A serum protein fingerprint in the diagnosis and prognosis of Wilms' tumors in children☆

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Abstract

Objective. To establish specific fingerprints of serum markers for the diagnosis and prognosis of childhood Wilms' tumors. *Methods.* A total of 120 serum samples include 30 samples of Wilms' tumor patients after diagnosis, 70 samples collected after nephrectomy and 20 samples collected from healthy children. The sera were analyzed by Ciphergen ProteinChip coupled with surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) to characterize the mass spectrometric profiles. The data were then classified by support vector machine (SVM) analyses. *Results.* A protein with mass/charge (M/Z) ratio of 6984.5 (pM/Z6984.5) and a protein with M/Z ratio of 6455.5 (pM/Z6455.5) were identified based on their progressive down-regulation in Wilms' tumor. In addition, a protein with M/Z ratio of 4153.9 (pM/Z4153.9) and a protein with M/Z ratio of 3257.6 (pM/Z3257.6) were found to be specifically associated with the transition from Wilms' tumor from stage I/II to stage III/IV. These data were cross-validated using the leave-one out method. The accuracy of clinical staging afforded by the protein chip technique was 100%. This result was consistent with pathological staging and was superior to computed tomography (CT) scan. Both pM/Z6984.5 and pM/Z6455.5 were weakly not expressed in pre-surgical serum specimens. However, their levels were elevated in 19 of 21 samples from patients who just underwent radical resection to a level comparable as found in normal controls. Interestingly, the expression of pM/Z6984.5 and pM/Z6455.5 in five cases remained low even at three and at six months after nephrectomies. *Conclusion.* The combination of SELDI-TOF-MS and SVM led to serum protein fingerprint comprised of pM/Z6984.5, pM/Z6455, pM/Z4153.9 and pM/Z3257.6 for Wilms' tumor. This fingerprint model may prove to be highly specific and sensitive for the diagnosis and prognosis. [Life Science Journal. 2009; 6(1): 27 – 32] (ISSN: 1097 – 8135).

Keywords: SELDI-TOF-MS; support vector machine; tumor stage; Wilms' tumor

1 Introduction

Wilms' tumor, or nephroblastoma, is one of the most common childhood tumors, occurring in 718 out of 1000000 children mostly under the age of 7 in the world. The incidence of Wilms' tumor peaks at about two to three years of age[1,2]. The early onset combined with the lack of convenient screening parameters, missed diagnoses and misdiagnoses are often, hampering effective treatments. Currently, the diagnosis, clinical staging and postoperative monitoring of Wilms' tumor rely heavily on clinical symptoms and imaging techniques such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), plain X-ray pyelography of the urinary tract (urography). However, often than not, imaging diagnosis are not as accurate and informative as pathological examinations for tumor staging.

Equally urgent is to find a sensitive and convenient method to monitor patients' prognoses. Although ultrasound is the choice of postoperative monitoring, the technical aspect of this method is involving. Moreover, it fails to detect tumors with a diameter less than 2 cm[3]. In this regard, the more superior method CT scan still does not have sufficient sensitivity to detect small nodules of lymph node metastases[4]. The overall false negative diagnosis rate by CT scan is about 7%[5]. Although

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not frequent, the heterogeneous mixture of inferior vena cava contrast media administered into patients for CT scan may be mistaken as tumor thrombi, therefore leading to misdiagnosis[6]. Given together, it is of great clinical significance to develop highly specific and sensitive biological markers for early diagnosis and postoperative monitoring of Wilms' tumor.

Common treatment schemes include nephrectomy (radical and partial) or the combination of nephrectomy with postoperative chemotherapy. A study led by US National Wilms' Tumor Study Group (NWTS) showed that the combination of nephrectomy and postnephrectomy chemotherapy lead to 45.0% – 98.9% 8-year relapse-free survival for Wilms' tumor. The survival rate inversely correlates with the stage of the diseases (stage I to IV)[7]. Therefore, sensitive early diagnosis and accurate prediction of prognosis are of paramount significance in the treatment and management of Wilms' tumor patients.

In this study, we screened serum proteins in patients with Wilms' tumor at various stages, postnephrectomy patients and healthy subjects by using surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and support vector machine (SVM) and identified a set of specific markers that mark Wilms' tumor onset and progression.

2 Materials and Methods

2.1 Subjects and reagents

A total of 120 serum samples include 30 samples of Wilms' tumor patients right after diagnosis (6 cases of stage I, 10 cases of stage II, 10 cases of stage III and 4 cases of stage IV), 24 samples collected two weeks after nephrectomy (21 radical/3 partial), 23 samples collected three months after nephrectomy; 23 samples collected six months after nephrectomy and 20 samples collected from healthy children. All these cases were from the Department of Pediatric Surgery of the First Affiliated Hospital of Zhengzhou University. Tumor staging was confirmed by pathological examination. The staging criteria in the present study were based on those proposed by NWTS-5[8]. Of the 30 patients with Wilms' tumor, there were 21 males and 9 females with a mean age of 2.8 ± 0.9 years. Among the 24 patients who underwent only nephrectomies, 16 were males and 8 were females with a mean age of 2.2 ± 0.1 years. The 20 healthy subjects were age- and gender- matched to the patients with Wilms' tumors. Fasting blood samples were collected in the early morning and were kept at room temperature for 1 to 2 hours. Then the blood samples were centrifuged at 3000 rpm for 10 minutes. The resulting supernatants were stored at – 80 °C before further analyses. CHAPS, urea, DTT, NaAC, and inapinic acid (SPA) were purchased from Promega (Madison, USA).

2.2 Ciphergen ProteinChip/SELDI-TOF-MS

The serum samples were centrifuged at 4 °C at 10000 rpm for 2 minutes. The resulting supernatants, were diluted 1 : 2 with the U9 sample buffer (9 mol/L urea, 2% CHAPS and 1% DTT) and loaded on 96-well plate and agitated at 4 °C at 600 rpm for 30 minutes in a chromatography refrigerator (4 °C). Next, 185 μl of WCX2 binding buffer (100 mmol/L sodium acetate, pH 4.0) was added into each of the samples. Subsequently, 100 μl of each mixture was applied to pre-pretreated WCX2 ProteinChips (Fremont, USA) and incubated with shaking for 60 minutes in a Bioprocessor (Ciphergen, USA). Then 1 L of 50% saturated solution of SPA was loaded on each spot and air-dried for SELDI-TOF-MS analyses in our lab.

2.3 Data acquisition

The WCX2 ProteinChips were scan and quantified using the PBSII MS system (Ciphergen). The reproducibility of the SELDI system was confirmed by parallel detection with quality control serum. All data were calibrated by ProteinChip Software 3.1 that was programmed to offset sampling variations. The accuracy of protein masses of SELDI-TOF-MS was calibrated by using internal controls with molecular masses on the same ProteinChip. The ZUCI-Protein Chip Data Analyze System software package (Zhejiang University, China) was used for the following analyses. Discrete wavelet analysis and local extrema method were used to filter out noises and identify true serum protein M/Z peaks from the raw data. Next, these mass/charge (M/Z) peaks were clustered using 10% as the minimum threshold value. To evaluate the statistical significance of M/Z peak in differentiating tissue from pairs on the basis of the *P* values, Wilcoxon rank-sum test was performed. The testing standard α was set at 0.01.

2.4 SVM classification

Support vector machine is a new and powerful automatable training method based on statistical learning theory. It can automatically find the support vectors exhibiting good differentiating capacity for classification by learning algorithm. This method demonstrates better compatibility and higher accurate separation than conventional methods such as decision tree and artificial neural network[9–11]. In our study, a linear SVM classifier was used to identify peaks by adopting the following settings. Radial-based kernel function was adopted with its gamma value set at

0.6 and penalty function (C) set at 19. The selection of feature vectors was conducted by the combination of statistical filtration and model-dependent screening. In order to establish discriminating tumor markers, M/Z peaks with significant different signals were randomly combined and tested by SVM screen and subsequently cross-validated by the leaving-one out method.

3 Results

3.1 Identification of serum proteins differentially expressed in Wilms' tumor patients

Using Ciphergen ProteinChip/SELDI-TOF-MS, a total of 352 polypeptide species with distinct M/Z values were detected in 120 serum specimens from children with and without Wilms' tumors. The relative intensities of the M/S signals which represent the level of protein expression were analyzed by Wilcoxon Rank-sum test. Eleven polypeptide species with distinct M/Z peaks and *P* values of less than 0.01 were selected for further analyses. SVM was performed to screen for random combinations of different M/Z peaks to select those with the highest Youden index values for predict differential expression. Two protein species with M/Z ratios of 6984.5 and 6455.5, respectively, were identified based on their progressive down-regulation from normal controls to high stages of Wilms' tumors. The expression levels were low in patients with Wilms' tumor but were high in healthy children (Figure 1). These two protein species are designated as pM/Z6984.5 and pM/Z6455.5, respectively. Based on the analysis of two serum markers (pM/Z 6984.5, pM/Z 6455.5) in the early diagnosis model of Wilms' tumor, we found that their expression profiles in various clinical stages of Wilms' tumor were as follows: their expression followed a sequential decline manner from stage I, II, III to stage IV while their expression in stage I was low compared with healthy subjects who showed a relatively high expression of these two markers. Moreover, a more advanced clinical stage of Wilms' tumor was positively correlated with a decreased expression of these two protein markers. The average expression level of the protein marker with pM/Z 6984.5 and pM/Z6455.5 were shown in Figure 2. The differential expression of pM/Z6984.5 and pM/Z6455.5 was cross-validated by the leave-one out method. Furthermore, in the discrimination model of the test set, the combination of pM/Z6984.5 and pM/Z6455.5 was found to have 100% specificity and 100% sensitivity.

Overall, a total of 16 protein species with distinct M/Z values were identified to be differentially expressed at different stages of Wilms' tumor (Table 1). Of these characteristic M/Z peaks, two proteins with M/Z ratios of 4153.9 (pM/Z4153.9) and 3257.6 (pM/Z3257.6) could differentiate stage I/II Wilms' tumor from stage III/IV Wilms' tumor with a specificity of 93.75% and a sensitivity of 83.33%. The expression levels in patients with Wilms' tumor stage I/II were higher than in stage III/IV (Figure 3 and Table 2).

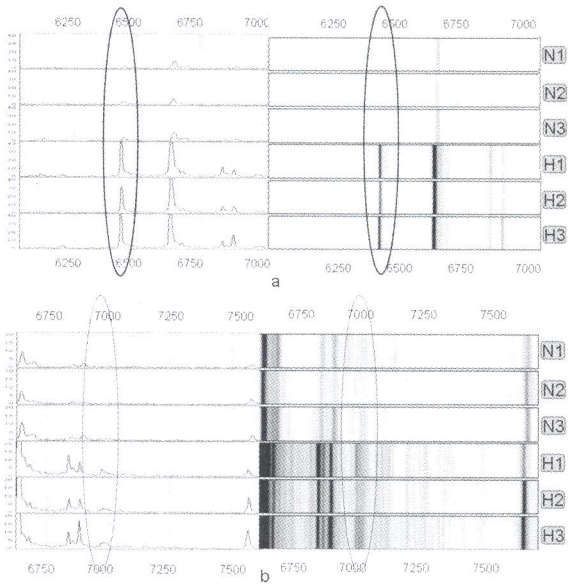

Figure 1. Differential expression of pM/Z 6455.5 (a) and pM/Z6984.5 (b) in the sera of health controls (H) and patients with Wilms' tumors (N). Each numerical number after H or N represents a different patient. In both (a) and (b), the left side showed the representative mass spectra, while the right sides showed the corresponding gel plots.

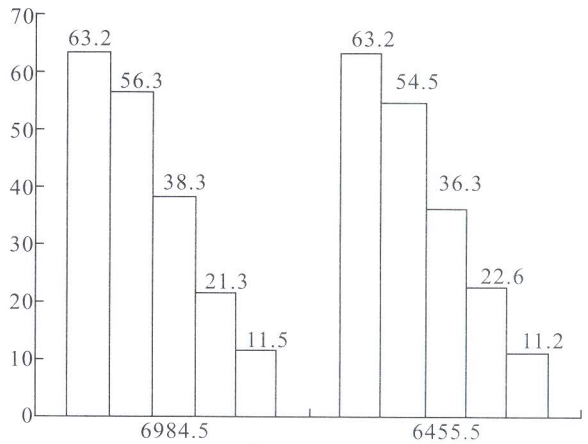

Figure 2. The expression levels of pM/Z6984.5 and pM/Z6455.5 in patients' sera. From left to right, the bards represent the data from sera of normal subjects, Stage I Wilms' tumor patients, Stage II Wilms' tumor patients, Stage III Wilms' tumor patients and Stage IV Wilms' tumor patients, respectively.

Table 1. Results of SELDI-TOF-MS

Group	Number of peaks	Number of specific peak	Sensitivity (%)	Specificity (%)
Stage I – Stage II	483	5	80.0	100.0
Stage I – Stage III	496	4	100.0	100.0
Stage I – Stage IV	565	4	88.9	100.0
Stage II – Stage III	490	3	88.9	100.0
Stage II – Stage IV	508	20	100.0	100.0
Stage IV – Stage III	504	12	93.8	100.0
Stage I + II – stage III + IV	519	3	83.33	93.75

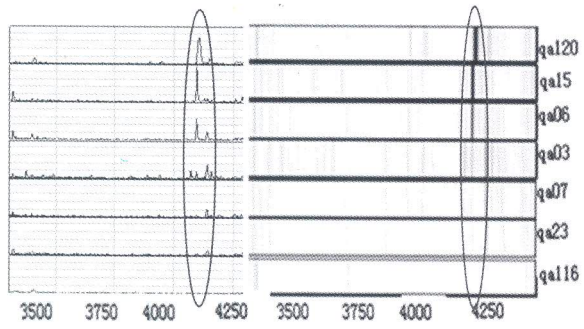

Figure 3. The stratification of Stage I & II from Stage III and IV Wilms' tumors with pM/Z4153.9 in the sera. The representative mass spectra (left) and gel plots (right) of sera samples from Stage I & II Wilms' tumor patients are labeled qa120, qa15 and qa06. The representative mass spectra (left) and gel plots (right) of sera samples from Stage III & IV Wilms' tumor patients are labeled qa03, qa07, qa23 and qa116.

Table 2. The expression of pM/Z4153.9 and pM/Z3257.6 in Wilms' tumors of different stages

Group	Case (*n*)	pM/Z4153.9	pM/Z3257.6
Stage I + II	16	1801.9 ± 427.2	4612.2 ± 1938.7
Stage III + IV	14	765.9 ± 591.0*	2454.1 ± 720.8#

*: *vs.* Stage I + II , $P = 0.011$; #: *vs.* Stage I + II, $P = 0.018$.

Using the average intensities of pM/Z6984.5 and pM/Z6455.5 in 30 samples, the staging of all 30 cases of Wilms' tumor was stratified into the following groups: six cases of Stage I, 10 cases of Stage II, 10 cases of Stage III and four cases of Stage IV. This pattern of clinical staging coincides exactly with that established by pathological examination with a concordance rate of 100.00% for each stage (Table 3). When compared to clinical staging by CT scan, the concordance rates of four stages between the two methods were 100.00%, 85.00%, 85.00% and 75.00%, respectively.

3.2 Construction of a serum protein fingerprint-based prognosis model of Wilms' tumor

To evaluate the value of pM/Z6984.5 and pM/Z6455.5 as markers for Wilms' tumor prognosis, their expression levels in serum at different post-nephrectomy time points were compared. Two weeks after the surgery, low levels of pM/Z6984.5 and pM/Z6455.5 were detected in 24 patients with their respective calibrated densitometric intensities being 525.21 ± 162.81 and 1406.62 ± 288.42. As compared with the levels of pM/Z6984.5 (1110.61 ± 213.80) and pM/Z6455.5 (2268.67 ± 1058.00) in healthy individuals, these levels were significantly lower ($P < 0.01$ for both proteins).

Two weeks after the surgery, the levels of pM/Z6984.5 and pM/Z6455.5 were significantly higher in 19 out of 21 cases with their respective calibrated densitometric intensities being 757.68 ± 194.50 and 2360.42 ± 696.28. No significant additional changes of these two proteins were noted in all the cases in the follow-up examinations three and six months after the surgery. As compared to the levels of pM/Z6984.5 and pM/Z6455.5 in healthy individuals, the postoperative levels of these proteins in 19 out of 21 patients were not significantly different ($P > 0.05$).

Interestingly, the serum levels of pM/Z6984.5 and pM/Z6455.5 in two patients remained largely unchanged ($P > 0.05$) and significantly lower than control subjects ($P < 0.01$). Such finding may be partly explained by the complexity of the biological characteristics of Wilms' tumor and individual differences. These data may also suggest the existence of residual or metastatic tumors. Indeed, in the re-examination by B mode ultrasound and CT scan performed six months after the nephrectomies, we found evidence of local recurrence in one case and lung metastases in another. One patient with metastasis eventually died of the disease.

4 Discussion

The development of Wilms' tumor is a dynamic pro-

Table 3. Clinical staging results by CT scan, protein chips and pathology

Group	Case (*n*)	Accuracy (%)	Stage I (%)	Stage II (%)	Stage III (%)	Stage IV (%)
CT scanning	30	86.7	100.0	85.0	85.0	75.0
Staging by ProteinChips	30	100.0	100.0	100.0	100.0	100.0
Pathological staging	30	100.0	100.0	100.0	100.0	100.0

cess involving a variety of genes and factors and is tightly associated with the aberrant regulation of oncogenes and tumor suppressor genes[12]. Because of the abnormal metabolic profiles arising from the changes of tumor genome, some tumor-related and/or -specific small proteins/peptides may be released into the body fluid and excreta, thus may serve as specific markers for diagnosis and prognosis. An ideal tumor marker must have high tumor specificity and quantitatively (or semi-quantitatively) reflect the tumor progression. Using the cutting edge technology of ProteinChip/SELDI-TOF-MS screening, we have identified a total of 16 specific M/Z peaks that may be potentially useful for differentiating various stages of Wilms' tumor. We further characterized two pairs of these polypeptide peaks in detail for their correlation with the stages and progression of the diseases.

The identification of the four specific serum protein markers for Wilms' tumor could have been a difficult task if the cutting-edge Ciphergen ProteinChip/SELDI-TOF-MS systematic screening were not employed. In these analyses, ionized polypeptides have different M/Z ratios, giving rise to well-resolved signals. This technique has the advantages of small sample size, easy operation, high sensitivity and high throughput[13–14]. Promising results have been reported using ProteinChip/SELDI-TOF-MS for the diagnosis of ovarian cancer[15], prostate cancer[16], breast cancer[17], lung cancer[18], liver cancer[19] and thyroid carcinoma. The key to clinical utility of the ProteinChip/SELDI-TOF-MS is the rigorous bioinformatic data processing. To ensure the accuracy of data acquisition and stratification, we used the powerful SVM for data analyses and automate training was used in combination with multiple data processing methods.

As compared with the normal controls, Wilms' tumor patients featured significantly lower levels of expression of both pM/Z6984.5 and pM/Z6455.5. The sensitivity of these two proteins for Wilms' tumor is 100% (30/30). Although it the origin of these two proteins is yet to be further confirmed, the down-regulation of pM/Z6984.5 and pM/Z6455.5 may be a gain of function in Wilms' tumor progression. If this hypothesis is validated, the recovery of these two proteins in post nephrectomy specimens may signify cancer eradication. Consistent with this notion, the steady high levels of pM/Z6984.5 and pM/Z6455.5, comparable to normal levels, coincide with tumor-free survival of the patients. In addition, ultrasound revealed that no relapse or metastasis occurred in these patients. In contrast, the persistent low post-operative levels of pM/Z6984.5 and pM/Z6455.5 correlates with tumor recurrence or metastasis. Since CT, a current method of detecting metastatic disease, may not distinguish metastasis from abnormalities of benign nature, serum detection of pM/Z6984.5 and pM/Z6455.5 alone or in combination with CT scan may significantly improve the accuracy and sensitivity in detecting recurring and metastatic Wilms' tumor.

As we continue to optimize the "fingerprint" models with all 16 M/Z peaks identified, based on the relative intensities of pM/Z4153.9 and pM/Z3257.6, Stage III and IV Wilms' tumors could be further differentiated from Stage I and II Wilms' tumors. Results from the present study indicated that the clinical staging established by ProteinChips/SELDI-TOF-MS of 30 cases was actually fully consistent with that by pathologist reports. This stratification may guide more tailored treatment strategies to improve the cure rates while minimizing unnecessary harsh treatments. The clear advantage of this serum protein "fingerprint" model is the elimination of invasive biopsy procedures.

5 Conclusion

The polypeptide fingerprints constructed in this study show great potential in the early diagnosis, clinical staging and prognosis monitoring of Wilms' tumor. The relatively small tissue samples required to conduct high throughput ProteinChips/SELDI-TOF-MS for these fingerprints makes it an attractive practical screening method in clinical application.

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