Differential proteomics analysis of oligodendrogliomas and astrocytomas using iTRAQ quantification

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Background: Astrocytomas and oligodendrogliomas are two types of primary Central nervous system (CNS) tumors. Because of the cytoarchitectural variability and lacking of accurate differential diagnosis biomarkers, distinguishing between these neoplasms remains a challenge. **Method:** In this study, we utilized the tumor tissue proteome to distinguish the astrocytomas and oligodendrogliomas. The protein samples from astrocytomas and oligodendrogliomas tumor tissues were analyzed by 2DLC/MS/MS and isobaric tags for relative and absolute quantitation (iTRAQ) quantification. The differential proteins were analyzed by IPA software, and three identified differential proteins were validated by Western Blot. **Results:** A total of 2189 proteins were identified, including 150 upregulated and 103 downregulated in astrogliomas compared to oligodendrogliomas. By bioinformatics analysis, compared to oligodendrogliomas, the astrocytomas is more likely tend to cell proliferation, migration and the tumor angiogenesis, indicating astrocytomas was more malignant than the oligodendrogliomas. Pathway analysis showed that members of Rho Family GTPases were remarkably changed between astrocytomas and oligodendrogliomas. Two member of Rho family of GTPases, Cell division control protein 42 homolog (CDC42) and Transforming protein RhoA (RHOA) were over-expressed in astrocytomas and oligodendrogliomas, respectively. **Discussion:** Above data indicated that the differential proteome could be useful to distinguish between astrocytomas and oligodendrogliomas, especially the Rho family of GTPases. Differential proteome could partially reflect the pathological characteristics of these two diseases.

Differential Proteomics analysis of Oligodendroglioma and Astrocytoma using iTRAQ quantifiction

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Abstract

Background:
Astrocytoma and Oligodendroglioma aretwo most commontypes of primary central nervous system (CNS) tumors. Because of the cytoarchitectural variability and lacking of accurate differential diagnosis biomarkers, distinguishing between these neoplasms remains a challenge.

Method:
In this study, we utilized the tumor tissue proteome to distinguish the Astrocytoma and Oligodendroglioma. The protein samples from Astrocytoma and Oligodendrogliomaclinicalspecimens were analyzed by 2DLC/MS/MS and isobaric tags for relative and absolute quantitation (iTRAQ) quantification. The differential proteins were analyzed by IPA software, and three identified differential proteinswere validated by Western Blot.

Results:
A total of 2189 proteins were identified, including 150 upregulated and 103 downregulated, in astrogliomas compared to Oligodendroglioma. By bioinformatics analysis, compared to Oligodendroglioma, the Astrocytoma is more likely tend to cell proliferation, migration and the tumor angiogenesis.These indicated thatAstrocytoma would bemore malignant than Oligodendroglioma. Pathway analysis showed that members of Rho Family GTPases were significantly differentbetween Astrocytoma and Oligodendroglioma. Two member of Rho family of GTPases, CDC42 (Cell division control protein 42 homolog) and RHO A (Transforming protein RhoA) were highly expressed in Astrocytoma and Oligodendroglioma, respectively.

Discussion:
Above data indicated that differential-proteome analysis could be useful to distinguish between Astrocytoma and Oligodendroglioma, especially the Rho family of GTPases. Differential proteome could partially reflect the pathological characteristics of these two diseases.

Key Words: Astrocytoma; Oligodendroglioma; Rho Family GTPases; Biomarker

Introduction

The astrocytic andoligodendrocytic carcinomasare most two important types of primarycentral nervous system tumor which constitute of approximately 30% of all adult patients suffering from brain tumor(Cairncross et al. 1998). **Astrocytoma**isthe most common type of glioma which originated from astrocytes,a group of star-shaped brain cells locating in the cerebrum(Cairncross et al. 1998). It has been well know that Astrocytoma may account for approximately 75% of neuroepithelial tumors. **Oligodendroglioma**is anothertype of glioma originated from oligodendrocytes or glial precursor cells (Cairncross et al. 1998).

Compared withAstrocytoma, Oligodendroglioma grows much slowly and patients suffering from Oligodendroglioma are with longer survival (Ohgaki & Kleihues 2005). In one series, median survival times for Oligodendroglioma were 11.6 years for grade II and 3.5 years for grade III(Ohgaki & Kleihues 2005);whereasthe median survival times for Grade II and III Astrocytoma were only 5.6 years and 1.6 years, respectively(Zhuang et al. 2011). Therefore, it is valuable to identify the pathological indicators or biomarkersbetween astrocytoma and oligodendroglioma(Louis 2006; Walker & Kaye 2001).

This work aims to identify unique-protein biomarker between astrocytoma and oligodendroglioma.Differential proteome analysis of clinical specimens, between grade II Astrocytoma and grade II Oligodendroglioma,were performed. PooledAstrocytoma and Oligodendroglioma protein samples were tryptic digested, labeled by 8-plex iTRAQ regents, mixed and analyzed by 2D-LC MS/MS. By iTRAQ quantification, proteins with an over 2-fold changes were considered as differential expression. After that, biological functions and canonical pathways of the differential proteins were annotated by Ingenuity Pathway Analysis (IPA). Furthermore, 3 selected differentialexpressing proteins were validated in individual sample by Western blot.

Materials and Method:

1. Case Selection criteria

The collection of tissues and the study protocol was all approved by the Ethics Committee of Chinese PLA General Hospital, with the informed consent of patients. Tumor pathological diagnosis was confirmed by two independent pathology experts according to WHO histological classification. Frozen samples were collected intra-operatively and immediately frozen in -80°C refrigerator and included 6 Grade II Astrocytoma and 6Grade II Oligodendroglioma. The study was approved by the review board in accordance with ethical norms. All clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by Chinese PLA General Hospital Ethics Committee (S2014-096-01).

2. iTRAQ Sample Preparation

Fifty-milligram samples from each of the ten frozen tissue samples were selected for proteomics analysis. Each sample was washed with PBS and then lyzed with lysis buffer (containing 2.5 M

thiourea, 8 M urea 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 quantified by the Bradford method.

Proteins from each sample were pooled together with the same total protein amount and digested with filter-aided sample preparation (FASP) method. Digested peptides from the Astrocytoma and Oligodendroglioma were desalted on C18 columns (3 cc, 60 mg, Oasis). The desalted peptides were lyophilized by vacuum centrifugation and stored at -80 °C.

The Astrocytoma and Oligodendroglioma samples were individually labeled with 115 and 116 iTRAQ, according to the manufacturer's protocol (ABsciex). After labeling, the labeled samples were mixed equally and dried by vacuum centrifugation

3. 2DLC-MS/MS

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

Each fraction was analyzed with a self-packed capillary RP-LC column (75 μm×100 mm, C18, 3 μm). The sample were loaded onto the column in buffer A (0.1% formic acid, 2% ACN), and gradient eluted by 5-30% buffer B (0.1% formic acid, 99.9% ACN; flow rate, 0.3 μL/min) for 40min. An LTQ-orbitrap Velos mass spectrometer was used to analyze the LC eluted peptides. Mass spectrometry data were obtained using the following parameters: 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).

4. Data processing

Mascot software (Matrix Science, London, UK; version2.4.01) was used for database searching of all samples. In Mascot, the database was set up to Swissprot human database and the digestion enzyme was set to Trypsin. The parent mass tolerance was 10 ppm and fragment ion was 0.5 Da. Carbamidomethyl of cysteine was set as a fixed modification, and 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 identification was set at FDR less than 1.0% on both peptide and protein level and contained at least 1 unique peptide. Proteins containing similar peptides and could not be distinguished based on MS / MS analysis were grouped separately in order to meet the principle of simplicity. Scaffold Q+ (version Scaffold_4.3.2, Proteome Software Inc., Portland, OR) was used for iTRAQ quantification. Acquired intensities in the experiment were normalized globally at all runs. The reference channels were normalized to produce a 1:1 fold change. All normalization calculations used medians multiply to normalize data.[14]

5. GO and IPA analysis

For GO analysis, all differential proteins were analyzed in 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 in Gene Ontology (GO) annotations.

For IPA analysis, the differential proteins were analyzed in 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, respectively, with Z-score and P-values rankings.[14]

6. Western blotting analysis

Three selected differential proteins including, GFAP (ab7260), CDC42 (ab64533) and RHOA (ab187026)) were validated by Western blot by the individual samples, with beta-actin as loading control. All the primary antibodies against these candidate proteins were purchased from the abcam company.

Results

1. Quantitative analysis of differential proteome

Protein sample from 6 Grade II Astrocytoma or 6 Grade II Oligodendroglioma was extracted and pooled respectively. Two pooled samples were iTRAQ-labeled and analyzed by 2DLC-MS/MS. By querying human Swissprot database with Mascot algorithm, at 1% false discovery rate (FDR) in both peptide and protein levels, 19793 spectrums were matched from 130959 spectrums. 9570 peptides were identified. 2189 proteins were identified from matched spectrums with>=1 peptides (Supplementary Table 1) and 2177 proteins were quantified by iTRAQ (Supplementary Table 2)(Raw MS data was shown in https://figshare.com/s/1497b062604de715a297). By a ratio-fold change>=2, 253 proteins were differentially expressed, including 150 upregulated (6.89%) and 103 downregulated (4.73%) in astrogliomas compared to Oligodendroglioma(Supplementary Table 3). By analyzing the distribution of the protein fold change (Supplementary Figure 1), the data set showed nearly symmetric distribution of fold change across the sample. T

2. Panther and IPA analysis

To further study the biological function of the differential expressing proteins between the Astrocytoma and Oligodendroglioma, the differential proteins were analyzed by GO and IPA.

To explore the possible function of differential proteins in Astrocytoma and Oligodendroglioma, PANTHER classification system(Mi et al. 2005) was used to search for the enrichment of the GO terms in differential proteins comparing to the whole human genome data. The cellular compartment, molecular function and biological process of the differentially expressed proteins were presented in Figure 1. In the molecular function category (Figure 1A), the percentage of structural molecular activity and the translational regulator activity were much higher, while the percentage of nucleic acid binding transcription factors and translational regulator activity were much lower compared with the whole genome data. In biological process category (Figure 1B), the cellular component organization was overrepresented, whereas the apoptosis process was remarkably underrepresented in the differential proteins. In cellular component category (Figure 1C), macro molecular complex was overrepresented whereas the membrane and extracellular matrix proteins was underrepresented.

To further analysis the detailed differential function between Astrocytoma and Oligodendroglioma, IPA analysis was performed. In disease and function analysis, function of neurotransmission, tumor cell adhesion, and proliferation of neuronal cells, formation of filopodia were activated in Astrocytoma; while the function of organism death were inhibited in Astrocytoma (Figure 2A, detailed data in Supplementary Table 4). These results indicated that

compared to Oligodendroglioma, the Astrocytoma is more likely tend to proliferation, and migration and the tumor angiogenesis. These results were consistent with the fact that the Astrocytoma was more malignant than the Oligodendroglioma.

Next, the detail molecular mechanism of the tumor development in Astrocytoma and Oligodendroglioma, pathway analysis was performed. The pathway analysis showed that Signaling by Rho Family GTPases was remarkably changed between the Astrocytoma and the Oligodendroglioma (Figure 3). The Rho family of small GTPases, including primarily, Cdc42, and RHOA are key signaling mediators of tumor cell invasion and cell migration. Among the Rho family members, the CDC42 was highly expressed in the Astrocytoma, whereas the RHOA was highly expressed in the Oligodendroglioma (Figure 2B, detailed data in Supplementary Table 4). These results indicated that the differences of tumor invasion characteristics between Astrocytoma and Oligodendroglioma might be related to the expression levels of Rho family of small GTPases in Astrocytoma and Oligodendroglioma.

In addition, we also analyzed the tissue origin of the differential proteins. The astrocytes originated proteins, such as Mesencephalic astrocyte-derived neurotrophic factor (MANF) and Astrocytic phosphoprotein PEA15 were over-expressed in Astrocytoma (Astrocytoma/Oligodendroglioma ratio were 1.66 and 3.44 respectively). On the other hand, the oligodendrocyte originated proteins, such as Myelin oligodendrocyte glycoprotein (MOG) and Oligodendrocyte-myelin glycoprotein (OMG), were over-expressed in Oligodendroglioma (Astrocytoma/Oligodendroglioma ratio were 0.33 and 0.44 respectively). The astrocyte and oligodendrocyte originated proteins could distinguish Astrocytoma and Oligodendroglioma effectively.

3. Western Blot Validations

By biological function and pathway analyzing, three differential proteins, including GFAP, CDC42 and RHOA, were chosen as potential biomarkers and selected for Western Blot validation. As shown in Figure 4A-4D and Table 1, by Western Blot validation, all the three proteins had the similar trends as iTRAQ analysis. GFAP, a previously reported marker, which has been used in the identification of astrocytic components of mixed tumors to distinguish Astrocytoma from oligoAstrocytoma, was highly expressed in Astrocytoma samples and low expressed in the Oligodendroglioma samples in our study. As newly reported candidate biomarkers, CDC42 were significantly overrepresented in the Astrocytoma samples, while the RHOA were significantly overrepresented in the Oligodendroglioma samples.

Discussion:

Because the prognosis was significantly different between Astrocytoma and Oligodendroglioma, it is necessary and valuable to reveal underling mechanisms and pathological-diagnosis biomarkers to distinguish Astrocytoma and Oligodendroglioma. Often, distinguishing between Astrocytoma and Oligodendroglioma is based on tissue morphology, cytoarchitecture and immunohistochemical features. These may not be able to make a clear or accurate decision only based on the pathology of astrocytoma and glioblastoma (Zhuang et al. 2011). It is still a difficult task to identify the two sub-types gliomas, astrocytic and oligodendrocytic components. Therefore, molecular-diagnostic panels were useful to overcome this difficult task. To address this issue, we performed proteomic profiling of these glioma sub-types to uncover differentially

expressed protein markers.

In this work, two members of Rho family which is a group of small GTPases belonging to Ras superfamily, *i.e.*Cdc42 and RHOA, were identified differentially expressing in Oligodendroglioma or Astrocytoma. It is known that CDC42 was highly expressed in many malignant tumors, including liver cancer, lung adenocarcinoma, and gastric cancer_(Fortin et al. 2013). CDC42 is involved in many cellular processes, including cell morphology, migration, endocytosis and cell cycle progression(Etienne-Manneville & Hall 2001; Kozma et al. 1995; Wang et al. 2007). Many results showed that CDC42 is involved in the invasion and progression of glioma cells via mediating Rac1's activation. As an upstream of Rac1, the TWEAK-Fn14 ligand receptor axis-induced activation of Rac1 is dependent upon a functional and activated Cdc42 protein; the depletion of Cdc42 inhibited migration and invasion of glioma cells *in vitro* (Fortin et al. 2012). Cdc42 activation has been shown to promote the cell migration along with Rac1 activation in glioblastoma multiforme (GBM) cells (Feng et al. 2012). In addition, CDC42 would also participate in a pathogenic crosstalk between tumor cells and pericytes in GBM (Caspani et al. 2014). In this work, a high level of CDC42 was identified in Astrocytoma than that in Oligodendroglioma. This result suggested that CDC42 would participate in the more aggressive feature of Astrocytoma compared with Oligodendroglioma.

As another Rho GTPases, RHOA would be a negative regulator in the progress of GBM. Decreased RHOA activity may relate to enhancement of glioma cell's metastasis (Johnston et al. 2007; Malchinkhuu et al. 2008; Tran et al. 2006; Yan et al. 2006). Functional studies suggest that the inhibition of RHOA effector ROCK, resulted in the invasion of glioma cells and the promotion of Rac1 activation (Salhia et al. 2005). In addition, Activation of RhoA in astrocytoma cells results in the reduction of Rac1 activity (Seasholtz et al. 2004). In presence work, a high level of RHOA was found in Oligodendroglioma than that in Astrocytoma. This may indicate that members of Rho family would participate in brain tumor regulation and be potential biomarkers for to distinguish the differentially pathologic feature of sub-types of glioma.

Conclusions:

In this study, we utilized proteomic methods to distinguish the Astrocytoma and Oligodendroglioma specimens. By function analysis, Astrocytoma is more likely tend to more aggressive proliferation, and migration and the tumor angiogenesis, compared with Oligodendroglioma. This result confirmed that Astrocytoma was more malignant than Oligodendroglioma. Next, by pathway analysis, expression of two members of Rho Family, CDC42 and RHOA, were remarkably different between Astrocytoma and Oligodendroglioma. The expression of CDC42 and RHOA, two new candidate biomarkers, were validated by WB. Therefore, proteomic study identified some new differential proteins which could reflect cellular pathological functions and the tissue origin, and could be useful to distinguish between Astrocytoma and Oligodendroglioma.

Acknowledgement

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

Figure 2 IPA analysis of differential proteins in between Oligodendroglioma and Astrocytoma.A and B: Function analysis (A) and top enriched pathways (B) in Oligodendroglioma and Astrocytoma. Z-score>2: significantly activated; Z-score<-2, significantly inhibited. -Log(p-value)>1.5: significantly enriched.

Figure 3 Differential proteins in Signaling by Rho Family GTPases pathway between Oligodendroglioma and Astrocytoma. Red: over-expressed in Astrocytoma. Green: under-expressed in Astrocytoma. Image credit:The Canonical Pathway Figure were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA®,QIAGEN Redwood City, www.qiagen.com/ingenuity).

Figure 4 Western blot validation for five differential proteins. A: Western blot figure of the three candidate biomarkers in Oligodendroglioma and Astrocytoma. Scatter plot of GFAP (B), CDC42 (C) and RhoA (D) were shown.

Table 1: Quantitative value in Astrocytoma vs Oligodendroglioma in iTRAQ quantitation and Western Blot quantitation methods.

316 Table 1

Protein name	Accession Number	iTRAQ quantification (oligo:astro)	Western blot (oligo:astro)
Glial fibrillary acidic protein (GFAP)	P14136	1: 2.25	1: 3.22
Cell division control protein 42 homolog (CDC42)	P60953	1: 2.37	1: 3.91
Transforming protein RhoA (RHOA)	P61586	1: 0.42	1: 0.41

Supplementary Figure 1: Distribution of proteins fold change in Astrocytoma vs Oligodendroglioma.

Supplementary Table 1: Qualitative protein list of Oligodendroglioma and Astrocytoma proteome.

Supplementary Table 2: Quantitative protein and peptide list of Oligodendroglioma and Astrocytoma proteome.

Supplementary Table 3: Differential proteins between Oligodendroglioma and Astrocytoma.

Supplementary Table 4: Detail protein list classified by biological functions and pathway analysis by IPA in Oligodendroglioma and Astrocytoma

Figure 1

GO analysis of differential proteins between oligodendrogliomas and astrocytomas.

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

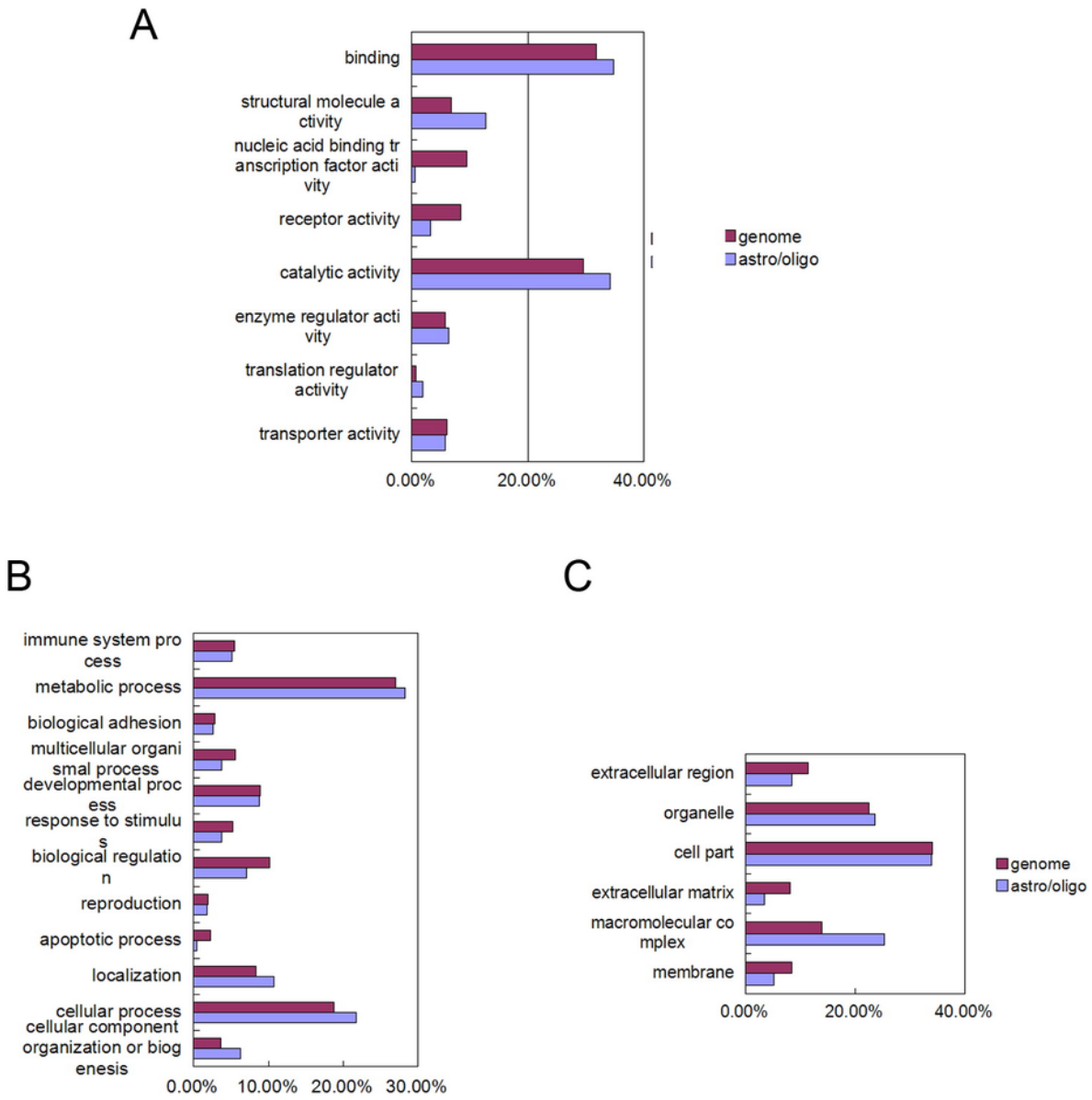

Figure 2

IPA analysis of differential proteins in between oligodendrogliomas and astrocytomas.

Figure 2 IPA analysis of differential proteins in between oligodendrogliomas and astrocytomas. A and B: Function analysis (A) and top enriched pathways (B) in oligodendrogliomas and astrocytomas. Z-score>2: significantly activated; Z-score<-2, significantly inhibited. -Log(p-value)>1.5: significantly enriched.

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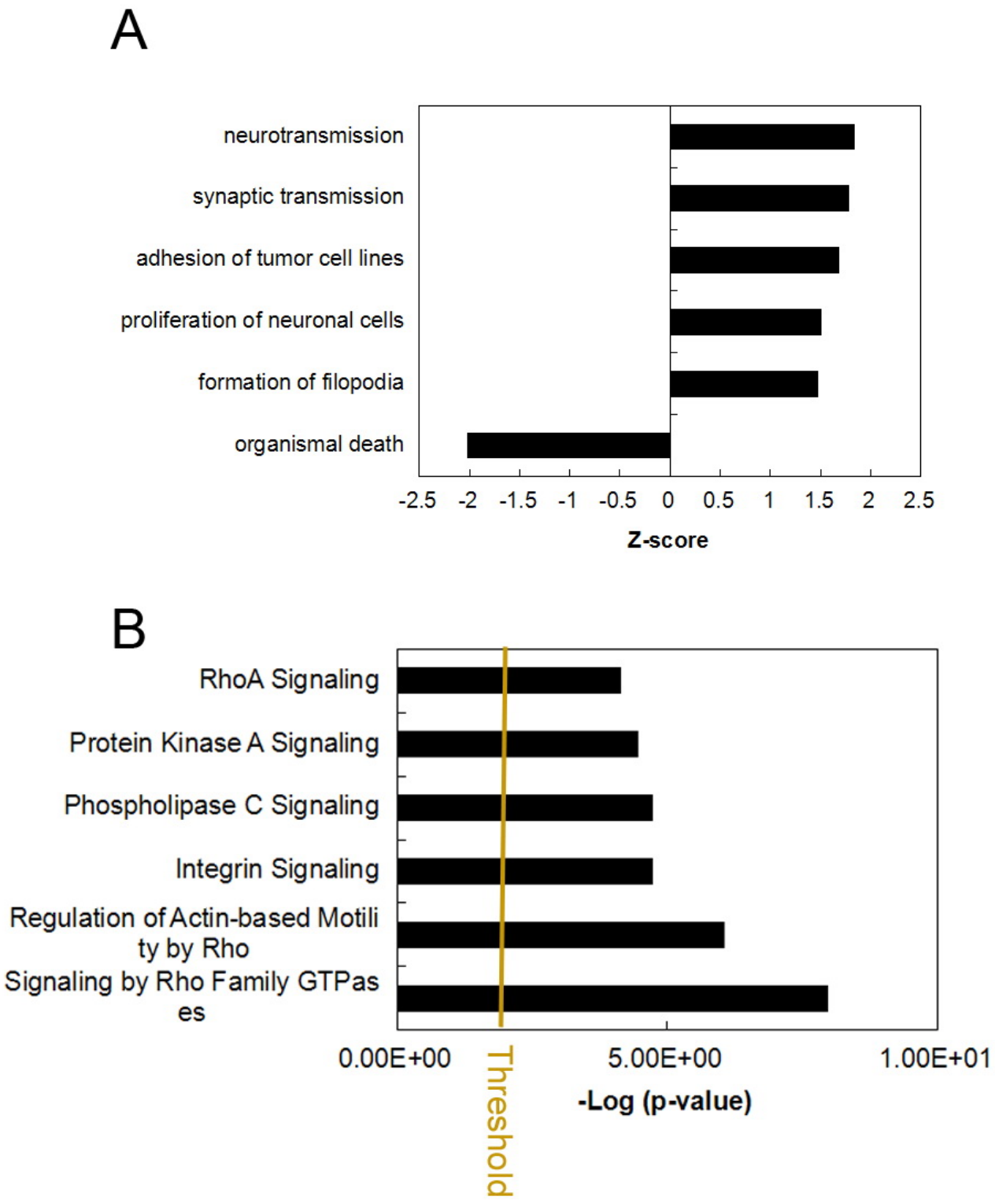
A
neurotransmission
synaptic transmission
adhesion of tumor cell lines
proliferation of neuronal cells
formation of filopodia
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Z-score
B
RhoA Signaling
Protein Kinase A Signaling
Phospholipase C Signaling
Integrin Signaling
Regulation of Actin-based Motility by Rho
Signaling by Rho Family GTPases
0.00E+00
5.00E+00
1.00E+01
-Log (p-value)
Threshold

Figure 3

Differential proteins in Signaling by Rho Family GTPases pathway between oligodendrogliomas and astrocytomas.

Figure 3 Differential proteins in Signaling by Rho Family GTPases pathway between oligodendrogliomas and astrocytomas. Red: over-expressed in astrocytomas. Green: under-expressed in astrocytomas.

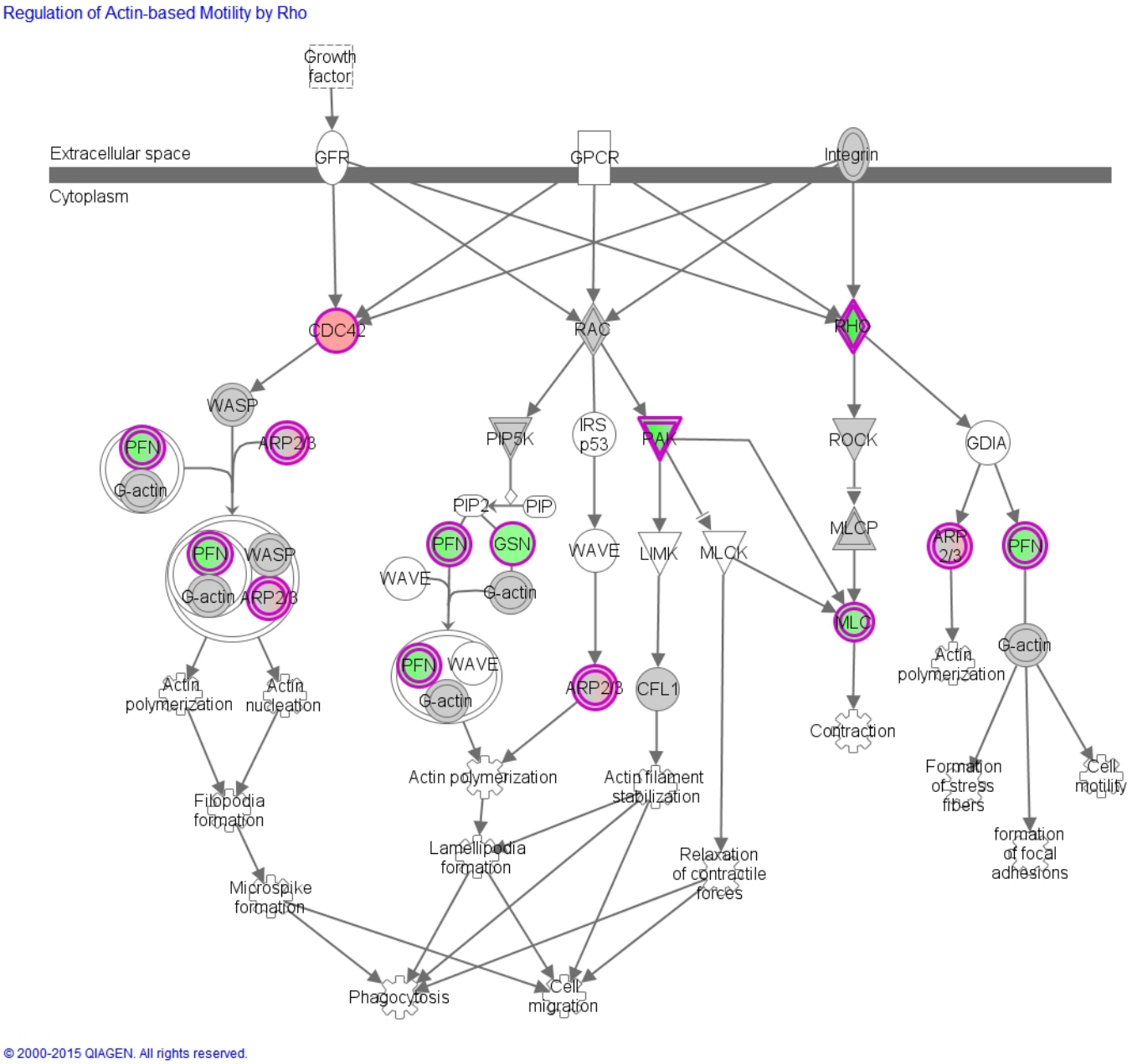

Figure 4

Western blot validation for five differential proteins. A: Western blot figure of the three candidate biomarkers in oligodendrogliomas and astrocytomas.

Figure 4 Western blot validation for five differential proteins. A: Western blot figure of the three candidate biomarkers in oligodendrogliomas and astrocytomas.

Scatter plot of GFAP (B), CDC42 (C) and RhoA (D) were shown.

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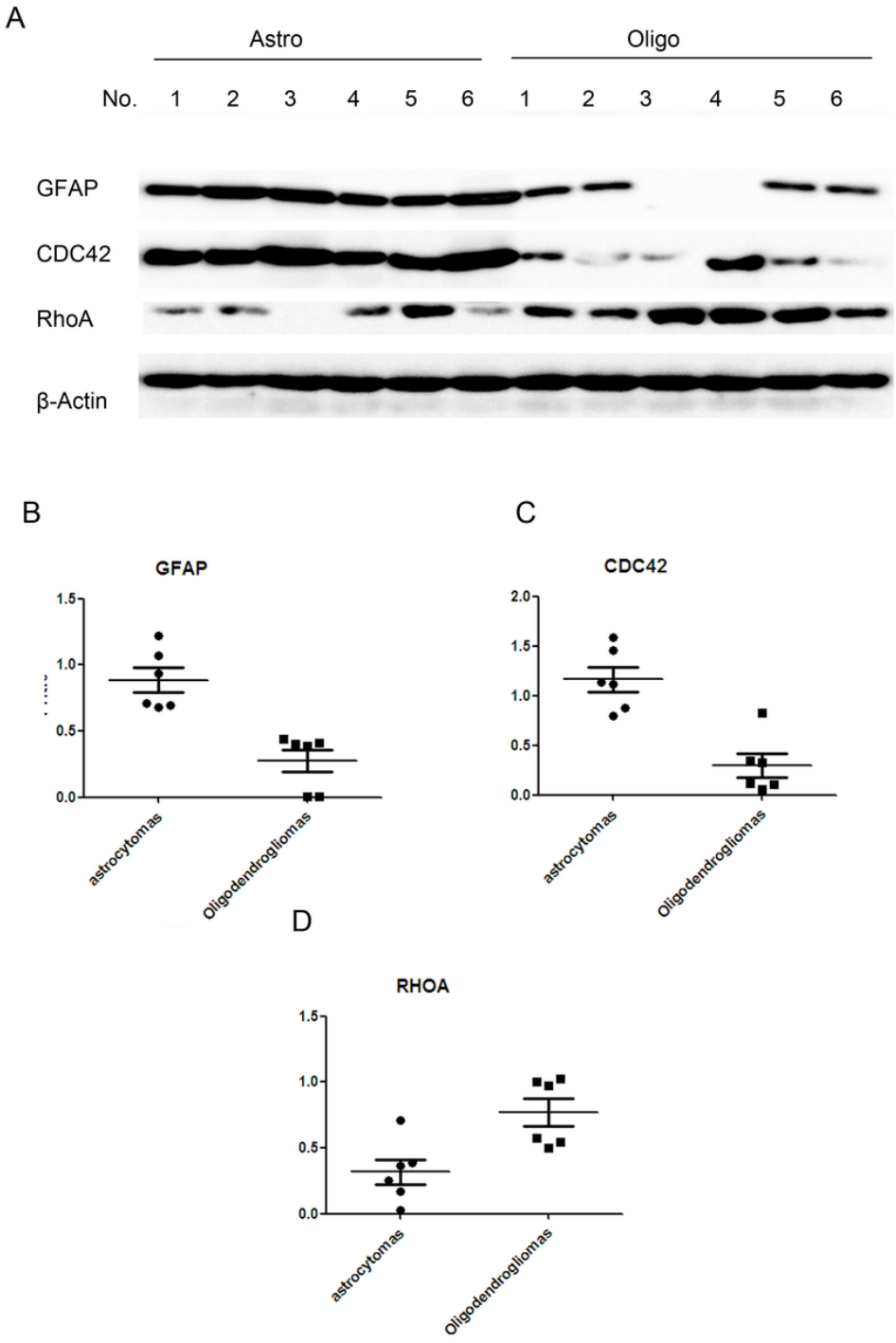
A
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0.5
0.0
astrocytomas
Oligodendrogliomas
C
CDC42
2.0
1.5
1.0
0.5
0.0
astrocytomas
Oligodendrogliomas
D
RHOA
1.5
1.0
0.5
0.0
astrocytomas
Oligodendrogliomas