

Screening and identification of apolipoprotein A-I as a potential hepatoblastoma biomarker in children, excluding inflammatory factors

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Abstract. The aim of the present study was to identify a child hepatoblastoma serum biomarker that is unaffected by inflammatory factors, with the ultimate aim of finding an effective method for the early diagnosis of hepatoblastoma. The magnetic bead-based weak cation exchange chromatography technique was used to process serum harvested from 30 children with hepatoblastoma, 20 children with systemic inflammatory response syndrome (SIRS) and 20 healthy children. Proteins differentially expressed in SIRS were excluded from consideration as biomarkers for hepatoblastoma. Proteins differentially expressed in hepatoblastoma and healthy controls were screened using surface-enhanced laser desorption/ionization-time of flight-mass spectrometry (SELDI-TOF-MS). Target proteins were purified by SDS-PAGE, and matrix-assisted laser desorption/ionization (MALDI)-TOF-MS was used to determine their amino acid sequences. Protein matches were searched in the SwissProt database. Quantitative polymerase chain reaction (qPCR) and ELISA were employed to confirm the expression of target proteins. Following screening to exclude inflammatory factors, SELDI-TOF-MS revealed a protein with a mass-to-charge ratio of 9,348 Da that was expressed at significantly lower levels in the serum of children with hepatoblastoma compared with healthy controls ($P < 0.01$). Sequence analysis identified this protein as apolipoprotein A-I (Apo A-I). qPCR and ELISA confirmed that the expression of Apo A-I mRNA and

protein were significantly lower in children with hepatoblastoma compared with healthy controls ($P < 0.05$). These results indicate that Apo A-I is a non-inflammatory protein marker for hepatoblastoma with the potential for use in early diagnosis of hepatoblastoma. In addition, the present study demonstrates the feasibility of proteomic screening for the identification of proteins that can serve as markers for a specific tumor.

Introduction

Hepatoblastoma is a common liver malignancy in children, with an incidence ranking third among abdominal solid tumors in children. This disease is often observed in boys aged between 6 months and 3 years and is rarely diagnosed in adults. Annually, ~0.5-1.5 children per million are diagnosed with hepatoblastoma, accounting for 1% of malignancies in children and 50-79% of liver malignancies in children (1). Hepatoblastoma is a curable malignancy in children and early diagnosis and therapy are effective in increasing survival rates (2). However, this type of cancer grows rapidly and has no specific clinical manifestations. Children with hepatoblastoma often present initially with a giant mass in the abdominal cavity, when the hepatoblastoma is at stage II or higher, and with metastases to the porta hepatis, portal vein or brain (3), leading to a poor prognosis. Color Doppler ultrasound, CT and hepatic arteriography are routinely used to diagnose hepatoblastoma, but these modalities are less effective for early diagnosis (4). Thus, the development of novel diagnostic methods is necessary for the early diagnosis of hepatoblastoma.

Proteomics has been used previously in the field of oncology to identify tumor-associated markers for the early diagnosis of cancer, to investigate mechanisms of pathogenesis and to identify therapeutic targets (5,6). Biomarkers have been identified for a variety of tumor types (7-9), including nephroblastoma (10-12), ovarian (13), prostate (14), pancreatic (15), colon (16) and breast cancer (17). The expression of certain serum proteins may be altered in patients with cancer due to focal or systemic inflammation; these proteins or inflammatory

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factors may confound biomarker screening for malignancies in these patients (18). Thus, these inflammation-associated proteins must be excluded when screening for biomarkers specific to malignancies.

The present study aimed to identify a pediatric hepatoblastoma serum biomarker that is unaffected by inflammatory factors. Serum was harvested from healthy children and those with hepatoblastoma or systemic inflammatory response syndrome (SIRS). A serum marker for hepatoblastoma was screened and identified following the exclusion of interfering inflammatory factors.

Materials and methods

Clinical information. Participants were recruited from the First Affiliated Hospital of Zhengzhou University of Henan Province (Zhengzhou, China) between December 2011 and December 2013. There were 30 children with pathologically proven hepatoblastoma, including 19 males and 11 females, with a mean age of 39.80 ± 16.50 months (range, 2-70 months). There were 20 children with SIRS, including 12 male and 8 female, with a mean age of 47.26 ± 19.76 months (range, 6-84 months). In addition, 20 healthy children were recruited as controls, including 10 males and 10 females, with a mean age of 38.53 ± 18.46 months (range, 6-72 months). Fasting blood was harvested from these subjects in the morning (05:00-06:00) and placed at room temperature for 1 h, followed by centrifugation at $3,000 \times g$ for 20 min. Supernatants were collected and stored at -80°C . Prior to the study, informed consent was obtained from the parents of these children. The study was approved by the ethics committee of Zhengzhou University.

Screening of differentially expressed proteins in the serum using SELDI-TOF-MS. Frozen sera were thawed in cold water, centrifuged at $10,000 \times g$ for 5 min at 4°C and the supernatants were collected. MB-WCX binding buffer (10 μl), MB-WCX magnetic beads (10 μl) (Profiling Kit 100 MB-WCX, Bruker Corporation, Ettlingen, Germany), and serum (5 μl) were mixed in an eppendorf tube and incubated at room temperature for 5 min. The tube was then placed in a bead separator for 1 min. The solution (but not beads) was removed, and the beads were washed twice with 100 μl MB-WCX wash buffer. The solution was removed, and 5 μl MB-WCX elution buffer was added. The tube was then placed into a bead separator for 2 min. The supernatant was transferred to a 0.5-ml tube, to which 5 μl MB-WCX stabilization buffer was added, followed by mass spectrometry (MS) analysis.

The parameters for CIPHERGEN PBS II+ SELDI-TOF-MS (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were set as follows: Maximal molecular weight, 30,000 Da (optimal molecular weight, 2,000-20,000 Da); optimal laser intensity, 190; and optimal sensitivity, 7. The extracted proteins were added to a CIPHERGEN WCX2 protein chip (Bio-Rad Laboratories, Inc.) that was placed in the instrument for analysis. Following bioprocessor analysis, different protein peaks and the corresponding mass-to-charge ratios (m/z) were further analyzed; peaks with a difference in m/z of $<0.3\%$ were regarded as representing the same protein. The data were analyzed using the Wilcoxon rank sum test, and the proteins

differentially expressed between healthy children and those with hepatoblastoma were identified based on the protein peaks and the corresponding m/z . The proteins differentially expressed between healthy children and those with SIRS (inflammatory proteins) were identified based on protein peaks and the corresponding m/z . These proteins were then excluded from those with potential as biomarkers during comparison of the protein expression between healthy children and those with hepatoblastoma.

Identification of target proteins by MALDI-TOF-MS. Serum proteins (5 $\mu\text{l}/\text{lane}$) were separated by SDS-PAGE on a separation gel with 12% acrylamide bis and a stacking gel of 5% acrylamide bis (10x Tris/Glycine/SDS, pH 8.8, Bio-Rad Laboratories, Inc.) at a voltage of 30 V through the separation gel and at 90 V through the stacking gel. The gels were then stained with Coomassie brilliant blue (Bio-Rad Laboratories, Inc.). The target protein bands were cut from the gel and stored at -20°C for further identification. Gel slices containing the target proteins were placed in 80 μl wash buffer (acetonitrile, Sangon Biotech Co. Ltd., Shanghai, China), centrifuged at $10,000 \times g$ at 37°C for 3-5 min 3 times, and dried at 90°C for 15 min. A total of 20 μl of digest buffer [Dithiothreitol (10 mmol/l) / NH_4HCO_3 (100 mmol/l), Sigma-Aldrich, St. Louis, MO, USA] and 2 μl reducing agent [iodoacetamide (55 mmol/l) / NH_4HCO_3 (100mmol/l) (2 μl), Sigma-Aldrich] were added to the dried proteins, followed by incubation at 37°C for 10 min. After cooling to room temperature, 2 μl blocking agent [NH_4HCO_3 (20mmol/l), Sangon Biotech Co. Ltd.] and 0.5 μl diluted trypsin were added. The mixture was incubated at 37°C with constant vortexing, followed by centrifugation at $10,000 \times g$ at 4°C for 10 min. The supernatants were transferred to fresh tubes to which 0.1% formic acid (0.2 μl) and 50% acetonitrile (0.2 μl) were added. Following centrifugation at $10,000 \times g$ for 10-15 min. Next, the supernatants were harvested for identification of proteins.

Nano high-performance liquid chromatography was used to purify supernatant proteins for application to a protein chip, which was then loaded into the MALDI-TOF-MS instrument (Bruker Corporation). Peptides were identified following protein digestion. Mascot searching software (www.matrix-science.com/, Matrix Science Inc., Boston, MA, USA) was used to search for corresponding proteins in the SwissProt database (www.expasy.org/proteomics).

Confirmation of target protein expression using quantitative polymerase chain reaction (qPCR). The Whole Blood RNA Purification Mini kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract total RNA from the serum of children with hepatoblastoma and healthy controls. To detect the mRNA expression of target proteins, first-strand cDNA was synthesized from RNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.). First-strand cDNA was amplified by qPCR using the Scientific Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, Inc.). The PCR primers for the amplification of the target protein apolipoprotein A-I (Apo A-I) were produced by Sangon Biotech Co. Ltd. and the sequences were as follows: Forward, 5'-ACAGCGTGA CCTCCACCTT-3' and reverse, 5'-CTTGCTCATCTCTG

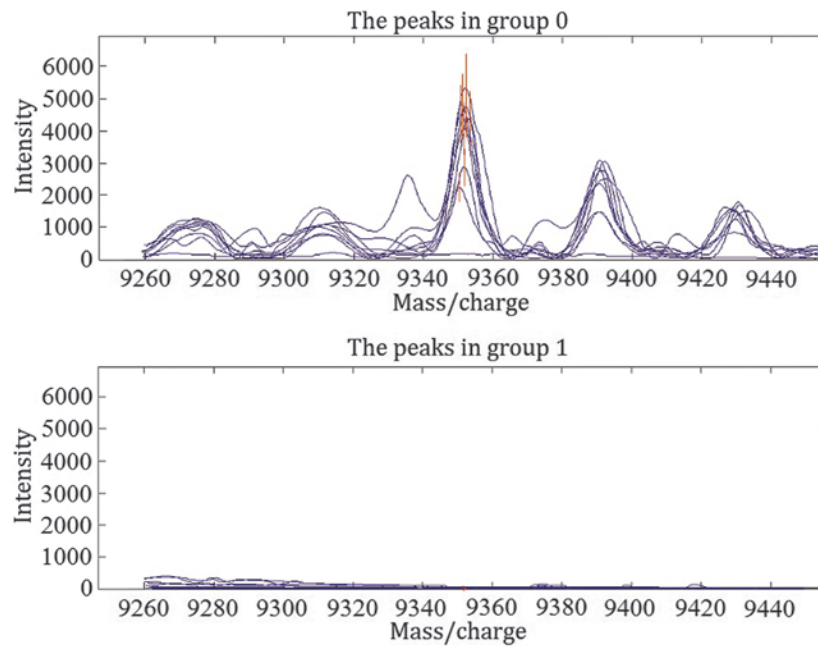


Figure 1. Screening of proteins differentially expressed between children with hepatoblastoma and healthy controls. A protein with m/z of 9348 Da demonstrated reduced expression in children with hepatoblastoma compared with healthy controls (29.0 ± 0.9 vs. 2036.7 ± 881.5 , respectively; $P < 0.01$). Group 0, healthy control group; group 1, hepatoblastoma group.

CCTCA-3'. PCR was carried out as follows: Pre-treatment with uracil-DNA glycosylase (Sangon Biotech Co. Ltd.) at 50°C for 2 min, pre-denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec, and extension at 60°C for 30 sec. Molecular Analyst ABI 7500 Software, version 2.0.1 (Bio-Rad Laboratories, Inc.) was used to analyze the PCR products, and the target protein mRNA expression levels were normalized against β -actin.

Enzyme linked-immunosorbent assay (ELISA). ELISA was used to detect the expression of Apo A-I protein in the serum of children with hepatoblastoma and healthy controls, using a Human apoprotein A1, apo-A1 ELISA kit (Sangon Biotech Co. Ltd.). Standard Apo A-I protein solutions (0, 62.5, 125, 250, 500, 1,000, 2,000 and 4,000 pg/ml) were prepared, and triplicate $100\text{-}\mu\text{l}$ samples at each concentration were added to a 96-well plate coated with monoclonal rabbit anti-human Apo A-I antibody (1:100 dilution). Serum collected from children with hepatoblastoma ($n=10$) and healthy controls ($n=10$) was diluted 1:100 with the ABC working solution from the apo-A1 ELISA Kit, and triplicate $100\text{-}\mu\text{l}$ serum samples were added to the same plate. Following incubation at 37°C for 90 min, the supernatant in each well was removed, and biotin-conjugated anti-human Apo A-I antibody ($100\text{ }\mu\text{l}$) was added, followed by incubation at 37°C for 60 min. After washing, ABC working solution was added for visualization and the samples were incubated at 37°C for 30 min. The samples were washed, 3,3',5,5'-tetramethylbenzidine ($90\text{ }\mu\text{l}$) was added to each well, and stop solution ($100\text{ }\mu\text{l}$) was added. The optical density was measured at 450 nm, and a standard curve was generated. The concentration of each sample was calculated according to the standard curve.

Statistical analysis. To distinguish between the differentially expressed proteins in the serum, as screened by MS, a nonlinear support vector machine (SVM) classifier was used as previously described (19). It was originally developed by Vladimir Vapnik, with a radialbased function kernel, a parameter γ of 0.6, and a cost of the constrain violation of 19. The leave-one-out crossing validation approach was applied to estimate the accuracy of this classifier (19). The capability of each peak in distinguishing data of different groups was estimated by the P-value of the Wilcoxon t-test. $P \leq 0.01$ was considered to indicate a statistically significant difference.

In confirming the Apo A-I expression by qPCR and ELISA, statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The independent samples t-test was used to compare mean values between two groups, and $P < 0.05$ was considered to indicate a statistically significant difference for all analyses.

Results

Screening of proteins differentially expressed between children with hepatoblastoma and healthy controls. The data collected following MS from children with hepatoblastoma and healthy controls were standardized and the protein peaks and corresponding m/z values were compared using the Wilcoxon rank sum test. A total of 10 peaks with differences were identified ($P < 0.01$). The results demonstrated that 4 highly expressed proteins and 6 proteins with low expression were identified in the serum of children with hepatoblastoma. Following screening with the SVM, a combined model with the highest Youden index was screened, and a protein with an m/z of 9,348 Da was identified as a marker. The Youden index is a method which evaluates the reliability of the screening test. Assuming that the false negative (the missed diagnosis)

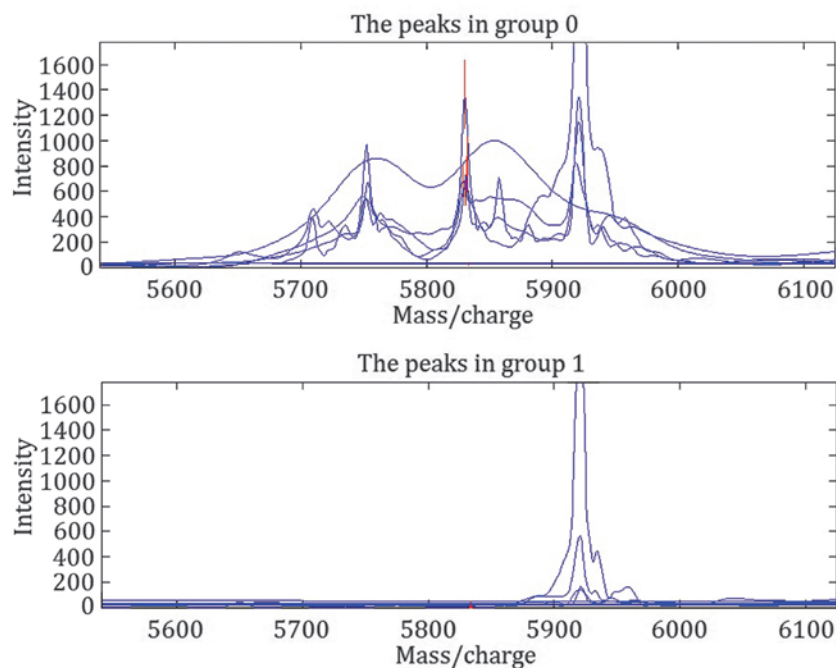


Figure 2. Screening of proteins differentially expressed between children with SIRS and healthy controls. A protein with an m/z of 5,833 Da demonstrated increased expression in children with SIRS compared with healthy controls ($1,283.0 \pm 943.3$ vs. 75.7 ± 75.1 , respectively; $P < 0.01$). Group 0, SIRS group; group 1, the healthy control group. SIRS, systemic inflammatory response syndrome.

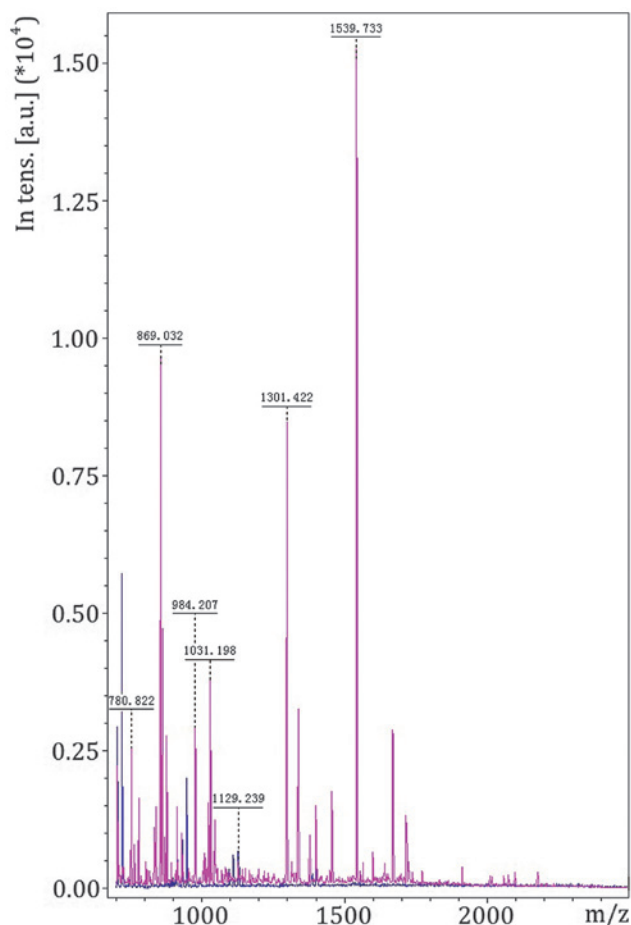


Figure 3. MS of peptides yielded by digestion of the 9,348 Da protein identified by MALDI-TOF-MS. MS of the peptide segments obtained following enzymatic digestion of the proteins and peptide segments with m/z of 9,348 Da. MS, mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization-time of flight-MS.

and the false positives (the misdiagnosis) result in the same outcome, the Youden index may be used. The Youden index is the sum of the sensitivity and specificity minus 1, and in this contexts refers to the total capacity of the screening method in identifying the difference between the real patients and the false negative and positives. The greater the Youden index value, the larger and more significant the effect and the accuracy of the screening experiment (20). As presented in Fig. 1, the expression of this protein was significantly reduced in children with hepatoblastoma compared with healthy controls (29.0 ± 20.9 vs. $2,036.7 \pm 881.5$, respectively; $P < 0.01$).

Screening of proteins differentially expressed between children with SIRS and healthy controls. Following processing and statistical analysis of data collected following MS, 10 protein peaks were identified that differed significantly ($P < 0.01$) between children with SIRS and healthy controls. The results indicated 6 proteins with increased expression and 4 proteins with reduced expression compared with controls in the serum of children with SIRS. Following SVM screening, a combined model with the highest Louden index was screened, and a protein with m/z of 5,833 Da was identified. As presented in Fig. 2, the expression of this protein was significantly increased in children with SIRS compared with healthy controls ($1,283.0 \pm 943.3$ vs. 75.7 ± 75.1 , respectively; $P < 0.01$).

Exclusion of interfering inflammatory factors. SELDI-TOF-MS was performed to identify protein peaks that differed between children with hepatoblastoma and healthy controls, and between children with SIRS and healthy controls. Paired comparison indicated that one protein with an m/z of 9,348 was not observed in the serum of children with SIRS (range, $\pm 0.3\%$), indicating that this protein was a serum biomarker for hepatoblastoma.

Table I. Amino acid sequence of each peptide yielded by protein digestion as determined by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry.

M/Z	Protein	Peptides identified	Sequence
9,348	Apolipoprotein A-I	K.WQEEMELYRQK.V R.QKVEPLR.A R.AELQEGARQK.L K.LSPLGEEMR.D R.AHVDALR.T R.THLAPYSDEL.R R.LAARLEALK.E	KWQEEMELYRQKVEPLRAE LQEGARQKLHELQEKLSPLGE EMRDRARAHVDALRTHLAPY SDEL.RQLAARLEALKENG

M/Z, mass to charge ratio.

Table II. Amino acid sequence of the full-length protein obtained by matching and recombination of peptides.

M/Z	Protein	Confirmed peptide	Coverage rate (%)	Score
9,348	Apolipoprotein A-I	KWQEEMELYRQKVEPLRA ELQEGARQKLHELQEKLS PLGEEMRDRARAHVDAL RTHLAPYSDEL.RQLAAR LEALKENG	45.0	88

M/Z, mass to charge ratio.

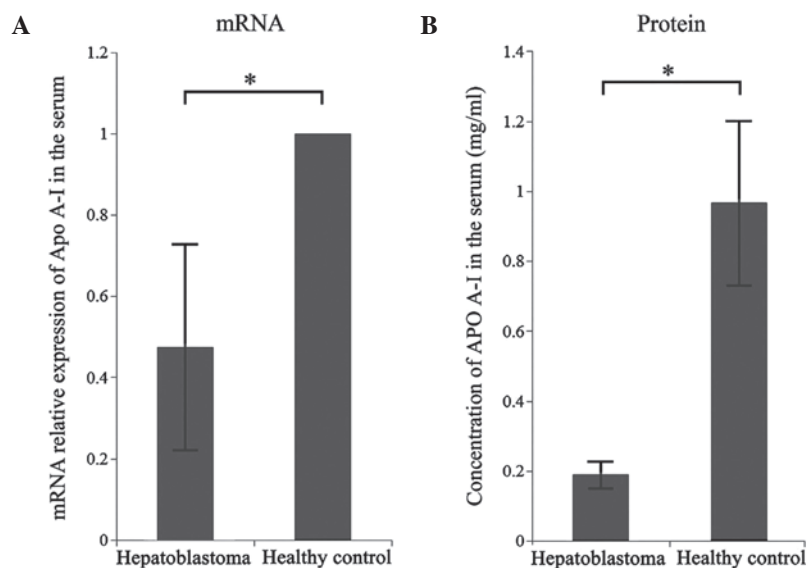


Figure 4. Apo A-I mRNA and protein expression in the serum of children with hepatoblastoma and healthy controls assessed by reverse transcription-quantitative polymerase chain reaction and ELISA. (A) The $\Delta\Delta C_t$ method was used to calculate the expression level of Apo A-I mRNA, with the expression of the Apo A-I mRNA of the healthy control group defined as 1. The relative expression of Apo A-I of the hepatoblastoma group was 0.4744 ± 0.2530 ; $^*P < 0.05$ vs. the healthy control group. (B) A standard curve was constructed using standard samples at different concentrations and the concentration of Apo A-I protein in the serum of children with hepatoblastoma and healthy children was calculated according to the standard curve. The Apo A-I concentration was 0.1898 ± 0.0387 mg/ml in children with hepatoblastoma and 0.9707 ± 0.2372 mg/ml in healthy controls ($^*P < 0.05$ vs. healthy control group).

Target protein identification. Following gel electrophoresis, the target protein with an m/z of 9,348 Da was harvested. The

protein band corresponding to the protein peak was collected and treated using in-gel digestion. MALDI-TOF-MS was

used for MS of peptides following digestion (Fig. 3), and the amino acid sequences were determined (Table I). The peptide sequences were analyzed for matching and recombination to generate the amino acid sequence of the complete protein (Table II). Mascot searching software was used to search for a corresponding protein in the SwissProt database, revealing a protein with an *m/z* of 9,348 Da and a coverage rate of 45.0%; the match score with Apo A-I was 88.

Confirmation of Apo A-I expression by qPCR and ELISA. To confirm that the protein identified by MALDI-TOF-MS was Apo A-I, fluorescence qPCR was performed to detect Apo A-I mRNA expression in serum. The results demonstrated that Apo A-I mRNA expression levels were significantly reduced in children with hepatoblastoma compared with healthy children ($P < 0.05$; Fig. 4A).

In addition, ELISA was employed to detect the levels of Apo A-I protein in the serum of children with hepatoblastoma and healthy children. A standard curve was constructed using standard samples at different concentrations, and the concentration of Apo A-I in the serum of children with hepatoblastoma and healthy children was calculated according to the standard curve. The Apo A-I concentration was significantly reduced in children with hepatoblastoma compared with healthy controls (0.1898 ± 0.0387 vs. 0.9707 ± 0.2372 mg/ml, respectively; $P < 0.05$; Fig. 4B).

Discussion

In the present study, SELDI-TOF-MS revealed a protein with an *m/z* of 9,348 Da that was expressed at significantly reduced levels in the serum of children with hepatoblastoma compared with healthy controls ($P < 0.01$); the interference of inflammatory proteins in this result was excluded. Sequence analysis identified this protein as Apo A-I. qPCR and ELISA demonstrated that the expression levels of Apo A-I mRNA and protein were significantly reduced in children with hepatoblastoma compared with healthy controls ($P < 0.05$). Collectively, these findings indicate that Apo A-I is a potential biomarker for hepatoblastoma.

A variety of inflammatory factors and proteins are important in the occurrence and development of malignancies, and their expression may further augment the inflammatory response. The association between inflammation and malignancies is therefore complex (21,22). Inflammatory proteins may confound the identification of markers that are solely specific to tumors (18). Previous studies have demonstrated that high serum levels of IL-10, IL-1, IL-6, and TNF- α are closely associated with renal, colon and oral cancer (23-25). To overcome the influence of inflammatory proteins in the search for biomarkers specific to hepatoblastoma, the present study identified a differentially expressed protein with *m/z* of 5,833 Da that was associated with inflammation by comparative screening of healthy controls and children with SIRS. This protein was different to the protein with an *m/z* of 9,348 Da that was identified to be differentially expressed between hepatoblastoma and healthy controls. The protein with an *m/z* of 5,833 Da identified in this screening was then eliminated as an interfering factor, and the aim was to identify a biomarker of hepatoblastoma that is not associated with inflammation.

SELDI-TOF-MS analysis demonstrated that the Apo A-I protein was expressed at significantly reduced levels in the serum of children with hepatoblastoma compared with healthy controls. Since this protein was not eliminated in the SIRS inflammatory protein screening, this protein may be considered to be a specific marker for hepatoblastoma. Apo A-I is an important structural protein in the high-density lipoprotein family, and the most abundant member of the Apo A family. This 28 kDa, 243-amino-acid protein is predominantly synthesized in the liver but is expressed in the majority of tissues (26). Apo A-I is involved in several steps of lipid metabolism, stabilization of lipoprotein structure and the regulation of lipoprotein metabolism (27). Abnormalities in the structure or concentration of Apo A-I can lead to lipid metabolism disorders and induce pathological changes, including hypertension and insulin resistance (28,29).

An association between Apo A-I and malignancies has been reported: A previous study demonstrated that Apo A-I is a serum biomarker specific for early-stage ovarian cancer that can be used for diagnosis (32). In order to study Apo A-I function further, 18 amino-acid peptides (Apo A-I mimetic peptides) have been designed (31). These peptides are of different subtypes (2F, 3F, 4F, 5F, 6F and 7F); all have helical structures similar to that of Apo A-I and anti-oxidative and hydrophilic properties. Gao *et al* (32) demonstrated that Apo A-I mimetic peptide L-4F regulates hypoxia inducible factor-1 α expression to inhibit the expression of vascular endothelial cell growth factor and angiogenesis in a number of different types of cancer. L-4F also induces the apoptosis of ovarian cancer cells, exerting antitumor effects on ovarian cancer (32). The expression of Apo A-I in early stage ovarian cancer does not preclude the use of Apo A-I as a specific biomarker for the early diagnosis of hepatoblastoma in children as ovarian cancer does not occur in preschool children.

In conclusion, the present study demonstrates that Apo A-I expression is reduced in children with hepatoblastoma compared with healthy controls. These findings indicate that Apo A-I may be used as a serum biomarker for hepatoblastoma in children. Additional studies are required to determine the actual efficacy of Apo A-I as a biomarker in clinical practice. Studies with a large patient cohort are required to determine the specificity and sensitivity of Apo A-I in diagnosing hepatoblastoma in children, and to identify any advantages of Apo A-I over other diagnostic tools. In addition, the association between Apo A-I and the occurrence and development of hepatoblastoma should be investigated to determine the role of Apo A-I in the pathogenesis of hepatoblastoma.

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