



Original Article

The identification of sialuria with different degrees of intellectual disabilities in children and adolescents

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Abstract

Background: Single nucleotide polymorphism/mutation in the R263L region of the allosteric site of the GNE gene produces a phenotype with an overproduction of intracellular levels of sialic acid and causes sialuria. In sialuria, a defective GNE gene, synthesized with lost feedback inhibition mechanism, produces many developmental delays and varying degrees of intellectual disabilities in children and adolescents. Several mutations in the epimerase and kinase domains exist that cause difficulty in getting a precise and exact effect of the GNE gene on the disease severity and sialic acid levels. This is the first study investigating the molecular basis of neuronal disorders exhibiting sialuria in Pakistani children/ adolescents.

Methodology: The current study quantified the mRNA expression of the GNE gene and urinary sialic acid concentration by Realtime-qRT-PCR and Fluorimetric assays, respectively. The correlation between relative mRNA and urinary sialic acid levels was evaluated by using Pearson Bivariate correlations.

Results: The data show that severely intellectually disabled (I.D.) patients showed significantly reduced mRNA expression levels of the GNE gene compared to controls. The concentrations of free sialic acid in urine were significantly reduced in severe I.D. patients compared to controls. Whereas patients with mild I.D. showed a two-fold increase in sialic acid levels when compared to controls. A significant correlation was found between an increased GNE mRNA and low urinary sialic acid levels from severe I.D. patients.

Conclusion: The effect of the GNE gene is beyond hyposialylation that could hinder N-glycan structure and sialic acid biosynthesis. The study highlighted the possible involvement of sialic acid levels with different degrees of intellectual disabilities in Pakistani children and adolescents.

Keywords

Sialuria, Intellectual Disability, Metabolic Error.



Introduction

Sialuria, an autosomal dominant disorder found in patients with a defective synthesis of a key enzyme UDP-N-acetylglucosamine-2-epimerase N-acetylmannosamine kinase (GNE) due to the mutation in the R263L region of the GNE gene. Due to this mutation, the negative feedback inhibition is lost, disrupting sialic acid synthesis in mammals¹. In sialuria, an increased overproduction of free sialic acid is found in the cytosol. Therefore, patients who exhibit an increased excretion of sialic acid in urine show many difficulties with developmental delays. Previously a transgenic mouse line that expresses GNE having a mutation in the same region R263L has been shown to produce and excrete 400 times higher RNA expression of mutated GNE gene than the wild type mice. N-acetylneuraminic acid levels were also higher in the brain cytoplasm with an increased polysialylation of neural cell adhesion molecule (NCAM) in transgenic mice than in wild type. However, the same study showed minor differences in membrane-bound sialylation in many organs. In contrast to this, a significantly higher expression of sialylation was observed on the surface of leukocytes. Kreuzmann and colleagues (2017) proved that the developmental delays associated with sialuria patient are due to increased intracellular levels of sialic acid that causes polysialylation on NCAM¹.

Sialic acids are composed of glycoproteins and glycolipids responsible for important cellular functions, infection, and metastasis. Its catabolism is important for a healthy heart and skeletal muscle functions not only in humans but in zebrafish as well². Siblings with sialuria, exercise intolerance/muscle wasting, and cardiac symptoms were reported previously having heterozygous mutations in N-acetylneuraminic pyruvate lyase gene (NPL) at [chr1:182775324C>T

(c.187C>T; p.Arg63Cys) and chr1:182772897A>G (c.133A>G; p.Asn45Asp)]. The effect was observed on sialic acid catabolism and cell-specific reductions in N-acetyl mannosamine (ManNAc) levels. Knockdown NPL in zebrafish leads to severe skeletal myopathy and cardiac edema, resembling the human phenotype. However, the phenotype was rescued by expressing wild-type human NPL. However, there was no change when p.Arg63Cys or p.Asn45Asp mutants were expressed. Surprisingly the phenotypes in zebrafish were rescued by feeding catabolic products of NPL: N-acetyl glucosamine (GlcNAc) and ManNAc2, suggesting monosaccharide replacement therapy for humans. As sialuria regulates neural development, neural regeneration, learning, and memory^{3,4}, any alterations can alter humans' intellectual levels. Therefore, in this study, we have investigated the mRNA expression levels of the GNE gene in blood and sialic acid levels in urine, of the patients with different intellectual disabilities.

Methodology

Subject recruitment and Ethical Approval

This study was approved by the Human Ethics Committee of the National Institute of Child Health and Rehabilitation Center (NICH), Jinnah Postgraduate Medical Center (JPMC), with the ethic number of HEA NO. F.2-81/2008-GENL/4086/JPMC. Parents of every subject were requested to read and understand the consent form before signing. The data was collected from 15th Jan to 30th July 2018. Overall 102 subjects were used for mRNA expression i.e. Controls (n=51) and patients (n=51). All subjects were between the ages of 0-17 years (male/females).

These subjects were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (DMS-IV) and



International Classification of Diseases (ICD-10) criteria and presented in Out-Patient-Department (OPD) and Darul-Sukun, Karachi, Pakistan. Expert psychiatrists evaluated all subjects through history and clinical examinations.

Biochemical analysis of Urinary Sialic Acid

The levels of free sialic acid, N-Acetylneuraminic acid, and urine were measured using the fluorometric sialic acid kit method (BioVision's cat # K566-100). Fluorescence at Ex/Em 535/587nm was measured using the molecular device SpectraMax Plus M5e Microplate Reader. Morning urine samples from I.D. subjects having risk for sialuria (10-13 years of age) were analyzed and compared with age-matched control samples. Serial dilutions of sialic acid standards were performed according to the sialic acid assay kit. Urine samples collected from subjects and controls were centrifuged at 3000 rpm for 5 min to remove the metabolites.

Real-Time Polymerase chain reaction (RT-qPCR)

Total RNA was extracted from whole blood (500 µl) using a whole blood RNA purification mini kit (Thermo Scientific GeneJET cat# K0761). The quality of extracted RNA was determined via a spectrophotometer (260/280 nm). According to the manufacturer's specifications, total RNA was transcribed to cDNA using

RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific cat# K1621). The PCR reaction was initiated by an incubation step at 25°C for 5 min, followed by only one cycle of annealing step at 42°C for 60 min with a termination step at 70°C for 5 min.

PCR reaction performed with an ABI 7500 software V.2.0.6 real-time PCR detection system (Applied Biosystem Inc. USA) using the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). The thermal cycler program initiated by an incubating step at 95°C for 10 min, followed by 40 cycles of denaturation step at 95°C for 15 s, annealing step at 55°C for 30 s, extension step at 72°C for 30 s and a final extension at 72°C for 5 min (Table 1). No Template Control (NTC) and Reverse Transcriptase Minus (R.T.-) control along with PCR analysis was conducted in triplicate for each sample. The Delta Delta CT method used and selected genes' content was normalized to the housekeeping gene (HKG) GAPDH. The calculation was performed using the comparative Ct method according to the following formulas:

$$\Delta Ct_{WT} = Ct_{\text{selected gene WT}} - Ct_{\text{HKG WT}}$$

$$\Delta Ct_{KO} = Ct_{\text{selected gene KO}} - Ct_{\text{HKG KO}}$$

$$\Delta\Delta Ct = \Delta Ct_{KO} - \Delta Ct_{WT}$$

$$\text{Ratio} = 2^{-\Delta\Delta Ct} \text{ (maximum efficacy is presumed)}$$

Table 1: Thermal cycling conditions for RT-Qpcr.

Gene	Primer sequence	PCR parameters for thermal cycle
GNE mRNA	5'CTCCGAGTTGCAATAGTCAG	1. Initial temperature _____ 95°C for 10 min 2. Denaturing temperature __95°C for 15 sec 3. Annealing temperature __55°C for 30 sec 4. Extension Temperature __72°C for 30 sec 5. Go to repeat cycle _____ 2, 40 times
	3' (F) CATCCAGAGACACAACAAGG (R)	
GAPDH	5'GCATCCTGGGCTACACTGAG	
	3' (F)	



5'TTGCCCTCAACGACCACTTT
3' (R)

6. Final extension Temperature ___72°C for 5 min
7. Hold on _____ 4°C forever

*Thermal cycling conditions for RT-qPCR: The table is illustrating thermal cycling conditions for RT-qPCR with GNE and GAPDH primers.

Statistical Analysis

The data obtained in this study were analyzed using curve expert and SPSS software version 17. Differences between the mean values of different groups were identified by applying a student independent t-test. Each experiment was repeated three times. The correlation between relative mRNA and urinary sialic acid levels was evaluated by using Pearson Bivariate correlations. P-value <0.05 was considered significant.

Result

Quantitative Estimation of Sialic Acid in Urine by Fluorometric Assay

Free sialic acid (FSA) concentrations (Table 2) from mild (0.32 ± 0.17), moderate (0.07 ± 0.03) and severe (0.04 ± 0.01) intellectually disabled (I.D.) subjects were used to plot a bar diagram (Figure1). The result illustrates

an average two-fold increase in sialic acid levels in mild I.D. subjects. However, the levels were not significant when compared to controls. In contrast, there was a significant decrease in the sialic acid levels found in the severely Intellectually Disabled subjects compared to controls.

Fluorometric assay is used to quantify urinary free sialic acid (FSA) levels in ID patients. The table depicts no significant differences in sialic acid levels in mild and moderate ID. patients. However, there was a two-fold increase in the levels of sialic acid in mild ID. and a significant reduction in the free sialic acid levels in severe ID patients compared to controls. Statistical analysis was revealed by two independent t-test. Means values are \pm S.D. $p < 0.05$ was considered significant.

Table 2: Fluorometric assay to quantify urinary free sialic acid (FSA) levels.

Symptom Severity	Age (Years)	Free sialic acid (FSA) concentration in Urine μ l		p-value
		(Means \pm S.D.)		
		Controls	Subjects	
Controls	10-13			
Mild ID	10	0.16 \pm 0.13	0.32 \pm 0.17	<0.0005
Moderate ID	11		0.07 \pm 0.03	<0.0005
Severe ID	13		0.04 \pm 0.007	<0.0005

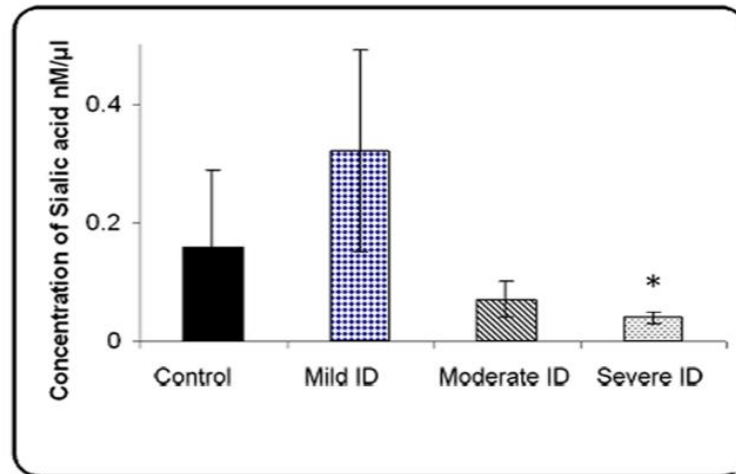


Figure 1: Sialic acid levels in the urine controls and patients from Pakistan.

The bar diagram shows the comparison of sialic acid levels among controls and ID patients. A significant difference was observed in patients with severe ID. Significant differences were evaluated by a two-tailed T-test (* $p < 0.0005$).

GNE mRNA Expression levels

The GNE gene expression in subjects with severe ID and controls was quantified concerning the housekeeping gene GAPDH. The results show a significant down-regulation in the expression of GNE gene levels in subjects with severe ($P < 0.003$) ID when compared to controls (Figure 2).

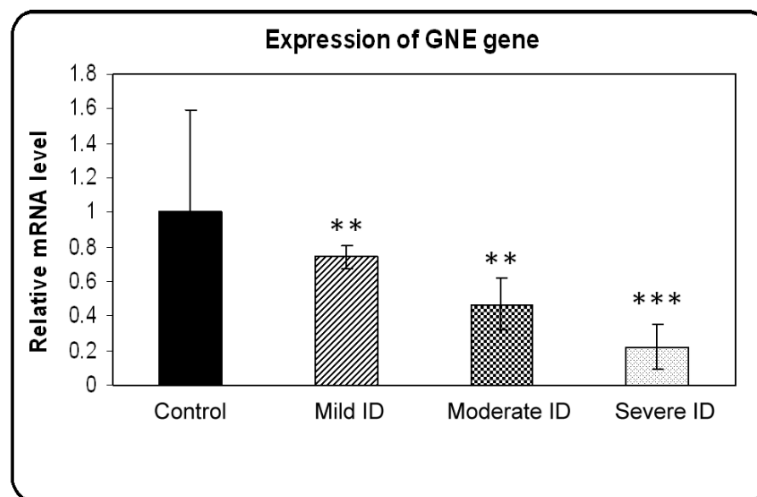


Figure 2: Relative expression levels of the GNE gene by real-time quantitative PCR.

Bar graph representing the fold changes of GNE mRNA levels quantified by normalization to the GAPDH as an internal control. Mild, moderate, and severe ID. patients showed down-regulation in the GNE gene expression compared to controls. However, Mild and moderate ID. patients showed 2- and 0.5-fold up-regulation compared to severe ID. patients, respectively.



Significant differences were evaluated by an independent T-test (** $p < 0.01$, *** $p < 0.003$). Significant correlation was found between GNE mRNA and sialic acid levels of severe ID patients (0.048 ± 0.0076) ($p = 0.021$).

Discussion

Previously we have identified G/A substitution (R263Q) mutations in SNP: rs121908623 of the GNE gene in the Pakistani Population⁵. As sialuria is a very rare disorder, up till now, only 10 ten patients have been reported worldwide. The first patient was identified in 1968 by (A), and the tenth patient was identified by Ishtiaq and colleagues (2020). In all of the patients, the symptoms were similar such as jaundice, low birth weight, coarse facies, hepatomegaly, seizures; however, they had normal birth and delivery⁵. Besides this, the neurological symptoms included speech and motor impairments. All these patients had mutations in the GNE gene at G→T: 849, G→A:848, G→T:839, G→A:839, G→C:250, T→C:51+34 positions⁶⁻¹⁵. Recent works on sialuria presented a link between intellectual disability with low household income, low maternal education, and consanguinity marriages¹⁶. Sialic acids are negatively charged amino sugars and are added to many glycoproteins during posttranslational modifications of proteins. Due to this, they actively participate in biological molecular interactions¹⁷.

Structural data of GNE/MNK homolog have developed by Kurochkina, Yardeni, and Huizing (2010) that exposes critical substrate binding sites on the enzyme¹⁸. This model helps explain the effects of missense mutations associated with HIBM or sialuria on enzyme actions, helix arrangement, and substrate binding. They confirmed that all reported mutations so far are, in fact, due to the mutations in the active site or secondary interfaces of the GNE/MNK enzyme. It was

reported that a Persian-Jewish HIBM has mutation p.M12T at the interface of alpha4 alpha10 that affected GlcNAc, Mg²⁺, ATP binding. Structural data helps develop the therapeutic options that target the misfolding of GNE/MNK in HIBM or Sialuria¹⁸. In this study, a few experiments were carried out separately to determine the sialic acid levels in urine and GNE mRNA expression levels in the blood of different subtypes of I.D. subjects. The data show a significant increase in urinary sialic acid in mild I.D. subjects and reduced GNE gene expression in severe I.D. subjects. It has been reported previously that patients with Sialuria tend to excrete more S.A. in urine than controls. It has been shown that the levels range from 10 to 30 folds is associated with several inborn errors of metabolic diseases such as salla disease, sialidosis, ISSD, and neuraminidase deficiency^{5, 6, 19, 20}. In sialuria, free sialic acid levels can be elevated up to 70 to 200 folds^{7, 8, 21, 22}. An earlier study reported a sixth subject of sialuria with mild developmental impairment showing an increased level of sialic acid in his urine. This is consistent with the current study that showed an increased sialic acid level in the urine. Subjects with mild Intellectual Disability exhibiting a 2-fold increase in free sialic acid levels than controls proved that mild phenotype of Intellectual Disability might be associated with sialuria. Moreover, data on the single-family also showed about 10-fold increased sialic acid levels in urine; however, the reductions in free sialic acid levels in subjects with severe Intellectual Disability showed that severe I.D. might not be linked with sialuria disease in Pakistani children and adolescents.

For confirmation, we analyzed and compared the expression pattern of the GNE gene in controls and severe ID children and adolescents of Pakistan. All subjects with severe ID showed down-regulation when



compared to controls. In earlier studies, subjects with mild impairments showed high expression of the GNE gene in their different tissue organs¹⁰. In contrast, when we analyzed the expression levels of GNE in subjects with severe I.D. who were suffering from severe developmental delay, our results show significantly low expression levels in these subjects, suggesting no association of Sialuria with severe mental illnesses.

Nevertheless, only GNE mRNA and urinary sialic acid levels from severe I.D. patients have shown a significant correlation. This means an increased GNE mRNA expression levels positively influence the low excretion of sialic acid levels in urine. As sialuria is known to cause an accumulation and urinary excretion of Neu5Ac sialuria, it differs from sialdoses, characterized as a defect in the storage and excretion of bound Neu5Ac from the body. The same GNE gene is involved in causing Nonaka myopathy (NK; MIM:605820) as well, besides sialuria and sialdoses. In Nonaka myopathy, muscle wasting and weakness of the distal and anterior tibial muscles takes place^{23,24}. Several mutations in the GNE gene in epimerase and kinases domains alter the enzymatic activity in a very minute and precise way. It is very difficult to describe and identify the actual effect.

Consequently, sometimes, the activity of the enzyme did not correlate with the severity of the disease²⁵⁻²⁹. The predominant function of GNE is to regulate the sialylation of cell surface glycoproteins and glycolipids¹²⁻¹⁵. However, the correlation between hyposialylation and the severity of the disease is inadequate because many human patients suffering from GNE myopathy have sialic acid levels not much different from the controls. This is the main reason why a poor correlation exists between sialic acid levels and symptom severity. These results were

also seen in animal models with D176V mutation in GNE gene³⁰. The symptoms in patients and mutant mice show normal and early birth but develop muscle weakness as they age, which can be relieved by providing them sialic acid or ManNAc.

Nevertheless, the sialic acid levels in animals remained low than in control animals³¹. They suggested a lack of correlation between the severity of the disease and the sialic acid levels, emphasizing that GNE gene mutation has effects beyond hyposialylation. The mutation can affect the N-glycan structure by changing the sialic acid biosynthesis and flux UDP-GlcNAc levels via hexosamine biosynthetic pathway^{32,33}. Our results propose that early effective avoidance from severe cognitive disabilities will obtain better information and averting the risk factors such as low birth weight, hypoxia, poverty, and serious diseases.

Conclusion

The urine analysis of sialic acid showed a significant reduction in severe mental retarded samples. There was an average two-fold increase in the urinary sialic acid levels in mild I.D. subjects compared to controls. Results from RT-qPCR showed a significant reduction in the I.D. mRNA expression of the GNE gene in mild, moderate, and severe I.D. subjects. There was a fivefold up-regulation of GNE gene expression in mild I.D. subjects compared to severe I.D. subjects. Only GNE mRNA and urinary sialic acid levels from severe I.D. patients showed a significant correlation. Thus data suggest that mild I.D. might be associated with sialuria in Pakistani children and adolescents. Therefore, Intellectually Disabled subjects and their immediate family members must be monitored for the polymorphisms in codons 263 to 266 of the GNE gene.



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