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# Protein expression in sera of patients with amoebic liver abscess (ALA): potential use of haptoglobin as a surrogate disease marker

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**Abstract.** The protein profile of serum samples from patients with amoebic liver abscess (ALA) was compared to those of normal individuals to determine their expression levels and to identify potential surrogate disease markers. Serum samples were resolved by two dimensional electrophoresis (2-DE) followed by image analysis. The up and down-regulated protein spots were excised from the gels and analysed by MS/MS. The concentration of three clusters of proteins i.e. haptoglobin (HP), $\alpha_1$-antitrypsin (AAT) and transferrin in serum samples of ALA patients and healthy controls were compared using competitive ELISA. In addition, serum concentrations of HP and transferrin in samples of patients with ALA and pyogenic liver abscess (PLA) were also compared. The results of the protein 2-DE expression analysis showed that HP cluster, AAT cluster, one spot each from unknown spots no. 1 and 2 were significantly up-regulated and transferrin cluster was significantly down-regulated in ALA patients' sera ($p<0.05$). The MS/MS analysis identified the unknown protein spot no.1 as human transcript and haptoglobin and spot no. 2 as albumin. Competitive ELISA which compared concentrations of selected proteins in sera of ALA and healthy controls verified the up-regulated expression ($p<0.05$) of HP and the down-regulated expression ($p<0.01$) of transferrin in the former, while there was no significant difference in AAT expression ($p>0.05$). However, when ALA and PLA samples were compared, competitive ELISA showed significant increased concentration of HP ($p<0.05$) while transferrin levels were not different. In conclusion, this study showed that HP is a potential surrogate disease marker for ALA.

## INTRODUCTION

Amoebic liver abscess (ALA) is the most common manifestation of extraintestinal amoebiasis. The disease is caused by the infection of *Entamoeba histolytica* through oral-faecal route, mainly from water and/or food that have been contaminated with the amoebic cysts. Poor sanitation, unsafe sex and travelers to endemic areas are also among the mode of transmissions (Jalan & Maitra, 1988; Walsh, 1988). Worldwide approximately 500 million people per year are infected, with symptoms seen in about 10%. This third leading parasite cause 100,000 people death per year (WHO, 1997). The clinical symptoms of ALA include fever, cough, dull and aching abdominal pain in the right upper quadrant (Petri & Singh, 1999). Diagnosis of ALA is usually performed using radiology and serology, however there are limitations to these methods. Thus availability of surrogate disease markers may be useful to assist in the diagnosis of ALA.

Comparison studies between serum from disease and non-disease groups had been performed in the study of the differences in protein expression levels (Anderson & Anderson, 1977; Omenn, 2004). The

advantage of this kind of study is that an alteration of a specific serum protein can be visualized. This may provide an early indication of an altered physiology that may be indicative of a disease (Poon & Johnson, 2001). In infectious disease, the identification of serum biomarkers using this technology platform is increasing because of the current awareness among researchers that the studies can be very useful for the prediction, detection and diagnosis of disease (He *et al.*, 2003; Lee *et al.*, 2006; Gangadharan *et al.*, 2007).

Albumin was reported to be useful for prognosis of patients with ALA, with most patients exhibiting hypoalbuminemia (< 2 g/dL) (Sharma & Ahuja, 2003). There are probably other host serum proteins which are potentially useful as surrogate markers for the diagnosis of ALA. To identify such proteins, the present study used proteomic technology (2-DE and MS/MS) and immunoassay to analyse expression of abundant host serum proteins of ALA patients.

## MATERIALS AND METHODS

**Sample collection**

Fifteen pairs of serum and pus samples from patients with ALA were collected from Hospital Universiti Sains Malaysia, Kelantan. The symptoms of the patients included pain and tenderness in the right upper quadrant of the abdomen, hepatomegaly and/or fever. Ultrasound showed abscess in liver parenchyma tissue. As controls, nine serum samples from healthy individuals were obtained from the serum bank of the Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia. In addition, serum samples from four patients with pyogenic liver abscess (PLA) were also used. All the serum samples were analysed using Ridascreen ELISA (R-Biopharm, Darmstadt, Germany) to detect the presence of specific anti-*E. histolytica* antibodies. Meanwhile, the pus aspirates from ALA patients were tested using real-time PCR assay to detect the presence of *E. histolytica* DNA, as previously reported by our group (Othman *et al.*, 2010).

**Two-dimensional electrophoresis (2-DE)**

Ten microlitres (~20 µg) of each serum sample from ALA patients and healthy individuals was used to rehydrate (overnight) a 13 cm of Immobiline Drystrip (GE Healthcare, Sweden) with pH range 4-7. The rehydrated strip was subjected to first dimensional electrophoresis using isoelectric focusing equipment (Ettan IPGphor3, GE Healthcare, Sweeden) under the following conditions: 200 V for step 1, 500 V for step 2, 1000 V for gradient 1, 8000 V for gradient 2 and 8000 V for the final step. The focused sample strip was then equilibrated with buffer containing DTT and iodocetamide, followed by SDS-PAGE using 8-13% gradient gel. The second dimension electrophoresis was ran at 50 V for 30 minutes followed by 500 V for 230 minutes at 16°C. The analytical gel was silver stained, while the gel used for coring out selected gel plugs was stained with Coomassie blue. All samples were ran in duplicates. The gel plugs were destained with three 45 min washes with 25 mM ammonium bicarbonate in 50:50 (ACN:water). Then, the gel pieces were vacuum-dried and stored at -20°C prior to in-gel tryptic digestion.

**Image analysis for protein expression patterns**

The analytical silver-stained gels were captured with 16 bits resolution using an image documentation system (Alpha Innotech, FC2, USA). The gel images were analysed for protein expression patterns using Progenesis SameSpots software (Nonlinear Dynamic, UK). Before performing any alignment or spot analysis, gel images each from sera of patients with ALA (disease) and healthy individuals (control) were subjected to a quality control (QC) of the Progenesis Samespots software. The QC includes an analysis of image compression, image saturation (spot exposition), image bit depth, available dynamic range (from 85% to 96%) and intensity level resolution and will detect any alteration to edited image. After passing all the QC parameters, one of the images from a healthy individual was selected to be the reference. The best five images from each serum samples from patients with ALA and healthy individuals

were chosen for the analysis. Then, the alignment of each gel image was performed using the automated mode or, if the result was not satisfactory, manual alignment was performed.

After getting an accurate alignment, the protein spots were normalized, detected and filtered. The normalization of spots was performed based on abundance ratio that was chosen in the algorithm developed by Progenesis Samespots. For each spot, the background corrected abundance is calculated and abundance ratio is determined by dividing the sample abundance by the reference abundance. The filtering step was performed to remove artificial spots. The analysis was designed to select gel images which fall into the test group or control group. The protein spots that were up-regulated or down-regulated were identified by one-way ANOVA test ($p<0.05$) analysis based on average normalized volumes. The selected protein spots were further analysed by looking at 2D montages, 3D montages and the location of an interest spot commonly found at the same position in each gel. In the statistical analysis, a list of the p- and q-values were exhibited for determination of the false positive rate (FPR). The FPR was calculated by multiplying the last q value in the list with number of spots that have been detected as $p<0.05$. The cut-off must be less than one ($FPR<1$). Finally, the expressed protein spots were selected for the mass spectrometry analysis.

**Mass spectrometry analysis and database search**

The selected protein spots were cored out and sent for mass spectrometry analysis at Proteomic International Pty Ltd, Australia. Protein samples were trypsin digested and peptides extracted according to standard techniques (Bringans *et al.*, 2008). After the destaining step, 10 µl of trypsin digest solution containing 12.5 µg/ml in 25mM ammonium bicarbonate was added to each gel piece and incubated at 37ºC for overnight. The peptides were extracted by 20 min incubation (twice) with 10-20 µl ACN containing 1% TFA, depending on the size of the gel piece. The pooled extracts were dried by rotary evaporation and stored at -20ºC prior to MS/MS analysis. Peptides were analysed by MALDI TOF/TOF mass spectrometer using a 5800 Proteomics Analyser (AB Sciex). The peptides were reconstituted in 2 µl of 30:70 (ACN:water) prior to addition with matrix solution (CHCA, 10 mg/ml) at ratio 1:10. Then, the mixture was spotted on a 384-well Opti-TOF stainless steel plate. For the MS mode, the system was set up using a standard TOF/MS protocol. For the MS/MS mode, the system was set up to select 15 most intense peaks from the MS run (excluding known peaks such as trypsin). The laser was set to shoot 400 times per shot in MS mode and 2000 times per shot for MS/MS mode. Laser intensity was 2800J (MS) and 3900J (MS/MS). A focus mass of 2100 amu was used in a mass range of 400-4000 amu. The acquired spectra were analysed to identify proteins of interest using MASCOT sequence matching software (Matrix Science) which was incorporated in the ProteinPilot software (5800) Proteomic analyser (ABI Sciex) against Ludwig NR Database and taxonomy set to 'Human'. The search parameters used were N-terminal acetylation, cysteine C-terminal carbamido-methylation and M-methionine oxidation. Hundred fifty ppm and ± 0.4 Da were set for peptide mass tolerance and the fragment mass tolerance, respectively.

**Competitive ELISA**

Comparison of serum concentrations of haptoglobin chains (HP), $\alpha_1$-antitrypsin (AAT) and transferrin were performed on all individual samples of patients with ALA and healthy individuals. In addition, serum concentrations of HP and transferrin were also compared between ALA and PLA patients. Available commercial competitive ELISA kits (Abcam, USA) for each protein were used according to the manufacturer's instructions. The results were analysed statistically using one-way ANOVA, then followed by Tukey HSD test for Post-ANOVA pair-wise comparison (HP and transferrin) and T-test (AAT) (SPSS version 12.0).

RESULTS

**Image analysis of serum samples**

The 2-DE gel separations are able to resolve almost all cluster spots of high abundant serum proteins that could be visualized in serum samples from patients with ALA and healthy individuals (Figures 1 and 2). Besides albumin and IgG, the previously reported clusters of proteins such as $\alpha_2$-HS glycoprotein (AHS), $\alpha_1$-HS glycoprotein (ABG), AAT, HP, clusterin (CLU2), transferrin and IgA were also observed. These appeared as well-separated distinct protein spots, and were consistent with serum profiles from reported images which utilized the same length and pH of IPG strips (Chen *et al.*, 2010; Emelia, 2010).

The protein expression patterns analysis between ALA (disease) patients and healthy individuals (control) serum samples showed the HP cluster was significantly up-regulated ($p<0.05$), with 1.8 fold in sera of patients with ALA (Figures 2.0, Table 1.0). Meanwhile, the

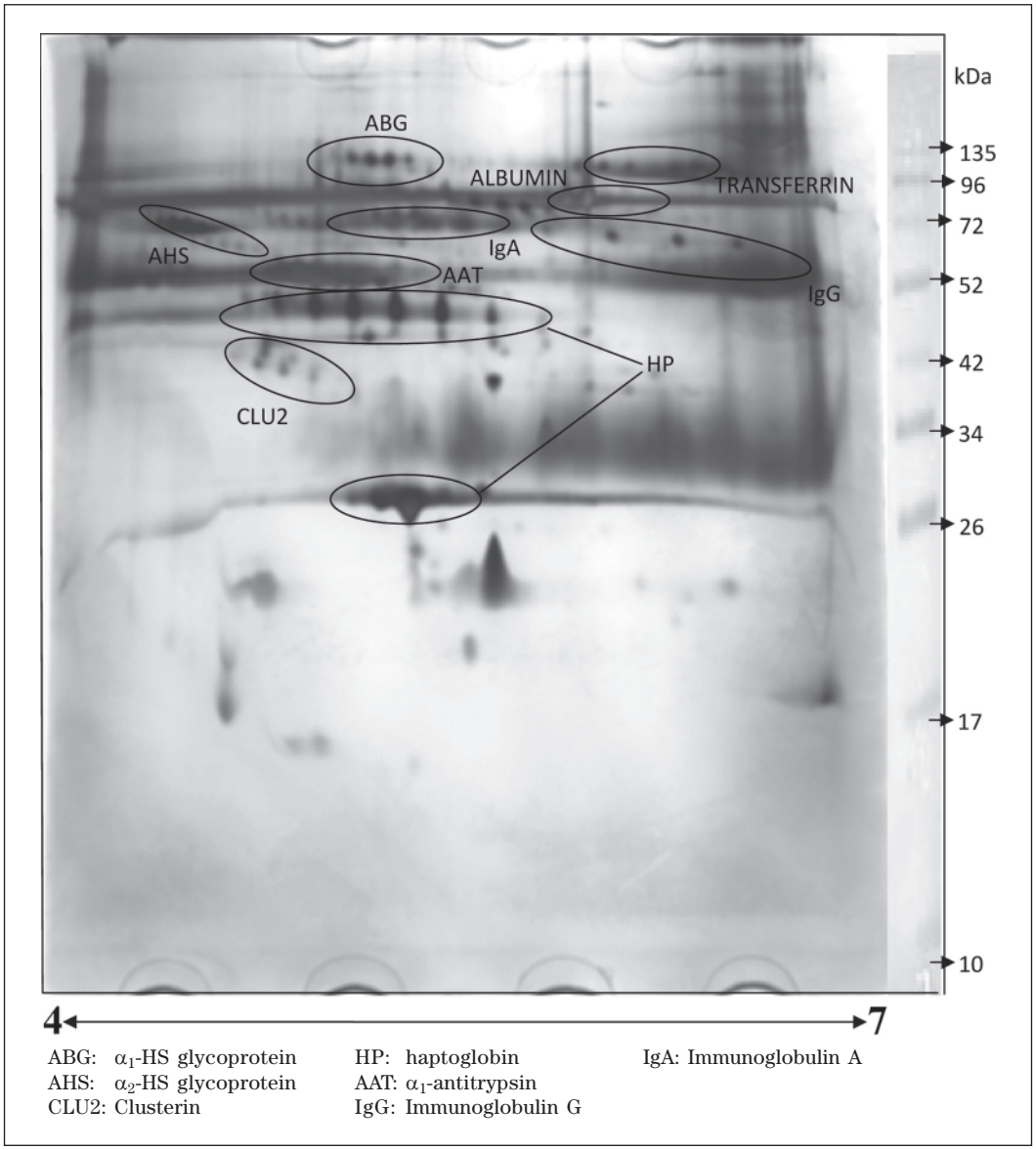


Figure 1. A representative 2-DE protein profile of serum sample from a healthy individual (control) showing the presence of high abundant protein clusters in the human serum (circled)

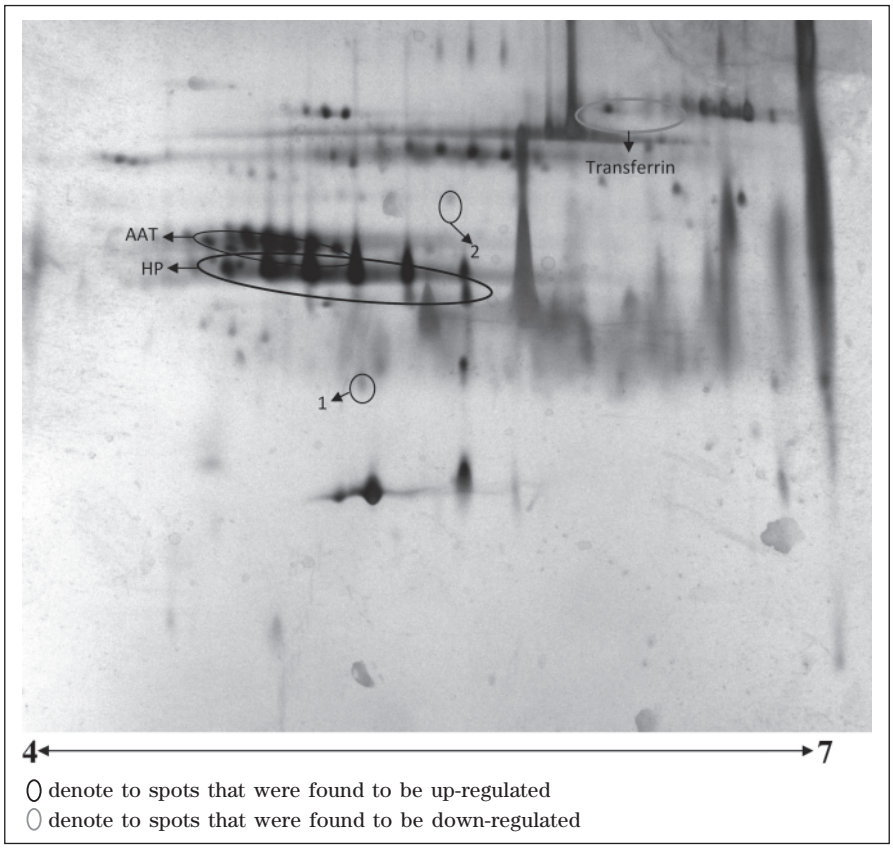


Figure 2. A representative 2-DE protein profile of serum sample from a patient with amoebic liver abscess (ALA) showing the up/down-regulated protein spots (circled)

Table 1. Summary of results of the protein expression patterns in sera of patients with ALA and sera from healthy individuals

| Name of cluster/ protein spot | ANOVA (p) | Fold | Average Normalised Volumes | | Up/down regulated |
|---|---|---|---|---|---|
| | | | Control (Healthy) | Disease (ALA) | |
| HP(haptoglobin) | 0.017 | 1.8 | 8.5542e+006 | 1.4383e+007 | Up |
| AAT ($\alpha_1$-antitrypsin) | 0.005 | 1.5 | 7.0930e+006 | 1.0508e+007 | Up |
| Unknown no. 1 | 0.004 | 2.0 | 6.4600e+005 | 1.3040e+006 | Up |
| Unknown no. 2 | 6.136e-004 | 1.9 | 1.1440e+005 | 2.1530e+005 | Up |
| Transferrin | 0.027 | 2.0 | 2.9280e+006 | 1.5509e+006 | Down |

AAT cluster was also significantly up-regulated ($p<0.05$), with 1.5 fold in the sera of ALA patients (Figure 2, Table 1). Furthermore, two unknown protein spots (no. 1 and 2) were also found to be significantly up-regulated ($p<0.05$), with 2.0 and 1.9 folds, in sera of patients with ALA, respectively (Figure 2, Table 1). In addition, the transferrin cluster was significantly down-regulated ($p<0.05$), with 2.0 fold in patients' sera when compared to sera of healthy individuals (Figures 2, Table 1).

**Identification of proteins using mass spectrometry**

The mass spectrometry and database search analyses verified the presence of the up/down regulated cluster proteins of HP, AAT, and

Table 2. Summary of results of mass spectrometry and database search analyses of the up/down-regulated protein spots from sera of patients with ALA

| Spot | Accession no. | Protein description | Scores | Sequence coverage | Matched peptides |
|---|---|---|---|---|---|
| HP | tr\|Q0VAC5 | HP (haptoglobin) protein Tax_Id=9606 [Homo sapiens] | 334 | 13% | 5 |
| AAT | NP_000286.3 | AAT (alpha-1-antitrypsin precursor) Tax_Id=9606 [Homo sapiens] | 651 | 30% | 10 |
| 1 | ens\|ENSP00000 381199 | transcript:ENST00000398131 Tax_Id=9606 [Homo sapiens] | 238 | 5% | 3 |
| | sp\|P00738\| | HP(Haptoglobin) Tax_Id=9606 [Homo sapiens] | 236 | 8% | 3 |
| 2 | tr\|B4DPP6 | cDNA FLJ54371, highly similar to Serum albumin Tax_Id=9606 [Homo sapiens] | 269 | 11% | 7 |
| Transferrin | B4E1B2 | cDNA FLJ53691, highly similar to Serotransferrin Tax_Id=9606 [Homo sapiens] | 294 | 8% | 7 |

Individuals' ion scores > 36 or 37 show the identification is significant

transferrin in sera of patients with ALA as shown in the Table 2. Meanwhile, unknown protein spot no.1 was identified as human transcript and HP proteins and unknown spot no. 2 was identified as albumin (Table 2). All the identified proteins were above the cut-off level of MASCOT scores. Details of the mass spectrometry results are shown in the supplementary section.

**Competitive ELISA**

The average HP concentration in serum samples of ALA patients was 2.8 and 4.7 times greater ($p<0.05$) than that in sera of healthy individuals and PLA samples, respectively (Figure 3A). In the case of AAT, although the concentration in serum samples of ALA patients seemed to be higher than in healthy individuals, the difference was not statistically significant ($p>0.05$) (Figure 3C). Meanwhile, the concentration of transferrin in serum samples of ALA patients was 2.4 times lesser ($p<0.01$) than in sera of healthy individuals (Figure 3B). However, no significant difference in concentration of transferrin when serum samples of ALA and PLA patients were compared ($p>0.05$).

## DISCUSSION

The diagnosis of amoebic liver abscess (ALA) is still challenging, especially in amoebiasis endemic areas. Radiological techniques such as ultrasound, computed tomography scanning or MRI imaging are used to detect the presence of the abscess. The laboratory investigation commonly performed is antibody detection test, however the commercially available tests often do not differentiate active and past infections. The available *E. histolytica* antigen detection tests which are sensitive for fecal samples are reported to be useful for patients with ALA provided they have not received treatment (Haque *et al.*, 2000; Zeehaida *et al.*, 2008). Due to the above limitations, it would be useful to have surrogate disease markers which may contribute to the diagnosis of ALA.

The technique of 2-DE followed by mass spectrometry analysis have previously been utilized to study the whole proteome of *E. histolytica* (Davis *et al.*, 2006; 2009; Tolstrup *et al.*, 2007). The application of the combined techniques for proteomic analysis of serum

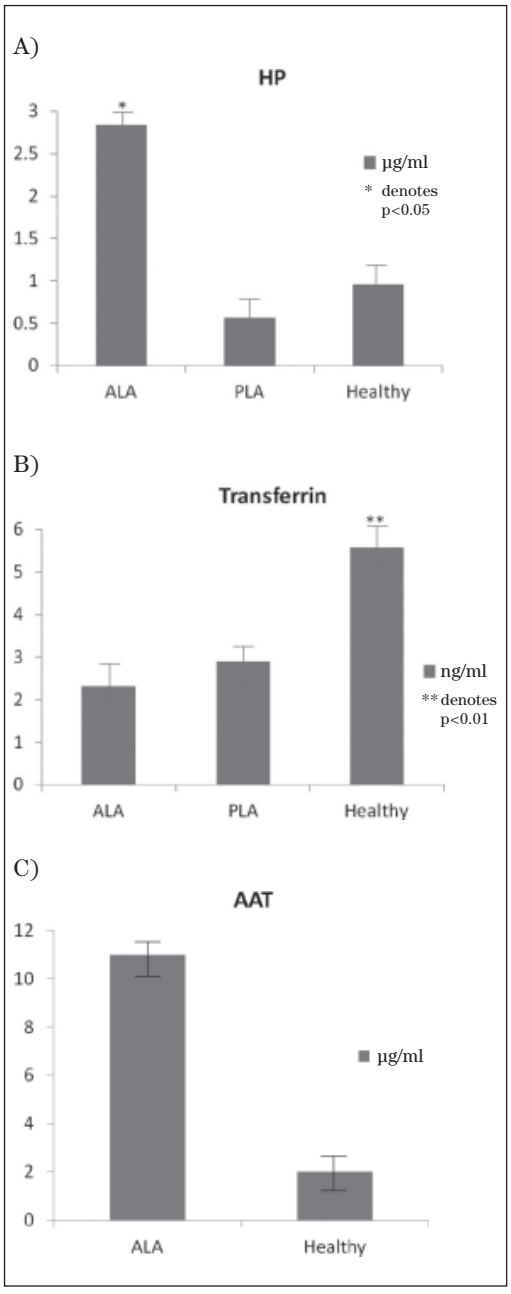


Figure 3. Total concentrations of selected serum proteins in samples of patients with amoebic liver abscess (ALA), pyogenic liver abscess (PLA) and/or healthy individuals determined using competitive ELISA: A) haptoglobin (HP); B) Transferrin; C) $\alpha_1$-antitrypsin (AAT)

samples has often been performed in cancer research and the results obtained were promising (Doustjalali *et al.*, 2004; Abdul-Rahman *et al.*, 2007; Mohamed *et al.*, 2008; Chen *et al.*, 2008; Seriramalu *et al.*, 2010). Furthermore, using a similar approach, several serum protein signatures were reported in patients with infectious diseases including hepatitis and toxoplasmosis (He *et al.*, 2003; Chen *et al.*, 2010). However, serum proteomic analysis of samples from patients with ALA is still lacking.

In this study, highly abundant proteins in unfractionated serum samples were investigated since these proteins may serve as promising infection indicators. Previously, several serum high abundant proteins such as clusterin, AAT, HP and leucine rich glycoprotein were found to be expressed (up-regulated) in patients with acute stage epithelial ovarian carcinoma (Chen *et al.*, 2008; 2009). Meanwhile, AHS and CLU were found to be highly expressed in sera of patients with toxoplasmosis (Emelia, 2010). Similarly, in the present study, several spots in cluster of high abundant proteins such as HP, AAT, albumin and transferrin appeared to be up/down-regulated in sera of patients with ALA.

HP, ApoA-I and AAT are among the most abundant serum glycoproteins secreted by liver, and have been reported to be associated with liver diseases (He *et al.*, 2003; Tan *et al.*, 2011). In this study, analysis by 2-DE showed that HP cluster was significantly up-regulated in sera of patients with ALA when compared to sera of healthy controls. Although fewer serum samples from healthy individuals were used, it was sufficient to be analysed statistically. Furthermore, the 2-DE serum profiles of healthy individuals are well established and are generally consistent from one sample to another (Chen *et al.*, 2010; Emelia, 2010). The up-regulation of HP cluster was verified using another assay (competitive ELISA) which showed the HP concentration in the ALA patients' sera was also significantly up-regulated when compared to the concentrations in sera of healthy individuals and PLA patients. This is similar to an early report by Migasena *et al.*,(1979) who used electro-immunoassay to show that estimation of HP level in serum samples was very useful as an indicator to differentiate patients with ALA from patients with primary hepatic carcinoma and normal

subjects. In another report, He *et al.* (2003) analysed sera from patients with liver inflammatory disease caused by HBV (hepatitis B virus ) and reported that the level of haptoglobin $\alpha 1$ and $\beta$ chains were slightly up-regulated in the low necroinflammatory score group when compared to the normal subjects.

Transferrin is an iron binding blood plasma glycoprotein that is produced in the liver. Routinely, the level of transferrin determined using total-iron binding capacity test (TIBC) along with serum iron test is used to evaluate a patient's nutritional status or liver function, its level is usually low in a patient with liver disease. In a previous report, the low level of transferrin in serum of ALA patients was identified as one of the useful indicators to differentiate them from primary hepatic carcinoma patients and with normal individuals (Migasena *et al.*, 1979). This is consistent with the results of this study whereby, using 2-DE and competitive ELISA, the transferrin cluster protein was found to be down-regulated in sera of patients with ALA when compared to sera of healthy controls. However, when the concentrations of transferrin in sera of ALA and PLA patients were compared, they were found not to be statistically different. Therefore transferrin is not suitable to be used as a surrogate marker for ALA.

AAT is the most prominent protease inhibitor in human serum and functions by inhibiting many of proteases that are released from dying cells, thus protects the normal tissues during period of stress such as inflammation (Van Molle *et al.*, 1999). Using mass-spectrometry and/or western blot analysis, AAT was reported to be highly expressed in sera of patients with severe chronic hepatitis and hepatic carcinoma caused by HBV (Tan *et al.*, 2011). In this study, AAT protein spots in the cluster appeared to be up-regulated in sera of patients with ALA as compared to the healthy control sera. However, the difference in AAT concentrations between the patient and control groups in competitive ELISA was not statistically different.

From the unknown protein spot no. 1, two proteins were identified by mass-spectrometry as human transcript and HP proteins, with almost similar search scores. In 2-DE gel, it is quite common to have one protein spot with more than one protein isoforms. This may occur when a spot has two or more proteins with similar molecular weights and isoelectric points. Furthermore, this may be exhibited by multigene families and or when protein isoforms are nearly identical (Diamandis *et al.*, 2003). The transcript protein identified in this study, was one of the seven products that has been translated from a gene which encoded whole HP protein (accession no. ENSG00000257017), the protein product is called HP-205. A protein-coding transcript is a spliced mRNA that leads to protein product; and based on the amino acid sequence of this transcript protein, two short exon regions seemed to have been removed during the transcription process. Similar to unknown spot no.1 which was not located in HP cluster, the unknown spot no. 2 (identified as albumin) was also seen not to be located in the albumin cluster. These resulted from fragmentation or degradation of the proteins in the cluster, thus had lower molecular weights. This is supported by fact that the sequence coverage of matched peptides, with individual ion scores more than cut-off levels, were located at only a portion of the whole protein sequence.

In conclusion, 2-DE and MS/MS showed that HP, AAT, and transferrin were up/down-regulated in sera of ALA patients as compared to the healthy control sera. Out of these, competitive ELISA verified the up -regulation of HP expression as compared to those of healthy individuals and PLA patients, thus HP is a potential surrogate disease marker for ALA.

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