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Analysis of the raw serum peptidomic pattern in glioma patients

Zheng Li [a], Hongmei Lu [b], Jing Yang [a], Xi Zeng [c], Lian Zhao [d], Hongdong Li [b], Qianjing Liao [c], Shuping Peng [a], Ming Zhou [a], Minghua Wu [a], Juanjuan Xiang [a], Yanjin Wang [e,*], Guiyuan Li [a,**]

[a] Cancer Research Institute, Key Laboratory of Carcinogenesis of Ministry of Health, Central South University, 110 Xiangya Road, Changsha, Hunan, 410078, PR China
[b] Research Center of Modernization of Chinese Medicines, College of Chemistry and Chemical Engineering, Central South University, Changsha, Hunan, 410078, PR China
[c] Cancer Research Institute, University of South China, Hengyang, 421001, PR China
[d] Department of Gastroenterology, Third Xiangya Hospital, Central South University, Changsha, 410013, PR China
[e] Department of Neurosurgery, Xiangya Hospital, Central South University, Changsha, 410013, PR China

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Background: Glioma is a common and lethal type of brain tumor. Serum peptides reflected the pathological changes of the body. Here we studied the serum peptide profiles to distinguish glioma disease and measure glioma staging.

Methods: Serum peptides were captured by WCX magnetic beads and were analyzed by MALDI-TOF mass spectrometer. Sera from 53 glioma patients and 69 age-matched healthy controls were analyzed. Clinpro Tools software was used to obtain a common peak *m/z* list from all measured samples. An optimal subset of peptides was selected to establish a predictive classification model with the newly developed competitive adaptive reweighted sampling (CARS) variable selection method. Serum peptide profiles were classified through a partial least-squares–linear discriminate analysis (PLS–LDA). We also searched for progressively different peptide peaks that correlated with an increasing malignancy of glioma.

Results: The following pattern recognition equation was established with selected peptide signals: $Y = -0.1113 - 0.113X_1 - 0.2916X_2 + 0.1128X_3 - 0.2057X_4 - 0.2047X_5 - 0.3048X_6 + 0.2835X_7 + 0.3121X_8 - 0.1458X_9 + 0.0354X_{10} - 0.2022X_{11}$. Using this pattern, classification sensitivity and specificity achieved were 0.9057 and 0.9855, respectively. Additionally, we detected 3 peptide signals that correlated with glioma grade. Among these, the intensity of peak 2082.32 Da correlated positively with the glioma progressing, and peaks with sizes of 3316.08 Da and 6631.45 Da show a decreasing intensity with increasing glioma grade.

Conclusions: 11 peptide recognition patterns and specific peak intensities might be useful for the early detection and tumor staging of glioma, but they need to be further validated and evaluated independently in clinical settings.

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1. Introduction

Glioma is the most common subtype of primary brain tumors in adult humans. Glioma is classified as astrocytomas, oligodendrogliomas and ependymomas, according to the affected glial cell type. Based on the differentiation and prognosis, gliomas are categorized into 4 grades by the World Health Organization (WHO). Glioma designated as grades I and II are well-differentiated and have better patient prognoses, with a median survival time of approximately 5–10 years [1]. Glioma designated as grades III and IV are undifferentiated or anaplastic with worse prognoses. In spite of advances in surgery, chemotherapy, radiotherapy and combined treatment approaches, the prognosis for grade IV glioblastoma is still very poor, with a median survival time of approximately 11 months [2]. The methods used to assist with glioma diagnosis include computed tomography (CT), magnetic resonance imaging (MRI), angiograms, skull x-rays, spinal taps, myelograms and histological examinations. However, glioma mortality rates remain high. Increasing evidence indicates that some biomarkers could be used as tools for the diagnosis and prognosis of glioma. Glial fibrillary acidic protein (GFAP) is expressed by the astroglial cells, neural stem cells, astrocytomas and glioblastoma multiforme [3–5]. Serum GFAP levels are noticeably elevated in glioblastoma multiforme patients, compared with the levels in non-GBM tumor patients and healthy controls [5]. Additionally,

Abbreviations: MALDI-TOF MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; MS, mass spectrometry; CARS, competitive adaptive reweighted sampling; PLS–LDA, partial least-squares–linear discriminate analysis; MB-WCX, weak cation exchange chromatography magnetic bead; SELDI, surface-enhanced laser desorption/ionization; HCCA, α-cyano-4-hydroxycinnamic acid.

* Corresponding author.
** Correspondence to: G. Li, Cancer Research Institute, Key Laboratory of Carcinogenesis and Cancer Invasion of Ministry of Education, Key Laboratory of Carcinogenesis of Ministry of Health, Central South University, 110 Xiangya Road, Changsha 410078, PR China. Tel.: +86 731 84805446; fax: +86 731 84805383.
E-mail address: ligy@xysm.net (G. Li).

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DNA mutations at codon 132 of isocitrate dehydrogenase 1 (IDH1) and codon 172 of IDH2 have been found in gliomas of different stages [6,7]. The discovery of applicable biomarkers for glioma diagnosis, treatment and prognosis has been an important area of research.

Blood appears to be suited to the non-invasive detection of biomarkers, as it contains a wide variety of molecules including DNA, RNA, proteins, peptides and metabolites. The serum peptidome refers to the low-molecular-weight proteins and fragments in the serum and could be a source of cancer-specific information, such as cleaved proteins and degradative enzymes from the tumor and surrounding tissue microenvironment [8]. The serum peptidome reflects the continued molecular activities that occur within cancer cells and might serve as promising biomarkers for early diagnosis [9,10]. Since 2002, several reports described the serum peptidome as the source of a biomarker pattern in various tumors, such as ovarian cancer [11], prostate cancer [12], oral cancer [13], breast cancer [14], colorectal cancer [15], and hepatocellular carcinoma [16]. These reports revealed that the serum peptidome could distinguish cancer patients from healthy controls with high specificity and accuracy.

In body fluids such as serum, plasma or cerebrospinal fluid (CSF), the concentrations of different proteins and peptides vary by more than a ten-fold difference. Therefore, the detection of low abundance protein or peptide is challenging [17]. Thus, a critical step in sample preparation of serum peptidome analysis is to isolate target serum peptides from the complex serum samples. In most of the current studies, coated magnetic beads are combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF) mass spectrometry to increase the binding capacity, improve serum purification, reduce complexity, and enrich low-abundance peptides and proteins [18,19]. In this study, we have identified possible biomarkers for the diagnosis and prognosis of glioma with coated magnetic bead purification and MALDI-TOF mass spectrometer (Ultraflex III MALDI-TOF/TOF, Bruker Daltonics).

2. Materials and methods

2.1. Patient enrollment, sample collection and preparation

Following ethical approval and written informed consent, serum samples were from 53 glioma patients and 69 age- and gender-matched healthy controls for this study. All samples were collected in consenting individuals from Xiangya Hospital, Central South University. The sample collection was performed according to the protocols approved by the ethics committee of Xiangya Hospital, Central South University. The blood samples were collected prior to any therapeutic procedures, such as surgery, chemotherapy and radiotherapy.

All serum samples were collected according to our previously published protocol [20]. Briefly, 4 ml of blood samples were collected in 5 ml of BD Vacutainer SST™ II advance blood collection tubes (no. 367955, Becton Dickinson). The tubes were immediately inverted gently 3 times to mix the clot activator and the blood samples. The sample tubes were stored vertically for 1 h at room temperature (RT) to allow clotting, followed by centrifugation at 1600 RCF, 4 °C for 10 min. Sera (supernatants) were collected and stored at −80 °C for further analysis.

Peptide extractions were performed with a weak cation exchange chromatography magnetic bead kit (MB-WCX, Bruker). All extractions in this experiment were performed with MB-WCX beads from a single batch. The samples were thawed gently at 4 °C. For each sample, 8 μl of serum was mixed with 10 μl of beads. The samples were purified in 3 steps: binding, washing, and eluting, according to the manufacturer's suggested protocol. A total of 5 μl of solid-phase peptide extraction were eluted, mixed with 5 μl stabilization buffer and stored at 4 °C prior to MS analysis. Generally, each serum sample was frozen and thawed twice prior to MS analysis.

2.2. Mass spectrometry and signal processing

All extractions were analyzed on a MALDI-TOF mass spectrometer (Ultraflex III MALDI-TOF/TOF, Bruker). For each sample, 1 μl of the peptide extraction eluate was deposited onto the stainless steel target surface and allowed to dry. Next, 1 μl of a matrix that contained 3 mg/ml saturated α-cyano-4-hydroxycinnamic acid (HCCA, Bruker) was spotted onto the same position as the dried elute. Peptide calibration standard (no. 206195) and protein calibration standard I (no. 206355, Bruker) were also mixed with the HCCA matrix and deposited onto positions that were centrally located to eight neighboring serum samples to form a 3 × 3 pattern on a standard 384-spot plate. An external calibration was performed for every sample by standardizing each separately to the corresponding protein or peptide. Every sample deposition was repeated randomly 8 times and the spectrum acquisition started within 2 h. The spectra were obtained in the linear mode and each spectrum was the result of an automatic multi-positioning of 15 × 100 laser shots/spot over a representative area of the sample spot (at 60% laser power). FlexAnalysis software (Bruker) was used to obtain the spectra and detect the peak m/z value and intensity (with $S/N = 5$).

2.3. Statistical analysis

All spectra were imported to a database and Clinpro Tools software (Bruker) was used for signal processing through a series of steps composed of smoothing, baseline correction, normalization, recalibration/alignment and peak labeling. For all samples, an identical set of signal coordinates (fraction number and m/z value) and the corresponding mass-spectrometric signal intensities were extracted and used for further statistical analysis.

Subsequently, an optimal subset of variables was selected with the newly proposed competitive adaptive reweighted sampling (CARS) method. Briefly, in each sampling run, CARS worked in three successive steps. First, the samples were randomly selected according to the Monte Carlo strategy. Next, some uninformative variables were eliminated through the enforced wavelength reduction by the exponentially decreasing function (EDF) and competitive wavelength reduction by adaptive reweighted sampling (ARS). Then, the subset with the lowest root mean square error of CV (RMSECV) was defined [21]. Finally, based on the importance level of each variable (m/z), a pattern recognition equation was established through the classical partial least-squares–linear discriminant analysis (PLS–LDA) method with the selected optimal subset. In addition, progressively different peptide peaks among grades I–II, III, and IV glioma groups were selected.

3. Results

3.1. Characteristics of the enrolled patients

Human sera from a cohort of 53 glioma patients and 69 healthy controls were analyzed. Twenty-three female and 30 male glioma patients were enrolled, including 24 grades I–II patients, 15 grade III and 14 grade IV patients. The grades and types of the tumors were assessed by a histological examination of the resected paraffin specimens. In the control group, the samples were collected from 69 healthy controls, of which 33 were female and 36 were male. The age distribution, gender distribution, and clinical characteristics are provided in Table 1. The median age of the glioma patients and healthy controls were 41.7 and 43.1 years, respectively, and the age distributions of the groups were similar. We investigated the correlation of gender with the serum peptidome in the two groups. The serum samples were prepared and analyzed according to a standard protocol [20]. The spectra were processed and aligned with the Clinpro Tools software package and analyzed by a principal component analysis (PCA) method. The first three principal components that account for most of the variance in the

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