

Quantitative proteomics analysis of FFPE tumor samples reveals the influences of novel targeting nanobubbles conjugated with NET-1 siRNA on tetraspanin protein involved in HCC

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Abstract

Background: Hepatocellular carcinoma (HCC) poses a severe threat to human health. The NET-1 protein has been proved to be strongly associated with HCC proliferation and metastasis in our previous study.

Methods: Here, we developed a label-free proteome mass spectrometry workflow to analyze formalin-fixed and paraffin-embedded HCC xenograft samples collected in our previous study.

Results: The result showed that 78 proteins were differentially expressed after NET-1 protein inhibited. Among them, the expression of 61 proteins up-regulated and the expression of 17 proteins were significantly down-regulated. Of the differentially expressed proteins, the vast majority of Gene Ontology enrichment terms belong to the biological process. The KEGG pathway enrichment analysis showed that the 78 differentially expressed proteins significantly enriched in 45 pathways. We concluded that the function of the NET-1 gene is not only to regulate HCC but also to participate in a variety of biochemical metabolic pathways in the human body. Furthermore, the protein-protein interaction analysis indicated that the interactions of differentially expressed proteins are incredibly sophisticated. All the protein-protein interactions happened after the NET-1 gene has been silenced.

Conclusions: Finally, our study also provides a useful proposal for targeted therapy based on tetraspanin proteins to treat HCC, and further mechanism investigations are needed to reveal a more detailed mechanism of action for NET-1 protein regulation of HCC.

Background

Worldwide, liver cancer is the fourth most common cause of cancer-related death and ranks sixth in terms of incident cases[1]. With a five-year survival of 18%, liver cancer is the second most lethal tumor after pancreatic cancer. Hepatocellular carcinoma is a major type of primary liver cancer. Changes in protein expression accompany HCC progress; thus, some proteins can be used as potential biomarkers for diagnosis and treatment[2].

Neuroepithelial transforming gene 1 (NET-1) is located at chromosome 10p15 and encodes a 54-kDa oncoprotein[3]. It is a guanine nucleotide exchange factor involved in cytoskeletal regulation and cancer cell invasion[4]. All NET genes have initially been identified as EST clones with sequences homologous to tetraspan, a superfamily which is distinguished by the presence of four transmembrane domains and has been implicated in signal transduction, cell adhesion, migration, proliferation, and differentiation[5, 6]. NET-1 protein was known to be a member of the tetraspanin family[7]. NET-1 protein has been identified in HCC, where it is a mediator of invasion and metastasis[8–10]. Our previous research proved that the NET-1 protein had an impactful role in the proliferation and stiffness of HCC[11, 12]. Besides, the low expression of NET-1 protein also reduced the migration and invasive ability of HCC[13]. However, the potential carcinogenic mechanism of NET-1 protein is still unclear.

Proteomics is an efficient research tool to reveal the mechanism and pathogenesis of diseases on the proteomic level. Because proteomics analysis could analyze quite a lot of expressed proteins in tissues or cells, this revolutionary technology has been applied to identify HCC-related proteins in many studies [14–16]. However, altered expression of proteins quantified with conventional label-free proteomic methods was limited by the fresh or rapidly frozen tissue samples. Most human tumor samples archived in hospitals for pathologic diagnosis are Formalin-Fixed Paraffin-Embedded (FFPE), which have been globally used for DNA, RNA, protein, and morphological measurements, and preanalytical factors affecting each type of measure have been identified [17]. Besides, multifarious enlightened techniques have been invented for genomic [18, 19], transcriptomic [20], proteomic and protein [21, 22] from FFPE samples. For the first time, FFPE tissues have been analyzed for protein using antibodies in 1991 as the invention of the heat-induced antigen retrieval (HIAR) technique for immunohistochemistry (IHC) [23]. Kinds of different technologies have been applied to extract proteins from FFPE samples, which have extended the research of proteins to a proteomic level [24–26]. These studies have initially confirmed that FFPE samples can be used in mass spectrometry-based proteomic analysis.

Here, we used proteomic analyses of FFPE HCC xenograft samples to characterize the global quantitative protein expression profile and identify the differential protein expressions after NET-1 siRNA transfection with the aid of low-frequency ultrasound (LFUS) irradiation. Furthermore, we aimed to shed light on the functions of tetraspanin protein involved in HCC development, and in this way, reveal HCC-related proteins valuable for targeted therapy.

Methods

Tissue samples

FFPE HCC xenograft samples were collected after sacrificed by isoflurane euthanasia from our previous research [11]. In our last study, BALB/c nude female mice (6–8 weeks, 10–25 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All the animals were housed in an environment with a temperature of 22 ± 1 °C, the relative humidity of $50 \pm 1\%$ and a light/dark cycle of 12/12 hr. Mice in group A were regarded as the negative control, which was injected with PBS. Mice in group E, which were injected with NET-1 siRNA-conjugated targeted nanobubbles and irradiated with low-frequency ultrasound, showed the best antitumor curative effect and the most extended median survival. The group A was renamed group 1, and group E was renamed group 2 to distinguish between two studies. Three pieces of FFPE samples in each group were selected for proteomic analysis.

Total Protein Extraction

The FFPE HCC xenograft samples were dewaxed with octane and then hydrated with graded ethanol. After hydration, the sample was washed twice with PBS. After removing the PBS solution, an appropriate amount of protein lysate (4% SDS, 100 mM Tris, pH = 8.5) was added and incubated at 95°C for 10

minutes at room temperature, mixed by shaking, and sonicated in an ice-water bath for 5 minutes. The samples were de-crosslinked with a refractive index at 95°C for 60 min, then reduced by adding an appropriate amount of TCEP and carboxyamidomethylated in CAA at 95°C for 5 min. The samples were sequentially centrifuged at 12000 g at 4°C for 15 min. Collecting the supernatant and adding four times volume of pre-cooling acetone at -20°C, and precipitated it at -20°C for at least 4 h. Centrifuging at 12000 g for 15 min at 4°C. Collect the precipitate and air drying. An appropriate amount of protein solution (6 M urea, 100 mM TEAB, pH = 8.5) was added to dissolve the protein pellet.

Trypsin Treatment

The protein solution was added to flat membrane ultrafiltration (cut off molecular is 10 kDa) tube and was centrifuged at 14,000 g at room temperature for 20 minutes, and the flow-through was discarded. 100 µL of 50 mM TEAB was added, and the sample was centrifuged at 14000 g at room temperature for 20 minutes. The washing procedure was repeated four times. 100 µL of 50 mM TEAB and an amount of 1:50 mass ratio of trypsin were added to the protein and incubated at 37°C overnight. After being centrifuged at 14000 g for 20 min, an equal volume of 2% formic acid was added. After mixing, the solution was centrifuged at 14,000 g for 20 min at room temperature. The supernatant of flow-through was slowly passed through a C18 desalting column, and then 1 mL washing solution (0.1% formic acid, 4% acetonitrile) was added to wash three times in succession, then 0.4 mL of eluent (0.1% formic acid, 75% acetonitrile) was added to elute twice in sequence, the eluent samples were combined and freeze-dried.

Lc-ms/ms Analysis

Mobile phase A (100% water, 0.1% formic acid) and B solution (80% acetonitrile, 0.1% formic acid) were prepared. The lyophilized powder was dissolved in 10 µL of solution A, centrifuged at 15,000 rpm for 20 min at four °C, and 1 µg of the supernatant was injected into a home-made C18 Nano-Trap column (2 cm × 75 µm, three µm). Peptides were separated in a home-made analytical column (15 cm × 150 µm, 1.9 µm) using linear gradient elution, as listed in Additional file 1: Supplemental Table 1. The isolated peptides were analyzed by the Q Exactive series mass spectrometer (Thermo Fisher), with ion source of Nanospray Flex™ (ESI), spray voltage of 2.3 kV, and ion transport capillary temperature of 320 °C. Full scan range from m/z 350 to 1500 with resolution of 60000 (at m/z 200), an automatic gain control (AGC) target value was 3×10^6 , and a maximum ion injection time was 20 ms. The top 20–40 precursors of the highest abundant in the full scan were selected and fragmented by higher-energy collisional dissociation (HCD) and analyzed in MS/MS, where resolution was 15000 (at m/z 200), the automatic gain control (AGC) target value was 5×10^4 , the maximum ion injection time was 45 ms, a normalized collision energy was set as 27%, and intensity threshold was 2.2×10^4 . The dynamic exclusion parameter was 20 s. The raw data of M.S. detection was named as ".raw".

Data analysis

The identification and quantitation of protein

The resulting spectra from each fraction were searched separately against the homo sapiens uniprot database by the search engines: Proteome Discoverer 2.2 (PD 2.2, Thermo). The search parameters are set as follows: mass tolerance for precursor ion was ten ppm, and mass tolerance for production was 0.02 Da. Carbamidomethyl was specified in PD 2.2 as fixed modifications. Oxidation of methionine (M) and acetylation of the N-terminus was specified in PD 2.2 as variable modifications. A maximum of 2 missed cleavage sites was allowed.

The identified protein contains at least one unique peptide with FDR no more than 1.0%. Proteins containing similar peptides that could not be distinguished by MS/MS analysis were identified as the same protein group. Precursor ion was quantified by a label-free quantification method based on intensity. Mann-Whitney Test statistically analyzed the protein quantitation results for proteins whose quantitation significantly different between experimental and control groups were defined as differentially expressed proteins (DEP).

The Functional Analysis Of Protein And Dep

Gene Ontology (GO) and InterPro (IPR) analysis were conducted using the interproscan-5 program against the non-redundant protein database (including Pfam, PRINTS, ProDom, SMART, ProSiteProfiles, PANTHER)[27], the databases of Clusters of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to analyze the protein family and pathway. The probable protein-protein interactions (PPI) were predicted using the STRING-db server[28] (<http://string.embl.de/>). The enrichment pipeline was used for enrichment analysis of GO, IPR, and KEGG[29].

Statistical analysis

All data are presented as mean \pm standard error of the mean (SEM). Statistical analysis was performed by unpaired, two-tailed Students t-test using the GraphPad Prism 8.0 software (GraphPad Software) if not denoted otherwise. Differences were considered statistically significant at p-value < 0.05 . Mann-Whitney Test statistically analyzed the protein quantitation results. Proteins were supposed to be significantly differentially expressed when the p-value < 0.05 , Fold Change (FC) ≤ 0.05 or p-value < 0.05 , FC ≥ 2.0 .

Results

Analysis of differential protein expression

In this study, we carried out a label-free proteome analysis using FFPE HCC xenograft samples. In total, 3389 proteins were quantified from the label-free analysis. Compared to Group 2 to Group 1, a total of 78 proteins were differentially expressed ($p\text{-value} \leq 0.05$, Fig. 1A). Among them, the expression of 61 proteins, such as P04350, M0QZR4, and A0A024R056, were significantly up-regulated ($FC \geq 2.0$, $p\text{-value} \leq 0.05$), and the manifestation of 17 proteins, such as A8K335, Q6NVC0 and A0A024QZY5, were significantly down-regulated ($FC \leq 0.05$, $p\text{-value} \leq 0.05$). Cluster analysis of differentially expressed mRNAs and lncRNAs was shown by a heat map (Fig. 1B). Red indicates high expression proteins, and blue indicates low expression proteins.

Analysis Of Go Functional Enrichment

Based on the GO enrichment analysis, we can explore the main biological functions of DEP. The representative five enriched GO details of DEP were shown in Table 1. The top 20 GO enrichment terms of DEP were illustrated in Fig. 2. The vast majority of GO enrichment terms belong to the biological process (B.P). The enriched GO terms of the biological process were exhibited detailed in Directed Acyclic Graph (DAG), as shown in Additional file 2: Supplemental Fig. 1A. Besides, the enriched GO terms of molecular function (M.F.) and cellular component (CC) were shown in Additional file 2: Supplemental Fig. 1B, C, respectively. The complete result of GO enrichment analysis was shown in Additional file 3: Supplemental Table 2.

Table 1
the representative 5 enriched GO terms

GO ID	GO Term	GO Class	P value	Protein ID
GO:0006468	protein phosphorylation	BP	0.001063999	Q00534, P27361, F8W6G1, A0A024QZY5, O14976, B4E2L0, P12931
GO:0004672	protein kinase activity	MF	0.001220283	Q00534, P27361, F8W6G1, A0A024QZY5 O14976, B4E2L0, P12931
GO:0034329	cell junction assembly	BP	0.002098766	A0A2R8Y5A3, P78357
GO:0042803	protein homodimerization activity	MF	0.003455028	B7Z9B1, B4DL07
GO:0033270	paranode region of axon	CC	0.019097794	P78357
CC represents Cellular Component, MF represents Molecular Function, BP represents Biological Process.				

Analysis Of Kegg Pathway Enrichment

The dominant biochemical metabolic pathways and signal transduction pathways, which were regulated by DEP, could be identified by KEGG pathway enrichment analysis. The representative five enriched KEGG pathways of DEP were “Glutamatergic synapse”, “Endocrine resistance”, “GABAergic synapse”, “Gap junction” and “Melanogenesis” (Table 2). The top 20 enriched KEGG pathway terms were presented by a scatter plot (Fig. 3). The details of all KEGG pathway enrichment analysis were shown in Additional file 4: Supplemental Table 3.

Table 2
The representative 5 enriched KEGG pathway terms of DEP

Map ID	Map Title	P value	Protein ID	Description
map04724	Glutamatergic synapse	0.000434861	A0A024R056, P27361, B4E2L0, Q5U0L9	Guanine nucleotide binding protein (G protein), beta polypeptide 1, isoform CRA a, Mitogen-activated protein kinase 3,cDNA FLJ54730, highly similar to cAMP-dependent protein kinase, beta-2-catalytic subunit, Homer homolog 3 (Drosophila)
map01522	Endocrine resistance	0.001179526	P27361, P42773, B4E2L0, P12931	Mitogen-activated protein kinase 3,Cyclin-dependent kinase 4 inhibitor C, cDNA FLJ54730, highly similar to cAMP-dependent protein kinase, beta-2-catalytic subunit, Proto-oncogene tyrosine-protein kinase Src
map04727	GABAergic synapse	0.001725647	A0A024R056 ,B4E2L0 P12931,	Guanine nucleotide binding protein (G protein), beta polypeptide 1, isoform CRA a, cDNA FLJ54730, highly similar to cAMP-dependent protein kinase, beta-2-catalytic subunit, Proto-oncogene tyrosine-protein kinase Src
map04540	Gap junction	0.001771527	P04350, P27361, B4E2L0, P12931	Tubulin beta-4A chain, Mitogen-activated protein kinase 3,cDNA FLJ54730, highly similar to cAMP-dependent protein kinase, beta-2-catalytic subunit, Proto-oncogene tyrosine-protein kinase Src
map04916	Melanogenesis	0.003105093	A0A2R8Y5A3, P27361, B4E2L0	Catenin beta-1,Mitogen-activated protein kinase 3,cDNA FLJ54730, highly similar to cAMP-dependent protein kinase, beta-2-catalytic subunit

Analysis Of Ipr Enrichment

In this study, we identified 47 IPR terms with differential enrichment. The representative five enriched IPR IDs, and titles of DEP were shown in Table 3. The top 20 enriched IPR terms were explained in Fig. 4. The details of all IPR enrichment terms were explained in Additional file 5: Supplemental Table 4.

Table 3
The representative 5 enriched IPR terms of DEP

IPR ID	IPR Title	P value	Protein ID	Description
IPR001393	Calsequestrin	0.012354152	P31415	Calsequestrin-1
IPR002495	Glycosyl transferase, family 8	0.012354152	B2R5R5	cDNA, FLJ92583, highly similar to Homo sapiens glycogenin (GYG), mRNA
IPR003109	GoLoco motif	0.012354152	A0A0A0MRC4	G-protein-signaling modulator 1
IPR003585	Neurexin/syndecan/glycophorin C	0.012354152	P78357	Contactin-associated protein 1
IPR004134	Peptidase C1B, bleomycin hydrolase	0.012354152	Q13867	Bleomycin hydrolase

Protein-protein Interaction Analysis

PPI analysis indicated that the significantly up-regulated proteins P12931, A0A2R8Y5A3, Q00534, and P27361 and the significant down-regulated proteins P31415, A0A024QZY5, B7Z9B1, B4DL07, and A8K335 were interrelated and interacted with each other.

Discussion

In our previous study, the NET-1 protein functions of HCC were investigated in vivo[11]. The expression of NET-1 protein in HCC xenograft was merely detected by IHC staining. The tumor xenograft samples were formalin-fixed and paraffin-embedded for long-term preservation and further studies. In the present study, we hypothesized that the NET-1 gene could regulate the expressions of many other proteins with the aid of LFUS irradiation. Label-Free proteome analysis was conducted to identify the differentially expressed proteins. Surprisingly, a total of 78 DEP were sifted from 3389 quantified proteins (p -value ≤ 0.05). Moreover, 61 proteins were significantly up-regulated (red dots in Fig. 1A, $FC \geq 2.0$, p -value ≤ 0.05), and 17 proteins were significantly down-regulated (green dots in Fig. 1A, $FC \leq 0.05$, p -value ≤ 0.05). Our

hypothesis was preliminarily confirmed preliminarily. Encouraged by the proof-of-concept results, we analyzed the potential functions of DEP based on the mass spectra proteome analysis results.

Firstly, GO enrichment analysis for biological process, cell component, and molecular function was performed. A total of 78 GO enrichment terms belong to the biological process, which would indicate the most DEP was involved in regulating the biological process of HCC. On the other hand, NET-1 protein was a member of the tetraspanin family, which is a crucial point of HCC biological processes such as proliferation, differentiation, migration, and invasion[30, 31]. Consequently, silencing the NET-1 gene could regulate many HCC biological processes by depressing the NET-1 protein expression. Secondly, KEGG pathway enrichment analysis was performed to explore the pathways, which may be regulated by the NET-1 gene. A total of 45 significantly enriched pathways were identified ($p\text{-value} \leq 0.05$), as shown in Additional file 4: Supplemental Table 3. In organisms, different proteins co-ordinate their biological behaviors, and the pathway-based analysis helps to understand their biological functions further. Interestingly, the DEP enriched in multifarious pathways, not only cancer-related pathways but also many other pathways, such as "Olfactory transduction" (map04740), "Cocaine addiction" (map05030), "Morphine addiction" (map05032), "Kaposi's sarcoma-associated herpesvirus infection" (map05167) and "Human papillomavirus infection Human papillomavirus infection" (map05165). We concluded that the function of the NET-1 gene is not only to regulate HCC but also to participate in a variety of biochemical metabolic pathways in the human body. Thirdly, proteins are composed of IPR that are units of protein structure, function, and evolution. The study of the IPR of proteins is essential for understanding the biological role of proteins and their development. In this study, 47 IPR enrichment terms were found, as shown in Additional file 5: Supplemental Table 4 ($p\text{-value} \leq 0.05$). Each IPR had a specific protein corresponding to it. It could be proved that IPR can form new proteins by copying and combining. The combination distribution between different IPR does not conform to the random model but shows that some IPR has a powerful combination ability, some of which are rarely combined with other domains. Finally, PPI analysis revealed the direct and potential regulatory relationships between DEP. As shown in Fig. 5, all DEP formed a big circle, and each node represented a DEP. The up-regulated proteins are represented by red nodes, and the down-regulated proteins are represented by blue nodes. The interactions of DEP are incredibly sophisticated. For instance, the P27361 protein could regulate the other 13 DEP; the P12931 protein was regulated by the other 11 DEP. All the protein-protein interactions happened after the NET-1 gene has been silenced.

Conclusion

In summary, for the first time, our present study provides valuable insight into the regulation of NET-1 protein on other proteins in HCC on a proteomics level. It proved that the NET-1 protein, one of the tetraspanin proteins, participated in regulating many critical signaling pathways in HCC development. The potential antitumor mechanism is that the tetraspanin protein regulated other proteins involved in the biological processes of HCC cells to inhibit proliferation and invasion. Our results also provide a useful proposal for targeted therapy based on tetraspanin proteins to treat HCC, and further mechanism

investigations are needed to reveal a more detailed mechanism of action for NET-1 protein regulation of HCC.

Abbreviations

HCC

Hepatocellular carcinoma

siRNA

small interfering RNA

NET-1

neuroepithelial transforming gene 1

LFUS

low-frequency ultrasound

FFPE

Formalin-Fixed Paraffin-Embedded

GO

Gene Ontology

KEGG

Kyoto Encyclopedia of Genes and Genomes

COG

Clusters of Orthologous Groups

PPI

protein-protein interactions

FC

Fold Change

Declarations

Ethics approval and consent to participate

All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Harbin Medical University institutional animal care and conducted according to the AAALAC and the IACUC guidelines.

Consent for publication

All authors agree to publish.

Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author Contributions

Conceptualization, W.C. and B.W.; Methodology, B.W., and H.S.; Data Curation, X.L.; Formal Analysis, X.L., and H.J.; Resources, B.W.; Writing – Original Draft, Y.T.; Visualization, Z.L., and B.L.; Funding Acquisition, W.C., H.S., X.L., and B.W.; Supervision, W.C. and B.W.

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Figures

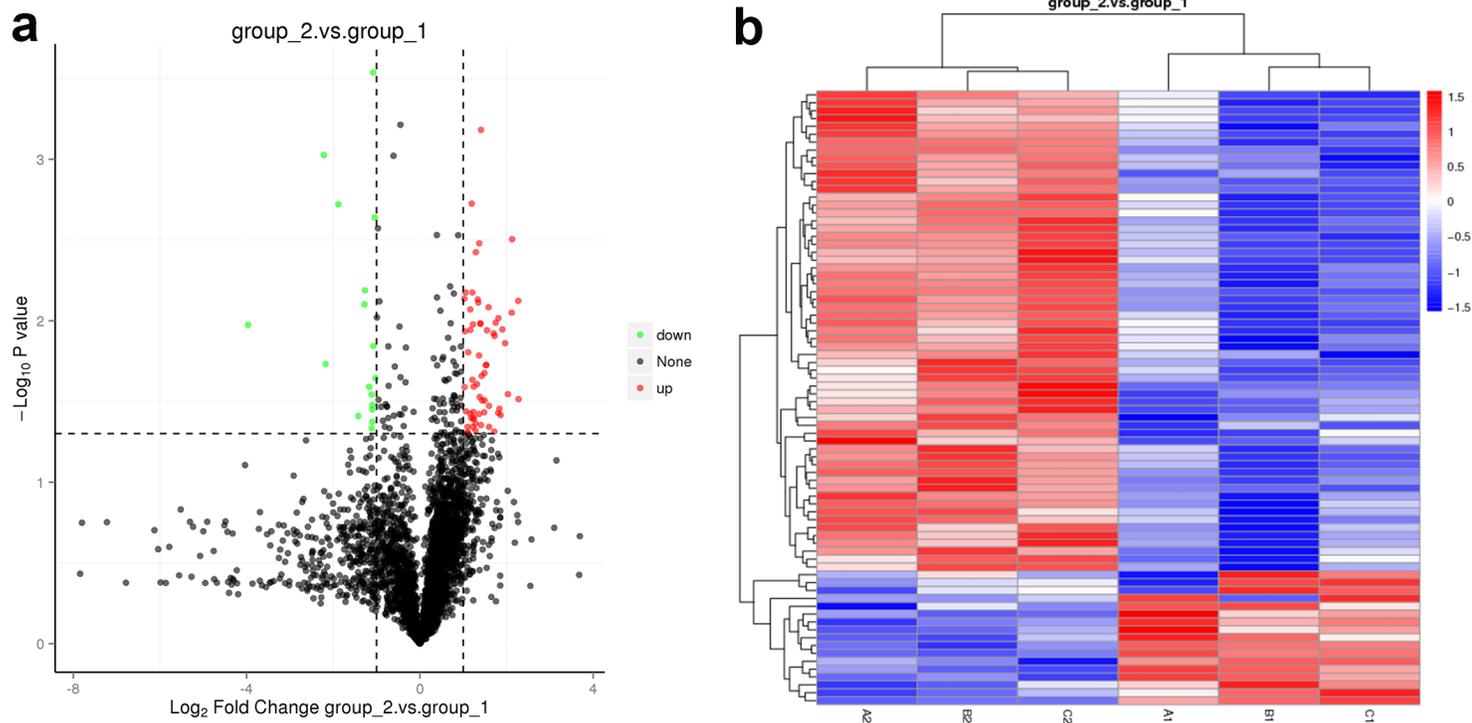


Figure 1

The illustration of DEP quantified from the label-free analysis. (A) Volcano plots of differentially expressed proteins between group 1 and group 2. Green dots represent down-regulated protein ($FC \leq 0.05$, $p\text{-value} \leq 0.05$), and red dots represent up-regulated protein ($FC \geq 2.0$, $p\text{-value} \leq 0.05$). (B) Heat map of hierarchical clustering of DEP. Red indicates high expression protein, and blue indicates low expression protein.

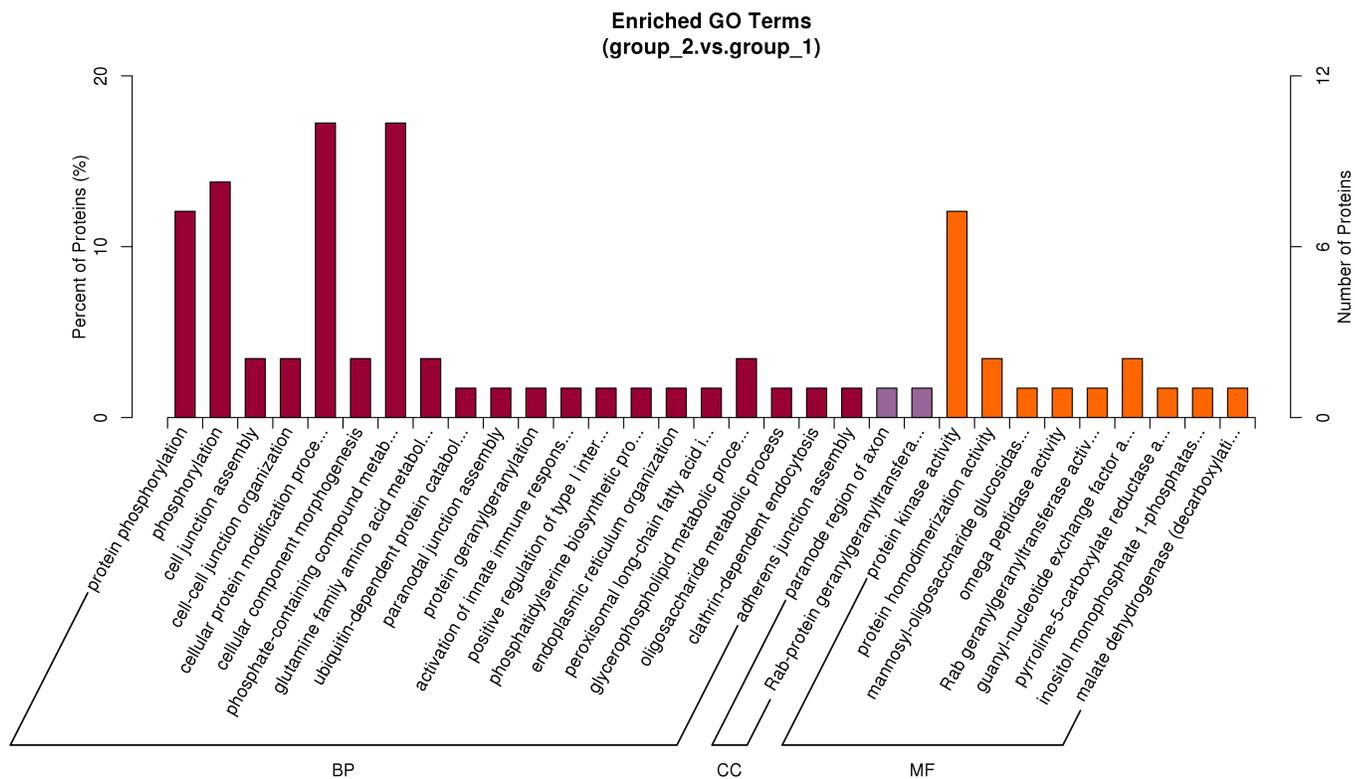


Figure 2

The illustration of the top 20 GO enrichment terms of all DEP. There are three categories of GO enrichment terms, biological process (B.P), molecular function (M.F.), and cellular component (CC). The most DEP enriched in biological process (dark red), and only two CC terms had DEP enriched (purple).

group_2.vs.group_1

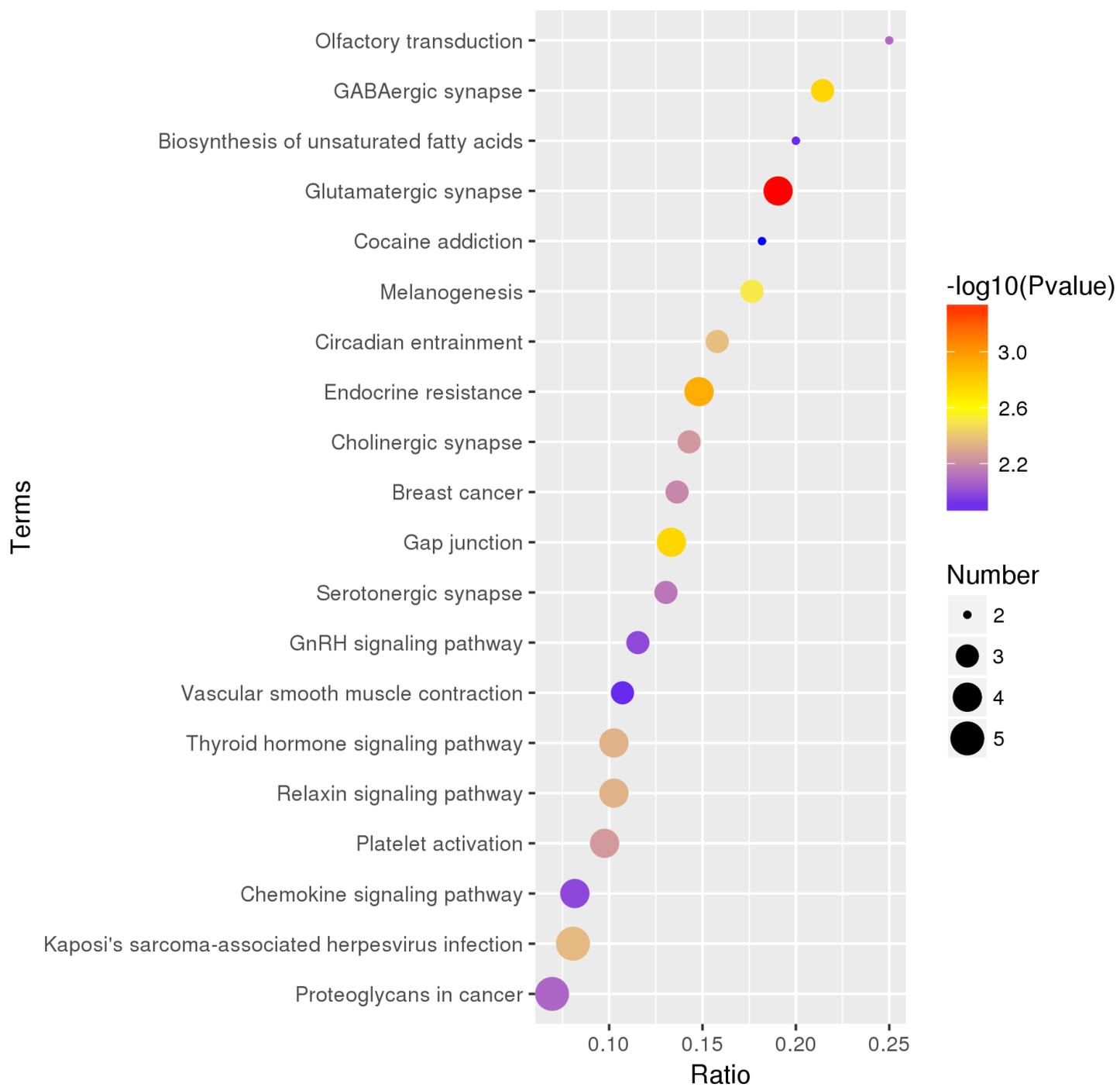


Figure 3

The KEGG pathway enrichment analysis of DEP. The top 20 KEGG enrichment terms of all DEP were shown in the scatter plot. The color of the dot represents the $-\log_{10}(\text{p-value})$, and the size of the dot represents the number of DEP.

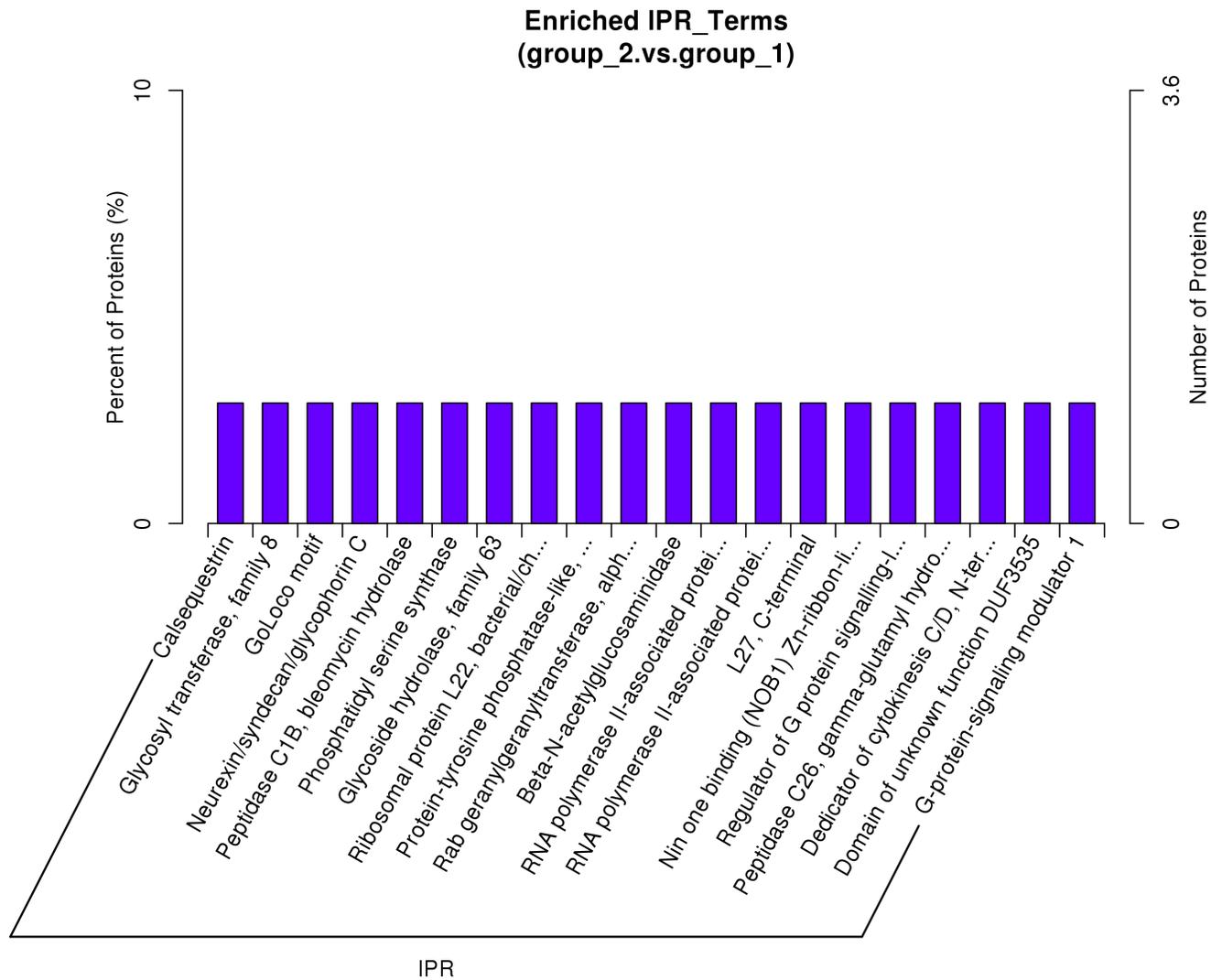


Figure 4

The IPR enrichment analysis. The top 20 IPR enrichment terms of all DEP were shown in the bar plot. Each IPR has only one DEP enriched.

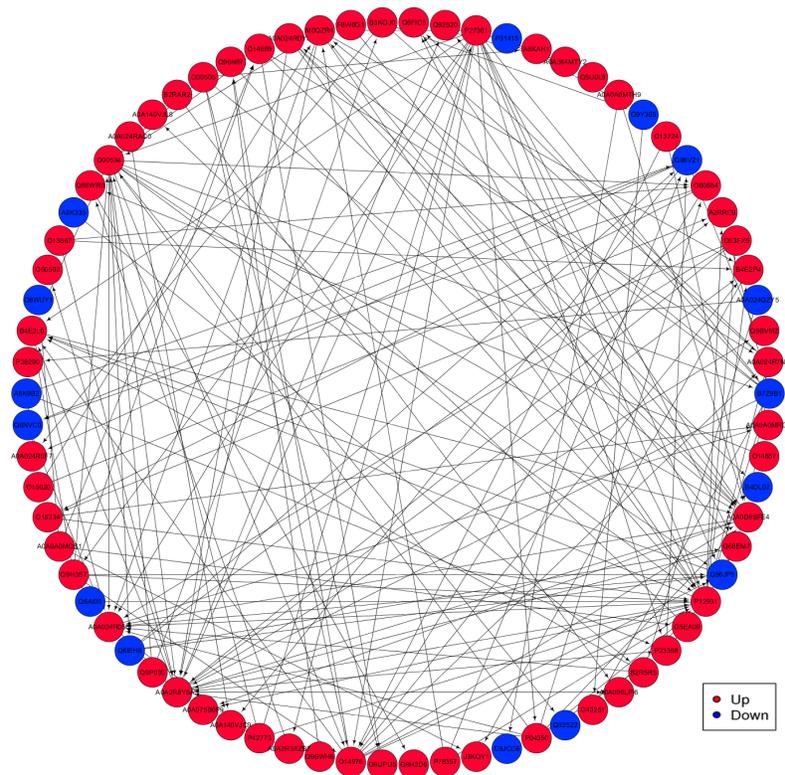


Figure 5

An interaction network of PPI analysis. The network revealed the direct and potential regulatory relationships between DEP. The up-regulated proteins are represented by red nodes. The down-regulated proteins are represented by blue nodes

Supplementary Files

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- [Additionalfile6SupplementalFigureLegends.docx](#)
- [Additionalfile2Supplementalfigure1.tif](#)
- [Additionalfile5SupplementalTable4.xls](#)
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- [Additionalfile7ARRIVEGuidelinesChecklist.pdf](#)
- [Additionalfile3SupplementalTable2.xlsx](#)
- [Additionalfile1SupplementalTable1.docx](#)