DOI 10.1002/pmic.200500472 *Proteomics* 2006, *6*, 3426–3435

RESEARCH ARTICLE

Identification of a plasma proteomic signature to distinguish pediatric osteosarcoma from benign osteochondroma

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Osteosarcoma (OS) is the most common malignant bone tumor in children. To identify a plasma proteomic signature that can detect OS, we used SELDI MS to perform proteomic profiling on plasma specimens from 29 OS and 20 age-matched osteochondroma (OC) patients. Nineteen statistically significant ion peaks that were differentially expressed in OS when compared with OC patients were identified ($p < 0.001$ and false discovery rate $< 10\%$). Using the proteomic profiles, we constructed a multivariate 3-nearest neighbors classifier to distinguish OS from OC patients with a sensitivity of 97% and a specificity of 80% based on external leave-one-out cross-validation. Permutation test showed that the classification result was statistically significant ($p < 0.00005$). One of the proteins (m/z 11 704) in the proteomic signature was identified as serum amyloid protein A (SAA) by PMF. The higher plasma level of SAA in OS patients was further validated by Western blotting when compared to that of osteochrondroma patients and normal subjects as reference. The classifier based on this plasma proteomic signature may be useful to differentiate malignant bone cancer from benign bone tumors and for early detection of OS in high-risk individuals.

Received: June 24, 2005
Revised: January 25, 2006
Accepted: January 28, 2006

Keywords:
Molecular classification / Osteosarcoma / SELDI / Serum amyloid protein A

1 Introduction

Osteosarcoma (OS) is a primary malignant bone tumor arising from primitive bone-forming mesenchymal cells and characterized by the production of osteoid material by malignant osteoblastic cells [1]. It accounts for approximately 60% of malignant bone tumors in the first two decades of life with an annual incidence rate of about 5.6 *per* million [2]. Approximately two-thirds of children with nonmetastatic OS are cured with surgery and chemotherapy. After initial diagnosis, patients usually receive multiagent preoperative chemotherapy and then surgical resection of the tumor followed by postoperative chemotherapy [3]. The response of the preoperative chemotherapy is measured by the extent of necrosis in the resected tumor. The challenge is that despite modifications in postoperative chemotherapy, patients who respond poorly to the preoperative chemotherapy (<90% of

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Abbreviations: NPC, nasopharyngeal cancer; **OC**, osteochondroma; **OS**, osteosarcoma; **SAA**, serum amyloid protein A; **SAM**, significance analysis of microarray

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tumor necrosis) have a worse outcome [4]. In fact, the long-term survival rate of these poor responders has not been improved in the past 20 years [5]. Therefore, it is important to identify these resistant tumors as early as possible, so that more aggressive therapy can be used upfront to improve the outcomes. As a first step of using a proteomic approach to classify these poor responder patients, we tested the feasibility of using SELDI to generate a plasma-based proteomic signature and classifier to distinguish OS patients from benign osteochondroma (OC) patients. Epidemiologically, patients with the hereditary form of retinoblastoma [6], and Rothmund-Thomson Syndrome patients with RECQL4 mutation have much higher risk of developing OS [7]. Currently there is no clinical test available that can be used routinely for early detection of OS in these high-risk patients. Therefore, an accurate classification method based on plasma proteomic signature will be useful for monitoring these high-risk patients.

SELDI is a rapid and sensitive proteomic technique that has been used to identify biomarkers in various cancers [8, 9]. When combined with bioinformatic approach, it can also be used to construct powerful multivariate classifiers to perform molecular classification. Promising results have shown the utility of SELDI-based molecular classifiers for early detection of various adult cancers, including prostate [10], ovarian [11], and breast cancers [12]. Although several genomics analyses have been performed in pediatric OS using primary tumor tissues [13–16], SELDI has not been used to classify OS based on patients' blood proteins. Furthermore, SELDI-based plasma proteomics has several advantages over RNA-based microarray analysis. For instance, it is a less invasive approach compared to microarray studies that require normal and tumor tissues. Also, plasma proteome is not as severely affected by RNA degradation as tissue specimens, and protein expression is better correlated with tumor phenotype.

In this study, we performed SELDI analysis of plasma samples from 29 OS and 20 benign OC patients. Because reproducibility is a key issue in SELDI experiments, we addressed this by including a human normal serum sample as a variability control in our study. 3-Nearest neighbor algorithm was used to classify OS patients from OC patients and attained an overall accuracy of 90%, which is statistically significant ($p < 0.00005$). We also identified one of the peaks in the proteomic signature that was used in the classifier as serum amyloid protein A (SAA). The differential expression of SAA was validated by Western blot analysis.

2 Materials and methods

2.1 Patients and samples

Plasma samples from 29 OS patients (age 10–22 years) were collected at the time of diagnosis. All samples were collected through IRB-approved protocols from three collaborative institutions, namely Texas Children's Hospital (TCH), Cook's Children Hospital in Fort Worth, Texas, and Oklahoma Children's Hospital after informed consent had been signed (Suppl. Table 1). These samples were used to create proteomic profiles. Plasma samples from 20 age-matched anonymized OC patients that were previously collected as discarded materials were used as benign bone tumor controls. We chose age-matched OC as a benign control because it is the most common benign bone tumor and these samples were readily available in our study. In terms of controlling for potential impact of nonspecific host response on plasma proteomic profiles, OC patients are probably more appropriate than normal subjects because both OS and OC patients share primary tumors that have similar anatomic location and tissue type. In addition, five human plasma samples from normal 18-year-old donors were purchased from Equitech-Bio (Kerrville, TX). The normal donor plasma samples were pooled and used as a normal control in Western blotting. All blood samples were collected in EDTA-containing tubes at room temperature and immediately centrifuged at 1000 rpm for 10 min. The plasma supernatant was collected and divided into aliquots and stored at −80°C until use.

2.2 SELDI profiling analysis

To optimize peak resolution and number of protein peaks detected in our experiments, an anion-exchange fractionation procedure was performed. Twenty microliters of each plasma sample was denatured by 30 μL of 50 mM Tris-HCl buffer containing 9 M urea and 2% CHAPS (pH 9). The proteins were fractionated in an anion-exchange Q Hyper D F 96-well filter plate (Ciphergen Biosystems, Fremont, CA). Six fractions, including those from the flow-through (Fraction 1), pH 7 (Fraction 2), pH 5 (Fraction 3), pH 4 (Fraction 4), pH 3 (Fraction 5), and organic eluant fractions (Fraction 6), were collected by stepwise decreases in pH gradient.

For ProteinChip array binding, 20 μL of each fractionated plasma was diluted in 80 μL of CM Low Stringency Buffer (0.1 M sodium acetate, pH 4.0) and profiled on Weak Cation Exchange (CM10) Arrays (Ciphergen Biosystems). Sinapinic acid (Ciphergen Biosystems), which served as an energy-absorbing molecule, was used to facilitate desorption and ionization of proteins on the ProteinChip arrays. Each sample was randomly assigned to a spot in a 96-well format. A commercially available pooled human control serum (Ciphergen Biosystems) was also randomly spotted on each chip to monitor the experimental reproducibility. Each plasma sample was analyzed in duplicate to minimize the effects of intraassay variation. All fractionation and on-chip spotting steps were performed on a Biomek 2000 Robotic Station (Beckman Coulter, Fullerton, CA).

Proteomic profiles on the ProteinChip arrays were detected by a Protein Biology System (Model PBSIIc, Ciphergen Biosystems) using three different laser spot pro-

tocols (low-, medium-, and high-power settings). The high mass setting for the low laser protocol was 25 kDa, with an optimization range from 1 to 7.5 kDa and a deflector setting of 1 kDa. The medium and high laser spot protocol had a high mass setting of 200 kDa and deflector setting of 10 kDa, with an optimization range from 10 to 50 kDa and from 10 to 75 kDa, respectively. MS profiles were generated by averaging 65 laser shots at laser intensities between 170 and 250 and detector sensitivities between 4 and 10, determined individually for each fraction to achieve maximum protein peak yield. Mass detection accuracy of PBSIIc was calibrated externally by using the All-in-1 peptide and All-in-1 protein II molecular mass standards (Ciphergen Biosystems).

Data analysis was performed initially with Ciphergen Express Software 3.0 (Ciphergen Biosystems). The spectra were baseline subtracted and then normalized to the TIC of m/z starting from 1500 for low-molecular weight proteins, from 9000 for medium-molecular weight proteins, or from 10 000 for high-molecular weight proteins. Peaks were autodetected with an S/N of >5, and the peaks were clustered using second pass peak selection with S/N of >2. A 0.7% mass window was selected for the optimal detection of peaks and minimization of noise. m/z ranges that were used for the analysis were set to 2000–10 000 for low-molecular weight protein, 10 000–30 000 for medium-molecular weight protein, and 30 000–200 000 for high-molecular weight proteins. Intensities from duplicates of the same sample were averaged before analysis. AUC calculations were preformed with Ciphergen Express software.

2.3 Reproducibility of SELDI profiling

The pooled normal human serum control was also fractionated and spotted randomly with patient specimens on each chip to measure the variability of fractionation, on-chip spotting, and data acquisition. Ion peaks in these control spectra with S/N >5 were selected for CV calculation, which was defined as the percentage of the SD divided by the mean peak intensity. All peaks used in the CV calculation were further inspected manually to ensure that the peak-picking algorithm correctly defined the centroid of the peak.

2.4 Tryptic digestion and protein identification

Four samples from Fraction 4 of OS plasma with high-level expression of m/z 11 704 biomarker were pooled. Four samples from the same fraction of OC plasma with low-level expression of the biomarker were also pooled as a negative control. The pooled samples were resolved by 4–20% Novex Tris-glycine SDS-PAGE gel (Invitrogen, Carlsbad, CA). The bands of interest that contained the desired biomarker were cut from the gel and subjected to overnight tryptic digestion in a reaction volume of 10 μL of 25 mM ammonium bicarbonate buffer containing 0.2 μg of trypsin (proteomics sequencing grade, Sigma, St. Louis, MO). Subsequently, 3 μL of the digestion mixture was analyzed on an RP H4 ProteinChip Array (Ciphergen Biosystems). CHCA (Ciphergen Biosystems) was used to facilitate desorption/ionization of the peptides generated from the tryptic digest. Masses of the trypsin-digested peptides were measured by PBSIIc. Background peaks were removed by comparing these with the spectrum of trypsin-digested gel without protein. The spectrum was calibrated internally by using two different protein standards: human angiotensin I (1296.5 Da) and beta-endorphin (3465.0 Da). Peptide masses were searched through Swiss-Prot and NCBI databases using three different search engines: ProFound [17], MASCOT [18], and PepIdent [19] to identify the corresponding protein.

2.5 Validation of SAA differential expression by Western blotting

The pooled OS and OC samples (see above) were used in Western blot analysis. The protein concentrations of these pooled samples were measured by DC protein Assay (BioRad, Chicago, IL). Fifteen micrograms of total protein of each sample was separated in 4–20% Tris-glycine SDS-PAGE gel (Invitrogen) and transferred to PVDF membranes. Membranes were blocked by 5% nonfat milk in PBS-0.1% Tween 20 buffer (PBS-T) for 1 h at room temperature, and then incubated overnight at 4°C with rat mAb of antihuman serum amyloid A (SAA, Biosource, Camarillo, CA) at 1:1200 dilution. The secondary antibody used was goat antirat IgG antibody conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotech, Santa Cruz, CA) at 1:5000 dilution. Signal was developed using ECL plus Western Blotting Detection Reagents (Amersham Bioscience, Piscataway, NJ). The PVDF membrane was dried and stained with SimplyBlue Safe Stain (Invitrogen) after Western blot transfer. Intensity of the 66 kDa band (albumin) on the membrane was used as a loading control.

To further validate the up-regulation of SAA in individual OS samples and the reproducibility of the fractionation procedure, raw plasma with high- or low-level expression of m/z 11 704 biomarker was independently fractionated using Q Ceramic HyperD F Spin Columns (Ciphergen Biosystems), which has the same anion-exchange chromatography media as the Biomek fractionation. As a normal control, a pooled sample of five normal human plasma samples was also fractionated at the same time. Expression levels of SAA from Fraction 4 of the fractionated samples were analyzed using Western blotting.

2.6 Bioinformatics and statistics

Statistically significant ion peaks were identified by using parametric t-test with p value of 0.001 by comparing the OS and OC samples. The expression values of all the ion peaks (n = 681) identified by Ciphergen Express 3.0 software (background subtracted and normalized) were log 2 transformed and imported to BRB ArrayTools 3.01 developed by Richard Simon and Amy Lam Pang (http://linus.nci.-

nih.gov/BRB-ArrayTools.html) to construct a multivariate classifier. Six different classification algorithms were used to construct an optimal classifier for OS, including compound covariate predictor [20], 1- or 3-nearest neighbors, nearest centroid, support vector machine, and linear discriminant analysis [21, 22]. The classification accuracy of different algorithms were measured by external leave-one-out cross-validation in which the feature selection step, was performed in each iteration at $p \leq 0.001$ to prevent underestimation of classification error [23]. Hierarchical clustering was performed using average dot product and average linkage. Significance analysis of microarray (SAM) was performed using two-class unpaired comparison of SAM-excel add in [24]. Other statistical analyses were performed using SPSS 12.0 (SPSS, Chicago, IL).

To calculate the performance of the classifiers, we defined the following:

A = The number of OS samples predicted correctly as OS
B = The number of OS samples incorrectly predicted as OC
C = The number of OC samples predicted correctly as OC
D = The number of OC samples incorrectly predicted as OS
Sensitivity = $A/(A + B)$
Specificity = $C/(C + D)$
Positive predictive value (PPV) = $A/(A + D)$
Negative predictive value (NPV) = $C/(B + C)$

3 Results

3.1 Reproducibility of the SELDI profiles

To increase the success rate of biomarker discovery, plasma specimens were first fractionated into six different fractions based on p*I*s of the proteins. Samples of each fraction were spotted randomly onto a weak cationic ProteinChip array CM10 using a 96-well microtiter plate adaptor and a robotic liquid handling system. We first tested the reproducibility of SELDI with an experimental reproducibility control, a pooled normal human serum control, at three laser power settings, and different fractions. This pooled normal human serum control was also fractionated and spotted randomly onto each array together with the patients' plasma samples. The CVs of reliable peaks in this serum control obtained from different fractions and laser power settings were calculated. The average CVs of different fractions ranged from 19.6 to 35.6% (Fig. 1A), which is comparable to two recent multi-institutional studies (15–36% CV, [25] and 15–43% CV [26]). The average CVs of all the fractions were similar to each other, except Fraction 1, which was significantly higher than the other fractions ($p < 0.01$, Games-Howell test) (Fig. 1A). This result suggests that Fraction 1, which was the flow through from the fractionation step was more variable than the other fractions. The average CVs of peaks detected using different laser powers were not significantly different

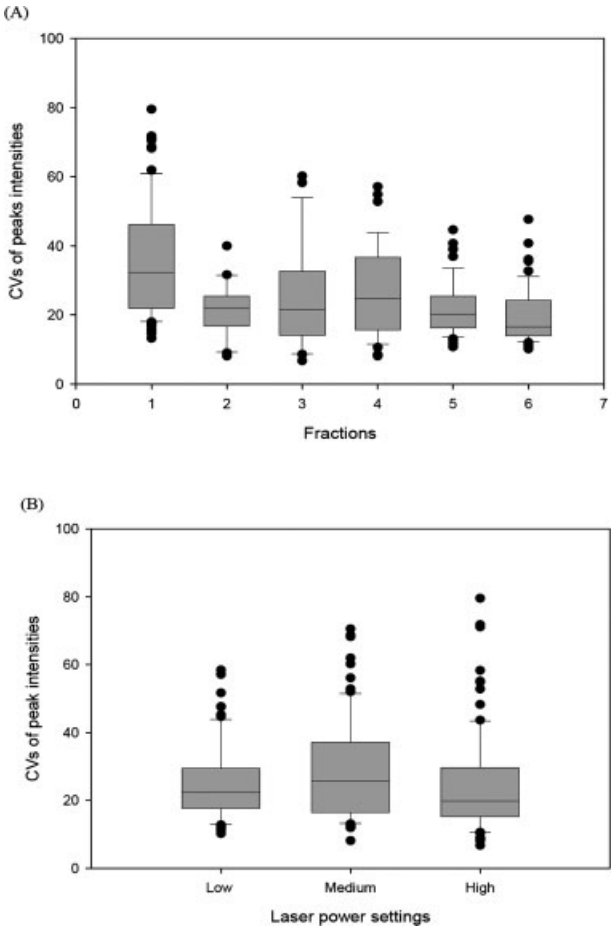

Figure 1. Box plots of CVs for ion peak intensities in (A) various fractions and (B) laser power settings for normal human control serum.

from each other, indicating that the variability of detecting low-, medium-, and high-molecular weight proteins were similar (Fig. 1B).

3.2 Classification of OS and OC

To test if we can use the plasma proteomic profiles to classify OS from OC, all the ion peaks ($n = 681$) identified by SELDI from six different fractions and three laser power settings were combined and used to construct a multivariate classifier. First, using $p \leq 0.001$, we identified 19 ion peaks, which were significantly differentially expressed in plasma of OS patients when compared to those of OC patients (Table 1, see also Suppl. Fig. 1 for the box plots of these peaks). The probability of identifying at least 19 false-positive peaks that were significant at p value ≤ 0.001 in the given dataset was 0.002, indicating that the identification of 19 peaks was statistically significant. To further confirm that these peaks were statistically significant by controlling the multiple testing problem, we performed multivariate permutation test and found that the 19 peaks had less than 10% false discovery rate at

Table 1. Nineteen statistically significant ion peaks differentially expressed in plasma samples of OS and OC patients

p-value[a]	*m/z*[b]	Fraction	AUC[c]	Peak intensity[d]				Fold change
				OS AVG	OS STD	OC AVG	OC STD	OS/OC
0.0000100	12 691	F1	0.817	1.343	0.759	0.558	0.271	2.407
0.0000119	34 274	F6	0.848	3.904	1.926	2.185	0.591	1.786
0.0000299	42 217	F5	0.167	1.094	0.371	1.748	0.543	0.626
0.0000323	43 229	F5	0.152	1.708	0.64	2.722	0.823	0.627
0.0000746	34 587	F6	0.848	3.678	1.527	2.397	0.504	1.535
0.0000750	42 770	F5	0.167	1.267	0.429	2.021	0.649	0.627
0.0000865	42 433	F5	0.183	1.168	0.394	1.869	0.625	0.625
0.0001003	10 838	F1	0.848	2.577	2.862	0.702	0.261	3.673
0.0001240	21 715	F5	0.167	0.68	0.192	0.951	0.216	0.716
0.0001286	31 925	F6	0.817	2.555	1.908	0.963	0.573	2.654
0.0001374	33 332	F6	0.802	6.492	2.333	4.495	0.989	1.444
0.0002388	108 914	F5	0.194	0.179	0.061	0.253	0.066	0.706
0.0002581	175 716	F2	0.697	0.042	0.011	0.029	0.015	1.444
0.0002915	123 203	F1	0.229	1.099	0.375	1.714	0.599	0.641
0.0003843	11 704	F4	0.833	3.955	8.198	0.295	0.191	13.409
0.0004371	44 468	F5	0.229	1.2	0.323	1.572	0.311	0.763
0.0006519	56 016	F6	0.245	2.553	0.67	3.338	0.771	0.765
0.0007267	10 862	F2	0.771	0.398	0.272	0.21	0.104	1.898
0.0009493	154 713	F1	0.245	0.774	0.337	1.435	0.774	0.539

a) *p* Values were calculated using parametric two-sample *t*-test.
b) *m/z* is the mass/charge ratio of the ion peak.
c) AUC (area under the ROC curve) values were calculated using OS as reference.
d) AVG and STD are the average and SD of the average peak intensity of the class. OS: osteosarcoma; OC: osteochondroma.

$p \leq 0.001$ with 90% probability. When we controlled for the number of false discovery, these 19 peaks had only 1 or less false discovery at $p \leq 0.001$ with 90% probability. Nine of the 19 peaks were up-regulated and ten were down-regulated in OS samples (Table 1). Figure 2 illustrates the SELDI spectra of four representative ion peaks (*m/z* 10 838, *m/z* 11 704, *m/z* 12 691, *m/z* 123 203) identified by this analysis. Using expression values of these 19 peaks to perform hierarchical clustering, most OS and OC samples were separated into two major groups (Fig. 3) suggesting that these 19 peaks contain information for classifying OSs and OCs.

To construct a multivariate classifier for OS, all 681 ion peaks were used to train six different classification algorithms, including feature selection and model building. Because our sample size was relatively small, we did not separate the samples into a training set and a testing set. Instead, we measured the accuracy of the classifiers by a well-accepted bioinformatic technique called external leave-one-out cross-validation [22]. Among different significance levels tested (p = 0.05, 0.01, 0.005, 0.001), $p \leq 0.001$ achieved the best classification result when combined with the algorithm of 3-nearest neighbors. It achieved an overall classification accuracy of 90%, sensitivity of 97%, and specificity of 80% in distinguishing OS plasma samples from OC samples. (Fig. 4). The positive predictive value and negative predictive value were 88 and 94%, respectively, for OS. In fact, the classifier only misclassified 1 of 28 OS patients, and 4 of 20 OC patients. Permutation analysis showed that the 90% classification accuracy was statistically significant ($p < 0.00005$).

3.3 Protein identification of *m/z* 11 704 plasma biomarker

We attempted to establish the protein identity of all the ion peaks used in the classifier and illustrate here the further investigation of one of the peaks (*m/z* 11 704) that we had successfully identified. This ion peak was highly up-regulated in OS samples and was statistically significant by parametric *t*-test ($p < 0.0003843$, average 13-fold change, Table 1). As an independent confirmation of the significance of this peak, it was also found to be statistically significant by the nonparametric Wilcoxon test ($p = 0.0000516$) and SAM (q-value = 0). It also has nice properties for protein purification such as high signal intensity, free of neighboring peak contaminations, and can be visualized by SDS-PAGE. For protein purification, four OS samples that overexpressed the ion peak and four OC samples that underexpressed the ion

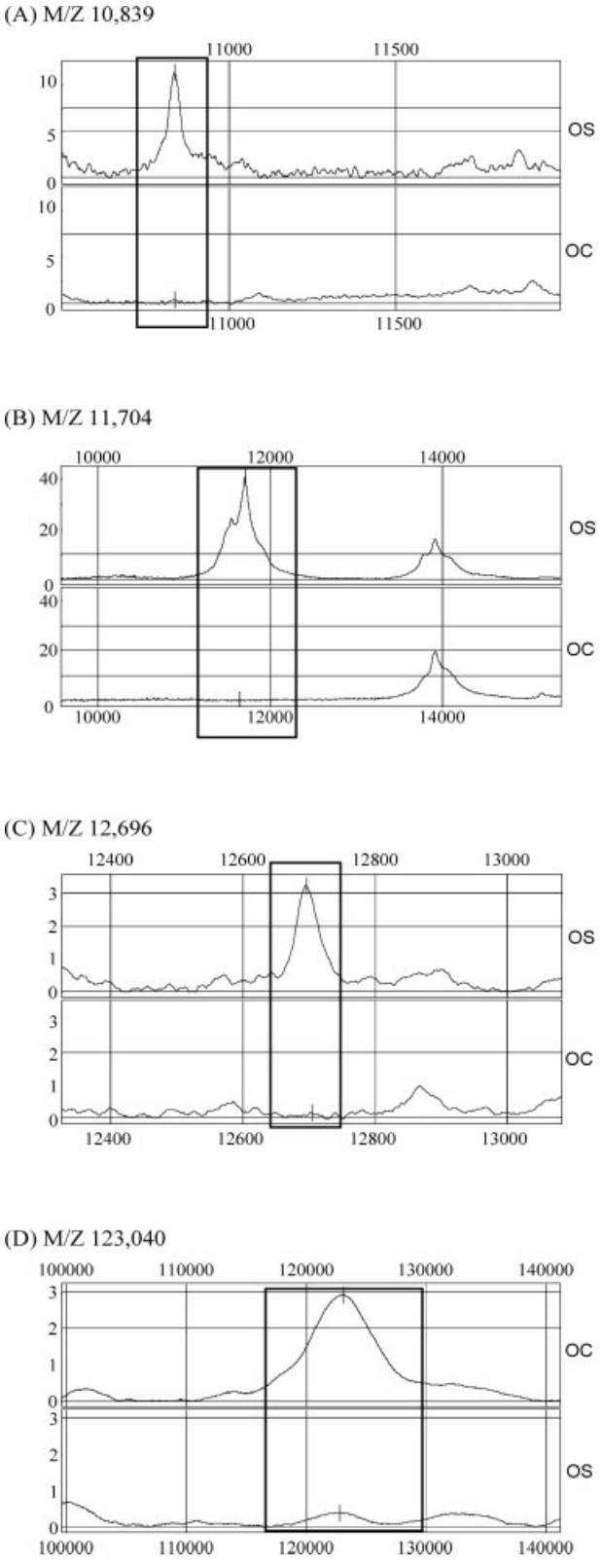

Figure 2. SELDI spectra of four representative proteins statistically significant ($p < 0.001$) in distinguishing OS from OC. *y*-Axis is the normalized peak intensity and *x*-axis is *m/z* ratio.

peak were selected to form positive and negative pools, respectively. The two resultant pooled samples (positive and negative) were resolved on SDS-PAGE. A band corresponding to 11.7 kDa protein was clearly overexpressed in the positive pooled sample when compared to the negative pooled sample (Fig. 5A). This 11.7 kDa band was excised and digested with trypsin (Fig. 5B). Using PMF, we determined the identity of the 11.7 kDa protein as SAA with high probability (z = 2.34, probability = 1.00 in Profound search engine) [17]. The trypsin-digested peptides of the 11.7 kDa protein matched 63% of the SAA proteins. Similar results were obtained by using MASCOT [18] and PepIdent [19].

3.4 Western analysis of SAA

To validate the differential expression of SAA in OS and confirm the protein identity of the *m/z* 11 704 biomarker, Western blotting was performed on the two positive and negative pooled samples used in protein identification by a specific anti-SAA antibody. Figure 6A shows that SAA was highly overexpressed in the pooled OS sample but not in the pooled OC sample. The Coomassie staining of the membrane after Western blotting indicated that similar amounts of a 66 kDa band (albumin) were present in the two pooled samples, suggesting that similar amounts of proteins were loaded in the two samples (Fig. 6B). However, to further validate and reproduce SAA overexpression in OS samples, we independently fractionated three plasma samples from each of the OS and OC patients using spin columns with anionic resin similar to the SELDI fractionation experiments. We also included a pooled normal control, which consisted of five age-matched normal subjects whose age was around 18 years old, in the column fractionation. Fraction 4 which contained the *m/z* 11 704 biomarker of these independent fractionated samples was used for Western blotting of SAA. The results showed that SAA was clearly overexpressed in samples from OS patients compared to those from osteochrondroma patients and normal subjects (Fig. 6C and D). In fact, the SAA expression levels in OC cases and healthy controls were almost undetectable, which is consistent with a previous report [27].

4 Discussion

4.1 Use of plasma specimens in proteomic profiling of pediatric cancers

SELDI is a relatively new proteomic technology that has impacted many areas of biological research [28]. The ease of use and high-throughput nature of this technology allow the processing of many samples simultaneously in a relatively short period of time. It requires only small amounts of sample for profiling, which makes it particularly suitable for clinical or translational studies. SELDI has been successfully applied in identifying early detection biomarkers in multiple

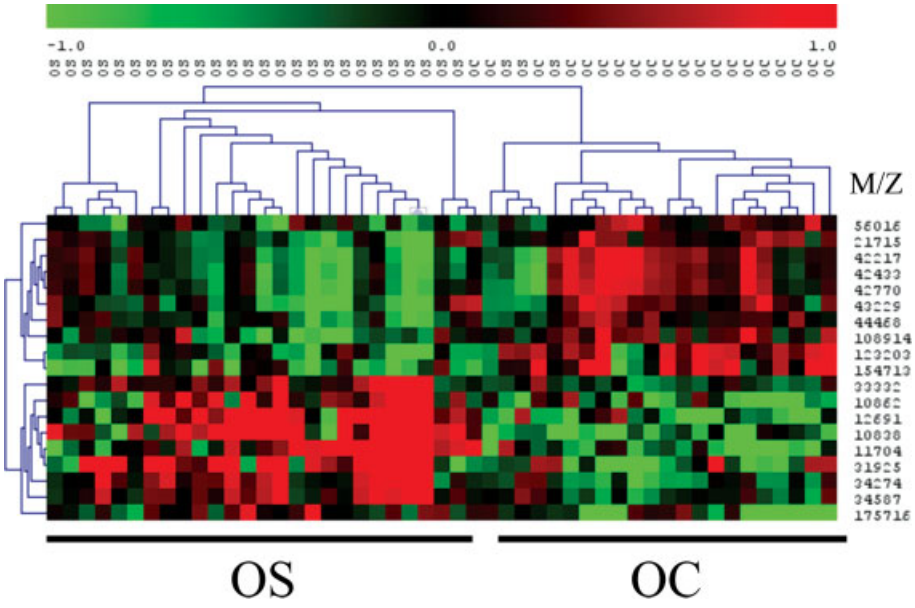

Figure 3. Hierarchical clustering of OS with 19 statistically significant discriminatory peaks in OS and OC ($p < 0.001$). Intensities of the peaks were median center. Average dot product and average linkage were used for clustering. Red to green scale represents log peak intensity from −1 to 1. Number labels on the right are *m/z* of the 19 significant peaks.

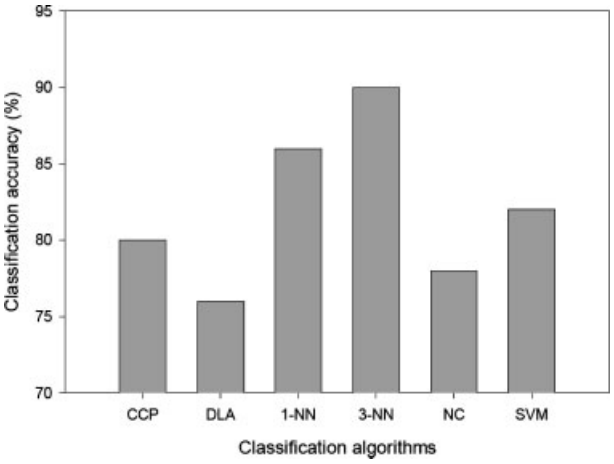

Figure 4. Classification accuracy of various supervised algorithms used to classify OS and OC. CCP: compound covariate predictor; DLA, diagonal discriminant analysis; 1- and 3-NN, 1- and 3-nearest neighbors; NC, nearest centroid; SVM, support vector machine.

adult cancers, including ovarian, prostate, and breast cancers [10–12]. However, SELDI technology is not widely used in pediatric oncology to identify biomarkers and perform molecular classification, partly due to the limited sample size when compared to adult cancers. Furthermore, SELDI can also be used as a complementary technology to RNA expression profiling using microarray because SELDI measures the proteomic expression in blood instead of RNA expression in tumor tissues. In principle, if biomarkers can be detected in blood, it is much easier and less invasive to measure than a biomarker that is only detectable in the tumor cells. One unique feature of this report is that we used plasma to perform proteomic profiling instead of serum, which is becoming the most commonly used specimen for proteomic profiling. Furthermore, in many cases, cells are spun down from the blood and DNAs from these blood cells can be used for genotyping analysis. The residual plasma may be used for proteomic studies, thus allowing for correlation between genetic polymorphism and protein expression.

4.2 The reproducibility issue

The reproducibility of the SELDI platform has been controversial [29–31]. Although some reports have suggested that SELDI results are not easy to analyze and noisy peaks may be used in classification, a recent report has shown that with proper experimental procedures and reader calibration, reproducible results can be obtained from multi-institutional studies [25]. Similar to the work of Semmes *et al.*, we have applied measures to ensure experimental reproducibility in our study. These include robotic liquid handling, randomization of samples to minimize the spot and chip effects, routine calibration of the ProteinChip reader using insulin and IgG, and introduction of normal human serum to monitor the intra and interexperimental reproducibility. The CVs of our normal serum control suggest that our experimental variability is comparable to other studies using meticulous procedures to minimize experimental variations [25, 32]. Our CVs of peak intensities are very similar in different fractions, except Fraction 1, a flow-through fraction. This suggests that no significant variability was introduced into different fractions during the fractionation step. The laser power settings did not affect the peak intensity reproducibility significantly (Fig. 1B), indicating that using SELDI peak intensities for low-, medium-, and high-molecular weight

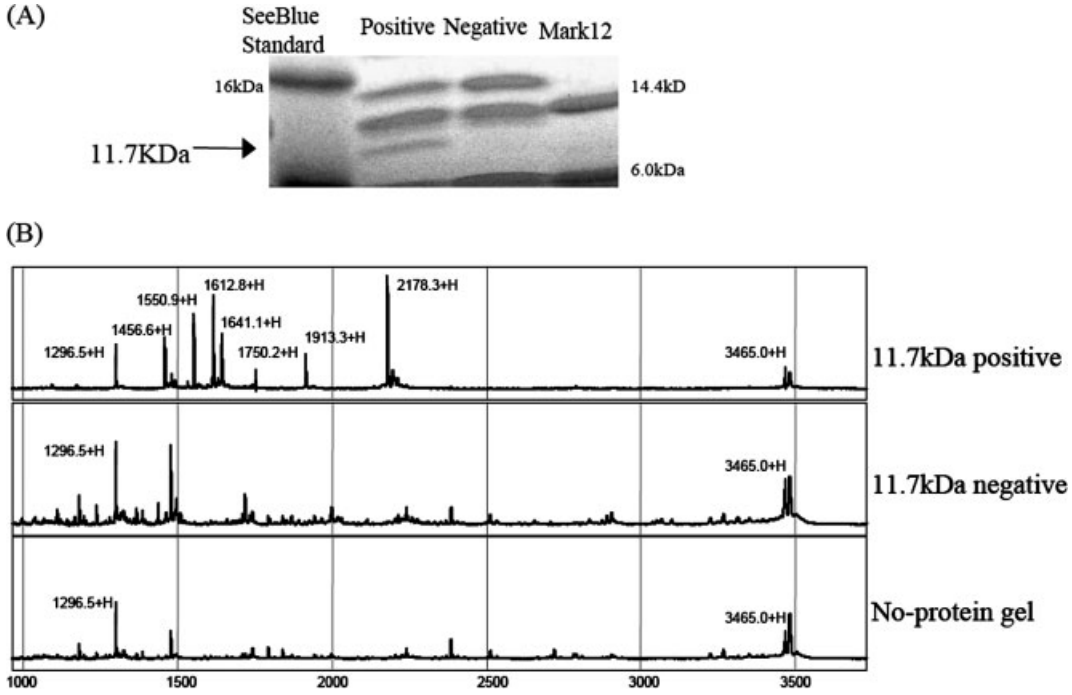

Figure 5. Protein identification of *m/z* 11 704 ion peak. (A) SDS-PAGE of the *m/z* 11 704 ion peak of the positive sample, which was a pooled sample from four OS samples with high expression of the ion peak. Negative sample was a pooled sample of four OC samples with low expression of the ion peak. See Blue and Mark 12 are two molecular weight standards (Invitrogen). (B) SELDI spectra of trypsin-digested 11.7 kDa biomarker from positive and negative controls. A gel with no protein was also excised as a reference.

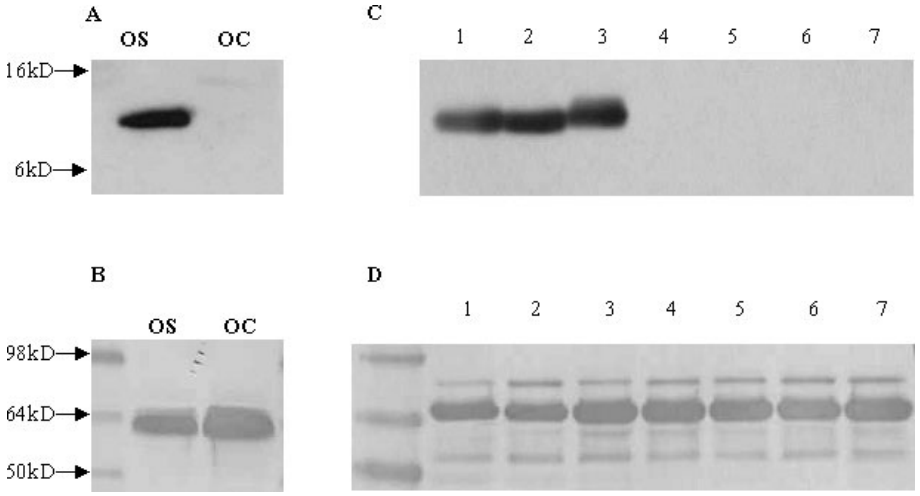

Figure 6. Western blotting of SAA. (A): SAA expressions in pooled Biomek fractionated plasma (11.7 kDa) in OS and OC. (B): Amounts of 66 kDa band (albumin) in the pooled OS and OC samples (CBB staining of the PVDF membrane). (C): SAA expressions in independently column-fractionated plasma samples. (D): Amounts of 66 kDa band (albumin) in column-fractionated plasmas (CBB staining of the PVDF membrane). Lanes 1–3: OS samples; Lanes 4–6: OC samples; Lane 7: a pooled normal human control plasma from five normal subjects.

proteins were consistent. This allows us to combine the data from different laser power settings and fractions for molecular classification.

4.3 SAA

We have used SELDI to identify 19 statistically significant plasma ion peaks (biomarkers) that are differentially expressed in OS when compared to benign OC ($p \leq 0.001$, 10% false discovery rate, and 1 or less false discovery). OC is the most common benign bone disease. Although typical OC does not progress to OS, occasionally there are rare cases that progress to malignant bone tumor, such as chondrosarcoma. In addition, OC samples could be a better control for acute phase response than normal subjects, though we did not observe many inflammatory reactions in both OS and OC specimens. One significant ion peak was identified as the 11.7 kDa SAA using peptide mass fingerprinting and vali-

dated by Western blotting using anti-SAA specific antibody. We also observed that there was a minor peak (m/z 11 500) at the shoulder of the SAA peak (Fig. 2B), which was probably the peak corresponding to SAA minus the amino-terminal arginine reported elsewhere [33]. SAA is a major component of the apolipoproteins in the high-density lipoprotein particle, but its function is still not clear [34]. Although SAA is mostly secreted by the liver and is not regarded a biomarker for a specific cancer, it has been found to be highly expressed in a number of cancers, including renal and colorectal cancers [35, 36]. In addition, it has also been shown to be a prognostic marker in some cancers [37], used to monitor disease and therapeutic response in prostate cancer [38], and associated with metastatic diseases [39]. SAA has recently been reported to be highly expressed in relapsed nasopharyngeal cancer (NPC) using serum proteomic profiling [40]. Therefore, the overexpression of SAA in OS is congruent with these previous findings in other cancers. Although increasing evidence suggests that SAA can be used as a biomarker for various cancers, some may still argue that the elevation of SAA in cancers is due to an acute phase response, such as infection, inflammation, trauma, rheumatoid arthritis, and amyloidosis [34]. However, Cho *et al*. have shown that SAA was only weakly associated with neutropenic fever in relapsed NPC patients, suggesting that the elevation of SAA in relapsed NPC is not merely the result of acute phase response [40]. When examining 621 cancer patients who were free of inflammation by a conventional RIA, Rosenthal and Sullivan [41] also showed that the level of SAA is highly increased in 95% (281 of 289) patients with metastatic solid tumors, in all myelocytic leukemia patients with high leukocyte counts, and in all advanced lymphoma patients, suggesting that SAA level is correlated with various cancers instead of inflammation [41]. Therefore, we believe that SAA and the other 18 ion peaks in the plasma form a proteomic signature, which can be used to classify OS from OC.

We have also attempted to identify the other 18 ion peaks in the proteomic signature. However, the success of our approach is dependent on the abundance of the protein, successful purification by the SDS-PAGE, and an unambiguous mapping of the digested peptides in PMF with a known protein in the protein databases. It is not uncommon that the peaks discovered from SELDI cannot be identified subsequently due to these technical limitations [42, 43]. In our case, SAA was the only protein that could be satisfactorily purified by SDS-PAGE and showed unambiguous results in protein identification using multiple protein databases.

4.4 The clinical significance of the classification

The multivariate classifier constructed with SELDI profiles correctly classified 90% of OS and OC samples (only 5 of 49 samples were misclassified) in the leave-one-out cross-validation. This percentage of correct classification is significant using a permutation test ($p < 0.00005$), suggesting that our classification result is not merely due to chance alone. Only 1 of 28 OS samples was misclassified (sensitivity of 97% and PPV of 88%), suggesting that our classifier is highly sensitive in detecting OS. Four of 20 OC samples were misclassified; however, we do not have enough clinical follow-up data to determine if any of these patients subsequently developed more malignant tumors, such as chondrosarcoma. Although we do not have enough specimens to perform an independent validation test, we have used a rigorous and well-accepted bioinformatic technique called external LOOCV to estimate the classification accuracy. We believe that if this classifier was used to classify another set of independent samples in the future, the classification accuracy will be similar to or may even be higher than this study. In our experience, LOOCV sometimes gives a more conservative estimate when the sample size is small [16]. Although the annual incidence of OS (5.6 *per* million) does not justify the use of the classifier to screen the general pediatric population and the specificity of the classifier for OS *versus* other cancers or diseases still needs to be determined, the classifier developed in this study may have clinical importance. First, the clinical diagnosis of OS is usually done by biopsy of the tumor specimen. Although it is a definitive method, obtaining a biopsy specimen requires an invasive procedure. If we can perform molecular classification with high sensitivity and specificity using blood specimens from the patients, it will be more convenient and less invasive to the patients at the initial diagnosis. Second, for patients who are at high risk for developing OS, such as those with hereditary retinoblastoma [6] and Rothmund-Thomson Syndrome with RECQL4 mutation [7], this molecular classification system could be used to monitor OS development as an adjunct to radiography. This would allow for early detection and treatment of OS in these high-risk patients.

We would like to thank Dr. Tai-Tung Yip for technical consultation on this project and critical review of the manuscript. We also wish to thank Dr. David Gorenstein (University of Texas Medical Branch, Galveston, Texas) and Ciphergen Biosystems for the use of the Biomek 2000. We are grateful to Jaya Visvanathan, Charlotte Nicholson, and Jonathan Lau for technical support and Carolyn Pena for manuscript preparation. The work is supported by grants from NIH CA81465, Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation, Gillson Longenbaugh Foundation, the Cancer Fighters in Houston (CL), and Carl C. Anderson, Sr. and Marie Jo Anderson Charitable Foundation (TKM).

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