Research Article

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Differential Proteomics Analysis of Oligodendrogliomas and Astrocytomas Using iTRAQ Quantification

Shi-de Lin^{1#}, Jing-zhe Li^{2#}, Peng Wang^{3#}, Yu-jie Zang^{1,4} and Ting-bao Zhao^{1*}

¹Department of Spinal Cord Injury, Institute of Orthopedics and Traumatology of Chinese PLA, Shandong, China ²Medical Research Center of China Academy of Chinese Medicine Sciences, Beijing, China ³Department of Neurosurgery, Chinese PLA General Hospital, Beijing, China

⁴The Second Military Medical University, Shanghai, China

Abstract

Background: Astrocytoma and oligodendroglioma are two histological subtypes of primary Central Nervous System (CNS) tumors. Because of the high cytoarchitectural variability and lacking accurate diagnosis biomarkers, distinguishing astrocytomas and oligodendrogliomas clinically remains challenging.

Methods: The total protein lysates from of astrocytoma and oligodendroglioma clinical specimens were analyzed by 2DLC/MS/MS and quantified via Isobaric Tags Relative and Absolute Quantitation (iTRAQ). Differentially expressed proteins were further analyzed by Ingenuity Pathway Analysis (IPA) software. Lastly, potential bio-markers' expression levels were validated by western blot.

Results: A total 1856 proteins were identified. Among them, 83 proteins were increased and 82 proteins were decreased in astroglioma specimens compared to oligodendroglioma. Our bioinformatics study showed this protein profile change in astrocytoma more likely tend to enhance cell proliferation, migration and angiogenesis. Moreover, pathway-analysis showed that protein level of Rho Family GTPases pathway components was remarkably different between astrocytoma and oligodendroglioma. Lastly, two members of Rho family of GTPases, cell division control protein 42 homolog (CDC42) and transforming protein RhoA (RHOA) were found highly expressed in astrocytoma and oligodendroglioma, respectively.

Discussion: Differential-proteomic analysis was validating to distinguish between astrocytomas and oligodendrogliomas. Two members of especially the Rho family of GTPases, CDC42 and RHOA, would be potential indicators to reflect the pathological characteristics of these two diseases.

Keywords: Proteomics; Astrocytoma; Oligodendroglioma

Introduction

Astrocytomas and oligodendrogliomas are two primary Central Nervous System (CNS) tumors, which account for approximately 30% of all adult brain tumors [1]. Astrocytoma, which originates from astrocytes, is the most common glioma; it occurs in most parts of the brain and occasionally in spinal cord. Oligodendroglioma originates from oligodendrocytes or glial precursor cells. Oligodendroglioma primarily occurs in adults or children, whereas Astrocytoma commonly occurs in teenager or young people.

The prognosis of patients suffering astrocytomas and oligodendrogliomas are different. Median survival times of oligodendrogliomas patients are 11.6 years for grade II and 3.5 years for grade III, while 5.6 years and 1.6 years for Grade II and III astrocytomas patients respectively [2,3]. Accurate pathological diagnosis of astrocytomas and oligodendrogliomas directly affects treatment and prognosis. However, high cytoarchitectural variability and the lack of established biomarkers maintain the obstacle in distinguishing those tumors [4,5]. Thus, novel and accurate biomarkers are required for achieving successful early diagnosis.

In this study, differential-proteomic analysis was used to analyze both astrocytomas and oligodendrogliomas specimens. By iTRAQ quantification, differentially expressed proteins were identified. We found 83 increased proteins and 82 decreased proteins in astroglioma compared to oligodendroglioma. After that, the biological functions and canonical pathways of those proteins were analyzed by Ingenuity Pathway Analysis (IPA); and Rho Family GTPases pathways were identified as the most influent ones with high differences. Furthermore,

Rho Family GTPases CDC42 and RHOA were confirmed highly expressed in astrocytoma and oligodendroglioma respectively, indicating that they can be specific biomarkers for distinguishing those tumors.

Materials and Methods

Case selection

All experimental protocols were approved by the Ethics Committee of Chinese PLA General Hospital, with patients' consent. Human study was approved by Ethics Committee of Chinese PLA General Hospital with 12 participants (No. 2012FC-TSYS-3045). Tumor pathological diagnosis was confirmed by two independent pathology experts according to WHO histological classification. Frozen samples were collected and were immediately frozen in -80°C refrigerator. 6 Grade II astrocytomas and 6 Grade II oligodendrogliomas were included. The study was approved by the review board according to ethical norms. All

*Corresponding author: Ting-bao Zhao, Department of Spinal Cord Injury, Institute of Orthopedics and Traumatology of Chinese PLA, General Hospital of Jinan Military Command No. 25, Shifan Road, Tianqiao District, Jinan, Shandong, China, Tel: 8613153032659; E-mail: doctorzhaotingbao@126.com

#Authors contributed equally

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clinical investigation was conducted according to the principles which expressed in the Declaration of Helsinki.

iTRAQ sample preparation

Fifty-milligram samples from each of the twelve frozen tissue samples were used for proteomics analysis. Tumor samples were washed with PBS to remove the blood and then lyzed with lysis buffer (containing 8 M Urea, 2.5 M Thiourea, and 65 mM DTT). Cell debris was removed by centrifugation at 14,000 g at 4°C for 10 min. The protein concentration of each sample was estimated using the Bradford method.

1 mg proteins from each sample were pooled together and digested using Filter-Aided Sample Preparation (FASP) method [6]. Digested peptides from the astrocytomas and oligodendrogliomas were desalted with C18 columns (3 cc, 60 mg, Oasis). The desalted peptides were lyophilized and immediately stored at -80°C.

 $100~\mu g$ of the astrocytomas and oligodendrogliomas digested peptide were individually labeled with 115 and 116 iTRAQ regents respectively, according to the manufacturer's protocol (ABsciex). After labeling, the labeled peptides were mixed and lyophilized by vacuum centrifugation.

2DLC-MS/MS

The mixed labeled samples were first separated with a high-PH PRLC column (Waters, 4.6 mm \times 250 mm, C18, 3 µm). The samples were loaded onto the column with buffer A (pH=10, 2% ACN), and eluted gradiently with 5-35% buffer B (90% ACN, pH=10; flow rate, 0.6 mL/min) for 60min. The elusions were collected in one fraction per minute, and a total of 60 fractions were collected. Then, the 60 fractions were pooled into 15 samples by combining fractions 1, 16, 31, 46; 2, 17, 32, 47; and so on. The 15 pooled fractions were analyzed by online LC-MS/MS.

In online LC-MS analysis, a self-packed capillary RP-LC column (75 μ m × 100 mm, C18, 3 μ m) was used for LC separation. The elusion condition was 5-30% buffer B (containing 0.1% formic acid, 99.9% ACN; flow rate, 0.3 μ L/min) for 40 min. LTQ-orbitrap Velos mass spectrometer was used for MS analysis. The mass spectrometry parameters was set as follows: HCD collision, 10 data-dependent MS/ MS scans per full scan, full scans acquired at a resolution of 30,000 and MS/MS scans at a resolution of 7,500, charge state screening (including precursors with +2 to +4 charge state), dynamic exclusion (exclusion duration 60 s). Each fraction runs once.

Data processing

Mascot software (Matrix Science, London, UK; version 2.4.01) was used for database searching. The database searching parameters was set as follows: Swissprot human database; Trypsin digestion; The mass tolerance of the parent ion was 10 ppm and the fragment ion was 0.05 Da; Fixed modification: Cysteine carbamidomethytion; A maximum of 2 miscleavage sites were allowed. For Protein identification, Scaffold (version Scaffold_4.0.7, Proteome Software Inc., Portland, OR) was used. Protein was identified at FDR less than 1.0% on both peptide and protein level and contained at least 2 unique peptides. Proteins containing similar peptides and could not be distinguished based on MS/MS analysis were grouped separately as protein groups. Scaffold Q+ (version Scaffold_4.3.2, Proteome Software Inc., Portland, OR) was used for iTRAQ quantification. The acquired iTRAQ intensities were normalized globally [7]. The protein quantitation results were statistically analyzed by Mann-Whitney Test.

Gene Ontology (GO) and IPA analysis

In GO analysis, the differential proteins were analyzed with the Panther database (http://www.pantherdb.org/), and compared with the whole human genome. Proteins were classified based on molecular function, biological processes and cellular component categories by GO annotations. Statistical overrepresentation test were analyzed using Bonferroni correction for multiple testing in Panther software. In IPA analysis, the differential proteins were analyzed by IPA software (Ingenuity Systems, Mountain View, CA). Proteins were mapped to the IPA database and other databases in the disease and functional category and the canonical pathways categories [7]. The statistical over representation tests were analyzed by IPA software.

Western blotting analysis

Three selected differential proteins, including GFAP (ab7260), CDC42 (ab64533) and RHOA (ab187026) were validated by Western blot in the individual samples, and beta-actin was used as loading control. All the primary antibodies to these three different proteins were purchased from the abcam company (ABcam, Cambridge, UK). The signal intensity of Western Blot bands of candidate proteins were quantitatively analyzed using Image J software by densitometry, and normalized by the respective beta-actin signal [8,9]. The relative intensities of these proteins between astrocytomas and oligodendrogliomas were statistical analyzed by SPSS 19.0 using student's t-test.

Real time PCR (RT-qPCR)

The total RNA was extracted from tumor tissues with the TRIzol reagents and converted to cDNA with a reverse transcription kit. The cDNA was amplified in the CFX96 Touch "Real-Time PCR Detection System (Bio-Rad) with the SYBR Green Master Mix. The results were calculated with the 2^{- $\Delta\Delta$}Ct method and presented as fold change against controls. The primers used in the present study include IL-10 (gttctttggggagccaacag and gctccctggtttctcttcct), HDAC11 (gtcttgcctgttcagtgcaa and tgcatccctgatttccacct) and β -actin (cgcaaagacctgtatgccaa and cacacagagtacttgcgctc). The primers were synthesized by Enke Biotech (Shenzhen, China).

Results

Quantitative analysis of differential proteome in astrocytomas and oligodendrogliomas

Protein samples extracted from 6 grade II astrocytomas and 6 grade II oligodendrogliomas clinical specimens were pooled respectively, iTRAQ-labeled and analyzed by 2DLC-MS/MS. By querying Swissprot Database with the Mascot algorithm, at 1% false discovery rate (FDR) both in peptide and protein levels, 25837 spectra were matched from 130959 spectra. In this step, 16632 peptides were detected. The raw data have been uploaded into Figshare Web (https://figshare. com/s/1497b062604de715a297), and named "Differential Proteomics analysis of Oligodendroglioma and Astrocytoma using iTRAQ quantification". We identified 1856 protein groups from matched spectra with ≥2 peptides. 24335 iTRAQ Reporter region containing spectra are usable for quantification (Supplementary Table 1) and, among them, we quantified 1697 proteins (Supplementary Table 2). By a ratio-fold change ≥2, 165 proteins were differentially expressed, including 83 proteins increased and 82 proteins decreased in astrogliomas compared to oligodendrogliomas (Supplementary Table 3). By stastical analysis of the differential proteins by Mann-Whitney Test, with Mann-Whitney

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Test P-value <0.05, 61 proteins were differentially expressed, including 26 proteins increased and 35 proteins decreased in astrogliomas compared to oligodendrogliomas by analyzing the distribution of the protein fold change (Supplementary Figure 1), we found symmetric distribution of fold change across the samples.

Bioinformatics analysis of differential proteins between astrocytomas and oligodendrogliomas

To explore the possible functional relevance of differentially expressing proteins in astrocytomas and oligodendrogliomas, we then used PANTHER classification system [10] to search for the enrichment of the GO terms in differentially expressing proteins comparing with that in whole human genome database. Selected differentially expressing proteins were presented in Figure 1 with their intracellular compartment, function and related biological processes. As to protein function, in the molecular function category (Figure 1A), the structural molecular activity (p=1.52E-02) and the protein binding activity (p=6.06E-04) were enriched in differentially expressing proteins compared with the whole genome data. In biological process and cellular component category (Figure 1B-1C), the macro-molecular complex was overrepresented (p=2.92E-02). To further analysis the detailed functional differences in the differentially expressing proteins between astrocytomas and oligodendrogliomas, we performed IPA analysis in disease and function: neurotransmission (z-score=1.83), tumor cell adhesion (z-score=1.68), neuronal cells proliferation (z-score=1.50) and filopodia formation (z-score=1.47) were activated in astrocytomas, while the function of organism death was inhibited (z-score=-2.01) (Figure 2A and Supplementary Table 4). Our results indicate that compared to oligodendroglioma, astrocytoma cells are



Figure 1: GO analysis of differential proteins between oligodendrogliomas and astrocytomas. By GO analysis, differential proteins in oligodendrogliomas and astrocytomas were classified into molecular function (A), biological process (B), and cellular component (C) categories for human genes, comparing with the entire human genome. Categories with constitution of at least 2% were displayed in the bar charts.

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more likely to tend to proliferate, and migrate and promote tumor angiogenesis. These results are consistent with the fact that astrocytoma is more aggressive than oligodendroglioma.

Next, we performed pathway analysis to further study the detailed molecular mechanisms of which those differentially expressing proteins lead to different phenotypic characteristics in astrocytomas and oligodendrogliomas. The pathway analysis showed that Signaling by Rho Family GTPases was remarkably different between the astrocytomas and the oligodendrogliomas (p=1.12E-08) (Figure 3). The Rho family GTPases, including Cdc42, and RhoA, are signaling mediators of tumor cell invasion and migration. Among the Rho family members, the CDC42 is highly expressed in the astrocytomas, whereas the RhoA is highly expressed in the oligodendrogliomas (Figure 2B and Supplementary Table 4). These results indicate that the differences of tumor invasion characteristics between astrocytomas and oligodendrogliomas might attribute by the expression variation of Rho family GTPases.

Moreover, the astrocytes expressing proteins, e.g. Mesencephalic Astrocyte-derived Neurotropic Factor (MANF) and Astrocytic

phosphoprotein PEA15 are highly expressed in astrocytomas (astrocytomas/ oligodendrogliomas ratio were 1.66 and 3.44 respectively); the oligodendrocyte expressing proteins, such as Myelin Oligodendrocyte Glycoprotein (MOG) and Oligodendrocyte-myelin Glycoprotein (OMG), are highly expressed in oligodendroglioma (astrocytomas/ oligodendrogliomas ratio were 0.33 and 0.44 respectively). This finding indicates that certain astrocyte and oligodendrocyte specifically expressing protein can be used to distinguish astrocytomas and oligodendrogliomas.

Western Blot validation of differentially expressed proteins

Next, we confirmed the expression levels of three selected proteins, GFAP, CDC42 and RhoA via immune-blotting. As shown in Figure 4A-4D and Table 1, all the three proteins had the similar trends as iTRAQ analysis showed. GFAP, which has been used to distinguish astrocytomas and oligoastrocytomas, was highly expressed in the astrocytomas and lowly expressed in the oligodendrogliomas samples (p<0.001 in student's t-test). The newly identified differentially expressed proteins, CDC42 and RhoA were significantly overrepresented in the astrocytomas samples (p<0.001 in student's t-test) and oligodendrogliomas samples

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(p<0.01 in student's t-test), respectively. To detect whether the decrease and increase of these proteins caused by transcription levels or not, we further validate the express levels of these three proteins in transcription levels using RT-PCR, Figure 4C showed that the mRNA expression levels of all the three genes the similar trends as the protein levels, indicating that the differential expression of the three proteins between astrocytomas and oligodendrogliomas might cause by transcription levels. The

Discussion

Given that the prognoses of astrocytomas and oligodendrogliomas are significantly different, the initial pathological diagnosis that distinguishes astrocytomas and oligodendrogliomas is necessary. Normally, clinical diagnosis of astrocytomas and oligodendrogliomas is dependent on tissue morphology, cytoarchitecture and immunohistochemistry. However, these tests may not be able to clearly distinguish astrocytoma and glioblastoma [3]. The accurate

mechanism of the differential expression of CDC42 and RhoA between

astrocytomas and oligodendrogliomas needs further study.

identification of gliomas with astrocytic or oligodendrocytic components is still a difficult task. Therefore, molecular diagnostics are necessary for accurate treatment. To identify the differential proteins between astrocytomas and oligodendrogliomas, we performed differential proteomic analysis of these glioma subtypes to discover differentially expressed proteins.

The Rho-family GTPases are differentially expressed in oligodendrogliomas and astrocytomas. CDC42 is highly expressed in many *malignant* tumors, including liver cancer, gastric cancer and lung adenocarcinoma; it is involved cell morphology, invasion/migration, endocytosis and cell cycle [11-13]. To date, several evidences have indicated that CDC42 participate in the invasion and progression of glioma cells. The TWEAK-Fn14 ligand receptor axis-induced activation of Rac1 was dependent upon the activation of Cdc42 protein; the knock-down of Cdc42 inhibited migration and invasion of glioma cells *in vitro* [14]. As a downstream of PDGFRa, Cdc42 activation promoted the cell migration along with Rac1 activation in Glioblastoma Multiforme (GBM) cells [15]. Both Cdc42 and Rac1 displayed high activity in GBM cells in the process of penetrating brain parenchyma and would be potential bio-sensors [16,17]. Furthermore, CDC42 participated in a

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Figure 4: Western blot and RT-PCR validations for three differential proteins. A: Western blot result of the three differential proteins in oligodendrogliomas and astrocytomas. B: Scatter plot of GFAP, CDC42 and RhoA were in Western Blot relative intensity. C: Scatter plot of GFAP, CDC42 and RhoA were in RT-PCR relative intensity. The relative intensities of these proteins between astrocytomas and oligodendrogliomas were statistical analyzed by SPSS 19.0 using student's t-test. **p<0.01, ***P<0.001.

Protein Name	Accession Number	iTRAQ Quantification (Astro: oligo)	Western Blot (Astro: oligo)	mRNA (Astro: oligo)
Glial fibrillary acidic protein (GFAP)	P14136	1: 2.25	1: 3.22	1: 2.78
Cell division control protein 42 homolog (CDC42)	P60953	1: 2.37	1: 3.91	1: 5.13
Transforming protein RhoA (RHOA)	P61586	1: 0.42	1: 0.41	1: 0.28

Table 1: Quantitative value in astrocytomas vs. oligodendrogliomas in iTRAQ quantitation, Western Blot quantitation and RT-PCR methods.

pathogenic crosstalk between tumor cells and pericytes in GBM [18]. In this work, we found that CDC42 was increased in astrocytomas than in oligodendrogliomas. These data provided the clue that high expression of CDC42 would lead to the more aggressive phenotype of related cancers.

Another Rho GTPases, RhoA plays a negatively regulatory role in GBM progression [19-22]. Decreased RHOA activity related to increase of glioma cell migration [19-22]. Functional studies suggested that the inhibition of RhoA's effector ROCK led to the invasion of glioma cells and the activation of Rac1 [23]. This inhibition also led to cell morphology changes [23]. In addition, the inhibition of LPA, the downstream of

Rho/Rho kinase downstream induced glioma cell chemotaxis, which led to the formation of elongated cells with extended processes [24]. Seasholtz et al. showed that activation of RhoA in astrocytoma cells was involved in cell contraction and rounding, with reduced Rac1 activity [25]. Also, pharmacologic inhibition of Ras in GBM resulted in decreased Rac1 activity, which was consistent with the findings under increased RhoA activation [26,27]. Treating glioblastoma cell with plant growth regulator Narciclasine led to increase ROCK activity and stress fiber formation, and these led to an increase in the survival rate of orthotopic transplanted tumor mice [16,28]. In the present work, we found RhoA is highly expressed in oligodendrogliomas but not astrocytomas. Therefore, we propose balance of two Rho GTPases, CDC42 and RhoA, would participate in the regulation of GBM and contribute to the different malignant features of oligodendrogliomas and astrocytomas.

Conclusion

Differential-proteomic analysis is valuable to identify biomarkers that distinguish astrocytomas and oligodendrogliomas. Our data show that astrocytomas cells are more likely to proliferate, migrate and lead to tumor angiogenesis, than oligodendrogliomas cells. This result indicates that astrocytomas are more malignant than the oligodendrogliomas. By pathway analysis, we found Rho Family GTPases were remarkably changed between astrocytomas and oligodendrogliomas. We identified two new differentially expressed proteins, CDC42 and RHOA, to be useful to distinguish between astrocytomas and oligodendrogliomas.

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