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Identification of potential serum biomarkers for Wilms tumor after excluding confounding effects of common systemic inflammatory factors

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Abstract Wilms tumor is the most common pediatric tumor of the kidney. Previous studies have identified several serum biomarkers for Wilms tumor; however, they lack sufficient specificity and may not adequately distinguish Wilms tumor from confounding conditions. To date, no specific protein biomarker has been confirmed for this pediatric tumor. To identify novel serum biomarkers for Wilms tumor, we used proteomic technologies to perform protein profiling of serum samples from pre-surgery and post-surgery patients with Wilms tumor and healthy controls. Some common systemic inflammatory factors were included to control for systemic inflammation. By comparing protein peaks among the three groups of sera, we identified two peaks (11,526 and 4,756 Da) showing significant differential expression not only between pre-surgery and control sera but also between pre-surgery and post-surgery sera. These two peaks were identified as serum amyloid A1 (SAA1) and apolipoprotein C-III (APO C-III). Western blot analysis confirmed that both proteins were expressed at higher levels in pre-surgery sera than in post-surgery and control sera. Using the method of leave-1-out for cross detection, we demonstrate that detection of these two candidate biomarkers had high sensitivity and specificity in discriminating pre-surgery sera from post-surgery and normal control sera. Taken together, these findings suggest that SAA1 and APO C-III are two potential biomarkers for Wilms tumor.

Keywords Protein biomarker · SAA1 · APO C-III · Inflammatory factor · Wilms tumor

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Introduction

Wilms tumor (nephroblastoma) is the fifth most common pediatric malignancy and the most common type of renal tumor in children, accounting for approximately 5.5% of all childhood solid tumors [1]. Over the past several decades, the multidisciplinary approach to this tumor has greatly improved survival rates for children with this neoplasm; however, a significant number of children still die from this disease [2–5]. Given that the prognosis of Wilms tumor is strongly correlated with tumor stage and patient's age [6], early accurate diagnosis and timely treatment are critical for improving long-term survival of patients with this disease. Currently, many traditional diagnostic tools have been used for Wilms tumor, such as sonography, computed tomography, magnetic resonance imaging, cytological examination and fine-needle aspiration; however, they either have questionable accuracy or are too invasive. Detection of serum molecular markers may provide a non-invasive and accurate diagnostic alternative to traditional diagnostic tools for Wilms tumor.

Recent advances in proteomics have introduced novel techniques for the screening of cancer biomarkers that permit early and accurate diagnosis of cancer diseases [7]. Surfaced-enhanced laser desorption/ionization

time-of-flight mass spectroscopy (SELDI-TOF-MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) are the two most popular approaches presently employed for detecting quantitative or qualitative changes in circulating serum or plasma proteins in relation to the presence of cancer [8]. In recent years, they have been successfully used to identify specific biomarkers for various types of cancer, such as ovarian cancer, prostate cancer, pancreatic cancer, colon cancer, and breast cancer [9–13].

Inflammation precedes and promotes tumor development and progression, while tumor development and progression induces inflammation [14]. Interactions between the immune system and malignant cells play a pivotal role in all stages of tumorigenesis [15]. Proteins involved in both local and systemic inflammatory responses can be detected in serum samples from cancer patients. However, most systemic response proteins are unspecific and may confound cancer detection or diagnosis [16]. Therefore, systemic inflammation as a confounding factor should be considered in cancer biomarker discovery and validation [14].

Our previous study has identified several potential serum biomarkers for Wilms tumor [17]; however, they lack sufficient specificity and may not adequately distinguish Wilms tumor from confounding conditions. To date, no specific protein biomarker has been confirmed for this tumor. In the present study, we used the SELDI-TOF-MS and MALDI-TOF-MS technologies to identify potential serum markers for Wilms tumor. To exclude the confounding effects of systemic inflammation, some previously characterized systemic inflammatory factors were included to control for confounding factors. The serum markers identified in this study may be useful for the detection of this pediatric tumor.

Materials and methods

Patients and serum samples

The study protocol was approved by the local ethics committee. Written informed consent was obtained from each patient or their guardian. In total, 103 patients with primary Wilms tumor who were treated from January 2006 to August 2010 in the Department of Pediatric Surgery of the First Affiliated Hospital of Zhengzhou University were included in the study. These patients, including 57 males and 46 females, had a median age of 39.12 months (range 2–183 months), and of them, 45 had stage I tumor, 28 had stage II tumor, 24 had stage III tumor, and 6 had stage IV tumor. All patients were treated with surgery first and then two-drug chemotherapy (vincristine and actinomycin) for stage I and II tumors, and three-drug chemotherapy (vincristine, actinomycin and adriamycin) plus radiotherapy for stage III and IV tumors. The pathological diagnosis of Wilms tumor was confirmed independently by at least two pathologists. All patients had favorable histology. Pre-surgery serum samples were collected from all patients during the week before surgery, while post-surgery serum samples were collected between 7 and 14 days after surgery from only 97 patients because two patients died and four were lost to follow-up. Serum samples from 103 healthy controls, consisting of 57 males and 46 females and having a median age of 44.31 months (range 3–194 months), were also used as controls. Blood samples were taken from peripheral veins in the early morning on an empty stomach, incubated for 1 h at 4°C, and centrifuged at 10,000 rpm for 5 min. The sera were then collected and stored at −80°C before use.

Serum protein profiling by SELDI-TOF-MS

Frozen serum samples were thawed on ice and spun at 10,000 rpm for 5 min at 4°C. Each serum sample (10 μl) was denatured and hybridized with pre-processed WCX2 proteinchip arrays (Ciphergen Biosystems, Fremont, CA, USA) [18]. Recombinant human inflammatory factors (PeproTech, Rocky Hill, NJ, USA) were pre-processed using the similar procedure. Some previously characterized systemic inflammatory factors, including macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-3α, MIP-3β, regulated on activation, normal T cell expressed and secreted (RANTES), interferon γ-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG), interleukin (IL)-10, IL-1, IL-6, tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF) were included in the study. The mass spectra of proteins were generated by SELDI-TOF-MS using the ProteinChip Biosystems (Ciphergen PBS II plus SELDI-TOF-MS, Ciphergen Biosystems). Mass peak detection was analyzed using ProteinChip Biomarker Software version 3.1 (Ciphergen Biosystems). Peaks of differential proteins which were similar to those of inflammatory factors were excluded. To distinguish data between the pre-surgery and post-surgery groups and between the pre-surgery and healthy control groups, a nonlinear support vector machine (SVM) classifier was used as previously described [18]. It was originally developed by Vladimir Vapnik, with a radial-based function kernel, a parameter Gamma 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. The capability of each peak in distinguishing data of different groups was estimated by the P value of Wilcoxson t-test. P-values less than 0.01 were considered statistically significant.

Purification of candidate protein markers by HPLC and MALDI-TOF-MS

Frozen serum samples were thawed on ice, and 100 μl of serum was mixed with 300 μl of ultra-pure water and 300 μl of acetonitrile (ACN), incubated at 4°C for 30 min, and centrifuged at 10,000 rpm for 5 min. The supernatant was collected into a new tube and was freeze-dried using a SpeedVac vacuum centrifuge enrichment system (SPD) (Thermo Electron, Waltham, MA, USA) for about 20 min. HPLC separation was performed using SCL-10AVP (Shimadzu, Nakagyo-ku, Kyoto, Japan) with a TSK-GEL C18 column (ODS-100S) (Tohoku Tosoh, Sakata, Yamagata, Japan). The eluate was detected at multiple wavelengths. The mobile phase consisted of solvent A (5% ACN, 0.1% trifluoroacetic acid (TFA)) and solvent B (95% ACN, 0.1% TFA). The HPLC separation was achieved with a linear solvent gradient: (100% A/0 min)-(20% B/15 min)-(40% B/30 min)-(70% B/80 min)-(100% B/100 min) at a flow rate of 1 ml/min. The fraction of each peak was stored in tubes. The purified protein fraction was freeze-dried in the SPD until the volume reached about 20 μl. After 1.5 μl of the purified protein fraction was mixed with 1.5 μl of acyanoacrylate-4-hydroxy-cinnamic acid and spotted onto the MALDI protein chip, the purity and mass of protein were determined using a MALDI-TOF-MS machine (Kratos Analytical, Manchester, UK).

Identification of candidate protein biomarkers by two-dimensional liquid chromatography-linear ion trap mass spectrometry (2D-LC-LTQ-MS)

Candidate protein biomarkers were broken down into peptides by enzymolysis before identification [18]. After terminate reaction, peptide sample was added to the C18 column (100 mm × 100 μm). After connecting sample column to chromatography column in series, sample was analyzed using the 2D-LC-LTQ-MS system (Thermo Electron). Constant gradient was used in analysis of process for 90 min. The peptide mass fingerprint and MS/MS data were analyzed using the SEQUEST program (Thermo Electron). Fraction of coverage and similarity of peptides are major factors contributing to identification of candidate protein biomarkers.

Confirmation of candidate protein biomarkers by Western blotting

To confirm the differential expression of candidate protein biomarkers among different groups, total protein (40 μg) was extracted from serum samples and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 103 volts using the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, Hercules, CA, USA). The proteins were transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) with the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at a constant voltage of 20 volts for about 5 min. The membranes were incubated with either mouse anti-human amyloid A1 IgG or rabbit anti-human apolipoprotein C-III IgG at 37°C for 2 h. Anti-mouse antiserum and anti-rabbit antiserum were used as secondary antibodies. All primary and secondary antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The immunoblot signals were visualized by chemiluminescence. The photographs were quantitatively analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA). Statistical analyses were performed using SPSS 13.0 (SPSS Corporation, Chicago, IL, USA). The independent samples *t*-test was used to compare mean gray values between two groups.

Results

Serum protein profiling and data processing

The preprocessed serum samples and tumor-related inflammatory factors were analyzed by SELDI-TOF-MS with WCX2 chip. Protein peaks were divided into three groups: pre-surgery, post-surgery, and control. Wilcoxon rank sum tests and the SVM classifier were used to analyze the different expression levels of proteins among the three groups. Seventeen peaks with a P value < 0.01 were obtained between the pre-surgery and control groups, and of them 16 were found up-regulated in the pre-surgery group (data not shown). Fourteen peaks with a P value < 0.01 were obtained between the pre-surgery and post-surgery groups, and of them nine were found up-regulated in the pre-surgery group (data not shown). The peaks with a m/z of 11,526 Da (Fig. 1; Table 1) and 4,756 Da (Fig. 2; Table 2) showed significant differential expression (both $Ps < 0.01$) not only between the pre-surgery and control groups but also between the pre-surgery and post-surgery groups. However, there was no statistical significance in the two peaks between the post-surgery and control groups. These two peaks could not be detected in the SELDI-TOF-MS spectrum of systemic inflammatory factors (Fig. 3). Using the method of leave-1-out for cross detection, we demonstrate that detection of the peak with a m/z of 11,526 Da had a sensitivity of 94.8% and a specificity of 93.2% in discriminating 103 pre-surgery and 97 post-surgery sera, and a sensitivity of 98.6% and a specificity of 97.3% in discriminating 103 pre-surgery and 103 control sera, while the corresponding values for detection of the peak with a m/z of 4,756 Da were 92.9 and 91.8%, and 95.4 and 93.8%.

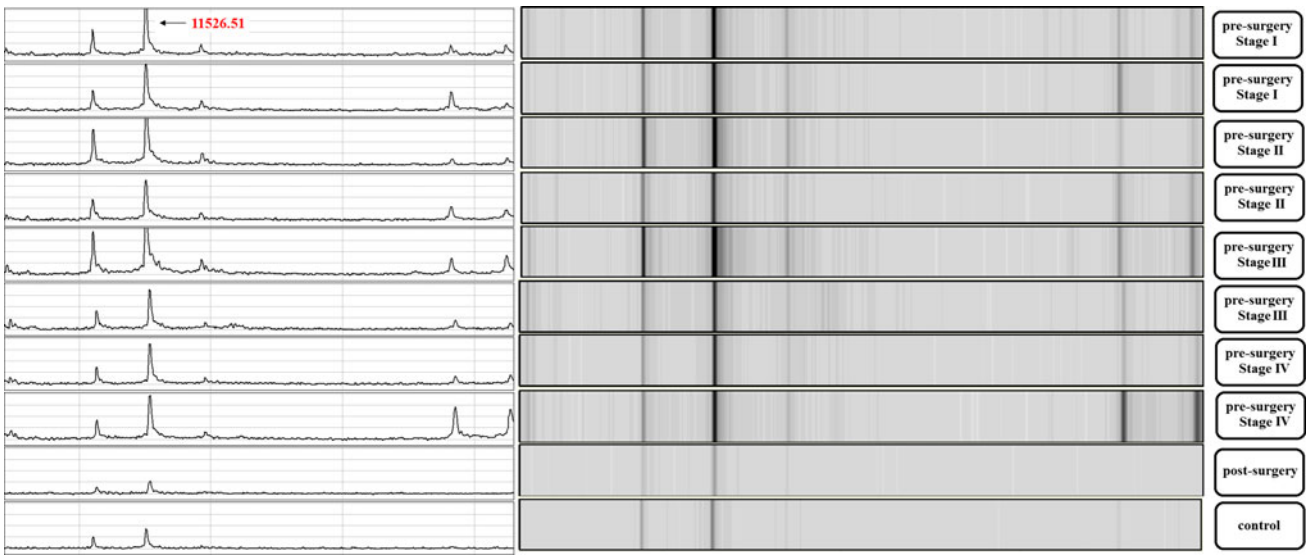

Fig. 1 A representative spectrum of SELDI-TOF-MS analysis of serum samples from the pre-surgery, post-surgery, and control groups. The expression level of 11,526 Da protein was significantly higher in the pre-surgery group than in the post-surgery and control groups

Table 1 The descriptive statistics for two candidate protein markers identified between pre-surgery and control sera

m/z	Pre-surgery (mean ± SD)	Control (mean ± SD)	*P*
4756	5082.64 ± 1342.18	2938.13 ± 876.21	0.001428
11526	4927.53 ± 1293.42	2312.43 ± 871.35	0.001143

Purification of candidate protein biomarkers

Serum samples of the pre-surgery group were used for purification of the two candidate biomarkers by HPLC (Fig. 4). The fraction of each peak in HPLC was collected into a tube, and the purified protein fractions was then analyzed by MALDI-TOF-MS. Figure 5 shows the results of MALDI-TOF-MS analysis of the two purified candidate biomarkers (11,526 and 4,756 Da).

Identification of candidate protein biomarkers

After enzymolysis with modified trypsin, the peptide mixture was analyzed by 2D-LC-LTQ-MS. Figure 6 shows the MS spectra of two identified peptides: (R.GNYDAAK

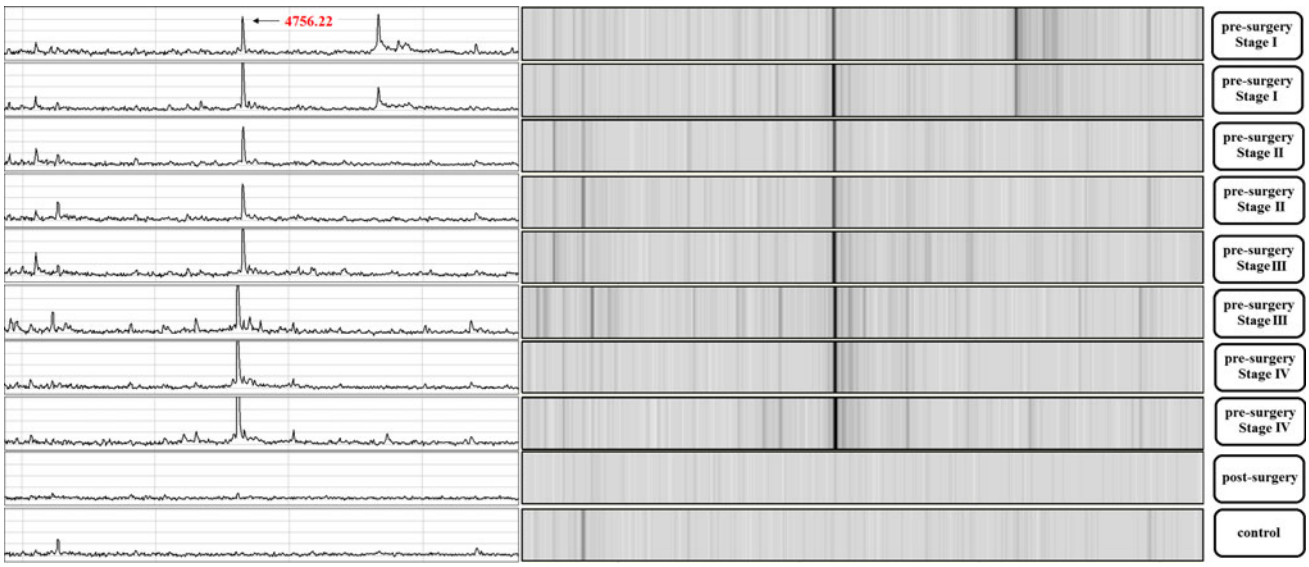

Fig. 2 A representative spectrum of SELDI-TOF-MS analysis of serum samples from of the pre-surgery, post-surgery, and control groups. The expression level of 4,756 Da protein was significantly higher in the pre-surgery group than in the post-surgery and control groups

Table 2 The descriptive statistics for two candidate protein markers identified between pre-surgery and post-surgery sera

m/z	Pre-surgery (mean ± SD)	Post-surgery (mean ± SD)	*P*
4756	4213.81 ± 1082.51	2507.15 ± 473.16	0.001721
11526	4383.15 ± 873.08	2043.44 ± 583.26	0.001539

RGPGGAWAAEVISDAR.E) from the 11,526-Da protein and (K.TAKDALSSVQESQVAQQAR.G) from the 4,756-Da protein). The obtained peptide sequence was used to search the Bioworks database with the SEQUEST program. The two candidate protein biomarkers were identified as serum amyloid A1 (SAA1) (NCBI: AAA64799.1) and apolipoprotein C-III (APO C-III) (NCBI: CAA25233.1).

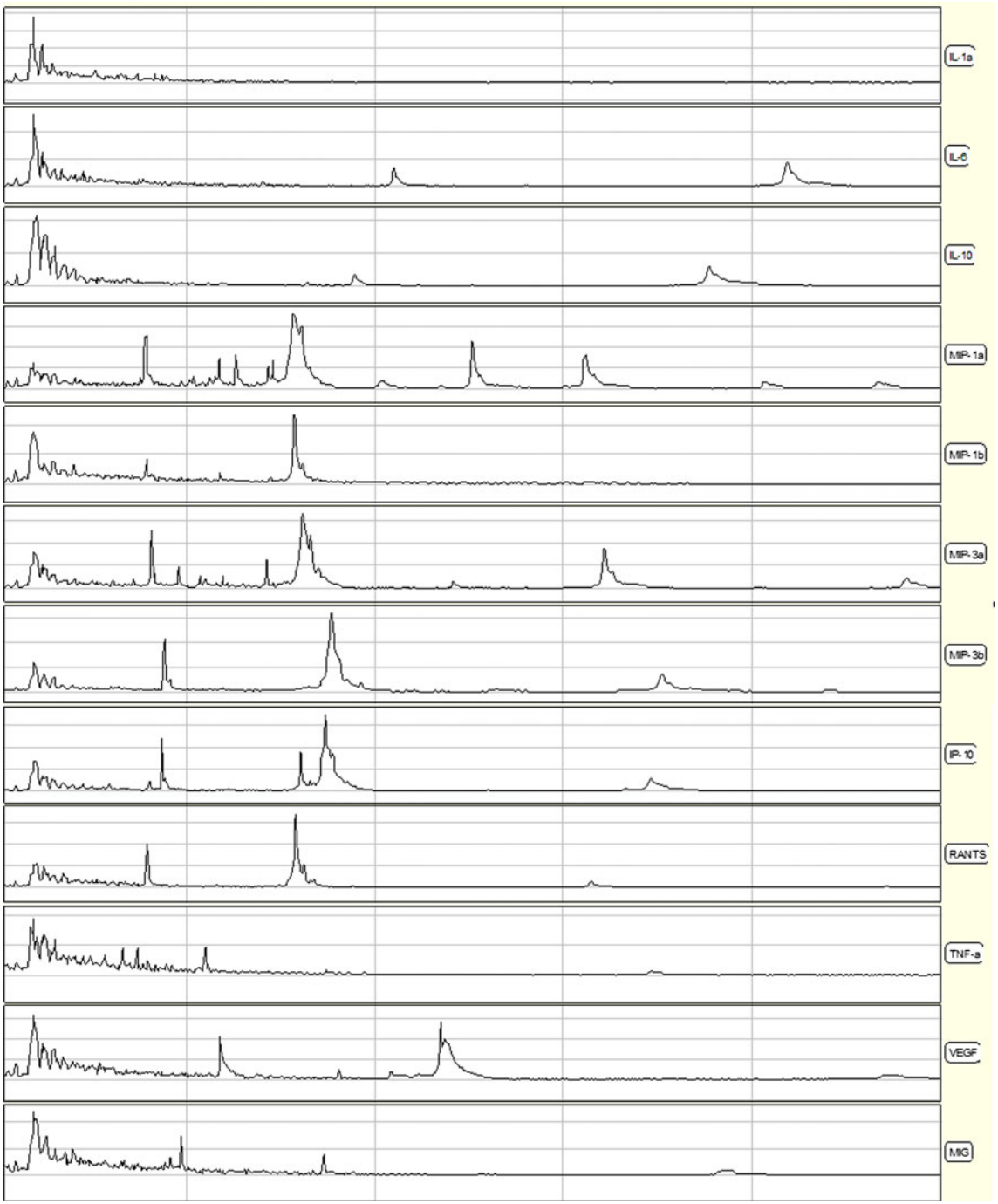

Fig. 3 A representative spectrum of SELDI-TOF-MS analysis of systemic inflammatory factors

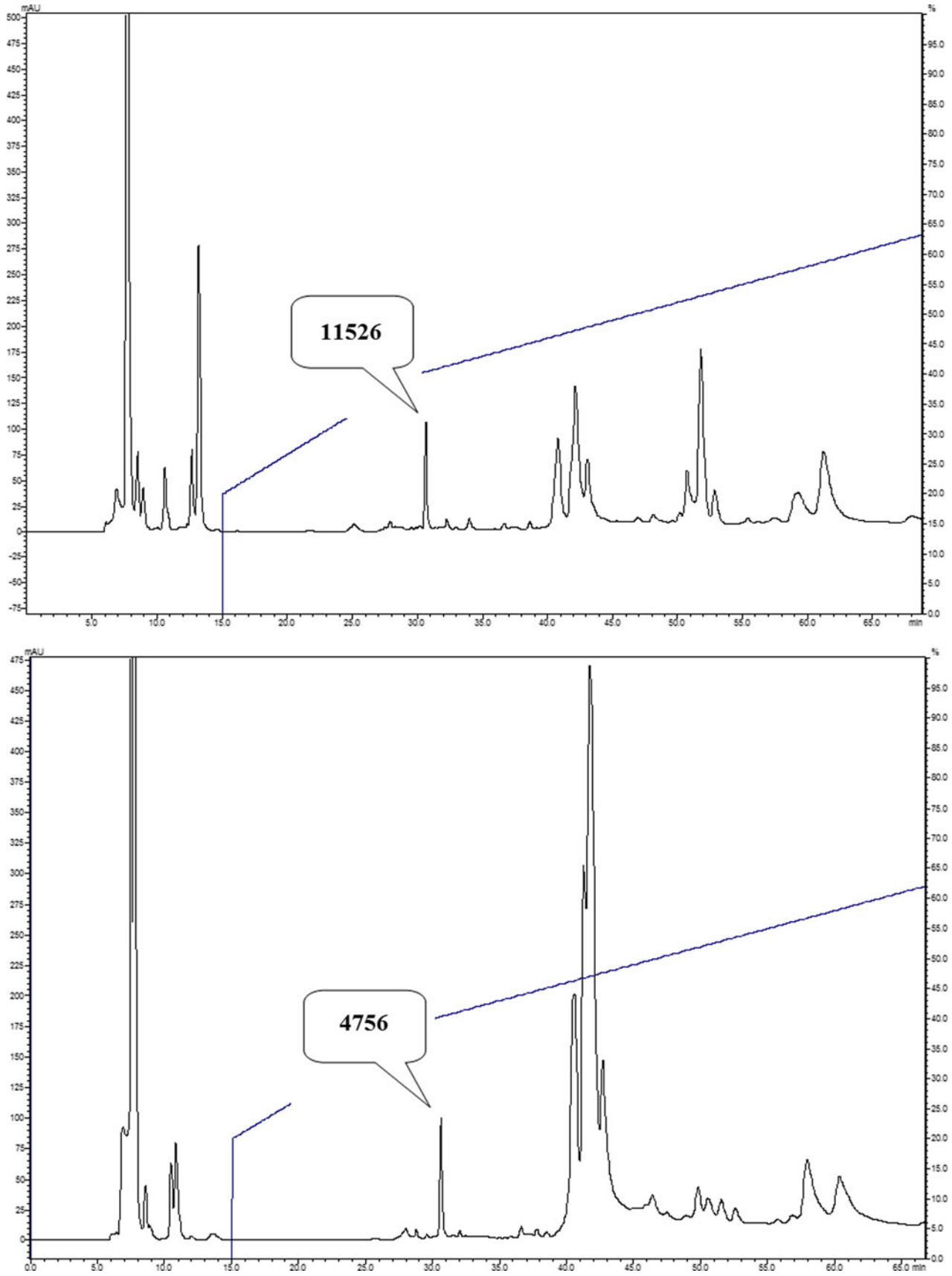

Fig. 4 Separation of serum samples of the pre-surgery group by HPLC. Each peak represents one protein

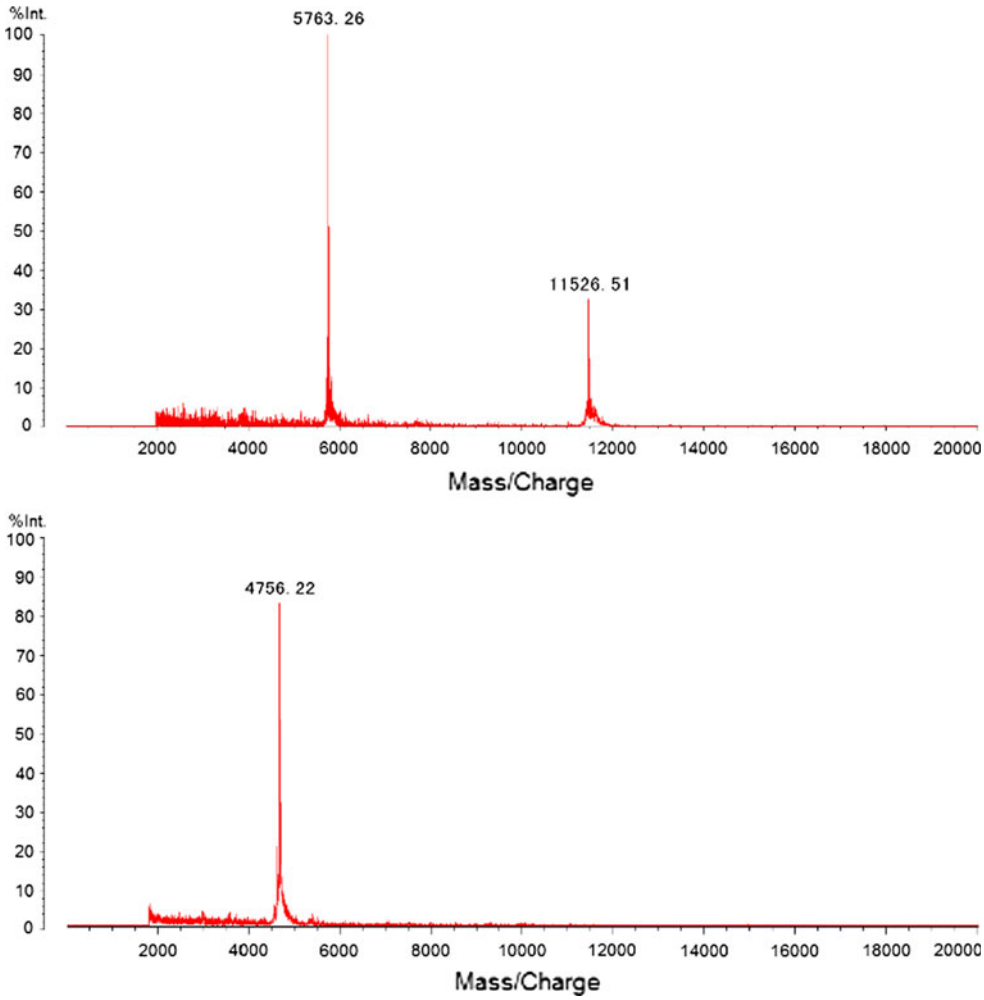

Fig. 5 MALDI-TOF-MS spectra of two purified candidate protein markers

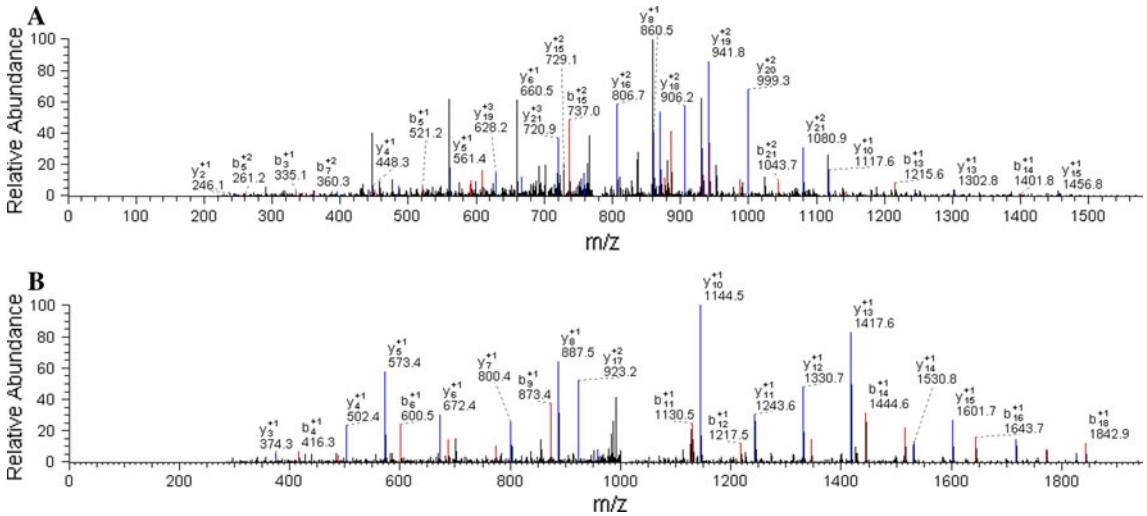

Fig. 6 MS spectra of a peptide (R.GNYDAAKRGPGGAWAAEVISDAR.E) from the 11,526 Da protein (A) and a peptide (K.TAKDALSSVQESQVAQQAR.G) from the 4,756-Da protein

Table 3 Identification of SAA1 and APO C-III as potential protein biomarkers for Wilms tumor

m/z	Protein	Sequence identified	Sequence coverage (%)	Score
11526	SAA1	R.SFFSFLGEAFDGAR.DMWR.AY SDMREANYIGSDK.YFHAR.GNY DAAK.R.GPGGAWAAEVISDAR.E NIQR.FFGHGAEDSLADQAANEW GR.SGKDPNHFR.PAGLPEKY.	85.33	86.35
4756	APO C-III	K.HATK.TAKDALSSVQESQVAQ QAR.GWVTDGFSSLKDYWSTVK. DK.F	43.83	40.31

The sequence coverage rate was 85.33% for SAA1 and 43.83% for APO C-III (Table 3).

Confirmation of candidate protein biomarkers

Twenty-one serum samples (9 from the pre-surgery group, 6 from the post-surgery group, and 6 from the control group) were randomly selected for Western blotting. Immunoreactive bands of SAA1 (11.5 kDa) and APO C-III (10.9 kDa) were detected in all serum samples. The expression levels of SAA1 and APO C-III proteins were higher in pre-surgery samples than in post-surgery and control samples (Fig. 7). The results of quantitative analysis revealed that the gray values of the bands corresponding to SAA1 and APO C-III proteins were significantly higher in pre-surgery samples than in post-surgery and control samples (Fig. 8).

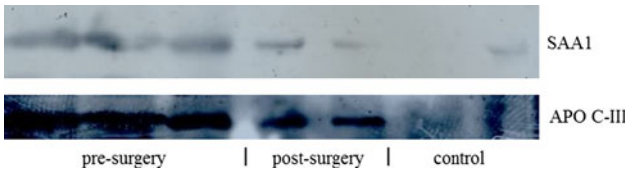

Fig. 7 Representative Western blot images showing differential expression of SAA1 and APO C-III proteins among different groups of serum samples

Discussion

In the present study, we used the SELDI-TOF-MS technology to perform protein profiling of serum samples from pre-surgery and post-surgery patients with Wilms tumor and healthy controls. By comparing protein peaks among the three groups after excluding confounding effects of common systemic inflammatory factors, we identified two peaks showing significant differential expression not only between pre-surgery and control sera but also between pre-surgery and post-surgery sera. We then identified these two peaks as SAA1 and APO C-II proteins by HPLC, MALDI-TOF–MS, and 2D-LC-LTQ-MS. Western blot analysis confirmed that both proteins were expressed at higher levels in pre-surgery sera than in post-surgery and control sera. Using the method of leave-1-out for cross detection,

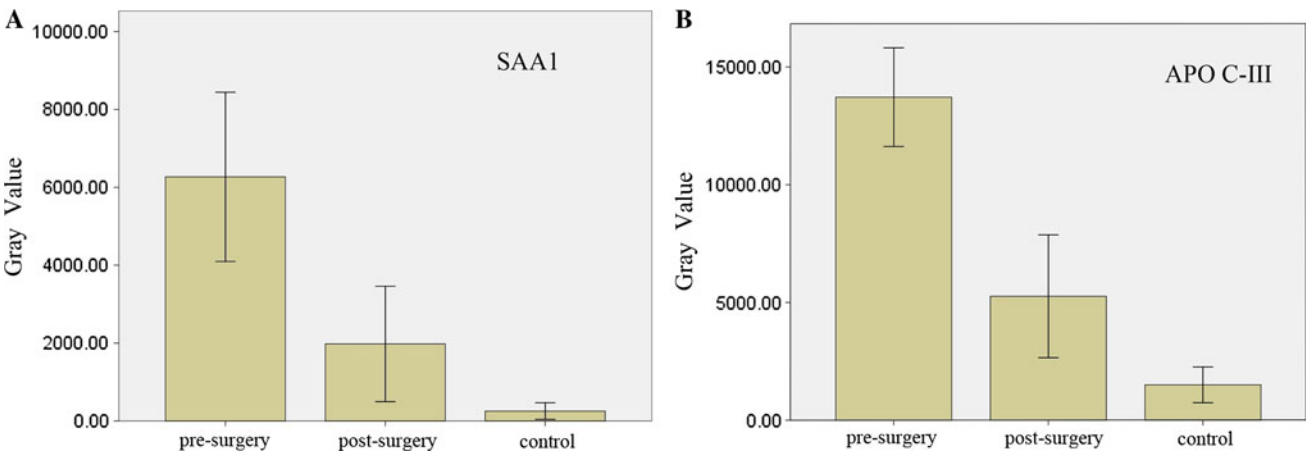

Fig. 8 A quantitative analysis of the gray values of the Western blot bands corresponding to SAA1 (**a**) and APO C-III (**b**) proteins in pre-surgery, post-surgery and control samples

we found that detection of these two candidate biomarkers had high sensitivity and specificity in discriminating pre-surgery subjects from post-surgery subjects and normal subjects. Collectively, these findings suggest that SAA1 and APO C-III are two potential biomarkers for Wilms tumor.

An ideal tumor marker should have both high specificity and sensitivity and is able to distinguish cancer from non-cancerous conditions. Since inflammation is a hallmark of cancer, most systemic response proteins present in cancer patients are unspecific and may confound tumor biomarker discovery and validation [16]. For example, elevated serum levels of interleukin-10 (IL-10), IL-1, IL-6 and tumor necrosis factor-α (TNF-α) have been correlated with renal cell carcinoma, colon carcinoma and oral cavity cancer [19–21]. To overcome this problem, we included some common systemic inflammatory factors to control for systemic inflammation in the current study. However, this strategy is limited by the number of inflammatory factors included. Alternatively, people with inflammatory conditions might be used as controls to exclude the confounding effects of inflammation [16].

Serum amyloid A (SAA) is an acute-phase protein that exists in various isoforms in a molecular mass range of 11–14 kDa [22] and occurs at low levels in sera of healthy individuals [23]. In humans, there are two nearly identical genes encoding SAA1 and SAA2 [24]. Acute-phase SAA levels are well known to increase during inflammation. However, recent evidence suggested a possible involvement of SAA in carcinogenesis [25]. SAA might play a role in the local inflammation of the malignant tissue [26]. New proteomic approaches have demonstrated that SAA1 may be a biomarker for cancers of the ovary [27], lung [22], kidney [28], gastric [29], nasopharynx [30], liver [31], colon [32] and placenta [33]. In this study, SAA1 was identified as a candidate protein biomarker for Wilms tumor. These data provide strong evidence for the involvement of SAA1 in this pediatric tumor. However, given that SAA1 is not a Wilms tumor-specific marker, it should not be used alone to detect this pediatric tumor. Instead, SAA1 might be included in a group of biomarkers or used in combination with established screening tests to detect Wilms tumor. In addition, considering that Wilms tumor is more common in children and that children have a narrower spectrum of malignancies, detection of serum SAA1 might have more important implications for the diagnosis of Wilms tumor than other adult malignancies.

Serum APO C-III levels were also found to be significantly elevated in patients with Wilms tumor in the present study. The mature ApoC-III protein is synthesized predominantly in the liver [34] and mainly involved in modulating plasma triglyceride levels by controlling the catabolism of triglyceride-rich particles. Plasma ApoC-III levels have been linked to several conditions associated with metabolic syndrome [35, 36]. Recently, altered serum levels of ApoC-III have also been observed in patients with papillary thyroid carcinoma [18], pancreatic cancer [37], or breast cancer [38]. However, the correlation between serum ApoC-III and other pediatric tumors has not been established so far. These findings suggest that serum APO C-III might be a potential useful marker for early diagnosis of Wilms tumor.

SAA is produced by hepatocytes [39], secreted into serum and rapidly binds to high-density lipoprotein, with 90% occurring in the bound form [40]. Multiple studies have suggested that SAA is involved in lipid metabolism [41]. Interestingly, APO C-III and apolipoprotein C–I (a previously identified potential serum marker for Wilms tumor [17]) have also been known to be regulators of lipid metabolism. These observations prompt us to speculate that Wilms tumor might affect lipid metabolism or that abnormal lipid metabolism might be involved in the pathogenesis of Wilms tumor. However, it remains to be investigated in future studies whether there is a causal link between Wilms tumor and abnormal lipid metabolism.

In conclusion, we have identified SAA1 and APO C-III as two potential serum biomarkers for Wilms tumor. However, there is still a gap between our findings and their application in clinical practice. Future studies including a sufficiently large sample size will be required to determine the sensitivity and specificity of these two serum biomarkers, alone or in combination with other biomarkers or established screening tests, in the diagnosis of Wilms tumor.

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Conflict of interest statement None.

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