Integrating Bioinformatics To Identify Potential Cytokines \textit{ALPL} /\textit{TNAP} In Children With Spastic Cerebral Palsy

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Research

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Abstract

Background: Several published studies have shown the significance of neuroinflammation in the pathophysiological development of cerebral palsy (CP). However, the underlying molecular mechanisms by which neuroinflammation is associated with brain injury in CP remain poorly understood. Spastic CP is the most common form of CP, comprising 80% of all cases. Therefore, identifying patterns of inflammatory and related biomarkers would serve to understand the etiology of spastic CP.

Methods: Related clinical parameters were assessed in 18 children with spastic CP and 20 age-matched healthy individuals. Blood samples of children with spastic CP and controls were analyzed with integrated transcriptomics and proteomics profiling to detect common differentially expressed mRNAs and proteins. Hypoxic ischemic encephalopathy was induced in postnatal day 7 rat pups. Behavioral testing was performed postinjury, and then, the differentially expressed markers and inflammatory cytokines were verified in the peripheral blood and cerebral cortex of the CP model rats by ELISA and Western blot. Independent sample $t$-tests, one-way analysis of variance and the Pearson correlation were used for statistical analysis.

Results: Through proteomic and transcriptome analysis, we identified common differentially expressed genes. Among them, $ALPL$, encoding tissue nonspecific alkaline phosphatase (TNAP), was downregulated in spastic CP. In addition, compared with those of the corresponding controls, significantly lower mRNA and protein levels of TNAP were found in children with CP and the CP model rats. In contrast, compared with the control rats, the model rats demonstrated a significant increase in osteopontin and proinflammatory biomarkers, such as interleukin (IL)-6, IL-17, and C-reactive protein (CRP), in both the plasma and cerebral cortex, while serum 25 hydroxyvitamin D and anti-inflammatory cytokines such as IL-10 were significantly decreased. Additionally, the level of serum TNAP was correlated with the content of serum CRP and IL-10 in model rats.

Conclusion: These results suggest that TNAP, the gene expression product of $ALPL$, is closely related to the pathogenesis and development of spastic CP, particularly in the upregulation of proinflammatory cytokines and the downregulation of anti-inflammatory cytokines, which contribute to the occurrence of inflammation in the progression of spastic CP.

Introduction

Cerebral palsy (CP) is the most common neurological dysfunction in children, frequently accompanied by cognitive, language and behavioral defects, and can be secondary to epilepsy and musculoskeletal problems. CP can be classified as spastic, dyskinetic or ataxic depending on the type and distribution of motor abnormalities and the location of the brain injury$^1$. Spastic CP is the most common form of CP, comprising 80% of all cases. At present, the etiology of CP remains poorly understood, making its clinical diagnosis difficult$^2$. 
In recent years, the integration of proteomics and transcriptome analysis has attracted increasing attention in multiomics studies. Transcriptome sequencing is a highly sensitive technique that can reveal specific biological processes and molecular mechanisms in the process of disease occurrence at the level of gene expression\[^3\]. Proteomic approaches are used to investigate the expression level of proteins as a whole as well as the interaction between proteins to further identify new targets to clarify the pathogenesis of diseases\[^4\]. Various bioinformatics analyses, such as GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, have been performed on differentially expressed genes and proteins to characterize their corresponding functions. It is generally accepted that this joint analysis of gene and protein expression data can provide a complete picture of disease status and may be a valuable strategic approach for biomarker screening and prediction in disease diagnosis\[^5\].

Recent studies\[^6,7\] adopting genome-wide arrays and next-generation sequencing technologies have begun to unravel the genetic contributions to CP. Currently, spastic CP-related genes are considered to be important potential factors leading to prenatal brain injury, and some target genes related to the pathogenesis of CP have been uncovered\[^8\]. For example, mutation of the protein regulator STXBP1, which may be related to neurotransmission disorders, has been found in ataxic CP\[^9\]. However, it is difficult to explain how some missense mutations predicted by bioinformaticians, without further verification of protein function or expression changes, lead to phenotypic changes\[^8,10\]. In addition, through high-throughput sequencing or protein mass spectrometry detection, the identification of disease-related gene or protein expression changes can be mutually verified via the assessment of clinical symptoms or pathological changes in disease-inducing factors, improving the value of the associated research.

In the present study, first, blood samples of children with spastic and dyskinesia CP were analyzed by an expression spectrum chip to select differentially expressed mRNAs. Then, we performed joint transcriptomics and proteomics profiling analysis on the blood samples of children with CP and controls to identify differential expression at both the mRNA and protein levels. By further analyzing the biological functions and the clinical manifestations in children with spastic CP, the present study provides insight into the development of spastic CP at the molecular level and identifies candidate biomarkers for future diagnosis treatment and prognosis. Finally, the differentially expressed proteins were further verified in CP model animals, and their functions and effects in the pathological process of CP were observed to further explore the pathological mechanism of CP.

**Materials And Methods**

1. **Study Participants**

A total of 22 children with CP aged 8 to 26 months were enrolled in this study between January 2019 and September 2021. According to the European Cerebral Palsy Monitoring Group’s classification system\[^11\], the participants were divided into spastic type CP (ST group; 18 participants) and dyskinesia type CP (DT group; 4 patients) (Table 1). The diagnoses and classifications of the children were determined by two independent clinicians. Twenty children aged 8-26 months, with no confirmed neurodevelopmental
disorders after careful physical examinations, were included into the study as the healthy controls (Table 1). We obtained the participants’ information from computer databases, electronic medical records, and collected plasma while excluding the patients with genetic, metabolic, and data insufficiency. The research protocol of this clinical study was approved by the ethics committee of Henan Province Children’s Hospital. Informed consent was obtained from the parents or the legal guardians of the child prior to onset of the study.

| Table 1: Clinical characteristics of our cohort |
|-----------------------------------------------|
| ST(n=18) | DT(n=4) | Control(n=20) |
| --- | --- | --- |
| **Sex** | **Male** | **Female** | **Male** | **Female** | **Male** |
| **Enhanced muscle tension** | + | +/- | - |
| **Decreased muscle tension** | - | +/- | - |
| **Hyperreflexia** | + | - | - |
| **Stereotypical movements** | - | + | - |

2. mRNA Microarray Analysis

All the samples included in this test were the plasma from the subjects. Six spastic CP, 4 dyskinesia CP and 5 control samples were analyzed. TRIzol reagent (Invitrogen, Gaithersburg, MD, USA) was used to extract total RNA from tissue samples, and a Nucleospin® RNA Clean-up Kit (Macherey-Nagel, Germany) was used for purification according to the manufacturer’s recommended procedure. Finally, a spectrophotometer (Thermo Science, Wilmington, DE, USA) was used to evaluate the quantity and quality of RNA, and a RNA Agilent 2100 system was used to evaluate the integrity of the total RNA.

RNA samples from the same group was used for cDNA library preparation. First, T7 mixed enzyme was used to synthesize cRNA. Then, using random primers and cRNA as templates, cDNA, obtained by reverse transcription, was purified and quantified with CbcScript II enzyme. The corresponding labeled cDNA samples were mixed, sequenced with an Agilent G2565CA microarray scanner (Agilent Technologies, USA), and analyzed with Agilent Feature Extract 10.7 software (Agilent Technologies, USA).

3. Proteomic Analyses

Four spastic CP samples and 4 control samples were used for proteomic analysis and mRNA microarray analysis. A 100 µg protein sample was taken from each sample and digested overnight at 37°C. Each sample was processed and labeled with the instruction of iTRAQ Reagent-8 Multiplexing Kit (AB Sciei, UK). The labeled sample was mixed in equal volumes, desalted, and lyophilized. High-performance liquid
chromatography was performed with a Rigol L3000 system and a C18 chromatographic column (Waters BEH C18, 5 mm). A Diane NCS3500 system (Thermo Science FicTM) equipped with a trap and an analytical column was used to fractionate the labeled samples, and the precursor ions decomposed by the higher-energy C-trap dissociation (HCD) method were sent to a tandem mass spectrometry Q Exactive HF-X MS (Thermo Fisher, Waltham, MA) for data acquisition and analysis.

4. Clinical Parameters of Cerebral Palsy Participants

4.1 Gross Motor Function Classification

The Gross Motor Function Classification System for cerebral palsy-Extended and Revised (GMFCS-ER)\textsuperscript{[12]} was used for gross motor function classification. The subjects involved in this motor function assessment covered 2 different age groups (0<2 and 2~4 years old). The motor indicators required by this system included maintaining erecting head, turning over, sitting alone, crawling, standing and walking. According to motor function performance, the subjects in each age group was divided into 5 grades, where grade I indicated slightest gross motor dysfunction, and grade V indicated the worst. GMFCS grades I to III were classified as mild to moderate motor dysfunction, and GMFCS grades IV to V were classified a severe motor dysfunction.

4.2 Neuroimaging Examination

All brain MRI scans were acquired on a 3.0T MR scanner (Discovery MR750, GE Medical Systems, USA). Imaging sequences included transverse T1-weighted (T1W), T2-weighted (T2W), T2-fluid attenuated inversion-recovery (FLAIR) and sagittal T1W imaging, with a section thickness of 4-5 mm. No enhanced scanning was performed in all cases. The obtained brain MRI images were evaluated by pediatric neuroradiologists and CP specialists who participated in our study. The findings were divided into periventricular white matter injury (PWMI): periventricular leukomalacia (PVL), ventriculomegaly; diffuse brain injury: subcortical softening foci, myelin dysplasia, cerebral atrophy, basal ganglia/thalamic lesions; focal lesions: focal cerebral ischemia, porencephalia; cerebral dysplasia: dysplasia of the corpus callosum, cerebellar dysplasia; and normal brain MRI imaging.

4.3 Additional Blood Sample Examinations

Three milliliters of venous blood samples were taken from all subjects in the morning on an empty stomach. An ADVIA2400 automatic biochemical analysis instrument produced by German Siemens was used to assess the percentage of lymphocytes (LYM) in the blood. An i-CHROMA Reader immunofluorescence analyzer from Boditech MED Inc., South Korea, was used to determine the C-reactive protein (CRP) level. A Roche cobas e602 analyzer (Roche Diagnostics, Switzerland), was used to detect 25 hydroxyvitamin D [25(OH)D], creatinine (Cr).

5. Animal Models
Forty-six 3-day-old Sprague Dawley pup rats were acquired from Shanghai Jihui Experimental Animal Breeding Co. Ltd. The pups were fed by their mothers until weaning. All rats were reared in an animal room with sufficient water and food under a 12-hour light-dark cycle. The animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the 3R principle.

Forty pups were randomly divided into Sham and Model groups (n=20). On postnatal day 7 (P7), the Model group was subjected to an operation to construct the hypoxic ischemic encephalopathy (HIE) model, while the pseudo operation was performed on the Sham group without occlusion of the artery on the same day (P7); 10 pups from each group were sacrificed on the 7th day post-operationally, and the remaining 10 pups were sacrificed on the 35th day post-operationally. Another 6 pups were sacrificed for triphenyl tetrazolium chloride (TTC) staining of brain slices 48 hours after the operation and nissl staining of brain slices 35 days after the operation. The pups of each group were weighed every other day; additionally, the righting reflex test was performed from P6 to P11, and the balance beam experiment was performed from P39 to P42 (Figure 6A).

6. HIE Model Construction

The HIE model CP has been widely accepted and recognized as an animal model of CP[13]. And the operation processes were as follows: on P7, the Model group pups were anesthetized with isoflurane, and the right common carotid artery was ligated. After waking, the pups were placed in a cabin with 8% oxygen and 92% nitrogen for 2.5 hours. Finally, the pups were sent back to their mother. As for the Sham group, the common carotid artery was isolated but not ligated.

7. Behavioral Test

Righting reflex test: The pups were placed on a platform with their back facing down, and the time for successful righting was recorded. Balance beam test: After three consecutive days of training, the pups successfully passed a crossbar with a width of 2 cm, length of 120 cm and height from the ground of 50 cm. On the 4th test day, the time to pass the crossbar and the number of slips of the hind limbs while passing the crossbar were recorded.

8. TTC and Nissl Staining

8.1 TTC staining: The two pups were euthanized 48 hours post-HIE model creation. After the heart was perfused with normal saline, the pup brains were dissected to make 2 mm thick sections for TTC (Sigma Aldrich, USA) staining.

8.2 Nissl staining: At 35 days post HIE model creation, the pups were perfused and fixed after sacrificed, and the brains were removed for sectioning. After rehydration, the brain slices were stained with 0.5% tar violet (Macklin, China), observed under an OLYMPUS-BX51 microscope (OLYMPUS, Japan), and photographed with the equipped cell Sens Standard 1.12 (OLYMPUS, Japan).
9. ELISA

Human fresh plasma was collected from children by centrifuging at 3000r, 10 min. After the rats were anesthetized, arterial blood was collected from the abdominal aorta into an EDTA anticoagulation blood collection tube, and plasma was obtained via centrifugation at 3000 rpm/min for 10 minutes. Huma n tissue nonspecific alkaline phosphatase (TNAP) (ml906210V), Rat tissue nonspecific alkaline phosphatase (TNAP) (ml497021V), Rat 25(OH)D (ml038318V), Rat osteopontin(OPN) (ml003147V), Rat C-reactive protein (CRP) (ml038253V), Rat interleukin (IL)-6 (ml102828V), Rat IL-10 (ml002813V), and Rat IL-17 (ml003003V) were detected according to the ELISA kit (Enzyme-linked Biotechnology Co., Ltd, Shang hai) manufacturer's recommended procedure.

10. Western Blot

The expression levels of TNAP, NF-κB, IL-10, and IL-6 in the right cortex tissues of the pups on the 7th and 35th days post-HIE model creating were measured by Western blot. The primary antibodies used were rabbit anti-TNAP (DF6225, Affinity), mouse anti-IL-6 (ab9324, Abcam), rabbit anti-NF-κB (#8242, Cell Signaling Technology), and rabbit anti-IL-10 (ab9969, Abcam). The internal control was rabbit anti-β-Actin (ab8227, Abcam). The gray values were quantified and analyzed by Image J software (NIH).

11. Data Analysis and Functional Annotations

The robust multiarray average (RMA) was used to normalize the results of the expression profiling chip, and then the logarithm base 2 (log2) of the normalized data was taken to generate a normalized value. We then used LIMMA [PMID25605792] (R software package (version 3.5.2) to screen differentially expressed mRNAs (|Log2Fc|>1 and FDR≤0.05) and differentially expressed proteins (|Log2Fc|>0.5 and FDR≤0.05). The cluster profiler package was used to perform GO and KEGG PATHWAY enrichment analysis for differential mRNA genes, and the items that were significantly enriched were identified (p.adjust <0.05).

The results were shown as the mean ± standard deviation. SPSS 23.0 statistical software was used for comparative analysis, the independent sample t-test was used for the analysis of differences between the two groups, and one-way analysis of variance (ANOVA) was used to evaluate the differences between multiple groups. The Pearson correlation coefficient was used to measure the correlation between two types of data. A p value <0.05 was considered to indicate statistical significance.

Results

1. Combined Analysis of mRNA Chip Sequencing and Protein Profile Detection to Screen the Related Factors of Cerebral Palsy.

After standardizing the mRNA data, batch effects and the changes in the expression (intensity) caused by the experimental technology were reduced (Figure 1A). First, the volcano map and heatmap (Figure 1C-D) show an obvious difference in the mRNA expression between the ST group and the Ctrl group;
specifically, with respect to those in the Ctrl group, 217 mRNAs were significantly upregulated and 2692 were downregulated in the ST group. Similarly, the DT group demonstrated 110 upregulated mRNAs and 72 downregulated mRNAs with respect to the Ctrl group (Figure 1E-F). GO enrichment analysis (Supplement Table S1) of the differential mRNAs between the ST group and the Ctrl group showed that the mRNAs were significantly enriched in 27 biological process (BP) items, 41 molecular function (MF) items and 19 cellular component (CC) items. The top 30 GO entries are shown in Figure 2A. Figure 2B shows the results of the KEGG enrichment pathway analysis of the differentially expressed mRNAs between the ST group and the Ctrl group, showing enrichment in the neuroactive ligand-receptor interaction pathway and the calcium signaling pathway, among others. Similarly, the differential mRNAs between the DT group and the Ctrl group were also subjected to GO and KEGG enrichment analysis, which revealed enrichment in 2 MF items and 1 KEGG pathway (Supplement Table S2). The ST group was subsequently subjected to further analysis.

Four blood samples from each group from the same batch of the ST group and Ctrl group were used for protein profile detection, which identified 10 upregulated proteins and 27 downregulated proteins (Figure 3A-B). Then, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database was used to construct a protein-protein interaction (PPI) network of differential proteins (Figure 3C), and the top 10 important proteins were identified by the MNC algorithm, including ALPL, FN1, SERPINA1, and PF4.

Finally, the 2909 differential mRNAs and 37 differential proteins were overlapped to obtain 3 common differential genes: ALPL (which was also among the top 10 genes of the PPI network), FABP1 and RP1. Additionally, we collected blood samples from 12 patients in the ST group and 15 children in the Ctrl group whose samples were not subjected to mRNA chip detection and mass spectrometry analysis. The results showed that compared with that of the Ctrl group, the expression product of ALPL, TNAP, was significantly decreased in the ST group (p<0.001, Figure 3E; Supplement Table S3).

2. GMFCS Levels, Blood Count and Biochemical Tests, MRI Results of Children with Spastic CP.

The severity of motor impairment in the ST group (n=18) was categorized by GMFCS level. According to the GMFCS, there was no patient at grade I; four patients (22.2%) were at grade II, 5 (27.8%) were at grade III, 6 (33.3%) were at grade IV, and 3 (16.7%) were at grade V. We grouped the participants at GMFCS grades II and III as moderate motor dysfunction (n: 9, 50%) and those at grades IV and V (n: 9, 50%) as severe motor dysfunction (Table 2). Additionally, the percentage of peripheral blood lymphocytes and the contents of serum CRP, 25(OH)D and Cr were measured in both the ST group (18 participants) and the Ctrl group (20 participants). The results showed that compared with that in the Ctrl group, the percentage of lymphocytes and CRP levels in the ST group were significantly increased (p<0.001, Figure 4A-B), while the 25(OH)D and Cr levels were significantly reduced (p<0.001, Figure 4C-D). Moreover, a direct association was also identified between the serum TNAP content and CRP and 25(OH)D levels in the ST group (Figure 4E; Supplement Table S4).

Table2 Classification of the gross motor function in children with spastic CP
Characteristics

|           | ST (n=18) | Control (n=20) |
|-----------|-----------|----------------|
| **GMFCS level** |           |                |
| MMI       | 9 (50%)   | \              |
| Level II  | 4 (22.2%) | \              |
| Level III | 5 (27.8%) | \              |
| SMI       | 9 (50%)   | \              |
| Level IV  | 6 (33.3%) | \              |
| Level V   | 3 (16.7%) | \              |

MMI: moderate motor dysfunction; SMI: severe motor dysfunction

The cranial MRI findings of the 18 patients in the spastic group were as follows (Table 3). PVL was observed in 10 patients (55.6%), including 3 with ventriculomegaly and/or irregular shape of the lateral ventricles, 4 with abnormal signal foci in the ventricular white matter, and 3 with dysplasia of the corpus callosum. Cerebral atrophy was observed in 2 patients, supratentorial hydrocephalus in 2 patients, multiple softening foci in the subcortical white matter in 1 patient, basal ganglia/thalamus lesions in 2 patients, and porencephalia in 2 patients (Figure 5, Table 3).

Table 3
The appearance of brain MRI in children with spastic CP

| Cranial MRI findings                  | ST (n=18) | Control (n=20) |
|--------------------------------------|-----------|----------------|
| PVL                                  | 10 (55.6%)| \              |
| ventriculomegaly                      | 3         |                |
| abnormal signal foci                 | 4         |                |
| dysplasia of corpus callosum         | 3         |                |
| Diffuse brain injury                 | 6 (33.3%) | \              |
| cerebral atrophy                     | 2         |                |
| supratentorial hydrocephalus         | 2         |                |
| basal ganglia/thalamus lesions       | 2         |                |
| Focal lesion                         | 2 (11.1%) | \              |
| porencephalia                        | 1         |                |
| periventricular hemorrhagic infarction| 1         |                |
| Normal                               | \         | 20 (100%)      |
3. Changes in TNAP and inflammation-related cytokines in the peripheral blood of rats with CP.

After HIE model creation, infarction foci and liquefaction or atrophy of the injured brain tissue (Supplement Figure S1A-B) were observed in the Model group, and the number of Nissl bodies was reduced relative to the Sham group (Supplement Figure S2). From the day of modeling (P7), the weight of the pups in the Model group was significantly lower than that in the Sham group ($p<0.05$; Figure 6B; Supplement Table S5), and the righting reflex time of the Model group after modeling (P7-11) was significantly longer than that of the Sham group ($p<0.05$; Figure 6C; Supplement Table S6). On the 35th day post-HIE model creation, the time for the Model group to pass the balance beam and the number of leg-slips were significantly larger than those of the Sham group ($p<0.05$; Figure 6D; Supplement Table S7). In sum, these results suggest that the HIE model rats can reproduce the clinical and pathological changes in children with spastic CP.

On the 7th day and 35th day post-HIE model creation, the levels of serum TNAP and IL-10 in the Model group were significantly lower than those in the Sham group (Figure 7A, 7I), while the levels of IL-6 and CRP were significantly higher (Figure 7D, 7E). Furthermore, the levels of OPN and IL-17 in pups in the Model group were significantly higher than those in pups in the Sham group (Figure 7C, 7H), while the serum 25(OH)D content was significantly reduced day 7 post-HIE (Figure 7B). In addition, 7 days and 35 days after HIE model creation, the level of serum TNAP was correlated with those of serum CRP and IL-10 (Figure 7F, 7G, 7J, 7K; Supplement Table S8).

4. Changes in TNAP and Inflammation-Related Cytokines in the Brain Tissue of Rats with Cerebral Palsy.

It was observed that the Model group displayed signicantly lower expression levels of TNAP and IL-10 in the cortex of the injured side than the Sham group ($p<0.001$; Figure 8D, E), while the IL-6 ($p<0.05$; Figure 8F) and NF-κB ($p<0.001$; Figure 8G) expression levels were significantly higher in Model group day 7 post-HIE. The changes in protein expression on the 35th day after HIE model creation were the same as those on day 7 after HIE model creation. Additionally, there was a strong correlation between the TNAP content in the serum and brain tissue of HIE model rats both on the 7th and 35th days post HIE model creation (Figure 8B, 8C; Supplement Table S9, Supplement Figure S3).

Discussion

A wide range of emerging biological data and multiomics approaches now provide unprecedented opportunities to further explore the key pathophysiological processes of various complicated diseases through different molecular layers, and which may help provide new diagnostic and therapeutic targets. Therefore, the analysis of multiomics data has potential value in the clinical diagnosis, treatment and prognosis which may improve the survival rates and therapeutic outcome [14, 15].

However, in the process of biological information analysis, many data will be widely dispersed; consequently, one of the goals of the current study was to investigate ways to quickly and effectively identify useless data and select core genes [16]. In this study, we found that the original data from the
transcriptomic analysis of the spastic CP group was less dispersed than those of the dyskinesia CP group, making the results of our analysis more credible and therefore, help direct the subsequent multiomics analysis and screening. The wide dispersion of the original data of the dyskinesia CP group may have been related to the small sample size of the enrolled patients; even if we had performed genetic data homogenization, there would still have been differences with the spastic CP group in the enrichment of unrelated genes and signal pathways. Spastic CP has the highest incidence and is the most common classification in CP. Its main manifestations are a hyper-stretch reflex, clinically characterized by increased muscle tension, active tendon reflex and positive pathological reflex\cite{17}. Therefore, the subsequent analysis mainly focused on spastic CP and explored the core genes and pathways related to its pathogenesis.

Integrated transcriptome and proteome analysis can be used, not only to explore the nature of the physiology and pathology of disease can be explored in the transcription level and the protein level, but also to reveal the mutual regulation and connection between the two levels\cite{18,19}. In our study, we used a combination of proteomics and transcriptomics to analyze the transcriptional activity and protein expression levels of genes involved in the mechanism underlying spastic CP.

First, compared with the control group, the spastic CP group had 2909 differentially expressed mRNAs, 217 upregulated and 2692 downregulated. We performed GO and KEGG enrichment analysis of the differentially expressed genes and found that they were mainly involved in BP items, such as immune response, neurotransmitter transmission, angiogenesis, and neurogenesis. KEGG signaling pathway analysis showed that differentially expressed genes were mainly enriched in inflammatory response and neurotransmitter transduction-related signaling pathways, such as the neuroactive ligand-receptor interaction pathway, the IL-17 signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway, and the calcium ion signaling pathway. Second, through protein mass spectrometry, 37 differentially expressed proteins were identified in the spastic CP group, including 10 upregulated proteins and 27 downregulated proteins. Through proteomic and transcriptome analysis and development of a PPI network, we identified molecules closely related to CP and further analyzed whether TNAP, the expression product of $ALPL$, may be closely related to spastic CP.

According to the ELISA results, we found that the TNAP expression level in the plasma of children with spastic CP was significantly lower than that of the control participants. The human $ALPL$ gene is located on chromosome 1 (1P36), with a genome span of approximately 50 kb and a total of 12 exons and 11 introns. Its mRNA length is 2580 bp, and it encodes TNAP, which contains 524 amino acids\cite{20}. The main biological function of TNAP is to hydrolyze the extracellular substrates inorganic pyrophosphate (PPi), pyridoxal-5-phosphate (PLP) and phosphor-ethanolamine (PEA). PLP is the main active form of vitamin B6 that exerts biological functions in the body\cite{21}. TNAP is an ectoenzyme that is anchored to the outer cell membrane and to extracellular vesicles via its glycosyl-inositol-phosphate (GPI)-anchor. TNAP not only has a high expression in the liver, kidney, and bone tissues but also plays an important role in the proliferation and differentiation of neurons during the development of the brain\cite{22}.
To confirm whether TNAP and CP were directly correlated, we evaluated the changes in TNAP in the plasma and cerebral cortex of HIE model rats. Among the many CP models, the HIE model is stable, widely used, and demonstrates long-term ischemia and hypoxia model, consistent with the characteristics of spastic CP\textsuperscript{23}. Through TTC staining, Nissl staining and other methods, brain injury was observed in the model rats with CP. The righting reflex and balance beam tests showed impaired motor function and muscle coordination in the model rats. Together, these findings showed that the HIE model rats simulated the clinical manifestations of CP well. We found that compared with that in the Sham group, the expression of TNAP in the brains and peripheral blood of model rats was significantly decreased. The results of the animal experiments were consistent with the changes in the plasma of the children with CP, and there was a strong correlation between the content of TNAP in plasma and the expression level of TNAP in the injured side cerebral cortex on the injured side. Therefore, we suggest that the changes in TNAP are not only related to the occurrence of CP but also directly involved in its pathogenesis.

Recently, TNAP activity has been linked to some previously unconnected processes, including metabolism and inflammation, which may be the result of ALPL genetic variants\textsuperscript{24, 25}. Graser et al\textsuperscript{26} reported that TNAP contributes to the balance between proinflammatory ATP effects and the anti-inflammatory effects of its breakdown product adenosine, which has received attention in the field. Beck et al\textsuperscript{27} found that insufficient TNAP phosphatase activity leads to the accumulation of PPI and osteopathy, which can initiate the accumulation of calcium crystals in the joints and consequently initiate inflammatory processes. In our study, the plasma levels of TNAP, 25(OH)D, OPN, and Cr in children with spastic CP were significantly lower than those in the control group, which is consistent with the results of related studies. Akpinar et al\textsuperscript{28} showed that neuromuscular conditions such as CP may lead to vitamin D deficiency and undernutrition in general. The major circulating metabolite of vitamin D, 25(OH)D, is widely used as a biomarker of vitamin D status\textsuperscript{29}; it can regulate the level of immune inflammatory factors and plays an important role in the growth and development of the body. In addition, Huang et al\textsuperscript{30} observed that 25(OH)D can inhibit the production of inflammatory molecules in neuronal glial cells by inhibiting the MAPK pathway and the production of downstream inflammatory molecules. Recently, some studies reported that OPN is a physiological substrate of TNAP and identified at least two preferred sites of dephosphorylation by TNAP. Yadav et al\textsuperscript{31} found that low expression of ALPL led to elevated OPN, which can activate inflammatory factors such as IL-6 and IL-17 in bone cells, thus triggering inflammation. It has been reported\textsuperscript{32} that OPN not only promotes the secretion of proinflammatory cytokines but also inhibits the production of anti-inflammatory factors such as IL-10. In this study, we also observed that the expression of ALPL was decreased in children with spastic CP and in model rats, triggering an increase in OPN expression, resulting in upregulation of IL-6, IL-17 and other proinflammatory factors in peripheral blood.

Some studies\textsuperscript{33–35} have confirmed the existence of neuroinflammation in children with spastic CP, which may be one of the main causes of brain damage. Cranial MRI findings play an important role in the evaluation of the sites, timing, and severity of brain injury in children with CP\textsuperscript{11}. The European Cerebral
Palsy Study reported\[36\] abnormal cranial MRI findings in 88.3% of patients, of which PVL was the most common, accounting for approximately 42.5%. The main manifestations of brain damage in spastic CP are PVL, and the main imaging findings are irregular expansion of the lateral ventricles; paraventricular tissue softening; decreased white matter volume; and dysplasia of the corpus callosum. In our study, 10 (55.6%) of 18 children with spastic CP showed PVL on cranial MRI, and the lesion location was consistent with the abovementioned literature. The pathogenesis of PVL is multifaceted\[35,37–39\], including maternal infection, cerebral ischemia and vulnerability of brain white matter\[40\], among which the response of fetuses and newborns to inflammatory damage is the key. In this study, we also observed that the level of CRP and lymphocytes in the peripheral blood of children with spastic CP was significantly higher than that of the control group, indicating the occurrence of inflammatory reactions in the subject group. Similarly, the expression of CRP, IL-6 and IL-17 was significantly increased in the peripheral blood of model rats, while the level of the anti-inflammatory factor IL-10 was significantly decreased. We observed signs of brain damage, such as edema, atrophy and liquefaction, in brain tissue slices of the injured cerebral hemisphere of model rats. Similarly, upregulation of IL-6 expression and downregulation of IL-10 expression were also detected in the cerebral cortex of model rats, confirming that inflammation is also involved in the brain injury process of CP. More importantly, downregulation of TNAP expression was observed in both children with spastic CP and model rats. In addition, the expression level of TNAP correlated well with CRP in both children with CP and HIE model rats, while some degree of correlation was observed between TNAP expression and IL-10 level in HIE model rats.

Hu et al\[41\] showed that TNAP is an inhibitor of NF-κB activity, and low expression of TNAP can induce NF-κB pathway activation. It is currently believed that NF-κB is involved in the transcriptional regulation of gene expression and inflammatory cytokines and plays an important role in the regulation of the cytokine network in the inflammatory response\[42\]. It has been confirmed that the NF-κB pathway is a key transcriptional pathway in neuroinflammation caused by cerebral ischemia and hypoxia, which may promote the abnormal expression of inflammatory factors\[23\]. There is a close relationship between NF-κB and IL-6, and previous studies\[43\] have suggested that cerebral ischemia and hypoxia can occur through the NF-κB/IL-6 pathway, resulting in rapid IL-6 release, direct induction of neuronal apoptosis and inhibition of nerve regeneration, ultimately aggravating neonatal brain injury. IL-17 is an early promoter of the T cell-induced inflammatory response, which can destroy the blood-brain barrier (BBB) and enter the site of the inflammatory response. By inducing other inflammatory cells, IL-17 can promote the secretion of inflammatory factors and produce a local inflammatory environment in the central nervous system\[44\]. In addition, IL-17 can activate downstream signaling pathways such as NF-κB, leading to the expression of proinflammatory chemokines and cytokines\[45\]. IL-10 is a synthetic inhibitor of human cytokines with immunomodulatory and anti-inflammatory effects\[46,47\]. Some studies have shown that the body can play an anti-inflammatory role and protect brain tissue by upregulating the expression level of IL-10\[48\]. In our study, we found low expression of TNAP and high expression of NF-κB in the cerebral cortex of model rats. Based on the analysis of the correlation between TNAP in peripheral blood and inflammatory factors and the high correlation between brain tissue and TNAP in peripheral blood, we suggest that the
downregulation of TNAP expression in CP might weaken the inhibition of NF-κB, leading to an abnormally high expression of NF-κB, the upregulation of the proinflammatory cytokines IL-6 and IL-17 and the downregulation of the anti-inflammatory factor IL-10, which contribute to the production of inflammation in the progression of CP.

A weakness of the present study is that TNAP shares a high degree of homology with several small proteins whose functions are unknown at this time. However, TNAP is a relatively small protein with no known regulatory domains, and we could not obtain ALPL knockdown mice for use as animal models. Therefore, there is an urgent need to evaluate inflammatory reactions and molecular mechanisms in ALPL knockdown mice and the regulatory relationship between TNAP and the occurrence of inflammation in the progression of spastic CP.

Conclusion

The results of this study indicate that TNAP, a gene expression product of ALPL, is closely related to the pathogenesis and development of CP, particularly in the occurrence of inflammatory reactions in spastic CP.

Abbreviations

CP: cerebral palsy
ST: spastic type cerebral palsy
DT: dyskinesia type cerebral palsy
Ctrl: control
ALPL: The liver/bone/kidney alkaline phosphatase gene
TNAP: tissue nonspecific alkaline phosphatase
HIE: hypoxic ischemic encephalopathy
IL-6: interleukin-6
IL-17: interleukin-17
CRP: C-reactive protein
IL-10: interleukin-10
NF-κB: nuclear factor kappa-B
KEGG: Kyoto Encyclopedia of Genes and Genomes
**GO**: Gene Ontology

**GMFCS-ER**: The Gross Motor Function Classification System for cerebral palsy-Extended and Revised

**MRI**: Magnetic Resonance Imaging

**T1W**: T1-weighted

**T2W**: T2-weighted

**PVL**: periventricular leukomalacia

**PWMI**: periventricular white matter injury

**LYM**: percentage of lymphocytes

**25(OH)D**: 25 hydroxyvitamin D

**Cr**: creatinine

**P6(P7, P11,P39,P42)**: postnatal day 6

**TTC**: triphenyl tetrazolium chloride

**OPN**: osteopontin

**RMA**: robust multiarray average

**FC**: fold change

**FDR**: false discovery rate

**BP**: biological process

**MF**: molecular function

**CC**: cellular component

**PPI**: protein-protein interaction

**MAPK**: mitogen-activated protein kinase

**PPI**: inorganic pyrophosphate

**PLP**: pyridoxal-5-phosphate

**PEA**: phosphor-ethanolamine
GPI: glycosyl-inositol-phosphate

BBB: blood-brain barrier

MMI: moderate motor dysfunction

SMI: severe motor dysfunction

Declarations

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Authors’ contributions

Bing Li designed the study and revised the manuscript. Xiaokun Wang performed experiments, analyzed the data and drafted the manuscript. Chao Gao acquired the clinical data. Hequan Zhong, Xiangyu Kong, Rui Qiao performed the experiments and recorded the results. Huichun Zhang, Dongmei Yang and Yang Gao participated data analysis. All authors read and approved the final manuscript.

Data Availability

Most of the data generated or analyzed during this study are included in this published article. All the data and materials are available if required.

Ethics approval and consent to participate

This clinical protocol was authorized by the review board of Children’s Hospital Affiliated to Zhengzhou University, China. (protocol approval numbers 2019-K-051).

All animal procedures were performed in strict accordance with the institutional guidelines for the care and Use Committee (IACUC) at Shanghai Public Health Clinical Center. (protocol approval numbers 2019-A018-02).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Competing interests
The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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**Figures**
Figure 1

Differential expression of mRNAs in the Ctrl group, ST group and DT group: A: Distribution of gene expression values in each sample following data standardization. As shown above, the horizontal axis represented the number of each group of samples, and the vertical axis displayed the mRNA level of each sample. After normalization, the expression level of each group of samples was more consistent. B: Expression distribution of the transcription profile of each group of samples. The principal component
analysis chart showed the mRNA expression of the ST group (green dots), DT group (red dots) and Ctrl group (blue dots); the transcription profiles of ST group and DT are significantly different from those of the Ctrl group. C-D: Differential expression of mRNA between the ST group and Ctrl group, depicted as C: a volcano graph of the differential mRNA distribution and D: a heatmap of the 2909 differential mRNAs between the groups, with red and blue representing upregulated and downregulated mRNAs, respectively (|Log2Fc|>1, FDR\textless{}0.05), and green representing mRNAs with no significant difference; E-F: Differential mRNA expression between the DT group and Ctrl group, depicted as E: a volcano map of the distribution of differential mRNAs and F: a heatmap of the 182 differential mRNAs between the groups, where red and blue represent upregulated and downregulated mRNAs, respectively (|Log2Fc|>1, FDR\textless{}0.05), and green represents mRNAs with no significant difference.
Figure 2

A-B: The GO and KEGG enrichment entries of the differential mRNAs between the ST group and Ctrl group. A: The vertical axis shows the first 30 entries of GO enrichment. The horizontal axis represents the number of enriched genes in each entry. The p.adjust value of each GO entry increases as the color becomes colder (i.e., from red to blue). B: The vertical axis shows the KEGG enrichment pathways, and
the horizontal axis represents the proportion of the entry among all KEGG entries. The dots represent the number of enriched genes in each entry.

Figure 3

A-B: Volcano map and heatmap showing 37 differentially expressed proteins between the ST group and the Ctrl group. The red and blue dots represent upregulated and downregulated proteins, respectively, and green represents proteins with no significant difference. The co-differential genes RP1, ALPL, and FABP1 identified by expression profile chip detection and mass spectrometry detection are marked in Figure. C: $|\text{Log2Fc}|>0.5$ and FDR$<0.05$ were used as the criteria for screening differentially expressed proteins. The PPI network diagram shows the differentially expressed proteins in the plasma of children in the ST group and the Ctrl group. Each dot in the network represents a protein, and the connection between the

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dots represents the relationship between them. The more connected lines there are, the more important the protein may be in the PPI network (minimum required interaction score >0.4); D: The top 10 genes in terms of degree in the PPI network screened by the MNC algorithm according to the test results of the plasma protein profiles of the children in the ST group and Ctrl group; the lines between the dots represent the relationship between them. E: The content of TNAP in the plasma of children in the ST group and Ctrl group. Compared with that in the control group, the expression level of TNAP in the plasma of children in the spastic type CP group was significantly lower (p<0.001, n=12 for ST; n=15 for Ctrl). ** p<0.001 vs. Ctrl.

Figure 4

Results of blood tests for the Ctrl group (n=20) and ST group (n=18). A: Compared with that of the Ctrl group, the percentage of lymphocytes in the ST group was significantly increased (p<0.001). B: The level of CRP in the ST group was significantly higher than that in the Ctrl group (p <0.001). C: Compared with
those in the Ctrl group, the levels of 25(OH)D in the ST group were significantly lower (p<0.001). D: The plasma Cr of the ST group was significantly lower than that in the Ctrl group (p<0.001). E: In the ST group, the level of TNAP was correlated with CRP (p<0.001, r=-0.96) and 25(OH)D (p =0.011, r=0.76). ** p<0.001 vs. Ctrl.

Figure 5
Cranial MRI findings of the 18 patients in the ST group. A1-2 (transverse): irregularly shaped bilateral ventricles with nearby, irregular, patchy T1 and T2 hypersignals. A3 (transverse): enlarged bilateral ventricles with nearby, patchy T2 signals. B1 (transverse). left frontotemporal parietal atrophy with subcortical softening foci. B2 (transverse): reduced volume of the left basal ganglia; B3 (transverse): decreased volume of the left thalamus. C1 (transverse): left partial cerebral perforation malformation with supratentorial hydrocephalus. C2 (transverse): right ventricular para-body brain penetration malformation with surrounding gliosis. C3 (sagittal): supratentorial hydrocephalus. D1 (transverse): thinning of the corpus callosum. D2 (sagittal): noticeably thinner and shorter corpus callosum. D3 (sagittal): significantly shorter corpus callosum. E1-3 (TI/T2 transverse/sagittal): normal brain MRI.

Figure 6

A: Flow chart of the HIE animal model experiments. B: After the operation, the Model group showed continuously significantly less weight than the Sham group (p<0.05, n=10). C: The time required for the Model group showed significantly longer righting reflex time from P7 to P11 than the Sham group (p<0.05, n=8). D: Compared with the Sham group, Model group showed increased time for passing the balance beam and the number of leg-slips were significantly increased in the Model group (p<0.05, n=10). *, p<0.05 vs. Sham
Figure 7

A-B, I: 7 days after HIE model creation, compared with those in the Sham group, the levels of TNAP, 25(OH)D and IL-10 were significantly reduced in the Model group (p<0.001, n=10). C-E, H: The levels of OPN, IL-6, CRP and IL-17 were significantly greater in the Model group than in the Sham group (p<0.001, n=10). F, J: Seven days post-HIE model creation, the plasma levels of TNAP were correlated with those of CRP (p =0.003, r=-0.83, n=10) and IL-10 (p =0.0143, r=0.74, n=10). D-F: Thirty-five days after HIE model creation, compared with the Sham group, the Model group had significantly higher levels of TNAP (p<0.05, n=10), IL-6 (p<0.05, n=10) and CRP (p<0.001, n=10), while the level of IL-10 was significantly decreased. (p<0.05, n=10). G, K: Thirty-five days post-HIE model creation, the plasma levels of TNAP were correlated with those of CRP (p =0.0004, r=-0.90, n=10) and IL-10 (p =0.0137, r=0.74, n=10). *, p<0.05 vs. Sham; **, p<0.001 vs. Sham
Figure 8

A: Representative Western blot images of TNAP, IL-10, IL-6 and NF-κB in the injured cerebral cortex. D-G: Compared with those in the Sham group, the cortex expression levels of TNAP and IL-10 were significantly reduced in the Model group (p< 0.001, n=6). Compared with those in the Sham group, the levels of IL-6 (p<0.05, n=6) and NF-κB (p<0.001, n=6) were significantly increased in the Model group. B-C: On day 7 (p<0.001, r=0.96, n=12) and day 35 post-HIE model creation (p<0.001, r=0.91, n=12), there was a positive correlation between the levels of TNAP in the peripheral plasma and the brain. *, p<0.05 vs. Sham; **, p<0.001 vs. Sham.

Supplementary Files

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