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RAPID COMMUNICATION

Potential glioblastoma biomarkers identified by mass spectroscopy and iTRAQ labeling

Glioblastoma multiforme (GBM) is the most common primary malignant tumor of the central nervous system. Grade IV GBM is lethal, and has a high rate of relapse despite aggressive therapy. The prognosis is poor because of therapeutic resistance and tumor relapse.[1] Very low rates of clinical responses and frequent treatment failures are common. Previous studies have focused on identifying genetic alterations in GBM that have diagnostic and prognostic values and may help to define the subclasses of the GBM patients,[2,3] including mutations in isocitrate dehydrogenase 1 (IDH1), amplification of epidermal growth factor receptors (EGFR), and mutations and polymorphisms of telomerase reverse transcriptase (TERT) promoter. These markers are useful for identifying the pathogenesis of malignant glioma formation, but they are all associated with tumorigenesis and do not currently influence the treatment of most GBM patients.

In this study, quantitative proteomics with isobaric tags for relative and absolute quantitation (iTRAQ) labeling and liquid chromatography/tandem mass spectrometry (LC-MS/MS) was used to identify novel GBM protein markers. The human tissue samples were confirmed as GBM (Fig. S1) by a neuropathologist. The extracted proteins from three GBM samples and three normal tissues of traumatic brain injury were iTRAQ-labeled and analyzed by two-dimensional LC-MS/MS. A total of 3856 proteins within the 95% confidence interval limits of the sample distribution were identified. Of those, 234 proteins were confirmed as differentially expressed proteins (DEPs) in the GBM and normal brain tissues ($P < 0.05$). The average protein ratio and *P*-value indicated that 76 were upregulated and 158 were downregulated in GBM compared with normal tissues (Fig. 1A and Table S1, 2). A hierarchical clustering diagram (Fig. S2) including the 234 DEPs ($P < 0.05$) was constructed using *R* statistical software (https://www.r-project.org). The biological functions of the 234 DEPs were investigated by GO annotation analysis and were found to be associated with biological processes, molecular functions, and cellular compartments (Fig. S3). Proteins in the biological process category (Fig. S3A) were involved in single–organism processes (10.84%), cellular processes (10.78%), biological regulation (8.38%), biological process regulation (7.67%), metabolic processes (7.38%), and localizations (7.09%). Proteins in the molecular function category (Fig. S3B) were associated with binding (48.31%), followed by catalytic activities, transporter activities, structural molecule activities, and enzyme regulator activities. Proteins in the cellular compartment category (Fig. S3C), *i.e.*, subcellular distribution, were cell part proteins (13.92%), cell proteins (13.92%), organelle proteins (12.26%), organelle part proteins (10.06%), membrane proteins (9.93%), and membrane part proteins (7.4%).

Western blot assays of five representative DEPs were performed to verify the quantitative results of iTRAQ labeling and LC-MS/MS analysis. HSPA5, also called GRP78, a previously reported biomarker, was found to be overexpressed in human malignant glioma. This observation of overexpression in HSPA5 is consistent with the results of Pyrko et al.[4] Compared with normal tissues, FAIM2, SYNGR3, SNAP25, and NDUFB7 were significantly downregulated in GBM tissues (Fig. 1B).

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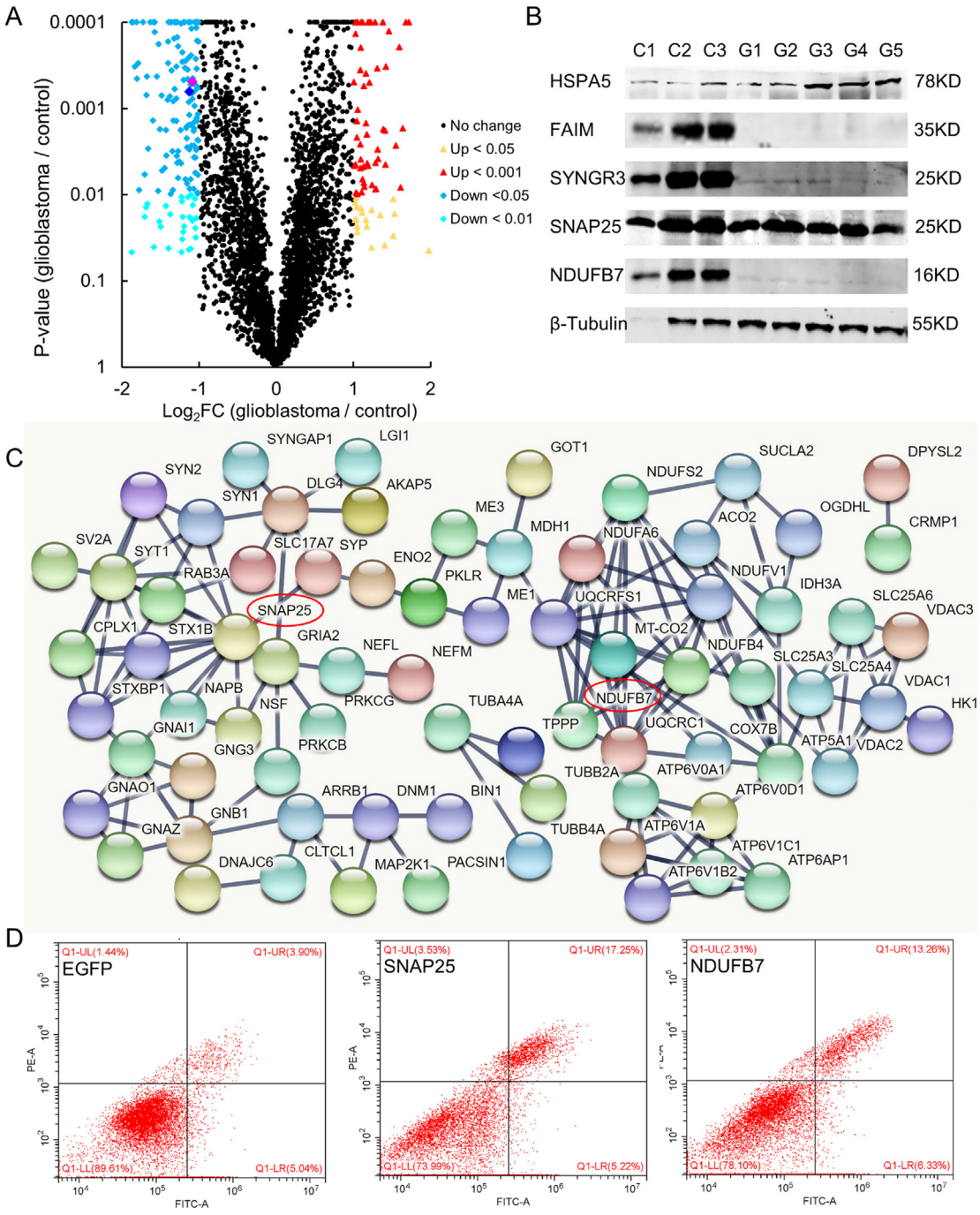

Figure 1 Identification and analysis of novel GBM protein markers. (**A**) Identification of DEPs. Log ratios and *P*-values for 3,856 proteins from three glioblastoma and three normal tissues are plotted. 6% of proteins with significant differences in the amount present in glioblastoma and normal tissue ($P < 0.05$, two-sided *t*-test) were considered differentially expressed. Black dots indicate non-significantly different proteins. $P < 0.05$ (yellow triangles) and $P < 0.01$ (red triangles) indicate significantly upregulated proteins; $P < 0.05$ (deep sky-blue diamonds) and $P < 0.01$ (aqua diamonds) indicate significantly downregulated proteins; magenta diamond indicates Snap 25 and blue diamond indicates NDUFB7. (**B**) Verification of five selected DEPs by western blotting. Representative western blots of the five candidate biomarkers in glioblastoma and control tissue. Box plots of the expression levels of HSPA5 (B), FAIM2 (C), SYNGR3 (D), SNAP25 (E), and NDUFB7. (**C**) Protein—protein interaction networks of DEPs. A high-confidence (0.900) PPI including the 144 DEPs was evaluated by String V11.5. Lines in the network display indicate evidence of association with data support and interaction score greater than 0.9. The nodes shown as opaque red circles are SNAP25 and NDUFB7. (**D**) Overexpression of NDUFB7 or SNAP25 in U251 cells promotes apoptosis of glioblastoma cells. U251 cells were transfected with pCAGGS-EGFP, pCAGGS-NDUFB7-EGFP, or pCAGGS-SNAP25-EGFP. Apoptosis was assayed by annexin V-FITC/PI flow cytometry 48 h after transfection. Compared with EGFP, overexpression of NDUFB7 or SNAP25 leads to a significant increase in apoptosis.

The analysis of the KEGG pathway revealed 215 GBM pathways. Most of the 234 DEPs were involved in organismal systems such as synaptic vesicle cycling and oxidative phosphorylation (Fig. S4). A high-confidence (0.900) protein—protein interaction network including 144 DEPs of the 234 DEPs was generated by String V11.5. SNAP25 and NDUFB7 had key roles in the DEP PPI network as shown in Figure 1C. SNAP25 was associated with 11 proteins, including SYP, SYN1, SYN2, SYT1, STXBP1, STX1B, CPLX1, RAB3A, NSF, NAPB, and GNAI1, most of which participated in synaptic vesicle cycling. NDUFB7 was associated with proteins including NDUFA6, NDUFV1, NDUFS2, NDUFB4, UQCRFS1, and UQCRC1, all of which were involved in oxidative phosphorylation (Fig. S4). Overexpression of NDUFB7 or SNAP25 in U251 cells promoted apoptosis as shown by Annexin V-FITC/PI flow cytometry (Fig. 1D).

In this study, iTRAQ-labeled two-dimensional LC-MS/MS identified DEPs in GBM and normal tissues. A total of 234 DEPs were identified, 76 of which were upregulated, and 158 were downregulated in GBM compared with normal brain tissues. Bioinformatic analysis revealed that most DEPs were functionally associated with cell processes, including protein binding, catalytic activity, and transporter activity, and the DEPs were matched with 215 pathways. Many DEPs were active in synaptic vesicle cycling and oxidative phosphorylation.

SNAP25 belongs to a family essential for synaptic and secretory vesicle exocytosis and regulation of neurotransmitter release, and associates with proteins involved in vesicle docking and membrane fusion. It regulates plasma membrane recycling by interacting with centromere protein F (CENPF) and modulates the gating characteristics of the delayed rectifier voltage-dependent KCNB1 potassium channel. The overexpression of SNAP25 exerting a tumor suppressor role was previously reported in human malignant glioma cell lines.[5] In this study, SNAP25 was significantly downregulated in glioblastoma, and transient expression of SNAP25 in U251 cells inhibited cell proliferation and promoted apoptosis of glioma cells. This is the first report that supports SNAP25 as a diagnostic marker and a target for glioma.

NDUFB7 and NDUFA8 are subunits of NADH—ubiquinone oxidoreductase (complex I) in the mitochondrial inner membrane, the first enzyme complex of the mitochondrial respiratory chain, and are located on the intermembrane surface of complex I. They have NADH dehydrogenase (ubiquinone) and oxidoreductase activities in the electron transport chain. NDUFB7 deficiency causes mitochondrial myopathy and mitochondrial encephalomyelopathies in humans, and decreased NDUFB7 activities associated with aging may cause Parkinson's disease, skin pigmentation diseases, ovarian cancer, and Wilson's disease. In this study, NDUFB7 expression inhibited proliferation and promoted apoptosis of glioma cells. NDUFB7 deficiency might promote GBM tumorigenesis because of a decrease in tumor suppression. The results support NDUFB7 as a GBM biomarker and candidate therapeutic agent for GBM.

In conclusion, iTRAQ and LC-MS/MS identified DEPs in GBM cells, and Western blot analysis confirmed the significance of the differential expressions of five DEPs (HSPA5, FAIM2, SYNGR3, SNAP25, NDUFB7) in GBM and normal brain tissues. Functional analysis indicated that SNAP25 and NDUFB7 influenced cell apoptosis of glioma cells. The glioma-associated activities support these proteins as potential diagnostic markers and therapeutic targets of GBM.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2022.11.010.

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