

Peptidome analysis of cerebrospinal fluid in neonates with hypoxic-ischemic brain damage

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Abstract

Background and Purpose To identify differentially expressed peptides in cerebrospinal fluid (CSF) of neonates with hypoxic-ischemic brain injury (HIBD) or controls, which may give a foundation for finding new promising drugs of neonatal HIBD. **Experimental Approach** CSF samples were collected from neonates with HIBD (n=4) or controls (n=4). ITRAQ LC-MS/MS was used to identify differentially expressed peptides between two groups. Effects of the peptide from heat shock protein 90-alpha (HSP90 α /HSP90AA1) on cell viability and pyroptosis under oxygen and glucose deprivation (OGD) were analyzed using cell counting kit-8 (CCK-8) assay and Annexin V-fluorescein isothiocyanate (FITC) Assay. Expressions of NLRP3, ASC and Caspase-1 were detected by quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. **Key Results** Compared with the control group, one peptide significantly up-regulated and thirty-four peptides significantly down-regulated in the CSF of neonates with HIBD. A fragment of HSP90 α /HSP90AA1 is the 2671.5Da peptide (HSQFIGYPITLFVEKER), one of the down-regulated peptides in neonatal HIBD. This peptide, we named it HIBDAP, inhibited pyroptosis of PC12 cells under OGD by suppressing expressions of NLRP3, ASC and Caspase-1. **Conclusion and Implications** The results of our study identified the characterization and expression profiles of peptides in CSF of neonatal HIBD. Several meaningful peptides such as HIBDAP may play significant roles in neonatal HIBD and may provide new therapeutic targets for neonatal HIBD.

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Keywords peptidomics; cerebrospinal fluid; neonates; hypoxic-ischemic brain damage; pyroptosis

Introduction

Hypoxic ischemic brain injury (HIBD) which could cause neuronal and white matter injury (Dixon et al., 2015) is one of the most common causes of neonatal death. HIBD accounts for 23% of global infant mortality (Lawn et al., 2005) and up to 25% survivors have permanent neuropsychological deficits, including cerebral palsy, learning and behavioral disabilities, epilepsy, and so on (Shetty, 2005; Nemati et al., 2018; Vannucci & Hagberg, 2004). However, until now, there are few effective treatments for HIBD except the hypothermia therapy (Johnston et al., 2011; Natarajan et al., 2016; Higgins et al., 2011). Therefore, looking for new HIBD therapeutic methods is urgently needed.

Peptides, low molecular weight fractions of proteins, play significant roles in physiological processes (Lau & Dunn, 2018), such as gene expression, substance metabolism and other processes (Soloviev & Finch, 2006). In recent years, peptides as drugs have attracted much attention because they are simpler and cheaper than protein-based drugs. Meanwhile, compared to small-molecule drugs, they are safer and more effective because they are highly selective and effective signal molecules. Until now, over 60 peptide drugs have reached the market for the benefit of patients, approximately 140 peptide drugs were in clinical trials and more than 500 therapeutic peptides were in preclinical studies (Fosgerau & Hoffmann, 2015). However, researches of peptide drugs are mainly in the fields of metabolic diseases, tumors and cardiovascular diseases; there were few reports in neonatal HIBD.

It is notable that some peptides have been found to play important roles in ischemic brain injury. TFP5, a modified 24-aa peptide (lys254-ala277) derived from p35, could significantly reduce the cerebral infarction area after ischemic stroking (Ji et al., 2017). Another peptide named DEETGE-CAL-Tat peptide also plays a neuroprotective role and maintains cognitive function in ischemic brain injury (Tu et al., 2015). Particularly, several recent studies have demonstrated that cationic arginine-rich peptides (CARPs), which include many cell-penetrating peptides [e.g., transactivator of transcription (TAT) and poly-arginine-9 (R9; 9-mer of arginine)], possess intrinsic neuroprotective properties in perinatal hypoxic-ischemic (Edwards et al., 2018). Therefore, it is interesting to find useful peptides in neonatal HIBD and may provide new treatments for it in the clinic.

Peptidome, an emerging proteome technique, is a qualitative and quantitative analysis of peptides in cells, tissues or body fluids. With the development of instrument performance, liquid chromatography-tandem mass spectrometry (LC-MS/MS) with high detection sensitivity and high throughput can detect low abundance proteins and any types of proteins, such as membrane proteins, nuclear proteins, extracellular proteins and so on. So, in this study, we used LC-MS/MS to analyze differentially expressed peptides between neonatal HIBD and normal control.

For diseases of the central nervous system (CNS), CSF is a valuable source of diagnosis biomarkers, disease mechanisms and novel therapy targets (Höltkä et al., 2012). Large amounts of peptides have been found in CSF (Höltkä et al., 2012; Zougman et al., 2008) and some of them are related to neurodegenerative diseases such as Alzheimer disease (Knopman, 2001). Therefore, peptidomic analysis of CSF from neonatal HIBD may give new clues to find new treatments of neonatal HIBD and has not been reported until now.

In brief, in this study, we aim to identify differentially expressed peptides in CSF of neonatal HIBD using LC-MS/MS. Through studying the function of one of these peptides, we hope to find new therapeutic peptide for neonatal HIBD.

Methods

Sample collection

Four HIBD infants from neonatal intensive care unit (NICU) of Children's Hospital of Nanjing Medical University were selected. Inclusion criteria included (1) term infants with acute fetal distress (prolonged resuscitation need, and/or cord pH<7.0, and/or Apgar score at 5min<5); (2) appearing neurological complication;

(3) clear brain injury which diagnosed by magnetic resonance imaging (MRI) or computed tomography (CT). Exclusion criteria included serious brain injuries caused by infection, intracranial hemorrhage, genetic metabolic diseases or others. Four matched controls were enrolled. All CSF samples were centrifuged at 3000rpm for 10min to acquire supernatants which were then stored in liquid nitrogen with protease inhibitor cocktail (Complete mini EDTA-free, Med Chem Express, USA). This study was approved by the ethics committee of Children's Hospital of Nanjing Medical University and achieved agreements from infants' parents.

Sample treatment

All CSF samples were grinded under liquid nitrogen conditions, mixed with protein lysate (7M urea, 2M thiourea, 4% SDS, 40mM Tris-HCl, pH8.5), 1mM phenyl methyl sulfonyl fluoride (PMSF), 2mM ethylene diamine tetraacetic acid (EDTA) and 10mM dithiothreitol (DTT). The mixtures were ultra-sounded on ice for 10min and then centrifuged at 12000g, 4°C for 30min to collect liquid supernatant. Equal amounts of proteins were treated by reductive alkylation. Then, equal amounts of proteins were ultrafiltrated using molecular weight cut-off (MWCO) filters (Millipore, USA) of 10kDa according to the manufacturer's recommendation. The flow-through from the filters containing peptide fractions was recycled, desalted, concentrated using C18 solid phase extraction (SPE) (Strata-X, 33µm, 2g/20mL, Phenomenex), and finally lyophilized.

Labelling

Peptide samples were dissolved in 0.5M Triethylammonium bicarbonate (TEAB) for peptide labeling after desalting. All peptides from samples were labeled with iTRAQ-8 standard kit (AB SCIEX Inc., Framingham, MA, USA). Then, the labeled samples were fractionated using a high-performance liquid chromatography (HPLC) system (Thermo DINOEX Ultimate 3000, USA) with a Durashell C18 (5µm, 100A, 4.6×250mm), and 12 fractions were collected.

SDS-PAGE

The protein samples and protein marker (Fermentas, St. Leon-Rot, Germany) were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and protein bands were visualized by Coomassie brilliant blue staining.

Liquid chromatography/Mass spectrometry (LC-MS/MS)

Reverse-phase HPLC-ESI-MS/MS was used to analyze the fractionated samples. The data were collected and identified using the TripleTOF 5600 plus mass spectrometer with the Eksigent Ultra Plus nano-LC 2D HPLC system system (AB SCIEX, USA). The freeze-dried peptide samples dissolving in 2% acetonitrile (ACN)/0.1% formic acid (FA) were loaded to a C18 trap column (5µm, 100µm×20mm, LC packings) and separated using the C18 analytical column (3µm, 75µm×150mm, LC packings) over a 90min gradient at 300nL/min. The two mobile phases of contained A, 2% ACN/98% of 0.1% FA (v/v) in water and B, 98% ACN/2% of 0.1% FA (v/v) in water.

For information-dependent acquisition (IDA), MS1 were scanned in 250ms, and MS/MS of 30 precursor ions were scanned in 50ms. MS1 spectra were collected in the range of 350-1500m/z, and MS/MS spectra were collected in the range of 100-1500m/z. Precursor ions were excluded from reselection for 15s. For MS analysis, ProteinPilot™ database search engine (V4.5, AB SCIEX, USA) was used to protein identification based on the MS/MS spectra data which provides an automatic mass recalibration of the data and obtains more data.

Bioinformatics analysis

The LC-MS/MS data were searched using the Mascot search engine (Matrix science) (<http://www.matrixscience.com>) against the SwissProt sequence database with the *Homo sapiens* subset including the following variable modifications: phosphorylation, amidation, deamidation, pyroglutamic acid, oxidation, acetylation, sulfation, oxidized and reduced cysteines. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analysis were used to study potential functions of differentially expressed peptides and their precursor proteins. The protein-protein interaction networks were mapped using STRING

database (<https://string-db.org/>) and UniProt database (<http://www.uniprot.org/>). The properties of peptides were checked through the website <http://www.expasy.org> and <http://smart.embl-heidelberg.de/>.

Cell culture and treatment

PC12 rat pheochromocytoma cells were obtained from American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 culture medium supplemented with 10% v/v horse serum (HS), 5% v/v fetal bovine serum (FBS) and appropriate antibiotics in a humidified chamber (5% CO₂ and 37°C), all of which were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA).

For the induction of oxygen and glucose deprivation (OGD), cells were switched to RPMI 1640 without glucose after washing twice with glucose-free RPMI 1640. TAT-HIBDAP (YGRKKRRQRRR-HSQFIGYPITLFVEKER) and the FITC tagged TAT-HIBDAP (Shanghai Science Peptide Biological Technology Co., Ltd., China) were dissolved in sterile water and added to the glucose-free culture medium. After 1h, cells were placed into an atmosphere of 2% O₂, 5% CO₂ and 93% N₂ at 37°C for 6h. Control cells were maintained in glucose-containing RPMI 1640 and incubated in a normoxic incubator for the same time.

Cell viability assay

Cell viability was measured using cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Tokyo, Japan) according to the manufacturer's protocol. Cells were inoculated into a 96-well plate (1 × 10⁴ cells/hole). Cells in 90 μL glucose-free culture medium of each well were added with 10 μL CCK-8 solution and incubated for another 1h in hypoxic environment. The absorbance was measured at 450nm using a Microplate Reader (Thermo scientific, Vantaa, Finland). All experiments were independently repeated three times.

Cell pyroptosis analysis

Cells were harvested by trypsinization and washed twice with ice-cold phosphate buffer saline (PBS). Then, cell pyroptosis was tested using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, NJ, USA). FACS Calibur Flow Cytometer (BD Biosciences, NJ, USA) was used to distinguish pyroptosis cells and the results were analyzed by FlowJo software (Tree Star Corp, Ashland, OR). All experiments were independently repeated three times.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA of cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. HiScript[®] II Q RT SuperMix for qPCR (Vazyme Biotech, Nanjing, China) was used for reverse transcription of mRNA following the manufacturer's instruction. The RT thermal cycle program was as follows: 50°C for 15min and 85°C for 5sec. The qPCR step was performed using a 7900HT Fast Real-Time PCR system with a TaqMan[®] MicroRNA Assay kit (Applied Biosystems, CA, USA) as the following conditions: 95°C for 5min, followed by 40 cycles (95°C for 10sec and 60°C for 30sec). The sequences of primers were: NLRP3 *F* : 5'-TGA AGA GTG TGA TCT GCG GAA AC-3'; *R* : 5'-GAA AGT CAT GTG GCT GAA GCT GT-3'; ASC *F* : 5'-AGT TGA TGG TTT GCT GGA TGC T-3'; *R* : 5'-GGT CTG TCA CCA AGT AGG GCT G-3'; Caspase-1 *F* : 5'-AAC CTT GGG CTT GTC TTT-3'; *R* : 5'-CAG GAG GGA ATA TGT GGG-3'; GAPDH *F* : 5'-AGA AGG CTG GGG CTC ATT TG-3'; *R* : 5'-AGG GGC CAT CCA CAG TCT TC-3'. The mRNA levels were calculated using the 2^{-T} method. All experiments were performed in triplicate.

Western blotting

After washed with ice-cold PBS, the cells were ultrasonically homogenized in a RIPA buffer and protease inhibitor cocktail, and then the homogenates were centrifuged at 12000xg for 15min at 4degC. The protein concentration was quantified using a BCA protein assay kit (Pierce, Rockford, IL, USA) and the supernatants of homogenates were boiled at 100degC in a laemmli sample buffer (Abcam, Cambridge, MA, UK) for 5min. Samples were separated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). Membranes were blocked with 5% (m/v) nonfat dry milk in 0.1% Tween 20 (TBS-T; 2mmol/L Tris-HCl, 50mmol/L NaCl, pH 7.4) for 2h at room temperature and subsequently incubated

overnight at 4degC in the blocked buffer with NLRP3 antibody (Cat: 19771-1-AP; Proteintech, Chicago, USA), ACS antibody (Cat: sc-514414; Santa Cruz, CA, USA), Caspase-1 p10 antibody (Cat: sc-514; Santa Cruz, CA, USA), and β -actin antibody (Cat: 8457S; Cell Signaling Technology, MA, USA), respectively. The membranes were washed with 0.1% Tween 20, and then treated with horseradish peroxidase-conjugated anti-mouse IgG (Abcam, Cambridge, UK) or goat anti-rabbit IgG H&L (HRP) (Abcam, Cambridge, UK) for 1h at room temperature. After washing thrice with TBST, proteins were visualized with an electrochemiluminescence detection system and quantified by an image analysis system (Image J, MD, USA).

Statistical analysis

All values are expressed as mean \pm SEM. The statistical difference was analyzed using the SPSS (version 22.0). GO and pathway analysis were considered to be significantly enriched when Hypergeometric P -value <0.05 . Quantitative analysis of our experiments was performed by Student's t -tests or one-way ANOVA. P -value <0.05 was considered statistically significant.

Results

Sample preparation

The peptidome constitutes only a minor part of the total protein contents of CSF. Because of the limited loading capacity of LC columns ($<1\mu\text{g}$), it is necessary to enrich the peptide fraction. Ultrafiltration using MWCO filters provides a simple means to remove high molecular weight proteins and retain a large part of peptides in the mass range of interest. The result of SDS-PAGE confirmed that most macromolecular proteins had been successfully removed (Fig. 1).

It was showed that lots of peptides of CSF bind to larger proteins and thus can be retained during the ultrafiltration step (Shores & Knapp, 2007). Therefore, urea, thiourea and DTT were used to pretreat CSF samples and dissociate peptides from carrier proteins prior to the ultrafiltration step. Salts had been removed by C18 solid phase extraction after ultrafiltration.

Figure 1

Peptide identification

Peptides extracted from CSF of 4 newborns with HIBD and 4 normal controls were analyzed directly by LC-MS/MS. A total of 35 differentially expressed peptides ($P < 0.05$ and fold change >1.5) from 25 protein precursors were identified (Table 1). Compared with the control group, 1 peptide was up-regulated and 34 peptides were down-regulated in the HIBD group.

Table 1

Characteristics of differentially expressed peptides

We initially analyzed the molecular weight (MW) (Fig. 2A), isoelectric point (PI) (Fig. 2B) and the distribution of MW versus PI distribution (Fig. 2C) of differentially expressed peptides. The MW of most peptides ranges from 1800-3000Da and the most widely distributed PI ranges from 9-10. Furthermore, the scatter plot of MW versus PI is shown in Fig. 2C. Points of these identified differentially expressed peptides are distributed around PI 10.

Next, we searched for cleavage sites in all differentially expressed peptides to study possible functional changes of CSF in HIBD. The cleavage sites of peptides are regular and we can study their rules to analysis the function of proteolytic enzymes (Fig. 3). Arginine (R) and Lysine (K) were the most common cleavage sites of the N-terminal amino acid of the preceding peptide and C-terminal amino acid of the identified peptide. Glicine (G) was the most common cleavage site of C-terminal amino acid of the preceding peptide. Lysine (K) and isoleucine (I) were the most common cleavage sites of N-terminal amino acid of the identified peptide.

Figure 2

Figure 3

Bioinformatics analysis

To preliminarily explore potential functions of these differentially expressed peptides in neonatal HIBD, GO and KEGG analyses were conducted based on their precursor proteins. The cellular components of peptide precursors were mainly as follows: nuclear chromosome, nuclear chromosome part, chromatin and so on (Fig. 4A). The most relevant biological processes were shown in Fig. 4B, including chromatin remodeling, chromatin modification, ATP-dependent chromatin remodeling and so on. The molecular functions of peptide precursors mainly included chromatin binding, chromatin DNA binding, structure-specific DNA binding and DNA binding (Fig. 4C). The pathway analysis showed that the most related pathways of these peptide precursors were hypertrophic cardiomyopathy (HCM), leukocyte transendothelial migration, gastric acid secretion, arrhythmogenic right ventricular cardiomyopathy (ARVC) and so on (Fig. 4D).

In order to find important peptides that play key roles in HIBD, the functions of differentially expressed peptides and their precursor proteins were studied using STRING database and UniProt database. The protein-protein interaction network of precursor proteins of differentially expressed peptides was shown in Fig. 5.

Figure 4

Figure 5

Effects of HIBDAP pretreatment on cell pyroptosis under OGD

The 2671.5Da peptide (HSQFIGYPITLFVEKER), one of the down-regulated peptides in neonatal HIBD, is a fragment of heat shock protein 90-alpha (HSP90 α /HSP90AA1). We named it HIBDAP (Hypoxic-Ischemic Brain Damage Associated Peptide). As given in Fig. 6A, HIBDAP derives from the 210st to 226st amino acids of HSP90AA1 is a hydrophilic peptide with high stability and has a long half-life of 3.5h in mammalian reticulocytes. We coupled HIBDAP to the transduction sequence of the TAT protein, allowing for the delivery of peptide into cells. It was demonstrated that FITC-labelled TAT-HIBDAP could successfully enter PC12 cells and further into the nucleus (Fig. 6B). After HIBDAP pretreatment and 6 hours of OGD treatment, we found that low concentrations of HIBDAP could increase the survival rate of cells revealed by results of CCK-8, except 40 μ M had a toxic effect (Fig. 6C). Safe concentrations of the peptide reduced pyroptosis of PC12 cells, except 20 μ M had no effect (Fig. 6D).

Then, we detected mRNA and protein expressions of the NOD-LIKE receptor (NLR) signaling pathway in PC12 cells pretreated with 5 μ M HIBDAP under OGD. Compared with the control group, expressions of NLRP3, ASC, and Caspase-1 were significantly increased in the OGD group whereas HIBDAP pretreatment significantly suppressed their expressions (Fig. 6E).

Figure 6

Discussion and Conclusions

In this study, we identified differentially expressed peptides between the CSF of neonatal HIBD and controls using LC-MS/MS and found a total of 35 differentially expressed peptides, among which only 1 peptide was significantly up-regulated and 34 peptides were significantly down-regulated in the HIBD group (Table 1). The MW of most peptides was around 1800-3000Da which confirmed that larger proteins were removed. The most widely distributed PI ranges from 9-10, which are in the alkaline group (Fig. 2).

Typical peptide precursors contain multiple sites for proteolytic processing that are usually pairs of basic amino acids, with Lys-Arg and Arg-Arg the most common. In our results, Lys-Arg was the most common cleavage site of N-terminal amino acid of the preceding peptide and C-terminal amino acid of the identified peptide (Fig. 3). The cleavage sites' distribution of our peptidome is consistent with that of neuropeptides (Zhang et al., 2008).

Most peptide precursors play roles on DNA binding in the nucleus and chromatin with the most related pathway including HCM, ARVC, dilated cardiomyopathy and so on (Fig. 4). Intriguingly, some significantly enriched pathways of precursor proteins were associated with cardiomyopathy. Studies have shown that molecular mechanisms of cardiomyopathy including dysregulated calcium flux, cardiomyocyte apoptosis and oxidative stress, which is also in the pathological process of neonatal HIBD (Chen et al., 2017).

Analysis of STRING database and UniProt database showed that precursor proteins of differentially expressed peptides were closely related to each other (Fig. 5). It was showed that the central protein is HSP90 α /HSP90AA1, which means this precursor protein closely interrelated with other precursor proteins. A fragment of HSP90 α /HSP90AA1 is the 2671.5Da peptide (HSQFIGYPITLFVEKER), one of the down-regulated peptides in neonatal HIBD. Previous studies have shown that HSP90 is able to bind and stabilize the transcription factor hypoxia-inducible factor1-alpha (HIF-1 α) (Liu et al., 2007; Isaacs et al., 2002; Katschinski et al., 2004; Baird et al., 2006), which acts as a cellular survival factor in response to tissue hypoxia (Pichiule et al., 2003; Chi & Karliner, 2004; Lee et al., 2004; Khan et al., 2006). Through the KEGG analysis, we found that HSP90 α was involved in the NLR signaling pathway. It was revealed that HSP90 and SGT1 bind to NLRP3, one of NLR sensors, to inhibit its activation (Gross et al., 2011; Mayor et al., 2007; Kadota et al., 2010). NLRs are best known for their ability to form inflammasomes, which biochemical function is activating Caspase-1 to induce the pyroptosis by forming pyroptosome, a complex of oligomerized ASC molecules. Therefore, we speculated that this 2671.5Da peptide, which we named it HIBDAP, may play roles in the mechanism of neonatal HIBD through pyroptosis, a novel mechanism of programmed cell death (Fernandes-Alnemri et al., 2007), by the NLR signaling pathway.

Results of bioinformatics analysis showed that HIBDAP with small molecular weight has high stability and long half-life, which revealed that it is suitable for the drug research. In accordance with other peptide drugs, the toxicity of HIBDAP increased with concentration. Our results showed that HIBDAP inhibited pyroptosis under OGD when its concentration below the harmful concentration (40 μ M) except 20 μ M. Beyond our expectations, groups of 10 μ M and 20 μ M had higher ratios of pyroptosis but also cell viability than the group of 5 μ M. We speculated that the higher pyroptosis of 10 μ M and 20 μ M than that of 5 μ M may due to its toxicity increasing. However, the number of living cells detected by CCK-8 can be influenced by various factors such as cell proliferation, pyroptosis, necrosis, apoptosis, autophagy and so on (Galluzzi et al., 2018). HSP90 α , the precursor protein of HIBDAP, plays an important role in promoting cell proliferation (Ory et al., 2016; Zhou et al., 2019). Therefore, groups of 10 μ M and 20 μ M had higher cell viability may because of the balance of pyroptosis and proliferation. We further studied the mechanisms of effects of HIBDAP on cell pyroptosis using the concentration of 5 μ M. The results of qRT-PCR and Western blotting showed that HIBDAP inhibited pyroptosis under OGD by reducing expressions of NLRP3, ASC and Caspase-1. We predicted HIBDAP may be a protective factor against neonatal HIBD through the pyroptosis mechanism (Fig. 7).

Figure 7

Using database analysis, *Leandro-Garcia et al. (2010)* identified 8 major beta-tubulins, including TUBB2B. Quantitative RT-PCR of 21 normal human tissues detected highest TUBB2B expression in brain. Much lower expression was detected in heart, kidney, colon, small intestine, tonsil, and skeletal muscle, with little to no expression in other tissues.

Using quantitative real-time PCR, *Breuss et al. (2012)* found that expression of TUBB2B was higher than that of other beta-tubulins in human fetal brain. TUBB2B expression was higher at gestational week 22 than at gestational week 13.

The 2699.6Da peptide (IINEPTAAAIAYGLDKK) originated from Heat shock cognate 71kDa protein (HSPA8) was another down-regulated peptide in neonatal HIBD. HSPA8 is a major component of HSP70 family (Kampinga et al., 2009). HSP70, another HSPs regulated by HIF-1 α during hypoxia, appears to interrupt both cell death and immune responses which leads to improved neurological outcome (Kim & Yenari, 2013). Analysis of KEGG pathway revealed that HSPA8 is involved in the mitogen-activated protein

kinase (MAPK) signaling pathway, which is involved in inflammatory responses (Sulistiyowati et al., 2018) and neuronal death/apoptosis (Kyriakis & Avruch, 2012). Therefore, we predict that this peptide may play roles in neonatal HIBD through the mechanism of cell death and inflammatory responses by the MAPK signaling pathway. However, it is unclear about detailed mechanisms and needs further studies.

Another interesting finding is that, of 35 differentially expressed peptides, 8 peptides come from the tubulin family, including Tubulin alpha-1A chain (TUBA1A), Tubulin beta-2B chain (TUBB2B) and Tubulin beta-4A chain (TUBB4A). The tubulin family is involved in the construction of cytoskeleton and is thought to be critical for the morphogenesis of axons and dendrites (Minoura, 2017). Recent studies revealed that tubulin isoforms caused a variety of neurological disorders (Magiera et al., 2018) and tubulin-binding drugs can activate a component of the hypoxic adaptive response, specifically the stabilization of HIF-1 α and its downstream targets (Aleyasin et al., 2015). Our results of the KEGG analysis found that Tubulin alpha and Tubulin beta were jointly involved in the regulation of gap junctions (GJs). In the central nervous system, GJs which were channels that connect the cytoplasm of cells form cellular syncytia and coordinate neural function. These channels have distinct functions within different cell types, and their expressions can change dramatically during neurodevelopment (Cina et al., 2007) and injury (Freitas-Andrade & Naus, 2016). Therefore, these 8 peptides from the tubulin family are interesting targets for further study as potential pharmacotherapeutic methods.

In summary, we present the first data to demonstrate differentially expressed peptides in CSF of neonatal HIBD and controls. Several meaningful peptides such as HIBDAP may play significant roles in neonatal HIBD. The molecular mechanisms and biological functions of these peptides may provide new pathogenesis and therapeutic targets for neonatal HIBD.

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Figure legends

Fig 1. SDS-PAGE analysis of neonatal CSF samples before and after ultrafiltration treatment.

1-4: CSF samples of neonatal HIBD infants without any treatment; U1-4: CSF samples of neonatal HIBD infants after ultrafiltration; 5-8: CSF samples of controls without any treatment; U5-8: CSF samples of controls after ultrafiltration; M: protein marker.

Fig 2. Characteristics of differentially expressed peptides.

(A) The MW of most differentially expressed peptides ranges from 1800-3000Da; (B) The PI of differentially expressed peptides most ranges from 9-10; (C) The distribution of MW versus PI of differentially expressed peptides. Points of these identified differentially expressed peptides are distributed around PI 10.

Fig 3. Cleavage sites in differentially expressed peptides.

Arginine (R) and Lysine (K) were the most common cleavage sites of the N-terminal amino acid of the preceding peptide and C-terminal amino acid of the identified peptide. Glicine (G) was the most common cleavage site of C-terminal amino acid of the preceding peptide. Lysine (K) and isoleucinel (I) were the most common cleavage sites of N-terminal amino acid of the identified peptide.

Fig 4. GO and KEGG analyses of peptide precursors.

(A) The main cellular components of peptide precursors were nuclear chromosome, nuclear chromosome part, chromatin, chromosome, chromosomal part and nucleoplasm; (B) The most relevant biological processes were chromatin remodeling, chromatin modification, ATP-dependent chromatin remodeling and so on; (C) The molecular functions of peptide precursors mainly included chromatin binding, chromatin DNA binding, structure-specific DNA binding and DNA binding; (D) The most canonical pathways of peptide precursors were HCM, leukocyte transendothelial migration, gastric acid secretion, ARVC and so on.

Fig 5. The protein-protein interaction network of peptide precursors.

Each node represents all precursor proteins produced by a single, protein-coding gene locus. Edges represent protein-protein associations.

Fig 6. Effects of HIBDAP pretreatment on cell pyroptosis under OGD.

(A) Bioinformatics analysis of HIBDAP; (B) FITC-labelled TAT-HIBDAP could successfully enter PC12 cells and further into the nucleus; (C) After HIBDAP pretreatment and 6 hours of OGD treatment, the cell survival rate was significantly increased in PC12 cells pretreated with 1 μ M, 5 μ M, 10 μ M and 20 μ M HIBDAP except that of 40 μ M group was significantly decreased; (D) The concentrations including 1 μ M, 5 μ M and 10 μ M of HIBDAP significantly reduced pyroptosis of PC12 cells, except 20 μ M had no effect; (E) Compared with the control group (NC), the mRNA and protein expressions of NLRP3, ASC, and Caspase-1 were significantly increased in the OGD group (OGD) whereas HIBDAP pretreatment (5 μ M+OGD) significantly suppressed their expressions (^a $P > 0.05$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$).

Fig 7. Diagrammatic Drawing.

HIBDAP inhibited pyroptosis under OGD by reducing expressions of NLRP3, ASC and Caspase-1 and may be a protective factor against neonatal HIBD through the cell pyroptosis mechanism.

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