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Serum protein fingerprinting coupled with artificial neural network distinguishes glioma from healthy population or brain benign tumor*

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Abstract: To screen and evaluate protein biomarkers for the detection of gliomas (Astrocytoma grade I–IV) from healthy individuals and gliomas from brain benign tumors by using surface enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) coupled with an artificial neural network (ANN) algorithm. SELDI-TOF-MS protein fingerprinting of serum from 105 brain tumor patients and healthy individuals, included 28 patients with glioma (Astrocytoma I–IV), 37 patients with brain benign tumor, and 40 age-matched healthy individuals. Two thirds of the total samples of every compared pair as training set were used to set up discriminating patterns, and one third of total samples of every compared pair as test set were used to cross-validate; simultaneously, discriminate-cluster analysis derived SPSS 10.0 software was used to compare Astrocytoma grade I–II with grade III–IV ones. An accuracy of 95.7%, sensitivity of 88.9%, specificity of 100%, positive predictive value of 90% and negative predictive value of 100% were obtained in a blinded test set comparing gliomas patients with healthy individuals; an accuracy of 86.4%, sensitivity of 88.9%, specificity of 84.6%, positive predictive value of 90% and negative predictive value of 85.7% were obtained when patient's gliomas was compared with benign brain tumor. Total accuracy of 85.7%, accuracy of grade I–II Astrocytoma was 86.7%, accuracy of III–IV Astrocytoma was 84.6% were obtained when grade I–II Astrocytoma was compared with grade III–IV ones (discriminant analysis). SELDI-TOF-MS combined with bioinformatics tools, could greatly facilitate the discovery of better biomarkers. The high sensitivity and specificity achieved by the use of selected biomarkers showed great potential application for the discrimination of gliomas patients from healthy individuals and glioma from brain benign tumors.

Key words: Astrocytoma, Artificial Neural Network (ANN), SELDI-TOF-MS, Protein fingerprint, Diagnosis
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INTRODUCTION

National Cancer Institute cancer incidence data and National Center for Health Statistics mortality data of the American Cancer Society were used as basis to estimate that brain tumor and other nervous system tumors are expected to account for 1.4% (18300) of all new cancer cases and that 13100 will die from this disease (Jemal *et al*., 2003). New biomarkers that could be used individually or in combination with an existing modality for cost-effective screening of brain tumor are still unavailable. Although modern imaging has greatly improved diagnosis of brain tumor, unfortunately, the effective biomarkers for diagnosis of brain tumor are still lacking, especially the biomarkers for the determination of tumor characteristic. Glioma is the most common malignant brain tumor, so this type of brain tumor is selected to serve as study object. Advances have been made in mass spectrometry to achieve high-throughput separation and analysis of proteins (Merchant and Weinberger, 2000; Wiesner, 2004), so study of proteomics presents a new horizon for bio-

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marker discovery. One of the recent advances is the ProteinChip® System manufactured by Ciphergen Biosystems, Inc. This system uses surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) to detect proteins affinity-bound to a ProteinChip Array. This system is a novel, extremely sensitive, and rapid method for analyzing complex mixtures of proteins and peptides. Initial studies established the potential of SELDI for discovery and profiling of many malignant tumors such as prostate cancer, breast cancer, ovarian cancer, etc. in body fluids and cell lysates (Cazares *et al*., 2002; Bast, 2003; Li *et al*., 2002; Mannes *et al*., 2003).

SELDI-TOF MS offers high-throughput protein profiling. Like many other types of high-throughput expression data, protein array data are often characterized by a large number of variables (the mass peaks) relative to a small sample size. An important issue in analyzing such data to screen for disease-associated biomarkers is to extract as much information as possible from a limited number of samples and to avoid selecting biomarkers whose performances are influenced mostly by non-disease-related artifacts in the data. The effective and appropriate use of bioinformatics tools has become a very critical issue (Srinivas *et al*., 2002).

We report the use of SELDI with H4 (hydrophobic interactions) ProteinChip Arrays to screen for potential serum biomarkers for Astrocytoma. One hundred and five retrospective serum samples were analyzed on a ProteinChip Reader Model PBS II (Ciphergen Biosystems). The complex protein profiles were analyzed using an artificial neural network (ANN) algorithm of bioinformatics tools and discriminant analysis SPSS 10.0 software.

MATERIALS AND METHODS

Sample preparation

A total of 105 retrospective serum samples from patients with Astrocytoma, brain benign tumors and healthy individuals were obtained from the Second Affiliated Hospital of Zhejiang University. All brain tumors consisted of 65 serum samples which were all confirmed pathologically (and included 28 cases of Astrocytoma, 32 cases of Mingioma, 2 cases of Neurillemmoma, 3 cases of Hemangioma). Specimens were obtained before treatment; normal controls consisted of 40 age-matched unaffected healthy individuals. The median age of the total 28 Astrocytoma patients was 43.4 years (range 5–82 years), in which grade I–II Astrocytoma occurred in 15 cases, grade III–IV occurred in 13 cases (grade definition based on WHO, 2000); the median age of the total 37 brain benign tumor was 52.0 years (range 28–77 years); the median age of the healthy individuals was 47.0 years (range 21–70 years); all samples were stored at −80 °C until used.

ProteinChip Array analysis

All serum specimen tubes were thawed in wet ice and centrifugated at 5000 rpm for 5 min, sampled for 10 μl and buffered with 90 μl of 0.5 percent CHAPS (pH 7.4) for 5 min, to which was added 100 μl Cibcron blue 3.0 G (Sigma, USA) and vortexed at 4 °C for 60 min on a platform shaker, then 50 μl samples were taken and diluted with 20 mmol/L HEPES to 240 μl (total reaction volume) and applied to each spot on the Protein Chip Array by a 96-well bioprocessor (Ciphergen, which can hold 12 pieces of chips). After the samples were allowed to bind at 4 °C for 60 min on a platform shaker, the array was washed twice with 200 μl of 20 mmol/L HEPES for 5 min, followed by two quick rinses with 200 μl of distilled H_2O. After air-drying, 0.5 μl of CHCA (saturation in 50% acetonitrile and 0.5% trifluoroacetic acid) were applied twice to each spot. Proteins bound to the H4 chips (through hydrophobic amino acids) were detected with the ProteinChip Reader. Data were collected by averaging 80 laser shots with intensity of 155 and detector sensitivity of 8.

Bioinformatics and biostatistics

All spectra were compiled, and qualified mass peaks (signal-to-noise ratio>5) with mass-to-charge ratios (m/z) between 2000 and 30000 were autodetected. Peak clusters were completed using second-pass peak selection (signal-to-noise ratio>2, within 0.3% mass window), and estimated peaks were added. The peak intensities were normalized to the total ion current of m/z between 2000 and 30000. All these were performed using Biomarker Patterns Software 3.1 (Ciphergen) to compute and rank the contribution of each individual peak toward the op-

timal separation of every diagnostic group. SELDI-TOF-MS can produce thousands of peaks that mostly represent the serum proteins and peptides but also contain the signals generated from the CHCA, the in-sample and sample-to-sample variations. To remove these signals, we excluded all the signals with m/z values below 2000. The in-sample variations observed at different times were within 10% and so was satisfactory for the reproducibility of the protein profiling in the study. The ANN used input and output data (samples for training set) to define (learn) the interrelationships among the data. Once the ANN has been trained, it could then predict outcomes from new sets of input data (samples for test set).

To identify potential biomarkers that can detect gliomas from healthy individuals, 28 protein profiles of specimens from Astrocytoma patients were compared against those of 40 cases from healthy individuals; in order to identify potential biomarkers that can detect Astrocytomas from brain benign tumors, 28 protein profiles of specimens from Astrocytoma were compared against those of 37 cases from brain benign tumors. The collected protein mass-dependent velocities (m/z) peaks were analyzed using an ANN algorithm. Once the panel of biomarkers was selected, their ability was evaluated using the set-aside independent test data set. In this procedure, the patient data set was repeatedly divided through random sampling into a training set (comprising 2/3 of total cases) to set up the diagnosis models and a test set (comprising 1/3 of total cases) for cross-validation and computation of sensitivities and specificities.

As the Astrocytoma sample size was small, the discriminant analysis derived SPSS 10.0 software was used to identify potential biomarkers that can detect grade I–II Astrocytomas from grade III–IV ones, 15 protein profiles from grade I–II Astrocytoma patients were compared against 13 protein profiles from grade III–IV Astrocytoma patients.

RESULTS

Peak detection and data preprocessing

Serum proteins retained on the H4 arrays were analyzed on a PBS II mass reader. The high mass to acquire was set to 30 kDa, with an optimization range from 2 kDa to 30 kDa. A mass accuracy of 0.1% was achieved by external calibration using the All-In-1 Protein Standard (Ciphergen). Among the total of qualified mass peaks (signal-to-noise ratio>5) detected, peaks had m/z values between 2 kDa and 30 kDa, Peak intensity was normalized to total ion current (2–30 kDa).

Biomarker selection

1. Biomarker selection based on Astrocytoma patients vs healthy individuals

To identify biomarkers with potential for detection of gliomas from healthy individuals, separability between the two groups was achieved by using ANN algorithm linered combinations of all 117 mass peaks. The glioma patients were separable from the healthy individuals group when the entire protein profiles were compared. A total of 68 cases were randomly divided into training set and test set; 45 cases (comprising 2/3 of total cases) were used as training set, 23 cases (comprising 1/3 of total cases) were used as test set; by mean of further auto-optimizing, a combination of fifteen peaks (8214.77 m/z, 8926.76 m/z, 4815.11 m/z, 8612.23 m/z, 2082.19 m/z, 4299.87 m/z, 2103.55 m/z, 7764.82 m/z, 2368.19 m/z, 3226.97 m/z, 2389.55 m/z, 2021.78 m/z, 4469.09 m/z, 6457.054 m/z, 8702.416 m/z) were selected to serve as final discriminating biomarkers. The sensitivities and specificities cannot be improved if the other mass peaks of the other 117 cases were added. On the test set (through cross-validation) the accuracy was 95.7% (22/23), the sensitivity was 88.9% (8/9, one case of grade I–II Astrocytoma was misdiagnosed as healthy individuals), the specificity was 100% (14/14) (Fig.1, Fig.2, Table 1).

2. Biomarker selection based on Astrocytomas vs brain benign tumors

To identify biomarkers with potential for detection of Astrocytoma from brain benign tumor as the control group, separability between the two groups was ANN algorithm linered combinations of all 223 mass peaks. The Astrocytoma was separable from the brain benign tumor when the entire protein profiles were compared. A total of 65 cases were randomly divided into training set and test set; 43 cases (comprising 2/3 of total cases) were used for training set, 22 cases (comprising 1/3 of total cases) were used for test set, by means of further auto-optimizing, a combination of twenty-two peaks (2256.76 m/z, 23481.05

Table 1 Grouping material of Astrocytoma patients and healthy individuals; mean and standard deviation (*SD*)

m/z	Astrocytoma patients (mean±*SD*)	Healthy individuals (mean±*SD*)	*P* value (*t*-test)
8214.774	0.70892±0.421845	1.784804±1.298162	5.12E-07
8926.763	2.78242±1.255198	4.009776±1.129363	5.74E-06
4815.106	4.237016±1.406863	7.01661±3.539101	2.40E-05
8612.234	1.568096±0.553037	2.407218±0.94228	0.000226
2082.189	4.20321±2.001461	8.937125±6.094717	0.000402
4299.871	2.807823±1.098875	3.915422±1.4436	0.000557
2103.549	1.945323±1.317936	3.139134±1.355257	0.000802
7764.823	0.596946±0.246297	0.977006±0.507807	0.001422
2368.193	7.877061±4.33783	5.041035±2.77071	0.003691
3226.972	6.0137±2.730695	4.442262±1.871277	0.010259
2389.547	2.728048±1.289943	1.91857±1.102702	0.01102
2021.778	13.93398±9.663456	17.32217±8.475961	0.012695
4469.088	3.583581±1.807865	4.78325±2.204768	0.013147
6457.054	7.442496±2.767541	5.882679±2.139846	0.013147
8702.416	1.740826±0.56574	2.144117±0.715842	0.016173

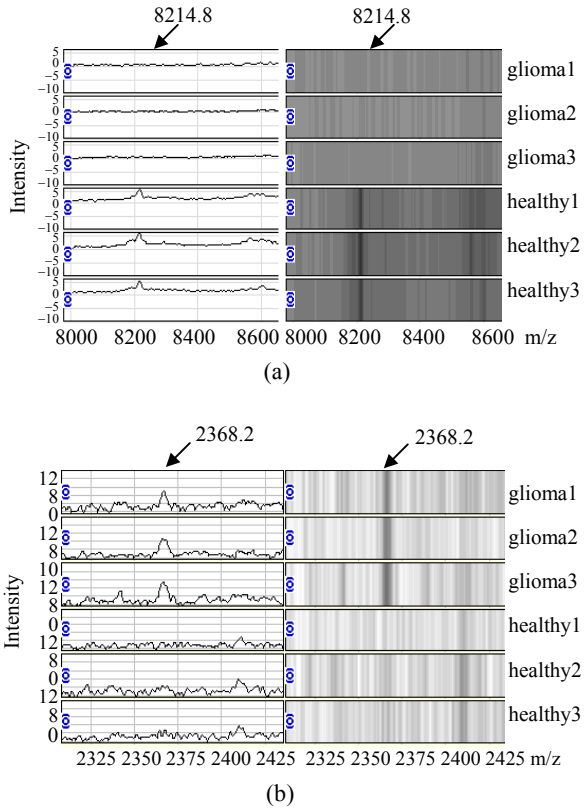

Fig.1 Representative spectra and gel views of the selected biomarkers of Astrocytoma patients and healthy individuals. (a) The biomarker of 8214.8 m/z; (b) The biomarker of 2368.2 m/z

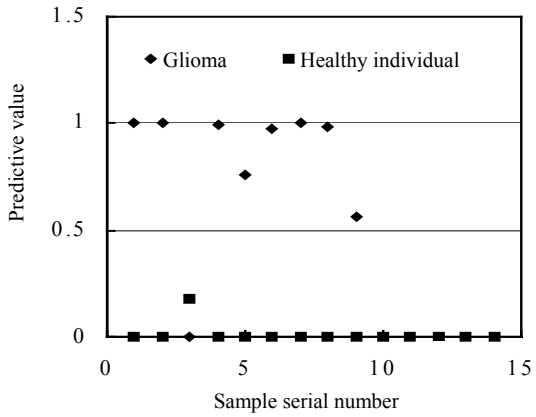

Fig.2 Distribution of the cases of test set across the couple of Astrocytoma patients and healthy individuals by the selected biomarkers of fifteen peaks (8214.77 m/z, 8926.76 m/z, 4815.11 m/z, 8612.23 m/z, 2082.19 m/z, 4299.87 m/z, 2103.55 m/z, 7764.82 m/z, 2368.19 m/z, 3226.97 m/z, 2389.55 m/z, 2021.78 m/z, 4469.09 m/z, 6457.054 m/z, 8702.416 m/z). When predictive value>0.5, the cases were judged as Astrocytoma; when predictive value≤0.5, the cases were judged as healthy individuals. In the figure, only one case of Astrocytoma was misjudged, and the rest were all judged correctly

m/z, 9198.31 m/z, 22513.91 m/z, 22888.73 m/z, 23087.61 m/z, 4155.28 m/z, 2489.11 m/z, 2246.47 m/z, 2617.14 m/z, 15099.38 m/z, 22666.41 m/z, 29047.13 m/z, 14378.33 m/z, 24002.85 m/z, 2891.43 m/z, 14047.77 m/z, 2006.00 m/z, 14951.04 m/z,

2267.22 m/z, 23672.58 m/z and 22331.29 m/z) were selected to serve as final discriminating biomarkers, the sensitivities and specificities cannot be improved if the other mass peaks of the rest of the 223 cases were added. The accuracy was 86.4% (19/22), the sensitivity was 88.9% (8/9, one case of grade I–II Astrocytoma was misdiagnosed as brain benign tumor), and the specificity was 84.6% (11/13, one case of brain benign tumor was misdiagnosed as grade I–II Astrocytoma) (Fig.3, Fig.4, Table 2).

3. Biomarker selection based on grade I–II Astrocytoma vs grade III–IV Astrocytoma

To identify biomarkers with potential for detection of grade I–II Astrocytomas from grade III–IV ones, separability between the two groups was achieved by using the discriminant analysis derived SPSS 10.1. linered combination of all 189 mass peaks. The grade I–II Astrocytomas were separable from the grade III–IV ones group when the combined biomakers of 2293.02 m/z, 2921.87 m/z and 16947.5m/z were used. A total accuracy of 85.7%, an accuracy of I–II Astrocytoma was 86.7%, an accuracy of III–IV Astrocytoma was 84.6% were obtained when comparing the grade I–II Astrocytomas versus grade III–IV ones (Table 3, Table 4).

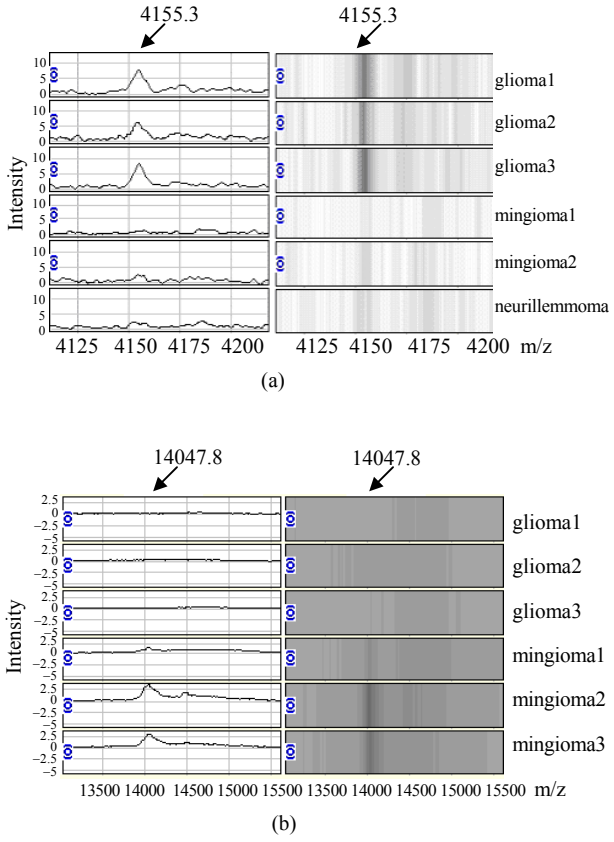

Fig.3 Representative spectra and gel views of the selected biomarkers of Astrocytoma and benign brain tumor. (a) The biomarker of 4155.3 m/z; (b) The biomarker of 14047.8 m/z

DISCUSSION

Because of the multifactorial nature of cancer, it is very likely that a combination of several markers will be necessary to effectively detect and diagnose cancer. To look for such "fingerprints" of cancer, it will require not only high-throughput genomic or proteomic profiling, but also sophisticated bioinformatics tools for complex data analysis and pattern recognition (Zhukov *et al*., 2003).

Taking advantage of the recent development in SELDI and ANN algorithm, we were able to simultaneously analyze the protein profiles of 105 serum samples from patients with glioma (Astrocytoma grade I, II, III, IV), brain benign tumor and healthy individuals. The Biomarker Patterns^{TM} Software allows evaluation of each mass peak according to its

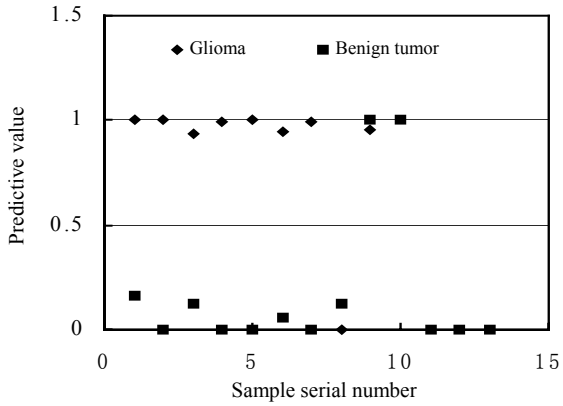

Fig.4 Distribution of the cases of test set across the couple of Astrocytoma and brain benign tumor by the selected biomarkers of 22 peaks (2256.76 m/z, 23481.05 m/z, 9198.31 m/z, 22513.91 m/z, 22888.73 m/z, 23087.61 m/z, 4155.28 m/z, 2489.11 m/z, 2246.47 m/z, 2617.14 m/z, 15099.38 m/z, 22666.41 m/z, 29047.13 m/z, 14378.33 m/z, 24002.85 m/z, 2891.43 m/z, 14047.77 m/z, 2006.00 m/z, 14951.04 m/z, 2267.22 m/z, 23672.58 m/z, 22331.29 m/z). When predictive value>0.5, the cases were judged as Astrocytoma; when predictive value≤0.5, the cases were judged as brain benign tumor. In the figure, one case of Astrocytoma and two cases of brain benign tumor were misjudged, and the rest of the cases were all judged correctly

Table 2 Grouping material of Astrocytoma and brain benign tumor; mean and standard deviation (*SD*)

m/z	Astrocytoma (mean±*SD*)	Brain benign tumor (mean±*SD*)	*P* value (*t*-test)
2256.757	2.893485±1.38871	4.052768±1.257162	0.001067
23481.05	0.109464±0.059989	0.141506±0.057924	0.002637
9198.313	1.267601±0.405923	1.552553±0.345406	0.003877
22513.91	0.282157±0.135811	0.363743±0.120654	0.004396
22888.73	0.201084±0.108307	0.263462±0.098198	0.00586
23087.61	0.169551±0.089946	0.213471±0.084181	0.006102
4155.284	4.653484±4.624575	5.916488±3.506948	0.006612
2489.105	2.096568±1.071251	1.389716±1.071954	0.007161
2246.465	2.55764±1.116384	1.839567±1.063831	0.00775
2617.139	1.538691±0.886599	1.012643±0.841443	0.008382
15099.38	0.323022±0.172705	0.43022±0.170605	0.00906
22666.41	0.244899±0.12878	0.314646±0.114315	0.009417
29047.13	0.029825±0.022439	0.047416±0.037989	0.009417
14378.33	0.388368±0.192627	0.505757±0.200205	0.010169
24002.85	0.06224±0.035671	0.07909±0.035296	0.011834
2891.429	1.361056±0.75447	0.890755±0.802495	0.013737
14047.77	0.624487±0.576495	0.863565±0.697355	0.015337
2006.001	2.745216±1.394223	1.932315±1.291929	0.015906
14951.04	0.412403±0.227632	0.539074±0.230404	0.017099
2267.218	2.801537±1.229789	2.046149±1.347649	0.019036
23672.58	0.084756±0.049776	0.106556±0.049661	0.019036
22331.29	0.340582±0.16395	0.414451±0.137132	0.023491

Table 3 The cross-validated result of discriminate-cluster analysis comparing the grade I–II Astrocytomas versus grade III–IV Astrocytomas

	Grade I–II	Grade III–IV	Total
Grade I–II (predicted)	13 (86.7%)	2 (15.4%)	15
Grade III–IV (predicted)	2 (13.3%)	11 (84.6%)	13
Total	15	13	28

Table 4 Grouping material of I–II Astrocytoma and III-IV Astrocytoma; mean and standard deviation (*SD*)

m/z	I–II Astrocytoma (mean±*SD*)	III–IV Astrocytoma (mean±*SD*)	*P* value (*t*-test)
2293.02	1.151773±1.030139	–0.07368±0.700751	0.001875
16947.52	0.052605±0.030456	0.081819±0.038258	0.032190
2921.87	1.032694±0.876525	1.762163±0.997416	0.045087

collective contribution toward the maximal separation of every two groups compared. These three models led to the identification of several discriminatory biomarkers respectively that, in combination, achieved both high sensitivity (>85%) and high specificity (>85%).

In this study, the ANN algorithm provided an efficient model to rank a large number of peaks collectively according to their contribution to the separation of two predefined diagnostic groups. The ANN models introduced random perturbations in multiple runs to test the consistency of the top-ranked peaks, measured by the *P* value of m/z peaks of computed ranks from multiple runs. The models established based on these selected biomarkers should be further validated independently, In such studies, validation data sets preferably should be from sources different from that of the original training data set. This is one way to ensure that the performance of the selected biomarkers is not influenced by systematic biases

between different groups (Ball *et al*., 2002).

The clinical diagnosis of brain tumor is mainly bases on imaging techniques, but sometimes there is still need to identify the characteristic of the lesions, especially before treatment. Unfortunately, the biomarkers for qualitative diagnosis of brain tumor are lacking (DeAngelis, 2001; Samoylova *et al*., 2003). In the study, we screened 22 biomarkers for identification of glioma from brain benign tumors whose sensitivity and specificity were 88.9% and 86.4% respectively. Meanwhile, because grade I–II Astrocytoma tend to be benign and grade III–IV Astrocytoma tend to be malignant in biological behavior, discriminating these two biomarkers is very important for treatment and prognosis clinically. We screened 3 biomarkers for identification of grade I–II Astrocytomas from grade III–IV ones, the total accuracy was 85.7%, accuracy for I–II Astrocytoma was 86.7%, accuracy for III–IV Astrocytoma was 84.6%, so these selected biomarkers may have great potential applications in the qualitative diagnosis of brain tumor before operation; and can provide helpful parameters for choosing chemotherapy, or radiotherapy before operation or the resecting range in the operation.

Although high-throughput profiling of complex protein expression patterns greatly facilitates the screening of a large number of potential markers simultaneously, for most currently available datum sets, the sample sizes are relatively small compared with the total number of detected mass peaks. The limited specimens size analyzed in this study may influence the validity of the results to some degree, so the diagnostic models established based on these selected biomarkers should be further validated independently by other new specimens, on the other hand, the early diagnosis of glioma is recommended (Petricoin and Liotta, 2004).

In conclusion, using proteomics approaches such as Ciphergen ProteinChip Arrays and SELDI-TOF-MS in combination with bioinformatics tools could facilitate the discovery of new biomarkers. High sensitivities and specificities could be achieved by using panels of selected biomarkers for the detection of gliomas from brain tumors or from healthy individuals, especially for application to qualitative diagnosis of brain tumor before operation.

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