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Indentification of Novel Biomarkers for Paroxysmal Non-Kinesigenic Dyskinesia Diagnosis Via Cerebrospinal Fluid and Plasma Proteomic Analysis

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ABSTRACT

Purpose: Paroxysmal non-kinesigenic dyskinesia (PNKD) is a rare autosomal dominant movement disorder characterized by spontaneous attacks. The purpose of this study was to find and analyze the differentially expressed proteins between PNKD patients and their healthy family members with matched age and gender, thus providing potential biomarkers for early diagnosis of patients with PNKD.

Methods: Four PNKD patients and four healthy family members were selected. Isobaric tags for relative and absolute quantitation (iTRAQ) coupled with LC-MS/MS were used to identify the differential proteins obtained from CSF and plasma of PNDK patients and healthy family members. Bioinformatic analyses of differential proteins were performed included Gene Ontology (GO) enrichment, pathway enrichment and protein-protein interaction network analysis.

Results: A total of 1242 and 512 unique proteins were identified in CSF and plasma respectively. The numbers of proteins that were differentially expressed between PNKD patients and their healthy family members were 42 in CSF and 57 in plasma respectively. Proteins that were differentially expressed were further analyzed based on bioinformatics. Two differentially expressed proteins in the CSF, CALML5 and PDGFA, and one in the plasma, ACTB, showed close links with the PNKD protein in the protein–protein interaction network.

Conclusion: CALML5 and PDGFA in the CSF and ACTB in the plasma may take part in PNKD pathogenesis and act as the potential biomarkers for PNKD diagnosis.

Key Words:

Paroxysmal non-kinesigenic dyskinesia (PNKD), Isobaric tags for relative and absolute quantitation (iTRAQ), Bioinformatics analysis, Protein–protein interaction network

Abbreviations

CALML5: Calmodulin-Like 5; CSF: Cerebro-Spinal Fluid; GO: Gene Ontology; iTRAQ: Isobaric Tags For Relative And Absolute Quantitation; KEEG: Kyoto Encyclopedia Of Genes And Genomes; MAPK3: Mitogen-Activated Protein Kinase 3; MR-1: Myofibrillogenesis Regulator 1; PDGFA: Platelet-Derived Growth Factor Subunit A; PNKD: Paroxysmal Non-Kinesigenic Dyskinesia; PPI: Protein–Protein Interaction

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Introduction

Paroxysmal non-kinesigenic dyskinesia (PNKD) is a kind of the familial paroxysmal dyskinesias which are a group of hyperkinetic movement disorders [1]. It was first reported by Mount and Reback in 1940 that PNKD is an autosomal dominant disorder with high penetrance [2]. PNKD is characterized by intermittent attacks with any combination of dystonia, chorea, athetosis or ballismus, which can be precipitated by stress, fatigue, coffee, alcohol and menstruation [1-5]. The disease is associated with single amino acid changes (A7V, A9V or A33P) in myofibrillogenesis regulator 1 (MR-1) on chromosome 2q35 which is the causative gene of PNKD [6-10]. We have previously reported the first large Chinese PNKD pedigree, and found a novel mutation of A7V on exon 1 of the PNKD/MR-1 gene [11].

Cerebrospinal fluid (CSF) reflects the ongoing biochemical changes occurring in the central nervous system, thus providing a potential source of biomarkers of neurodegenerative diseases such as Alzheimer's disease and progressive supranuclear palsy [12,13].

As PNKD is a rare neurological disorder characterized by paroxysmal movement disorders, CSF might also act as an ideal source for screening and identifying novel PNKD-related biomarkers. However, the collection of CSF is an invasive process. In this case, plasma, which contains thousands of proteins available for potential biomarker evaluation, offers a promising alternative choice for PNKD biomarker screening [12].

Proteomic technologies have been largely used to search for differentially expressed proteins, in order to clarify the diagnosis and prognosis of diseases such as tumor and neurodegenerative disorders [14-16]. Isobaric tags for relative and absolute quantitation (iTRAQ) technique is one of the most widely used approaches because it can simultaneously analyze 8 different specimens, thus increasing throughput while reducing experimental error [17-19]. In this study, we used iTRAQ labeling followed by 2D-LC-MS/MS for the quantitative proteomic analysis of the CSF and plasma samples from PNKD patients and their healthy family members to discover potential effective biomarkers for PNKD diagnosis.

Materials and Methods

■ Recruitment of Subjects:

Four patients with PNKD from previously reported Chinese family and four healthy family members were enrolled in 2015. Each patient was matched with a healthy family member of the same gender and the age gap of 0-3 years **(Figure 1)**.

■ Sample Preparation:

All plasma and CSF samples were obtained at 10 o'clock in the morning after 12 hours' fasting. Each sample was obtained within 30 minutes and centrifuged at 2,000 rpm for 10 minutes at 4℃. The supernatants were transferred into Eppendorf tubes and stored at -80℃.

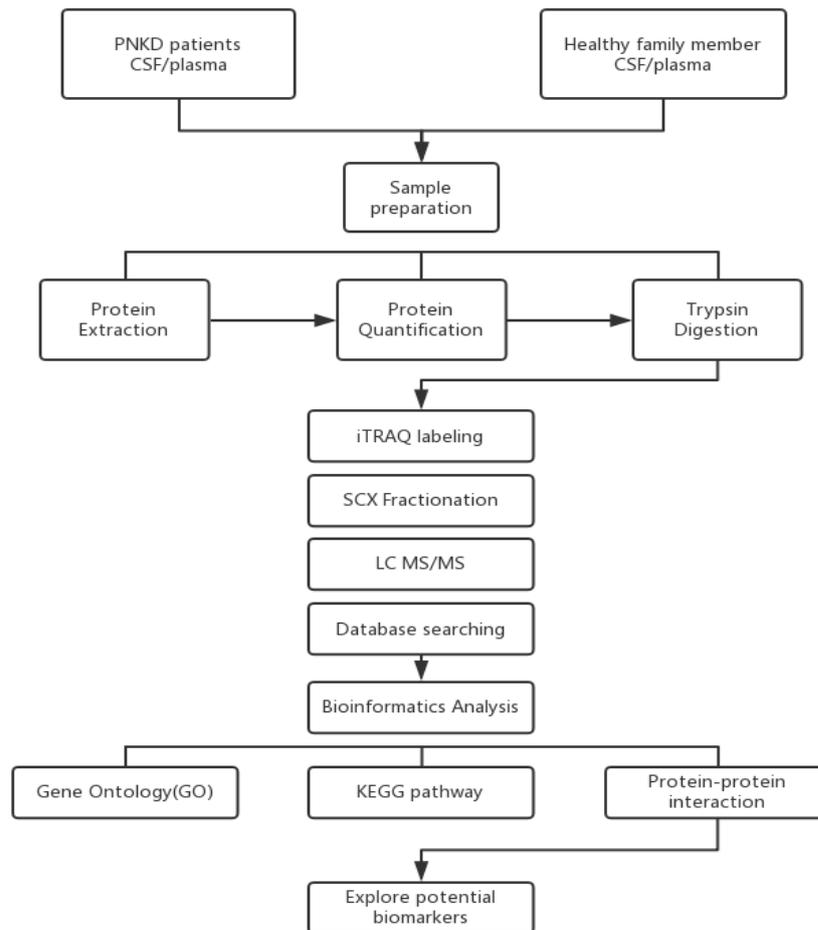### ■ Protein extraction, quantitation and digestion:

500 μl SDT lysates were added to each sample and homogenized, before being placed in a boiling water bath and undergoing ultrasonic lysis. The samples were then centrifuged, and the supernatant was collected and proteins with a molecular weight cutoff of 3 kD were extracted by ultrafiltration. After elution, the protein content of each subgroup was quantified with a BCA Kit (Thermo Scientific™). One hundred mictoliters of the processed protein were taken out of each sample solution and then the protein was digested with Trypsin Gold (Promega, Madison, WI, USA) at a ratio of protein : trypsin = 100 : 1 at 37ºC for 12 hours.

■ iTRAQ labeling and SCX Fractionation

80 μg of samples in each group were labeled according to the manufacturer's instructions (AB Company: iTRAQ Reagent-8plex Multiplex Kit, AB SCIEX). Briefly, one unit of iTRAQ reagent (defined as the amount of reagent required for the labeling of 40 μg of protein) was thawed and reconstituted in 24 μl isopropanol. Peptides were labeled with 113 to 119, and 121 iTRAQ tags by incubation at room temperature for 2 hours. The resulting labeled peptide samples were then pooled together, dried up and then, the mixtures were fractionated by SCX (strong cation exchange) separation.

■ LC-MS/MS analysis

After separation by SCX chromatography, equal amounts of digested protein were loaded into a 10-cm analytical C18 column packed in-house, and then washed with solvent A (0.5% FA / H2O), solvent B (0.5% FA / ACN), the peptides

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Figure 3: Workflow of the designed study used for searching important proteins associated with Paroxysmal non-kinesigenic dyskinesia (PNKD).

were eluted with a gradient 150 min at 300 nl/min flow rate. MS/MS was carried out with a Q-Exactive mass spectrometer (Thermo Finnigan, USA) setting in data-dependent mode with the previously reported ion scanning parameters [14]. Fragmented peptide masses were set in dynamic exclusion for 120 seconds and singly charged ions were excluded from MS/MS analysis.

■ Database Search

The resulting MS/MS spectra were searched against the Ensembl database Homo sapiens, IWGSC1.0 + popseq 28 with Proteome Discoverer 1.4 software (version 1.4.0.288, Thermo Fisher Scientific). Retrieval of peptides and spectrogram matching (PSM) was filtered with the Percolator algorithm and FDR<0.01.. Retrieving peptides were merged into protein using the maximum parsimony principle.

■ GO Enrichment Analysis

The Gene Ontology (GO) database (http://www.geneontology.org) was used to classify and group the differentially expressed proteins with a 1.5-fold change according to Fisher's exact test. The Parent-Child-Intersection method was used for enrichment analysis, and Benjamini-Hochberg was used for multiple test correction. Curated association (i.e., all evidence codes except IEA, ND, and NR) was used for enrichment analysis.

■ Pathway enrichment analysis:

Pathway analysis was used to determine the significant pathways of the differential proteins according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Fisher's exact test was performed to select the significant pathway, and the threshold of significance was $p < 0.05$.

■ Protein-protein interaction network:

Hypergeometric distribution was used in order to calculate pathway enrichment, and FDR was used to adjust the p-values for multiple

comparisons. After parsing the whole KEGG database, all of the pathways were extracted for the studied genes, and the pathway network was generated with the help of the pathway topology gleaned from the KEGG database. Network construction was performed by Cytoscape 3.4.0 (http:// www.cytoscape.org/).

Results

■ Identification of differential proteins in CSF and plasma between PNKD patients and healthy family members:

In the CSF proteome, peptide lengths were mainly distributed between 8 and 17 (mean 14.0), with most at a length of 10 peptides.

In total, 18258 PSMs were identified in the CSF, among which 17797 were labeled with the iTRAQ reagent, achieving a labeling efficiency of 97.5%. A total of 1424 proteins were identified. 22 up- and 20 down-regulated proteins in PNKD patients were found when compared against healthy with 1.5-fold change **(Table 1)**.

In the plasma proteome, peptide lengths were mainly distributed between 10 and 15(mean 13.0), with most consisting of 11 peptides. 6942 PSMs were labeled with the iTRAQ reagent, and 512 proteins were identified. 32 up- and 25 down-regulated proteins in PNKD patients were found when compared against healthy with 1.5-fold change **(Table 2)**.

Table 1: The differential expressed proteins in CSF.

Accession	Protein name	Gene symbol	Fold change (PNKD vs normal)
P31151	Protein S100-A7	S100A7	2.75
Q9NYL2-3	Isoform 3 of Mitogen-activated protein kinase kinase kinase MLT	MLTK	2.55
P04206	Ig kappa chain V-III region GOL	KV307	2.45
G3V1N2	HCG1745306, isoform CRA_a	HBA2	2.39
P06702	Protein S100-A9	S100A9	2.24
Q03188	Centromere protein C 1	CENPC1	2.05
P02538	Keratin, type II cytoskeletal 6A	KRT6A	1.92
P29508-2	Isoform 2 of Serpin B3	SERPINB3	1.90
A8MXB6	Dynamin-like 120 kDa protein, mitochondrial (Fragment)	OPA1	1.84
K7ERI9	Truncated apolipoprotein C-I (Fragment)	APOC1	1.80
B8ZZE5	Augurin	C2orf40	1.76
Q9NZT1	Calmodulin-like protein 5	CALML5	1.73
P47929	Galectin-7	LGALS7	1.69
D6REY1	Chitotriosidase-1	CHIT1	1.68
P22004	Bone morphogenetic protein 6	BMP6	1.66
P23083	Ig heavy chain V-I region V35	HV103	1.62
P01608	Ig kappa chain V-I region Roy	KV116	1.59
P86790	Vacuolar fusion protein CCZ1 homolog B	CCZ1B	1.58
H0Y6W5	Zinc finger protein basonuclin-2 (Fragment)	BNC2	1.53
Q504Y0-2	Isoform 2 of Zinc transporter ZIP12	SLC39A12	1.53
P78563-2	Isoform 2 of Double-stranded RNA-specific editase 1	ADARB1	1.51
P05109	Protein S100-A8	S100A8	1.51
F8VSC5	SCY1-like protein 2 (Fragment)	SCYL2	0.66
P04085-2	Isoform Short of Platelet-derived growth factor subunit A	PDGFA	0.65
E5RGY1	Disintegrin and metalloproteinase domain-containing protein 28	ADAM28	0.65
P01877	Ig alpha-2 chain C region	IGHA2	0.64
E7EPS8	Receptor-type tyrosine-protein phosphatase mu	PTPRM	0.64
C9J2Z9	Histone-lysine N-methyltransferase SETD1A (Fragment)	SETD1A	0.63
A6NEL2	Ankyrin repeat domain-containing protein SOWAHB	SOWAHB	0.62

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P13284	Gamma-interferon-inducible lysosomal thiol reductase	IFI30	0.61
H0Y987	Phosphoacetylglucosamine mutase (Fragment)	PGM3	0.61
C9JRE9	C-reactive protein(1-205)	CRP	0.60
O43493-2	Isoform TGN46 of Trans-Golgi network integral membrane protein 2	TGOLN2	0.59
O76061	Stanniocalcin-2	STC2	0.57
F8W8P9	Rho guanine nucleotide exchange factor 11	ARHGEF11	0.56
O14793	Growth/differentiation factor 8	MSTN	0.55
P01860	Ig gamma-3 chain C region	IGHG3	0.53
Q8N895	Zinc finger protein 366	ZNF366	0.46
Q9UI40-2	Isoform 2 of Sodium/potassium/calcium exchanger 2	SLC24A2	0.42
K7EQJ5	40S ribosomal protein S15 (Fragment)	RPS15	0.41
P01614	Ig kappa chain V-II region Cum	KV201	0.40
P01880	Ig delta chain C region	IGHD	0.39

Table 2: The differential expressed proteins in plasma.			
Accession	**Protein name**	**Gene symbol**	**Fold change (PNKD vs normal)**
P01814	Ig heavy chain V-II region OU	HV201	2.367
Q96M19	Transmembrane protein LINC00477	LINC00477	2.132
P05534	HLA class I histocompatibility antigen, A-24 alpha chain	HLA-A	2.110
P49326-3	Isoform 3 of Dimethylaniline monooxygenase [N-oxide-forming] 5	FMO5	2.042
P11226	Mannose-binding protein C	MBL2	2.027
O95810	Serum deprivation-response protein	SDPR	1.970
Q9H4B7	Tubulin beta-1 chain	TUBB1	1.892
P07737	Profilin-1	PFN1	1.887
Q12951-2	Isoform 2 of Forkhead box protein I1	FOXI1	1.839
P63267	Actin, gamma-enteric smooth muscle	ACTG2	1.827
P60709	Actin, cytoplasmic 1	ACTB	1.811
P01722	Ig lambda chain V-VI region NIG-48	LV602	1.795
E9PAQ1	Properdin	CFP	1.790
Q16610	Extracellular matrix protein 1	ECM1	1.787
P43652	Afamin	AFM	1.769
H7C2W2	E3 ubiquitin-protein ligase SHPRH (Fragment)	SHPRH	1.760
B8X2Z3	CARMIL2b	RLTPR	1.757
B4DV03	HCG1780060, isoform CRA_b	CCDC163P	1.738
P01612	Ig kappa chain V-I region Mev	KV120	1.712
F5H677	Nucleolar complex protein 3 homolog	NOC3L	1.682
Q96EU6-2	Isoform 2 of Ribosomal RNA processing protein 36 homolog	RRP36	1.650
F5H125	Cell adhesion molecule 1 (Fragment)	CADM1	1.635
P01699	Ig lambda chain V-I region VOR	LV101	1.624
Q5SZK8-2	Isoform 2 of FRAS1-related extracellular matrix protein 2	FREM2	1.614
Q86UX7-2	Isoform 2 of Fermitin family homolog 3	FERMT3	1.601
Q6UXB8	Peptidase inhibitor 16	PI16	1.594
P08514-3	Isoform 3 of Integrin alpha-IIb	ITGA2B	1.563
Q8NDW8-6	Isoform 6 of Tetratricopeptide repeat protein 21A	TTC21A	1.546
C9JPQ9	Fibrinogen gamma chain (Fragment)	FGG	1.531
B1ALQ8	Complement factor H-related protein 4	CFHR4	1.527

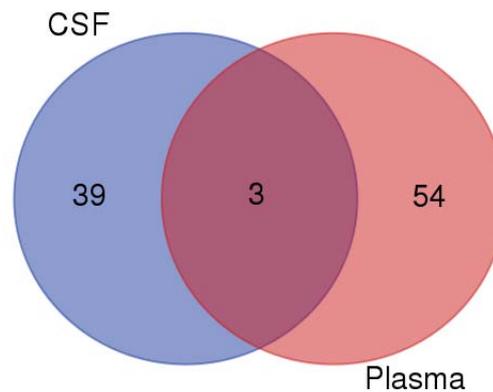| Q96JK4-2	Isoform 2 of HHIP-like protein 1	HHIPL1	1.512
P01703	Ig lambda chain V-I region NEWM	LV105	1.507
P42356	Phosphatidylinositol 4-kinase alpha	PI4KA	0.649
P18428	Lipopolysaccharide-binding protein	LBP	0.644
P02656	Apolipoprotein C-III	APOC3	0.639
Q9UJY5-4	Isoform 4 of ADP-ribosylation factor-binding protein GGA1	GGA1	0.632
P02042	Hemoglobin subunit delta	HBD	0.623
P02747	Complement C1q subcomponent subunit C	C1QC	0.619
Q9H4Z2-2	Isoform 2 of Zinc finger protein 335	ZNF335	0.613
P14780	Matrix metalloproteinase-9	MMP9	0.579
P02745	Complement C1q subcomponent subunit A	C1QA	0.563
P01860	Ig gamma-3 chain C region	IGHG3	0.538
M0QZV5	Endophilin-A2	SH3GL1	0.535
M0R1F2	Zinc finger protein 208 (Fragment)	ZNF208	0.527
F5GZM4	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	PCK1	0.522
E5RH81	Carbonic anhydrase 1 (Fragment)	CA1	0.476
A6NIW5	Peroxiredoxin 2, isoform CRA_a	PRDX2	0.448
P01816	Ig heavy chain V-II region DAW	HV203	0.407
P01859	Ig gamma-2 chain C region	IGHG2	0.399
P69905	Hemoglobin subunit alpha	HBA1	0.371
P00918	Carbonic anhydrase 2	CA2	0.357
P68871	Hemoglobin subunit beta	HBB	0.338
H0YMN4	SH2 domain-containing adapter protein F (Fragment)	SHF	0.326
P07996	Thrombospondin-1	THBS1	0.283
P02741	C-reactive protein	CRP	0.234
P01880	Ig delta chain C region	IGHD	0.194
A0FGR8-4	Isoform 4 of Extended synaptotagmin-2	ESYT2	0.124

Comparing the two groups of differentially expressed proteins , we found 3 co-down regulated proteins in CSF and plasma (*CRP,IGHG3,IGHD*) **(Figure 2)**, but further analysis showed no clear evidence of association with the PNKD.

■ Functional annotation of the differentially expressed proteins in CSF and plasma:

In order to obtain a global functional view of the differentially expressed proteins, GO enrichment analysis and KEGG pathway analysis were employed. The GO annotation was categorized including biological process, molecular function, and cellular component.

The up-regulated proteins in CSF participated in biological processes included neutrophil aggregation, chemokine production, neutrophil degranulation, positive regulation of peptide secretion etc., The molecular functions categories included RAGE receptor binding, Toll-like receptor 4 binding, arachidonic acid binding.

The top three cellular components were classified in extracellular space, extracellular region, extracellular exosome **(Figure 3a-3c)**. The down-regulated proteins' biological processes categorized positive regulation of B cell activation, phagocytosis, recognition, phagocytosis, engulfment, B cell receptor signaling pathway, complement activation, classical pathway etc.. Top three molecular functions categories included immunoglobulin receptor binding, antigen binding, phosphoacetylglucosamine mutase activity. The cellular components categories included immunoglobulin complex, circulating, blood microparticle, external side of plasma membrane **(Figure 3d-3f)**. The differentially expressed proteins were further defined based on KEGG. The up-regulated proteins in CSF were participated in IL-17 signaling pathway, phototransduction, African trypanosomiasis. And the down-regulated proteins were categorized in cytokine-cytokine receptor interaction **(Figure 3g, 3h)**.

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Figure 3: Venn diagrams of differentially expressed proteins in CSF and plasma.

■ PNKD protein–protein interaction networks:

In order to provide a landscape of what and how the differentially expressed proteins participate in PNKD pathogenesis, PPI network was constructed based on PNKD protein, identified proteins(include differentially expressed proteins) and PNKD associated proteins from STRING database in CSF and plasma respectively **(Figure 4a-4h)**.

Among the network in the CSF, calmodulin-like 5 (CALML5) and platelet-derived growth factor subunit A (PDGFA) were able to link together with PNKD through several different pathways with the interaction of certain identified proteins and the intermediate partner mitogen-activated protein kinase 3 (MAPK3) as shown in **Figure 5** and Supplementary **Figure 1**. This indicates that CALML5 and PDGFA might regulate PNKD through MAPK3 pathway and be the potential biomarkers of PNKD diagnosis in CSF.

Among the network in the plasma, 8 differentially expressed proteins (ACTB, ITGA2B, PFN1, FGG, PCK1, THBS1, MMP9 and FERMT3) were capable of linking together with PNKD through different pathways by interacting with the intermediate partners (grey) and/or identified proteins at the PPI level, as shown in **Figure 6** and Supplementary **Figure 2**. These results indicate that ACTB, ITGA2B, PFN1, FGG, PCK1, THBS1, MMP9 and FERMT3 are the potential biomarkers of PNKD diagnosis in plasma.

Discussion

Myofibrillogenesis regulator 1 (MR-1) gene is reported to be responsible for PNKD, and it is transcribed into three spliced isoforms:
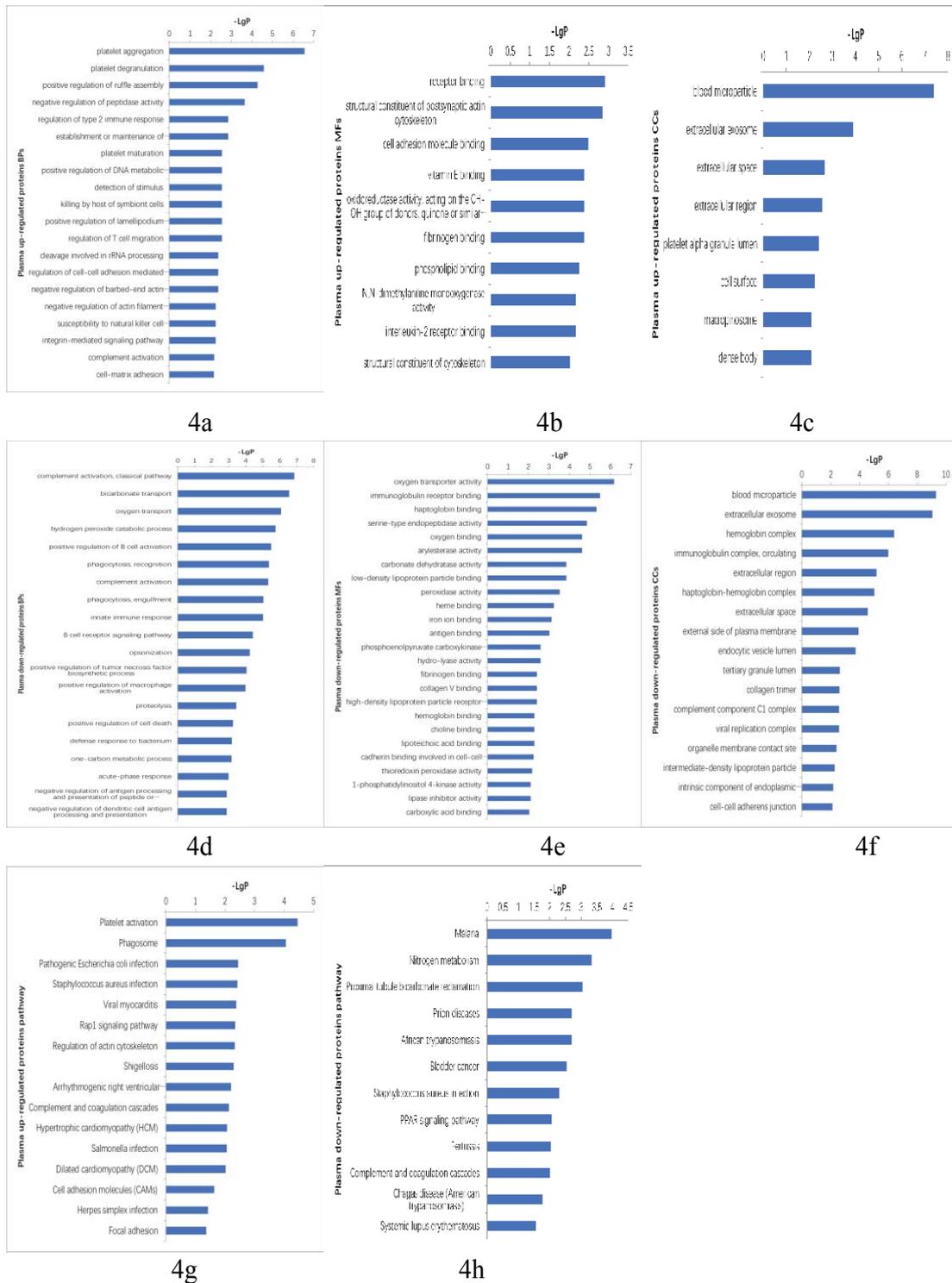
long (MR-1L/PNKD-L), medium (MR-1M/PNKD-M) and short (MR-1S/PNKD-S) [6,10]. We focused on the MR-1L/PNKD-L isoform in this study, because it is only expressed in the brain [6,10]. The results of LC-MS/MS analysis showed that neither wild type nor mutant PNKD-L proteins were found in the CSF or plasma, and confirmed that wild type and mutant PNKD-L proteins are specifically expressed in brain tissue.

The GO is a structured and controlled vocabulary of terms, and widely recognized as the premier tool for the organization and functional annotation of molecular characteristics [18,20,21]. By interpreting the GO of differentially expressed proteins and then analyzing them statistically, we have obtained 42 significant CSF proteins and 57 significant plasma proteins. Pathway analyses showed the distinct biological processes and significant pathways in which the differentially expressed proteins were involved. PNKD-related proteins were searched using the NCBI and STRING Database, and their relationships and pathways were merged the KEGG Database. KEGG is a knowledge base for systematic analysis of gene functions, linking genomic information with higher order functional information, and is widely utilized for pathway related analysis [22,23].

According to these pathways, we then constructed a PNKD-related protein-protein interaction network, which helped us to not only understand the interactions and inter-regulation of these integral genes, but also identify the key genes in these networks as determined by their Degree value. Among the PPI network in CSF, CALML5 (also known as CLSP) is reported as secreted bioactive peptide that inhibits neuronal cell death. [24], was identified as a potential

3a

3b

3c

3d

3e

3f

3g

3h
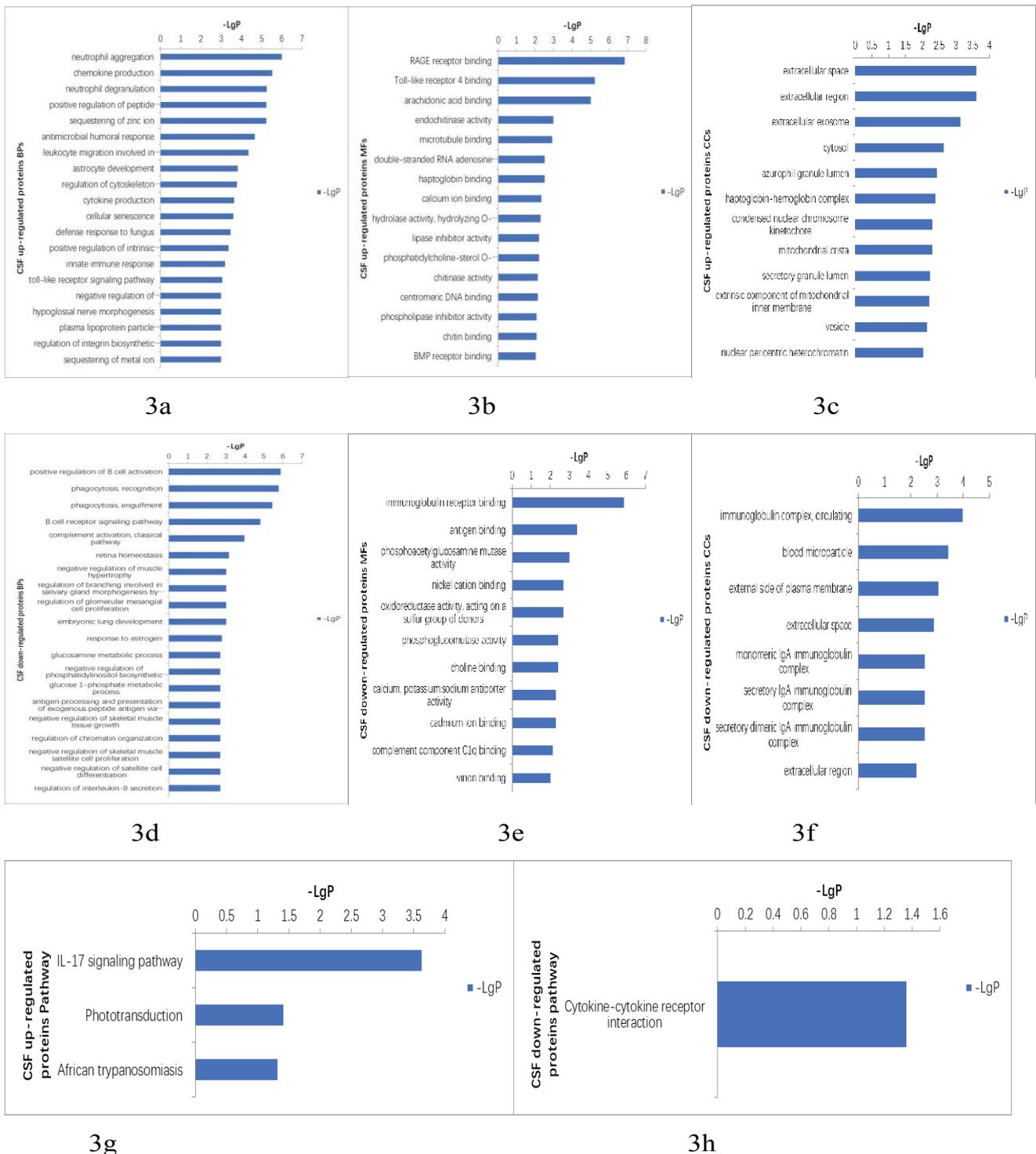
Figure 3: 3a: CSF up-regulated proteins GO biological processes Top20

3b: Molecular functions;

3c: Cellular components;

3d: CSF down-regulated proteins GO biological processes;

3e: Molecular functions;

3f: Cellular components;

3g: CSF up-regulated proteins pathway;

3h: CSF down-regulated proteins pathway.

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4a

4b

4c

4d

4e

4f

4g

4h

Figure 4: 4a: Plasma up-regulated proteins GO Top 20 biological processes;

4b: molecular functions;

4c: cellular components;

4d: plasma down-regulated proteins GO biological processes;

4e: molecular functions;

4f: cellular components;

4g: CSF up-regulated proteins pathway;
4h: CSF down-regulated proteins pathway.
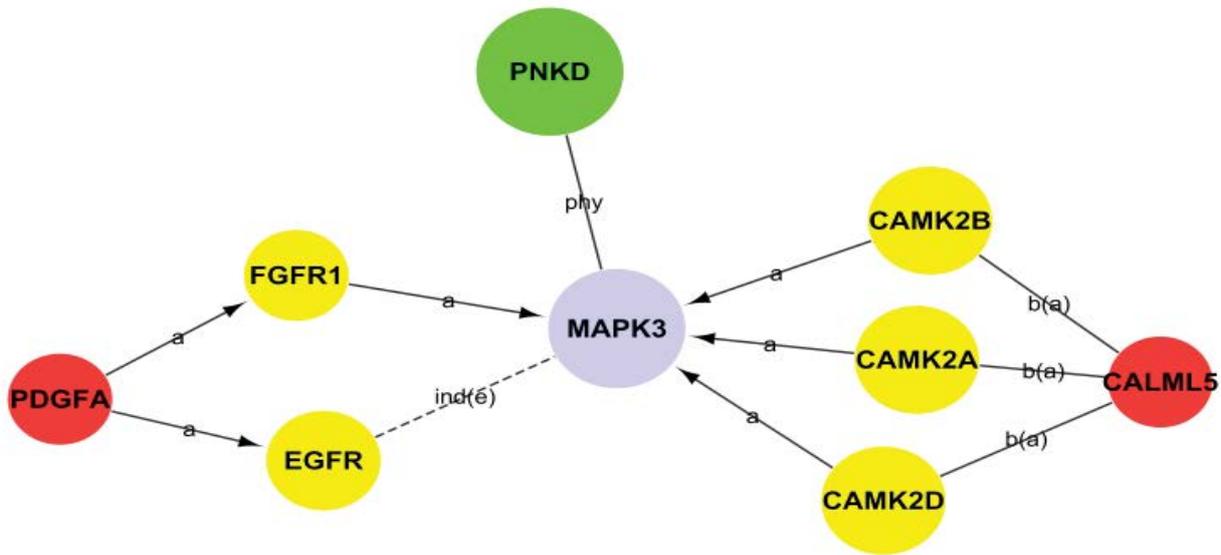
Figure 5: The PPI network of CSF differential expression proteins, (red nodes), identified but not differentially expressed proteins (yellow nodes), PNKD protein (green node), PNKD associated proteins from STRING database (grey).
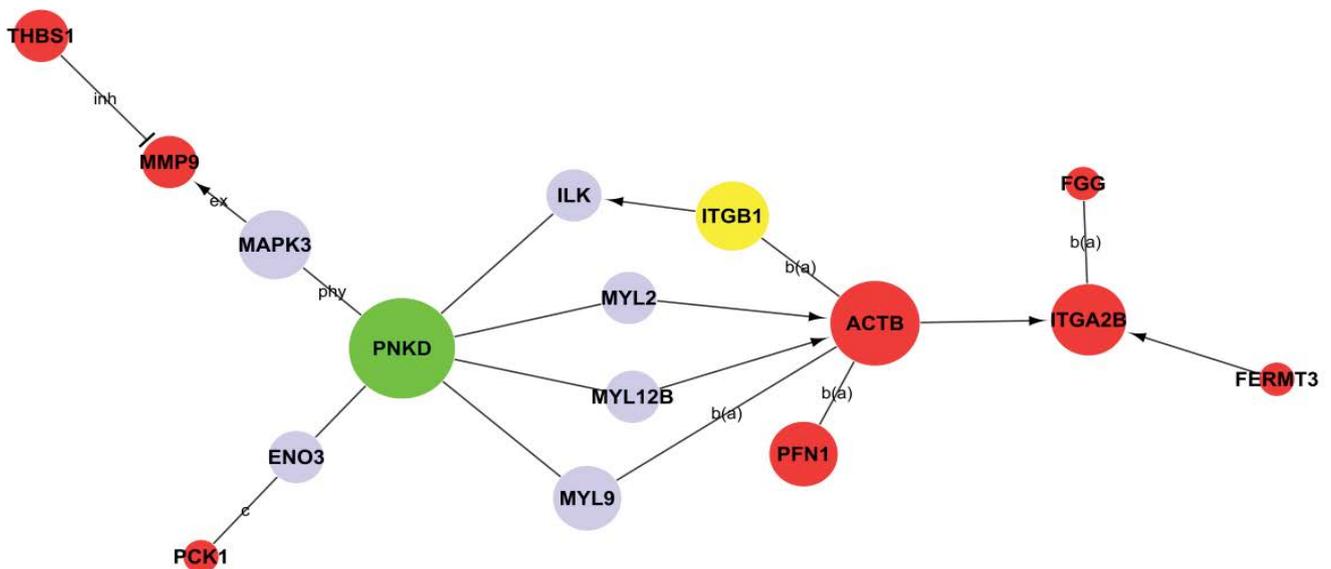
Figure 6: The PPI network of plasma differentially expressed proteins (red nodes), identified but not differentially expressed proteins (yellow nodes), PNKD protein (green node), PNKD associated proteins from STRING database (grey).

pathway component of PNKD pathogenic mechanism. Another potential biomarker in CSF of PNKD diagnosis was PDGFA, which regulates the proliferation and self-renewal in stem cells such as skin adipocyte stem cell and glioblastoma stem cell [25,26],and may participate in the development of malignant middle cerebral artery infarction (MMI) [27].

Furthermore, the major potential pathway component of the PNKD pathogenic mechanism in plasma is ACTB, which has been regarded as a housekeeping gene and a reference gene/protein in different diseases like cancers and Baraitser-Winter syndrome [28-31] and may have an important role in AD pathology [32]. ACTB can bind to MYL9 and MYL2 through the tight junction pathway. MYL9 and MYL2 are both able to bind to the PNKD protein. However, the relationships of these proteins and the pathogenic mechanisms of PNKD still require further research.

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In conclusion, 42 differentially expressed proteins in CSF and 57 differentially expressed proteins in plasma were found between PNKD patients and their healthy family members. Among the newly discovered differentially expressed proteins, CALML5 and PDGFA in the CSF and ACTB in the plasma are the potential biomarkers for PNKD diagnosis.

Acknowledgments

Ethical approval

The experiment complied with the Ethics Committee of the First Affiliated Hospital of PLA General Hospital Beijing, China.

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