of anti-A1BG antibody, at room temperature, overnight using an EnVision-system-HRP (Dako, Glostrup, Denmark) for one hour. The immunoreactivity was developed with diaminobenzidine tetrahydrochloride (Sigma-Aldrich) and 0.1% H_2O_2 in 50 mmol/L Tris-HCl, pH 7.8. PBS was used instead of primary antibody for negative control.

Statistical analysis

Continuous variables were expressed as mean $\pm$ SD and Student's t-test was used to compare continuous variables between groups. The Fisher's exact test was used to compare the differences in clinicopathological findings of CCA patients. Youden's index was used for selection of cut-off optical density and subsequently indicates diagnostic values of the test. The survival analysis was performed using the Kaplan–Meier method and compared using the log-rank test. All analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). P values <0.05 were considered as statistically significant.

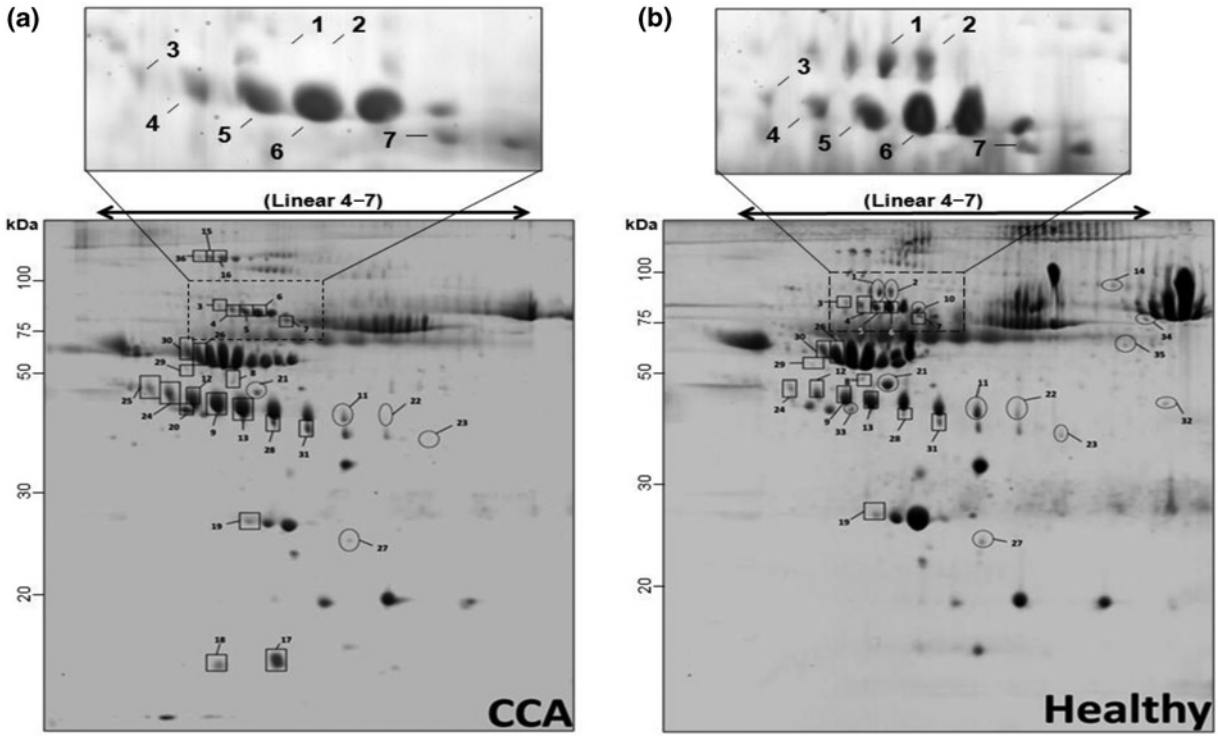

Figure 1 Representative 2D electrophoretic profiles of serum samples from CCA cases (a) and healthy controls (b). Spots (1–36) with different intensity between profiles are shown; squares indicate hyper- and circles indicate hypo-intensity in CCA samples. Inserts emphasize AFM (spots 1–2) and A1BG (spots 3–7) which were decreased and increased in CCA patients. CCA, cholangiocarcinoma; AFM, afamin; A1BG, $\alpha 1\beta$-glycoprotein

Results

Proteomic profiles of CCA and controls

After albumin and immunoglobulin depletion, the proteomic patterns of serum samples from individual CCA cases ($N = 6$) and healthy controls with age and sex matched to the CCA group ($N = 10$) were analyzed using 2-DE with colloidal CBB-G250 staining. The total protein spots obtained from CCA (152 ± 26 spots) and control serum samples (168 ± 29 spots) were compared with 109 ± 28 match spots ($67.66 \pm 10.34\%$). Out of 38 gels, 36 differential protein spots corresponding to 17 genes were found in CCA, with 23 spots of nine genes being expressed at higher levels compared with controls (Figure 1).

Protein identification

The 36 identified protein spots were subjected to LC-MS/MS and the results of the MASCOT web-based analysis are illustrated in Supplementary Table 1 (please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.012215/-/DC1). We were particularly intrigued by two low-abundance proteins that had been associated with cancer, i.e. AFM (Figure 1, spots 1–2) and A1BG (spots 3–7) that were selected and further validated by Western blot analysis. Of note, AFM and A1BG spots were lower and higher in CCA sera, respectively, compared with controls (Figure 1, insets).

Western blot analysis for AFM and A1BG

Expression levels of AFM and A1BG were further confirmed by Western blot analysis following the demonstration that the respective spots reacted with the specific antibodies (Figure 2a). As both AFM and A1BG are located above the positions of albumin and globulins, the depletion of these proteins before electrophoresis was not necessary. Following preliminary experiments, we determined that SDS-PAGE was adequate for protein discrimination using specific antibodies in a single-step Western blot. Patterns from CCA and control serum samples differed in the intensities of the AFM and A1BG reactivity (Figure 2b). The reactivity of AFM was significantly lower in CCA samples while that of A1BG was significantly higher, as expected following 2-DE. The intensity of each band was determined and analyzed as the A1BG/AFM ratio.

Serum A1BG/AFM ratio in CCA

Using the above-mentioned Western blot methods, A1BG/AFM ratios were determined in a larger series of serum samples from CCA ($n = 64$) and controls (healthy subject, $n = 20$; benign biliary diseases, $n = 4$) (Figure 2c) and values were significantly higher in CCA (mean $\pm$ standard deviation 4.32 ± 3.73; 95% confidence interval [CI] 3.51–5.26) compared with controls (1.39 ± 0.67; 95% CI 1.02–1.68; $P < 0.0001$) (Figure 2d). There was no association between the A1BG/AFM ratio of presurgery serum and age, gender, histopathology and tumor staging (data not shown).

A receiver operating characteristic curve (ROC) analysis was then performed to determine the best threshold to discriminate CCA from controls. We determined an area under the curve = 0.886 with a 95% CI for the area between 0.813

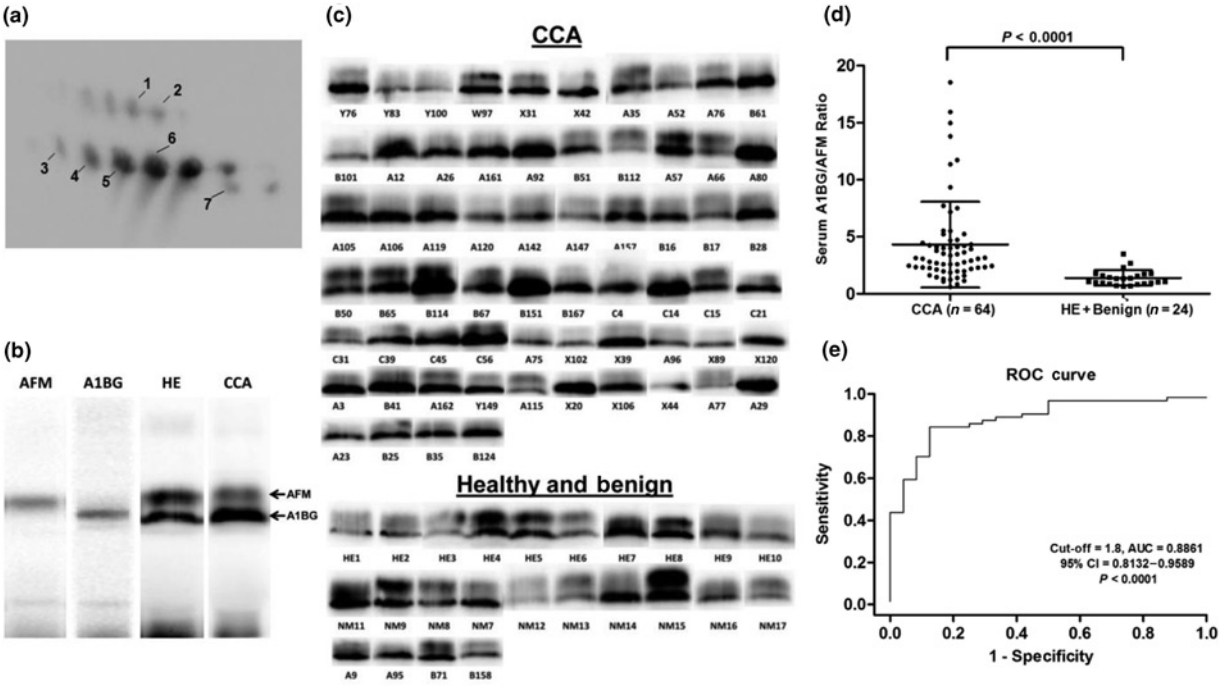

Figure 2 CCA discrimination by high A1BG/AFM ratio in presurgery serum samples. (a) Western blot of serum samples using antibodies against AFM and A1BG; (b) representative immunoblottings of whole serum samples separated by conventional SDS-PAGE using AFM and A1BG antibodies in separated strips and the combined antibodies for healthy (HE) and CCA patients' serum samples; (c) AFM and A1BG expressions in serum samples from 64 CCA cases and 24 controls; (d) presurgery serum A1BG/AFM ratio in patients with CCA were significantly different from those of controls ($P < 0.00001$); (e) ROC of serum A1BG/AFM ratio determining a threshold for CCA diagnosis. CCA, cholangiocarcinoma; AFM, afamin; A1BG, $\alpha 1\beta$-glycoprotein; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ROC, operating characteristic curve

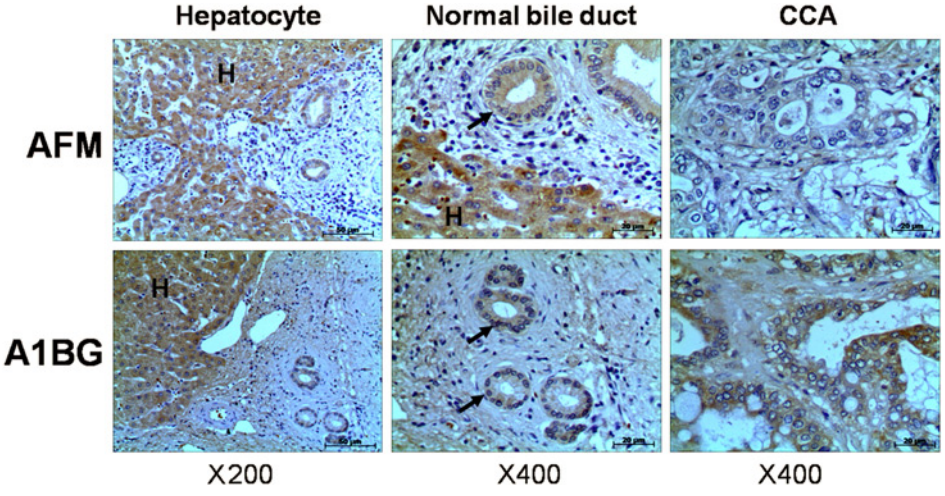

Figure 3 Immunohistochemistry staining for A1BG and AFM in CCA liver tissues. Hepatocytes (H) strongly express both proteins while normal bile duct epithelia are faintly positive. CCA tissue features are low positivity for AFM and high expression of A1BG. CCA, cholangiocarcinoma; AFM, afamin; A1BG, $\alpha 1\beta$-glycoprotein. (A color version of this figure is available in the online journal)

and 0.959 (Figure 2e). An A1BG/AFM ratio threshold of 1.8 thus provided 84% (54/64) sensitivity and 87.5% (21/24) specificity rates associated with 94.7% (54/57) positive and 67.7% (21/31) negative predictive values.

A1BG and AFM tissue expression

Immunohistochemistry was performed in paraffin-embedded liver tissues ($n = 10$) to determine A1BG and AFM expression and demonstrated that both proteins are strongly expressed in hepatocytes and, with lesser intensity, in normal bile duct epithelia (Figure 3). Conversely, compared with the matched normal bile duct epithelia, the staining intensity of AFM was reduced (10/10, 100%) whereas that of A1BG was increased (5/10, 50%) in CCA tissues. Of note, the expressions of A1BG and AFM in CCA tissues manifested the same trend of serum A1BG and AFM levels revealed by Western blotting.

Table 1 Univariate analysis of $\alpha 1\beta$-glycoprotein/afamin (A1BG/AFM) ratio in postsurgery serum of cholangiocarcinomapatients

Variable	No. of patients	Serum A1BG/AFM ratio		P
		Low (<1.8)	High (≥1.8)	
Age				
<56 years	12	5	7	0.387
≥56 years	14	4	10	
Sex				
Male	12	3	9	0.296
Female	14	6	8	
Histopathology				
PAP	15	5	10	0.598
Non-PAP	11	4	7	
Tumor stage				
I–II	10	4	6	0.225
III–IV	8	1	7	
Marginal status				
R0	12	9	3	<0.001
R1	14	0	14	
Chemotherapy				
No	17	7	10	0.302
Yes	9	2	7	

PAP, papillary

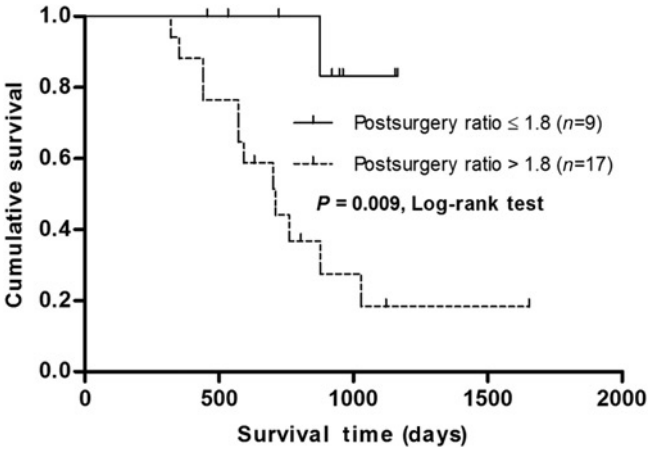

Figure 4 A1BG/AFM ratio in postsurgery serum samples is associated with patient outcome. The Kaplan–Meier analysis of postsurgery serum samples indicated that CCA patients with postoperative serum of A1BG/AFM ≤1.8 had a better survival than those with serum A1BG/AFM >1.8. CCA, cholangiocarcinoma; AFM, afamin; A1BG, $\alpha 1\beta$-glycoprotein

Prognostic significance of A1BG/AFM ratio in patients undergoing surgical resection

Presurgery and the matched postsurgery serum samples were available from 26 patients with CCA. The operative procedures for all patients were intended for cure; however, the pathology record showed 46.2% (12/26) of the cases were margin-free. In this series, 34.6% (9/26) of patients received chemotherapy after operation and none of the patients received postoperative radiotherapy. Postsurgery serum samples were arrayed based on low (≤1.8) and high (>1.8) A1BG/AFM ratios and were evaluated for the association with clinicopathological features of CCA patients using univariate analysis. High A1BG/AFM ratio of postsurgery serum samples was significantly associated with positive surgical margin ($P < 0.001$) but not with age, sex, histopathological grading, tumor staging and chemotherapeutic treatment (Table 1).

The log-rank analysis indicated that CCA cases with elevated serum A1BG/AFM ratios at the presurgery and postsurgery time points had worse survival rates; however, the survival rates were statistically significant only for postsurgery serum samples (Figure 4) ($P = 0.009$). The median survival time of CCA patients with postsurgery A1BG/AFM ratios ≤1.8 ($n = 9$) could not be defined, whereas those with A1BG/AFM ratios >1.8 ($n = 17$) was 710 days.

Factors which may affect survival were analyzed for the independent value in predicting overall survival using the Cox proportional hazard model (Table 2). High level of A1BG/AFM ratios (>1.8) of postsurgery serum ($P = 0.016$) was shown to be an independent prognostic factor for shorter overall survival in CCA. CCA patients who had high level of A1BG/AFM ratios (>1.8) had a 15-fold higher risk of death than patients who had low serum A1BG/AFM ratios.

The study of 31 postsurgery serum samples from 26 CCA patients (Figure 5a) revealed that A1BG/AFM ratios tended to decrease at 1–6 months (2.7 ± 1.1) and 7–12 months (2.3 ± 1.1) following resection compared with presurgery serum samples. However, only serum A1BG/AFM ratios were significantly reduced at 7–12 months after resection ($P < 0.05$). Of most interest, A1BG/AFM ratios were higher in the postsurgery serum samples of the 14 patients in which margin-free resection could not be achieved ($P = 0.0003$) (Figure 5b). Finally, postsurgery serum samples which were collected from five patients who visited the clinic more than one time, who were subgrouped according to the resection margin, indicated that serum A1BG/AFM ratio of patients who had free surgical margin had consistent low A1BG/AFM ratio values (≤1.8) till 25 months postsurgery compared with presurgery serum samples which were higher than 1.8 (Figure 5c). In contrast, serum A1BG/AFM ratios of patients who had positive surgical margin were reduced during the first few months and then increased beyond 1.8 (Figure 5d).

Table 2 Multivariate analysis of alpha-1-β-glycoprotein/afamin (A1BG/AFM) ratio in postsurgery serum of cholangiocarcinomapatients

Variable	N	Adjusted HR	95% CI	P
Postsurgery serum A1BG/AFM ratio				
Low (<1.8)	9	1		
High (≥1.8)	17	15.071	1.654–137.352	0.016
Age				
≤56 years	12	1		
>56 years	14	0.451	0.123–1.662	0.232
Gender				
Male	12	1		
Female	14	1.990	0.616–6.432	0.250
Histopathology				
PAP	15	1		
Non-PAP	11	1.379	0.436–4.362	0.584
Received chemotherapy				
No	19	1		
Yes	7	1.519	0.440–5.244	0.508

HR, hazard ratio; CI, confidence interval; PAP, papillary

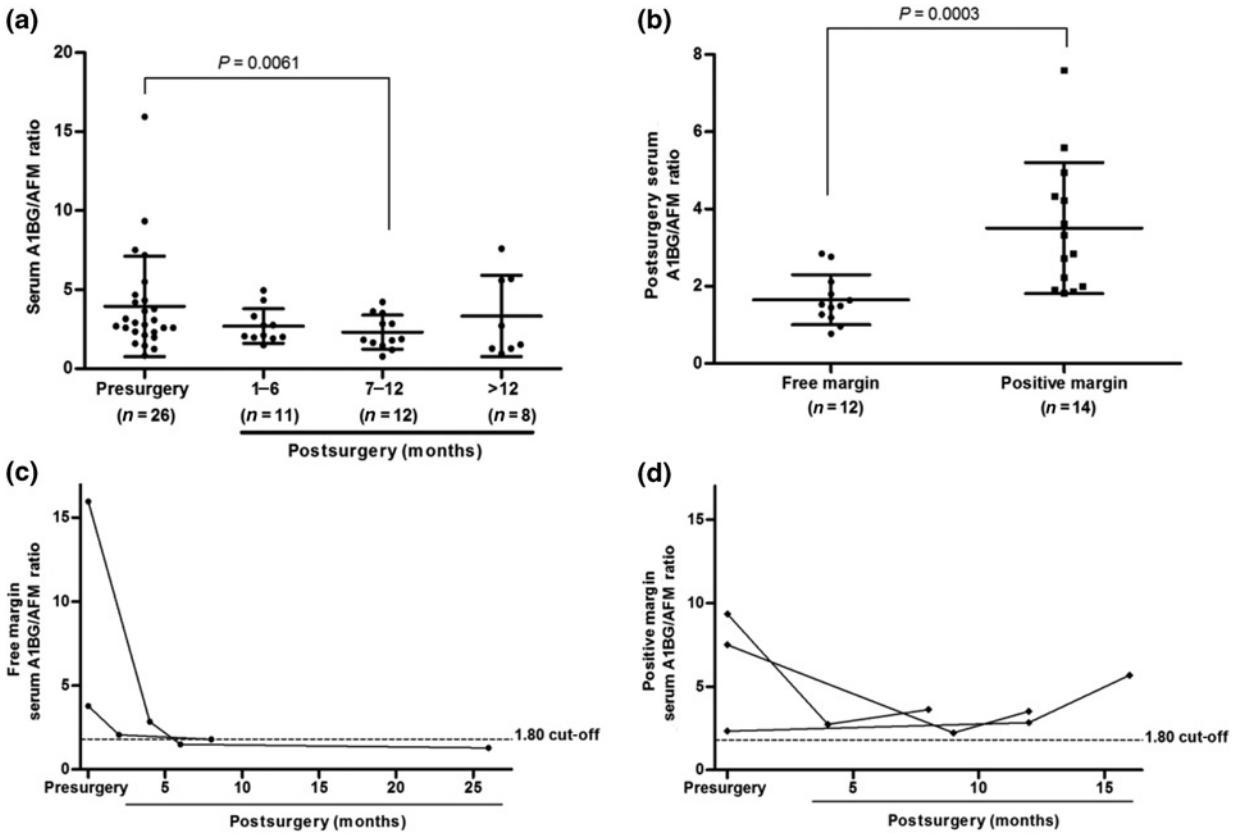

Figure 5 Postsurgery serum A1BG/AFM ratio is associated with the characteristics of resection margins in CCA. (a) Serum A1BG/AFM ratio is significantly reduced at 7–12 months after surgery; (b) Patients with resection margins free of disease have significantly lower serum A1BG/AFM ratio compared with patients with infiltrated margins; (c) patients with resection margins free of disease have persistently reduced serum A1BG/AFM ratio up to two years while (d) patients with affected margins have increasing ratios over time. CCA, cholangiocarcinoma; AFM, afamin; A1BG, $\alpha 1\beta$-glycoprotein

Discussion

In the continuing search for biomarkers for CCA, we report herein that the levels of two serum proteins, expressed as A1BG/AFM ratio, are indeed associated with a high diagnostic power and also associated with specific cancer clinical features that predict the disease outcome. Moreover, serum levels reflect the A1BG/AFM expression of CCA tissues and the presence of tumor.

Serum is an obvious target for the development of non-invasive biomarkers but protein concentrations are often characterized by wide inter- and intra-individual variability. To overcome these limitations, we utilized a two-tier approach to determine that A1BG and AFM may constitute candidates for the challenging role of clinical markers in CCA. First, we identified differentially expressed proteins in a subgroup of well-characterized serum samples from CCA cases and controls. At this step, we depleted serum samples of albumin and globulins before proceeding to 2-DE and mass spectrometry. We subsequently selected two low-abundance proteins, namely A1BG and AFM, out of the 36 differentially expressed in a consistent fashion across the two populations. A1BG was elevated and AFM was decreased in serum samples of CCA patients compared with controls, thus suggesting that a ratio between these two proteins may better reflect their respective levels measured using specific antibodies. Second, we developed a single-step Western blot method to measure both proteins without the need for albumin and globulin depletion based on the target proteins' molecular weights. The use of a single test has several advantages that include a lower intra- and inter-individual variability.

Following the first part of this study, we then tested if the serum A1BG/AFM ratio could be used in the management of suspect and established cases of CCA and be thus considered a reliable biomarker. First, we observed the high sensitivity and specificity of serum A1BG/AFM ratio in discriminating CCA and controls in a large cohort. The ROC analysis indicated the diagnostic values of serum A1BG/AFM with 84.4% sensitivity and 87.5% specificity, independent of sex, age, CCA histological type or staging. Second, the serum level of A1BG/AFM ratio corresponded with the expression levels in CCA tissues and was significantly reduced after tumor resection. Third, the A1BG/AFM ratio of postsurgery serum could predict patients' outcomes as supported by the comparison of serum samples obtained from patients with CCA after undergoing surgical resection. The level of A1BG/AFM ratio was an independent prognostic variable for unfavorable outcome of CCA patients. Patients who exhibited higher A1BG/AFM ratio after surgery had significantly shorter survival than those who had low levels. This poor outcome may be due to tumor recurrence since the level of A1BG/AFM ratio of postsurgery serum higher than 1.8 was significantly associated

with signs of disease infiltration. Accordingly, a more radical surgery (i.e. with resection margins free of infiltration at histology) was associated with significantly lower A1BG/AFM ratios, and remained at normal level during follow-up. In contrast, serum A1BG/AFM ratio values remained elevated above 1.8 in patients in whom the resection margins showed CCA infiltration.

A search for details on the two proteins utilized in the biomarker proposed herein may help understand their significance. A1BG is a protein with several isoforms but with no clearly defined function despite reports of its specific binding to the exocrine-secreted cysteine-rich secretory protein 3 that is up-regulated in inflammatory conditions including pancreatitis and prostate cancer.[12,13] Of note, changes in A1BG expression were reported in pancreatic and hepatocellular cancers.[14,15] Our findings in CCA appear to be in agreement with pancreatic cancer. The AFM gene was first identified as a member of the albumin, α-fetoprotein and vitamin D-binding protein family.[16] It is thus not surprising that AFM transports a wide variety of molecules, exerts a significant role in protecting against oxidative stress and contributes to major cell functions such as adhesion, proliferation and apoptosis.[17] Pertinent to oncology, low serum levels of AFM consistent with our results were also reported in ovarian[18,19] and hepatocellular[20] cancer, while the enhanced AFM expression was associated with cervical cancer.[21]

We recognize that the present study manifests major strengths. First, the number of cases that were included in this study is highly representative of a highly endemic region of CCA such as the north-east of Thailand. Second, we acknowledge that the use of serial samples from patients undergoing clinical resection with different outcomes (represented by both short-, i.e. resection margins and medium-term, i.e. survival indicators) is an ideal model for the first step of the development of a CCA biomarker. Third, the use of serum for the development of this biomarker is an obvious advantage compared with other more invasive biological samples that have been proposed in the past years.[22] On the other hand, we are also aware of two possible limitations of our study, namely of the fact that the vast majority of CCA cases in this area are secondary to parasitic infection and may not fully reflect the variable risk factors inducing the cholangiocyte neoplastic transformation.[23] It is of limited applicability to compare gene expression with proteomic profiles but we note that we could not identify the same patterns in our series as in the reported data from primary sclerosing cholangitis, a chronic cholestatic condition leading to CCA. Furthermore, we also submit that the limits of a largely cross-sectional study should be considered in the interpretation of our data but are convinced that this design is the only feasible first-tier approach to discover biomarkers for conditions with rare incidence in the general population.[24]

Based on our data obtained from a proteomic approach, a large number of cross-sectional and serial serum samples from patients with CCA, and the confirmation in liver tissues of the protein expression, we suggest that the serum A1BG/AFM ratio is a potential biomarker for CCA diagnosis and outcome. In particular, we are convinced that the observed association of the A1BG/AFM profiles in serum samples (as determined by Western blotting) and CCA tissue (as determined by immunohistochemistry) supports the tumor origin of the serum proteins. Furthermore, the determination of two proteins in a single test enhances the sensitivity and specificity of the test by reducing the intra- and inter-individual variability. Finally, the biomarker appears to apply to all histological subtypes of CCA as the association with serum A1BG/AFM ratios was independent of this variable as well as the sex and age of the patients.

In conclusion, our proteomic, serological and histological approach suggest that the ratio between two low-abundance serum proteins may be suitable to complement the medical decisions that are now based on physical examination, imaging and commonly used (but poorly reliable) tumor markers. We warrant prospective studies in subjects at high risk of developing CCA such as those carrying the liver fluke infection or those with primary sclerosing cholangitis to further determine the impact of the proposed biomarker in clinical practice. In these studies, we also encourage the head-to-head comparison with currently proposed and new putative serum markers[25] and long follow-up durations.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. AT, SP, AS and SR conducted the experiments; SW and NK collected specimens and analyzed clinical data; and AT and CW wrote the manuscript.

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