·Basic Research·

Application of surface-enhanced laser desorption/ ionization time-of-flight-based serum proteomic array technique for the early diagnosis of retinoblastoma

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Abstract

• AIM: To find new biomarkers in the sera of retinoblastoma (Rb) patients with surface-enhanced laser desorption/ ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and protein chip technique.

• METHODS: SELDI-TOF-MS, IMAC30 and CM10 protein chips were used to analyze the protein profiles from sera of 18 patients with Rb and 17 age-matched controls. The protein profiling was analyzed statistically by Ciphergen protein chip software 3.0.2. The Student's t-test was applied to compare the protein peak intensity. Fisher's exact test was used to compare the predominance of differential protein peaks appeared in patients.

• RESULTS: With IMAC30 protein chips, there were 26 proteins which appeared difference in sera of patients with Rb compared to normal children. Among them, 21 proteins, *i.e.* 7746, 7014, 11713, 3049, 7084, 7299, 5888, 2544, 12575, 5489, 9658, 9575, 9929, 10161, 8955, 1886, 10617, 6209, 2411, 7374, 6614m/z were up-regulated and 5 proteins, *i.e.* 8382,7923,7972, 8590, 66576m/z, were down-regulated ($P < 0.01$). Using the 7014m/z protein peak for statistical analysis, we could differentiate the patients with Rb from the healthy children with a sensitivity of 94.4% and a specificity of 82.4%. By CM10 protein chips, 4 proteins, including 3 up-regulated proteins (5888, 6097, 7798m/z) and 1 down-regulated protein (8590m/z), were detected in Rb patients ($P<0.01$). The sensitivity and specificity were 83.3% and 70.6% respectively when 7798m/z protein peak was selected for statistical analysis.

• CONCLUSION: There are a few candidates as Rb biomarkers in the sera of Rb patients. SELDI-TOF-MS protein chip technology could be a potential method in the clinical screening test of Rb.

• KEYWORDS: retinoblastoma; SELDI protein chip; differential protein

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INTRODUCTION

At present, the diagnosis and differential diagnosis of retinoblastoma (Rb) are mainly based on the history of the disease, clinical manifestation, computer tomography (CT) and type B ultransonography and other imaging methods. Some scholars have studied the diagnostic value of the serum neurone specific enolase (NSE) in Rb and the results showed that the sensitivity and specificity of NSE were limited[1,2]. So it is an essential and urgent task to find a better method that has higher sensitivity and specificity in the early diagnosis and screening of Rb.

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is a new, convenient and rapid method for the relative expression levels of proteins over a wide range of molecular weights in biological samples under different conditions. Compared with other proteomic technologies, it has many advantages, such as rapid way, high throughput, high sensitivity and specificity, low sample requirement. Furthermore, it can detect multiple changes of different proteins simultaneously, in particular lower-abundance proteins and some lower molecular weight proteins. As a result, it can be applied directly to detect complicated biological specimen like serum [3-5]. Our purpose is to look for protein markers in sera of Rb patients and to provide new markers for the screening, diagnosis and differential diagnosis of Rb. In addition, it can provide significant information for diagnostic accuracy of Rb and further determination of the candidate proteins in research.

MATERIALS AND METHODS

Serum Samples Thirty-five serum samples were taken from the Zhongshan Ophthalmic Centre of Sun Yat-sen University. Among them, seventeen were taken from healthy children and eighteen were taken from Rb patients confirmed by histologic examination. The healthy control group (n=17, range from 8 months to 8 years, mean 2.9 years) and Rb group (n=18) were age-matched. The Rb group was subsequently divided into the intraocular Rb group (n=14) and the extraocular Rb group (n=4). The parents of all subjects had given written informed consent to the study protocol, which was approved by the Ethics Committee of Sun Yat-sen University. All serum samples were collected in the morning before breakfast. The sera were left at 4℃ for 1 hour, centrifuged at 4 000r/min for 20 minutes and then stored at -80℃.

Reagents and Instruments Carbamide, acetonitrile, trifluoroacetic acid, sinapic acid (SPA), Tris-HCl and HEPES were purchased from Sigma Corporation (USA). The ProteinChip Biosystems (Ciphergen PBS II plus SELDI-TOF-MS), CM10 chip and IMAC30 chip were purchased from Ciphergen Biosystems Corporation (USA).

ProteinChip Array Analysis Immobilized metal affinity capture array 30 (IMAC30) were used and put into a bioprocessor. According to the reference [6], the chips were coated with 50μL of 100mmol/L $CuSO_4$ on each array and agitated for 5 minutes at room temperature, and then $CuSO_4$ was discarded. The chips were rinsed three times with deionized H_2O. 150μL of 100mmol/L sodium acetate buffer (pH 4.0) was added to each microarray, which was shaken for 5 minutes to remove the unbound Cu^{2+}. 5μL of serum was mixed with 10μL of 8mol/L urea and was vibrated for 30 minutes at 4℃. 10μL of the serum/urea mixture was applied to each array with 90μL of 100mmol/L sodium acetate buffer, incubated and shaken for 30 minutes. After the serum/urea mixture was discarded, the chips were washed twice, 5 minutes in each wash cycle, with 150μL of 100mmol/L sodium acetate buffer as described above. The chips were removed from the bioprocessor, washed twice with deionized H_2O, air-dried, and stored at room temperature until subjected to SELDI-TOF analysis.

At the same time, we employed the CM10 chip for detection. The chip was coated with 5μL serum and 10μL of 9mol/L urea. Above 5μL mixture was added to CM10 with 60μL of 100mmol/L sodium acetate buffer (pH 4.0) and 10μL of 10mmol/L HCl, placed in room temperature for 10 minutes. After washed twice, the chip was installed to bioprocessor. Next, CM10 chip was washed with 200μL buffer and shaken for 5 minutes, repeated again. 100μL serum sample was added to each spot on the chip , agitated and incubated for 1 hour at room temperature, discarded, then washed and agitated three times, 5 minutes every time. After each spot was washed with 200μL of 1mmol/L HEPES (pH 7.0) and air-dried, 0.5L SPA was applied to each spot, repeated twice, air-dried again until subjected to SELDI-TOF analysis.

Before SELDI-TOF MS analysis, the instrument was calibrated with standard polypeptide and protein marker of lower molecular mass to ensure deviation at 0.05%. Chips were analyzed by the PBS-Ⅱ plus mass spectrometer reader. Data were obtained by averaging the results from a total of 124 laser shots with an intensity of 210, a detector sensitivity of 9, a high mass of 150kDa and an optimized range of 3k-30kDa. Peaks were identified after mass calibration, and background subtraction and normalization were realized through the clustering and alignment function of ProteinChip Biomarker Wizard software 3.1 (Ciphergen Biosystems, Fremont, CA, USA).

Bioinformatics and Biostatistics Comparison between groups was performed by analysis of Student's t-test with the ProteinChip Biomarker Wizard software (Ciphergen Biosystems, Fremont, CA, USA). Values of P<0.01 were

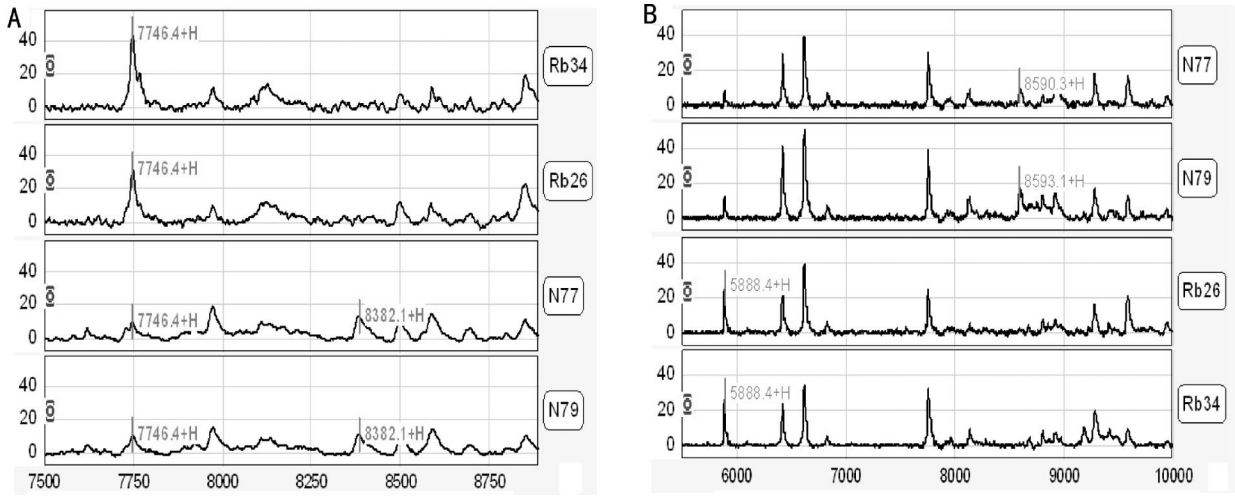

Figure 1 SELDI-TOF protein profiles of the Rb group and control group

considered statistically significant. Fisher's exact test was used to compare the predominance of differential protein peaks appeared in patients and normal children and the testing standard was set at α=0.05.

RESULTS

Two different chips, IMAC30 and CM10, were performed to analyze serum protein mass spectrogram of 18 Rb patients and 17 normal children. The results showed that there were different peaks between them.

In Figure 1A, IMAC30 protein chip showed the protein masses between 7250m/z and 8800m/z. The protein peak of 7746m/z was highly expressed in Rb group and the protein peak of 8382m/z was lowly expressed in healthy individuals. In Figure1B, CM10 protein chip showed the protein masses between 5000m/z and 10000m/z. The protein peak of 5888m/z was highly expressed in Rb group and the protein peak of 8590m/z was lowly expressed in healthy individuals. The X axis represents the molecular mass; the Y axis represents relative peak intensity. Rb represents retinoblastoma patients and N represents normal children.

We analyzed the distribution of the differential protein between Rb patients and normal children. From the distribution we can know that there was manifest regularity. For instance, in Rb group, the peak intensity at 7014m/z and 7084m/z by IMAC30 protein chip increased obviously, while the peak intensity at 7054m/z had no significant difference between Rb group and control group (Figure 2A). Otherwise, the peak intensity at 6097m/z by CM10 protein chip was up-regulated noticeably in Rb group (Figure 2B).

In Figure 2, the content of proteins of 7014, 7084 and 6097m/z increased obviously in Rb patients, but there was no statistically significant difference about content of the protein of 7054m/z between Rb patients and normal children. The result of IMAC30 chip was shown in Figure 2A and the result of CM10 chip was shown in Figure 2B. Rb represents retinoblastoma patients and N represents normal children. The X axis represents the molecular mass; the Y axis represents logarithm of relative peak intensity.

Our research results showed that 26 differential proteins were detected in the sera of Rb patients by contrast with healthy children by using IMAC30 protein chip. Of the 26 proteins, 21 differential proteins were up-regulated: 7746, 7014, 11713, 3049, 7084, 7299, 5888, 2544, 12575, 5489, 9658, 9575, 9929, 10161, 8955, 1886, 10617, 6209, 2411, 7374, 6614m/z and 5 differential proteins were down-regulated: 8382, 7923, 7972, 8590, 66576m/z. Through statistical analysis, all P values were less than 0.01(Table 1). From the results of CM10, 4 differential proteins were detected in Rb patients. Among them, 3 differential proteins increased and 1 protein decreased in the sera of Rb patients. Statistically, all P values were less than 0.01 (Table 2). In addition, we respectively used the differential proteins of 7014m/z and 7798m/z to carry out a statistical test, and results showed that the former had a specificity of 82.4% and a sensitivity of 94.4%, and the latter had a specificity of 70.6% and a sensitivity of 83.3%.

DISCUSSION

Retinoblastoma is the most common intraocular malignancy in infant and children, which can lead to the blindness, or even death. Currently, at clinic, most Rb has entered intermediate stage, or even terminal stage when it is diagnosed. For these Rb patients, enucleation is still the

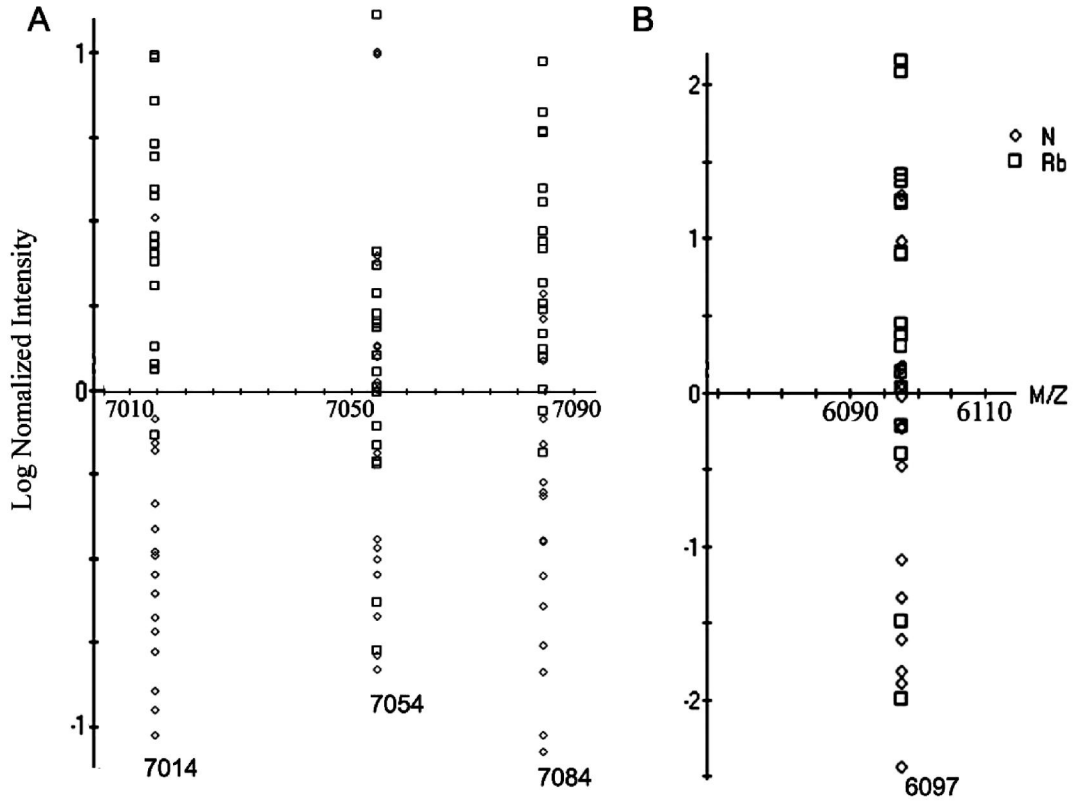

Figure 2 Protein peak intensity results of IMAC 30 chip and CM10 chip

Table 1 Differential proteins in sera of Rb patients screened by IMAC30 chip (mean±SD)

m/z	healthy adults	Rb patients	change tendency of marker protein in sera of Rb patients
7746	14.17±5.08	31.22±10.88	↑
7014	4.38±1.34	8.46±1.84	↑
11713	8.04±2.27	15.29±3.90	↑
3049	2.18±2.29	9.88±5.38	↑
7084	4.50±1.26	7.65±1.76	↑
7299	4.35±0.87	6.89±1.66	↑
5888	1.10±0.74	6.29±5.34	↑
2544	2.12±2.10	8.83±4.56	↑
12575	4.22±1.58	9.46±4.48	↑
5489	1.65±1.40	5.30±2.99	↑
9658	3.82±1.64	7.45±2.29	↑
9575	19.85±9.30	37.52±8.78	↑
9929	4.68±3.07	9.33±2.00	↑
10161	4.30±2.03	8.35±1.88	↑
8955	29.58±11.67	50.66±10.94	↑
1886	1.28±2.76	6.33±3.86	↑
10617	3.80±1.95	6.88±2.11	↑
6209	3.65±1.54	6.14±1.82	↑
2411	6.81±4.20	15.74±8.38	↑
7374	3.67±0.86	5.21±1.47	↑
6614	3.82±1.64	6.93±3.05	↑
8382	11.76±5.10	3.40±1.94	↓
7923	6.32±3.32	1.43±1.03	↓
7972	18.10±5.34	11.84±2.84	↓
8590	16.83±6.38	10.62±3.13	↓
66576	19.66±5.25	12.65±4.57	↓

Note: all $P<0.01$

Table 2 Differential proteins in sera of Rb patients screened by CM10 chip (mean±SD)

m/z	healthy adults	Rb patients	change tendency of marker protein in sera of Rb patients
5888	12.73±9.38	20.96±10.67	↑
6097	0.80±0.83	2.07±1.38	↑
7798	3.30±1.10	5.13±2.60	↑
8590	5.13±5.15	2.82±5.42	↓

Note: all $P<0.01$

main therapeutic approach, which results in disfigurement of face and physical disability, and the cure rate is relative low[7,8]. In addition, the differential diagnosis between some atypical Rb, especially ectogenous Rb, and Coat' s disease is very difficult, because these two diseases represent leukocoria[3,9]. If retinoblastoma was misdiagnosed as other disease, it would be threatening children's life on account of the delayed treatment; while if other diseases were misdiagnosed as retinoblastoma, the patients would be wrongly performed enucleation, which would lead to lifetime disability. So it is an urgent task for an ophthalmologist to make an earlier correct diagnosis and differential diagnosis of retinoblastoma. Regarding the molecular targets of Rb, some scholars had studied the relationship between lactate dehydrogenase, neuron specific enolase (NSE) and retinoblastoma in serum and aqueous humor. Hervas *et al*[1] measured the content of

NSE in sera from 17 retinoblastoma patients and 8 healthy adults by radioimmunity method and found that serum NSE level of 88% retinoblastoma patients was obviously higher than that of healthy adults. Wu *et al* [2] measured the content of NSE in sera from 23 retinoblastoma patients and 25 healthy adults by electrophoretic technique and found that NSE was detected in 78.2% retinoblastoma patients. Moreover, NSE exists not only in tumor tissue and blood of retinoblastoma patients but also in nervous tissue like retina. Besides, some diseases such as trauma, inflammation, ischemia, hypoxia in nervous system, small cell lung cancer, neuroendocine tumor and so on, can also give rise to the ascent of NSE content in blood [10-12]. So the value of NSE is limited in early diagnosis and differential diagnosis of retinoblastoma. Furthermore, lactate dehydrogenase has a lower specificity and it is difficult to be applied widely in clinic. And genetic diagnosis of retinoblastoma is only suitable for the patients with Rb genetic mutation in germinal cell. However, about 60% Rb patients are not genetic type.

In recent years, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) and protein chip technique have been widely applied in the field of searching biomarkers for early cancer detection [13-18]. The mechanism of this technology is laser desorption/ ionization time-of-flight mass spectrometry and protein chips made of a variety of chromatographic surfaces were used to selectively retain polypeptides and proteins over a wide range of relative molecular mass from 1kDa to 500kDa from crude extracts. It is of a rapid, simple, sensitive and high-throughput character and can detect many samples simultaneously. It can be applied directly to detect biological specimen, such as urine, blood, cerebrospinal fluid, cell disruption fluid and all kinds of secretion. Adam and his associates [13] reported promising results with sensitivity of 83% and specificity of 97% using the SELDI-TOF pattern for serum biomarkers of prostate cancer detection. Using the same technology in prostate cancer, Pan *et al* [19] found 18 marker proteins. Of the 18 proteins, four proteins of 15265, 15868, 16003, 16068m/z had high expression and the other 14 proteins had low expression. Based on the results, the classification tree for diagnosis of prostate cancer can distinguish 96.386% patients and 92.632% normal persons. Also, Kanmura *et al* [20] found 6 marker proteins with sensitivity of 83% and specificity of 76% when they made a study about early diagnosis of hepatocellular carcinoma (HCC) using the SELDI-TOF pattern. These studies demonstrated that the SELDI-TOF pattern had a broad clinical application prospect in the diagnosis of tumor, but its reproducibility and reliability need the standardization of detecting parameter and further clinical verification [21]. Yet, the proteomic approach has not been used to identify the protein markers of retinoblastoma at present. Our study chose two different protein chips which had different chemical modification surface in order to increase the number of captured proteins and provide valuable information about physicochemical property for marker proteins. IMAC30 chip can capture phosphorylated protein or/and metal-bound proteins while CM10 can capture mainly hydrophilic proteins with high isoelectric point. Our results showed that IMAC30 chip captured 26 differential proteins while CM10 chip captured 4 differential proteins. Among them, two proteins, 5888m/z and 8590m/z, were captured by the two different protein chips simultaneously, which demonstrated that some differential proteins had many different physicochemical properties and were captured by different chips because of its structural difference. Therefore, using different chips can make a supplementary effect and capture more differential proteins. Our study showed that there were rich phosphorylated and metal-bound differential proteins in sera of Rb patients.

Using the SELDI-TOF pattern, we detected serum samples from 18 Rb patients and 17 age-matched normal children and found that, among 28 differential protein peaks obtained from IMAC30 chip and CM10 chip, 23 peaks were expressed highly and 5 peaks were expressed lowly. We respectively used the differential protein 7014m/z and 7798m/z to carry out a statistical test, and results showed that the former had a specificity of 82.4% and a sensitivity of 94.4% and the latter had a specificity of 70.6% and a sensitivity of 83.3% . These results hint that there are specific marker proteins in sera of Rb patients and SELDI-TOF-MS and protein chip technique is possible to become a new important method for screening, early diagnosis and differential diagnosis of retinoblastoma. However, we still need to identify what on earth the differential proteins are by some other methods and increase the number of samples to further confirm its validity and reliability in clinical application.

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