The level of GNE and its relationship with behavioral phenotypes in children with autism spectrum disorder

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Abstract
Autism spectrum disorder (ASD) is a serious nervous system disease, and the cause is not known. Sialic acid (SA) is an indispensable nutrient for early brain development. In previous studies, it was found that the SA level of ASD group was lower than that of control group. However, the reason for this has not been explained. A case-control study was conducted to understand the association between the SA synthase enzyme regulatory gene and ASD. The study sample included 65 ASD children and 64 healthy children. The levels of the GNE gene were measured, which encodes UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE), a key enzyme in SA biosynthesis. The symptom severity, intelligence development level, and behavioral performance of ASD children were estimated. There was a significant difference in the levels of GNE between the ASD and control groups ($t = 2.028, P = 0.045$). Moreover, the levels of GNE were negatively related to stereotypical behaviors according to the Autism Diagnostic Observation Schedule (ADOS) assessment ($r = -0.386, P = 0.039$). However, there is no correlation between the levels of GNE and autistic severity. As evaluated through the Social Responsiveness Scale (SRS), the levels of GNE were negatively associated with autistic mannerisms scores, social cognition scores and SRS total scores in the children with ASD ($r = -0.314, P = 0.020$). These results indicate that the GNE gene may be associated with autism spectrum disorder, and it is also related to autistic behavioral performance, such as stereotypical behaviors, autistic mannerisms, and social cognition ability. Our data suggest that future studies to explore the causal relationship between GNE and the etiology of ASD may be needed.

Abbreviations: ABC = autism behavior checklist, ADI-R = autism diagnostic interview-revised, ADOS = autism diagnostic observation schedule, ASD = autism spectrum disorder, BTBR = BTBR T+Itpr3tf/J mouse, CARS = childhood autism rating scale, CK = creatine kinase, GNE = UDP-GlcNAc 2-epimerase/ManNAc kinase, NCAM = neural cell adhesion molecules, PPVT = peabody picture vocabulary test, SA = sialic acid, SRS = social responsiveness scale, VABS = vineland adaptive behavior scale.

Keywords: autism spectrum disorder, behavioral phenotypes, GNE gene

1. Introduction
Autism spectrum disorder (ASD) is a group of neurodevelopmental conditions involving social deficits, impaired communication, and repetitive stereotypical behaviors.\textsuperscript{[1]} The prevalence of ASD in children and adolescents is 1 in 59 individuals in the United States; although high rates have been reported, the etiology of ASD is still unknown.\textsuperscript{[2]} Moreover, patients with ASD often show phenotypic heterogeneity.\textsuperscript{[3]} Since the discovery of ASD, a large number of scholars have carried out etiological studies of ASD, including studies of neuroanatomy, brain imaging, genomics, proteomics, heredity, environmental factors, and epigenetics.\textsuperscript{[4–7]} So far, some scholars believe that ASD has a strong correlation with genetic factors.\textsuperscript{[8]} Although a large number of genes have been reported to correlate with ASD, the main effect gene of ASD has not been found, which brings great challenges to the diagnosis and treatment of ASD.\textsuperscript{[9]} However, there are fewer studies on the association between the expression of genes in the blood and their behavioral phenotypes in patients with ASD.

The GNE gene takes part in encoding UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE), which is a bifunctional enzyme that initiates and regulates the biosynthesis of sialic acid (SA).\textsuperscript{[10]} SA is an essential nutrient for brain development and cognition and is also a necessary component of brain gangliosides.\textsuperscript{[11]} Gangliosides are the most abundant sialoglycans expressed in nerve cells.
and may play an important role in axon-myelin interactions, axon stability, axon regeneration, and modulation of nerve cell excitability.\textsuperscript{12} Ganglioside levels are increased in the cerebrospinal fluid from children with ASD.\textsuperscript{13,14} Furthermore, SA could be synthesized into polysialic acid glycan, which associates with neural cell adhesion molecules (NCAM) to form PolySia-NCAM, which in turn impacts molecular interactions during synaptic plasticity and neural development.\textsuperscript{15,16} The expression of PolySia-NCAM was decreased in the brain tissue of both valproic acid induced autistic model rats and BTBR T\textsuperscript{+}Ipr\textsuperscript{3tf}f mouse (BTBR). An case-control study found that the levels of SA in the peripheral blood of children with ASD were lower than those of controls.\textsuperscript{17} Additional, 1 study found that levels of saliva SA in children with ASD was different from healthy children, and it was related to autistic behaviors.\textsuperscript{18} The above evidence suggests that children with ASD was different from healthy children, and it was peripheral blood of children with ASD were lower than those of controls.\textsuperscript{17} Additional, 1 study found that levels of saliva SA in children with ASD was different from healthy children, and it was related to autistic behaviors.\textsuperscript{18} The above evidence suggests that the SA signal pathway may be associated with ASD. Therefore, more comprehensive studies are still required to confirm this hypothesis.

Based on the above findings, the abnormal synthesis of GNE may lead to dysfunction and the inability to synthesize enough SA. Therefore, we suspect that abnormalities in the expression of the GNE gene may be involved in the unusual regulation of GNE synthesis in patients with ASD. In addition, we want to explore whether the level of GNE is associated with behavioral phenotypes of children with ASD. Therefore, we conducted a case-control study to confirm the above hypotheses.

2. Methods

2.1. Patients

The case-control study contained 65 children with ASD (10 girls, 55 boys, age 4.45 ± 0.98) and 64 typically developing children (15 girls, 49 boys, age 4.43 ± 0.72). All children with ASD were diagnosed by a psychiatrist based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders-Fifth Edition and the combined results of the Autism Diagnostic Interview–Revised (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS). The exclusion criteria were fragile X syndrome, which in turn impacts molecular interactions during synaptic neural cell adhesion molecules (NCAM) to form PolySia-NCAM, related to autistic behaviors.\textsuperscript{18} The above evidence suggests that children with ASD was different from healthy children, and it was peripheral blood of children with ASD were lower than those of controls.\textsuperscript{17} Additional, 1 study found that levels of saliva SA in children with ASD was different from healthy children, and it was related to autistic behaviors.\textsuperscript{18} The above evidence suggests that the SA signal pathway may be associated with ASD. Therefore, more comprehensive studies are still required to confirm this hypothesis.

2.2. Evaluation of behavioral symptoms in ASD children

Based on clinical history and neuropsychiatric assessment, the children with ASD or their caregivers completed the Peabody Picture Vocabulary Test (PPVT), the Autism Behavior Checklist (ABC), the Childhood Autism Rating Scale (CARS), the Vineland Adaptive Behavior Scale (VABS), the Social Responsiveness Scale (SRS) and the neuropsychological development examination table for children aged 0-6 years old. The PPVT is used to examine receptive vocabulary ability and to assess intellectual development in children.\textsuperscript{19} The ABC, which was completed by the parents, was developed to assess and screen for autism and to evaluate the severity of autism symptoms.\textsuperscript{20} CARS is a commonly used tool for diagnosing autism. Scores ranging from 30 to 36 were categorized as mild to moderate autism, and scores ranging from 37 to 60 were considered severe autism.\textsuperscript{21} The SRS has been validated, and has been shown to be reliable and have a good correlation with the gold-standard ADI-R.\textsuperscript{22} The VABS is used to examine core autistic behaviors and focuses on communication and adaptive behaviors.\textsuperscript{23}

2.3. Measurement of GNE levels

Blood samples were obtained between 8:30 and 11:00 in the morning, and RNA was immediately extracted from fresh blood. Total RNA was extracted from fresh blood of the children with ASD and healthy controls using the RNAprep pure Blood Kit (TIANGEN BIOTECH (BEIJING) CO., LTD). Clearance of DNA contamination in the RNA samples and cDNA synthesis was performed using the PrimeScript RT reagent Kit with gDNA Eraser according to the manufacturers instructions (TaKaRa Bio). Real-time PCR was subsequently performed using the ABI-7500 System employing the SYBR Select Master Mix (Applied Biosystems, Life-Technologies). The specific RT-PCR primers for GNE were synthesized by Sangon Biotech (Shanghai) Co., Ltd. Primer sequences were:

\begin{align*}
\text{GNE:} & \quad \text{Forward} & \quad 5’-\text{CAATGATGGCAACCTGTGCTG-3’} \\
\text{GNE:} & \quad \text{Reverse} & \quad 5’-\text{AATGCAACCCAGTGCTTGTG-3’} \\
\text{HS-ACTB:} & \quad \text{Forward} & \quad 5’-\text{CCTGGCACCCAGCACAAT-3’} \\
\text{HS-ACTB:} & \quad \text{Reverse} & \quad 5’-\text{GGGGCCTCTGCTACAT-3’} \\
\end{align*}

2.4. Statistical analysis

Data were prospectively collected and analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). For the descriptive data, we computed the means and standard deviations of demographic and outcome variables (independent t test and one-way ANOVA). We used the Chi-Squared test, Fisher exact test and the Kruskal-Wallis H test to determine differences in the distribution of categorical variables (PPVT, ABC, CARS scores) in different groups. The correlation between GNE levels and the behavioral phenotype of children with ASD was determined using Pearson and Spearman correlation analysis. For all analyses, statistical significance was set at a P value of .05.

3. Results

3.1. The levels of GNE in ASD and control groups

There was no age or gender difference between the ASD and the control groups (P = .931, .247, respectively). There was significant difference in the levels of GNE between the ASD and control groups (t = 2.028, P = .045) (Fig. 1). No statistically significant difference was found in different ages and genders between the children of ASD and control groups (P > .05).

3.2. The relationship between the levels of GNE and behavioral symptoms in ASD children

Through Spearman correlation analysis, it was found that GNE levels were negatively related to stereotypical behavior scores of ADOS scale (r = -0.386, P = 0.39, n = 29). It was shown that the lower the expression levels of GNE were, the higher the stereotypical behavior scores in the children with ASD who had...
more serious stereotype behavior problems (Fig. 2). There was no significant difference in the GNE levels among different ASD severity levels or different intelligence development levels of children with ASD (Table 1). Within the ASD group, the levels of GNE were negatively correlated with the autistic mannerisms scores and social cognition scores of the SRS scale ($P < .05$). It indicated that the lower the level of GNE was, the higher the autistic behavior score, and the more serious the autistic behavior problem. However, GNE levels were not correlated with various field scores on the VABS scale (see Table 2).

4. Discussion

To the best of our knowledge, this is the first study on GNE levels in peripheral blood and their association with behavioral phenotypes in children with ASD. There was a decrease in the GNE levels of peripheral blood in ASD group compared with the healthy controls. Additionally, the GNE levels were negatively related to autistic mannerisms scores and social cognition scores of SRS scale, which was used to evaluate social behaviors. This meant that the lower levels of GNE in children with ASD were associated with serious autistic and social problems. Moreover, ASD children with the lower the levels of GNE may perform more serious stereotypical behavioral problem.

Sialic acid, a family of 9-carbon sugar acids, is an integral structural and functional component of the nervous system, particularly as precursors for the synthesis of the polysialic acid glycan, which modify the cell membrane-associated NCAM.\cite{15} SA is synthesized in the cytoplasm and transported into the nucleus where it is activated with cytidine triphosphate to form the activated nucleotide donor of SA, cytidine monophosphate sialic acid.\cite{24} It exerts negative feedback inhibition on GNE, the key bifunctional cytoplasmic enzyme that regulates the biosynthesis of SA, thus limiting excess production of free SA.\cite{25} During childrens learning and cognition development, the surfaces of nerve cells need to form a large number of PolySia-NCAM complexes; so that the demand for SA also subsequently increases.\cite{15} Therefore, both GNE and SA levels might affect the development of learning and cognitive function. However, a low level of learning and cognitive ability is common in children with ASD, so it is important to carry out a study to understand the correlation between the SA synthetase regulating gene (GNE) and ASD.\cite{26,27}

![Figure 1. The GNE mRNA level in the ASD and control groups.](image1)

![Figure 2. The correlation between GNE mRNA level and stereotypical behavior scores of ADOS scale.](image2)

### Table 1

| Item | N | GNE (Mean ± SD) | F  | P   |
|------|---|----------------|----|-----|
| ABC  | 27 | 1.09 ± 0.37    | 1.698 | .192 |
| 50–66| 12 | 0.90 ± 0.32    | 0.978 | .328 |
| ≥67  | 24 | 0.97 ± 0.28    | 0.978 | .328 |
| CARS | <30| 1.02 ± 0.26    | 0.041 | .960 |
| 30–36| 44 | 0.99 ± 0.36    | 0.041 | .960 |
| 37–60| 3  | 0.97 ± 0.17    | 0.041 | .960 |
| PPVT | <70| 1.01 ± 0.34    | 1.776 | .179 |
| 70–85| 13 | 0.99 ± 0.20    | 1.776 | .179 |
| >85  | 6  | 1.27 ± 0.45    | 1.776 | .179 |

In this study, 2 ASD child did not complete the ABC scale, 3 ASD children did not complete the CARS scale, and 6 ASD children did not complete the PPVT test.

ABC = Autism Behavior Checklist, PPVT = Peabody Picture Vocabulary Test, CARS = Childhood Autism Rating Scale.

### Table 2

| Item | r   | P   |
|------|-----|-----|
| VABS total standard scores | 0.094 | .482 |
| Communication | 0.177 | .362 |
| Daily living skills | 0.055 | .681 |
| Socialization | 0.223 | .093 |
| Motor skills | 0.066 | .621 |
| SRS total scores | −0.306 | .023 |
| Social awareness | −0.085 | .538 |
| Social cognition | −0.272 | .044 |
| Social communication | −0.243 | .074 |
| Social motivation | −0.199 | .144 |
| Autistic mannerisms | −0.314 | .020 |

In this study, 7 ASD children did not complete VABS scale and 10 ASD children did not complete SRS scale.

VABS = Vineland Adaptive Behavior Scale, SRS = Social Responsiveness Scale.
GNE myopathy is caused by a mutation in the GNE gene, and the level of creatine kinase (CK) in these patients is increased. Abnormal expression of CK and the creatine kinase brain-hand isoenzyme was found in the peripheral blood of patients with ASD. CK will affect the function of skeletal muscle, exercise ability, and behaviors. Patients with ASD are often characterized by low levels of gross motor ability and social behavior disorder. In addition, 2 studies reported that motor skills (e.g., stability, motor accuracy, and object manipulation) were negatively related to social function in children with ASD. However, there is no report on whether the GNE gene is associated with motor ability, social behavior and other behaviors in patients with ASD. Interestingly, there was a difference in GNE gene expression between the children with ASD and control children in this study. The reasons for the low expression of the GNE gene in the ASD group still need more systematic study.

The main core symptoms of ASD are social impairment and stereotypical behavior. However, in this study, we found that GNE levels were negatively correlated with autistic mannerisms, social cognition and stereotypical behaviors according to the SRS and ADOS scales. Autistic-like behaviors have a great impact on the education, life and health of ASD children. Improving the core symptoms, providing a reasonable intervention ASD treatment and ameliorating the quality of life for children with ASD may have profound significance. Moreover, this study could provide new evidence for the etiological study of ASD.

There are some limitations to this study. First, the level of UDP-GlcNAc 2-epimerase/ManNac kinase in the children with ASD was not measured. Apart from this, an increased number of samples may be needed in a follow-up study. Second, as a nervous system disease, determining the levels of the GNE gene and GNE in the brain tissue of patients with ASD will provide more specific evidence to elucidate the relationship between GNE and ASD. In the follow-up study, the mutation of GNE in patients with ASD should be further evaluated. Third, this work was a case-control study; thus, there was a limitation in verifying the causal relationship between GNE and ASD. Therefore, an autistic mouse model will be used to further verify the exact association between GNE and ASD in subsequent studies.

Author contributions
Data curation: Xiaolei Yang and Jie Ge.
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Measurement of GNE levels: Xiaolei Yang and Jie Ge.
Project administration: Xiaolei Yang.
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Writing – review & editing: Zhongguang Zhou, Jicheng Liu.

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